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Isolation of a cDNA clone of gp-80 from a lambda gt-11 mammary tissue library

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ISOLATION OF A cDNA CLONE OF GP-80 FROM A LAMBDA gt-11 MAMMARY TISSUE LIBRARY

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ABSTRACT

ISOLATION OF A cDNA CLONE OF gp-80 FROM A LAMBDA gt-11 MAMMARY TISSUE LIBRARY.

By Shanavaz L. Nasarabadi

Gp-80 is an integral membrane protein with an apparent molecular weight of 80kD. A λgt-11 cDNA expression library of bovine mamary tissue was screened with; (1) affinity purified polyclonal antibodies made to gp-80 in a rabbit host and (2) nucleic acid probes made to the partly known amino acid sequence of the protein. The appearance of background on screening the library with antibody probes could not be determined. The inability in identifying a clone on screening with the antibody could be due to the inability of the antibody to recognize the fused protein, instability of the fused protein in the prokaryotic environment, or the presence of the insert DNA in an incorrect reading frame. The high degeneracy and low concentration of the nucleic acid probes could be the probable reason for the inability to screen the library with these probes.

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I would like to thank my advisor Dr. Greenwalt for his guidance and patience even when the tidings were low. I would also like to thank Dr. Stacks for her guidance and advice during my work. I would like to thank my husband for his support and encouragement.

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Introduction:

The membranes of a cell have the principal function of setting the boundaries between the cell and the environment, and between compartments within the cell. These boundaries prevent the movement of all polar solutes from one compartment to another, unless such movement is required for biological activity. Under these circumstances, special transport systems are required. Thus membranes can be considered as structures which are selectively permeable. The barrier to movement of polar solutes across the membrane is provided by one of the two major components of the membrane, i.e. the lipid component which makes up the bilayer of the membrane.

The second major component of the membrane, the proteins, which provide the permeability function, are present within or on the surface of the lipid component. Membrane proteins also determine most other properties of the membrane such as (a) determinants of specificity which distinguish one cell from another and allow recognition between cells, (b) determination of the shape and architecture of the membrane, (c) receptors for information about the environment such as antigen recognition, or hormone binding sites etc., and (d) enzymes involved in the catalysis of reactions within or on the cell.

During lactation, milk triacylglycerols are secreted from mammary epithelial cells in the form of discrete droplets coated with a surface layer of membrane derived largely from the apical cell surface (1,2). This membrane layer, commonly referred to as the milk-fat-globule membrane (MFGM), is derived from the apical pole of secretory alveolar cells by a budding process in which intracellular lipid droplets are progressively enveloped with apical plasma membrane (3,4). Some membrane may also be acquired directly from secretory vesicle membrane within the cell (5,6), although the magnitude of the contribution to MFGM from this source remains controversial (7,8). As may be expected, the cell-surface origin of MFGM is reflected in the enzyme and lipid composition of membrane preparations isolated from milk.

Typical plasma membrane components enriched in MFGM include sphingomyelin, 5'-nucleotidase, phosphodiesterase, and alkaline phosphatase (9,10). MFGM thus provides a convenient source of membrane protein and lipid from a specific epithelial cell type in an heterogeneous population of cells. Separation of the proteins of MFGM from several species by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) leads to the resolution of eight to ten major components. Two of these components, xanthine oxidase and butyrophilin, account for nearly 50% of the total protein detectable by staining gels with Coomassie Blue (7,11,12). Butyrophilin is firmly bound to MFGM and appears to be largely restricted to the apical plasma membrane of secretory epithelial cells (7,13). Other glycoproteins that appear largely restricted to the apical surface include a highly sialylated protein with an Mr of over 400,000 in several species (14,15,16) and glycoproteins of Mr 100,000 and 55,000 in the cow and guinea pig, respectively.

MFGM preparations from human, cow, and guinea pig also contain a glycoprotein with an Mr of approx. 75,000-80,000. Approximately 5% of the total Coomassie Blue-positive protein of bovine MFGM is composed of this component (PAS IV of reference 17). The distribution of this glycoprotein (GP-80) in bovine tissues has been determined by immunofluorescence microscopy (18). It was found to be present in the endothelial cells of the capillaries lining the heart, liver, muscularities and mucosae of the small intestine, spleen, salivary gland, and pancreas. The antigen was found to be concentrated on the apical surface of secretory epithelial cells in mammary tissue but is also present in apparently significant amounts, in mammary endothelial cells. In other tissues, with some exceptions, GP-80 is restricted to capillary endothelial cells and is firmly bound to cellular membranes, which is a reflection of the protein's integral membrane nature. The absence of the protein in the endothelial cells of the kidney, brain, lung or large blood vessels appears consistent with the general hypothesis that, endothelial cells from different tissues express different levels and

complements of proteins and that these differences reflect the distinctive origins of capillary cells in embryonic tissues during development (21).

GP-80 is very hydrophobic. Removal of detergent from preparations of the purified protein, by nonpolar adsorption chromatography, led to complete precipitation of GP-80 from solution (18). The protein also remains firmly bound to the MFGM after treatment of isolated preparations with sodium carbonate solution at high pH, a procedure found to effectively remove loosely bound and peripheral membrane proteins (19,20). In the membrane bound form, the glycoprotein is resistant to proteolysis under conditions that completely remove the cytoplasmic protein coat material routinely seen in electron micrographs of MFGM (18). However, when solubilized by various detergents, GP-80 is readily degraded by a variety of proteinases. These results imply that GP-80 is closely associated with the membrane lipid bilayer and is not a peripheral protein of the cytoplasmic coat or loosely adsorbed to the external membrane surface. The hydrophobic nature of the protein has been confirmed by amino acid analysis of the protein, wherein 50% of the amino acids have nonpolar residues and a further 20% have side chains with uncharged polar groups at neutral pH(18). The protein is also characterized by a low level of the sulphur-containing amino acids, cysteine and methionine, and a high percentage of branched chain amino acids, valine, leucine, and isoleucine. The hydrophobicity index of GP-80 has been estimated to be 1,174, which implies that the protein has a pronounced hydrophobic character. Approximately 5.3% (18) to 32% (49) of the glycoprotein is composed of carbohydrate on a weight basis. The principal sugars detected were mannose, glucose, galactose, and sialic acid.

GP-80 isolated from heart and lung is several thousand daltons larger than that isolated from MFGM. The hypothesis put forward to explain this discrepancy, is that GP-80 associated with MFGM is degraded by proteinases, either during or after secretion, and that this glycoprotein is initially the same size in all tissues (22).

In the present study, a lambda gt11 cDNA library made from the mRNA extracted from bovine mammary tissue, was screened with (1) polyclonal antibodies made to bovine GP-80 in a rabbit host, and (2) nucleic acid probes made to the amino acid sequence of the protein. The purpose of this study was to isolate a clone for the protein of interest (i.e. GP-80) in order to be able to get a cDNA sequence which could then be correlated with the amino acid sequence. The advantage of screening a cDNA library is that since it's made from mRNA, the clone obtained would lack the introns and hence would directly correlate to the authentic sequence of the protein. This study would thus facilitate the determination of the internal sequence of the protein which is difficult to sequence directly since it is very hydrophobic and the enzymatically digested protein fragments stick to the C-18 column on elution.

The genome of the entire family of lambdoid phages are similarly organized so that the central third, shown as the replaceable region in figure 1, contains genes that are entirely dispensable for lytic growth. For the purpose of DNA cloning, the genetic map is most simply the essential transcription to the right that leads to expression of DNA replication functions. The genes for cell lysis, then proceed across the cohesive end to express the genes for cutting and packaging the mature phage DNA.

Transcription from pR (right promoter gene) is normally terminated at tR2 (right terminator) until gene N product accumulates to anti-terminate and allow transcription to proceed to the right. Deletion nin5 removes tR2 and thereby enlarges the replaceable region to include gene N. The promoter pR defines one end of the replaceable region of the genome since any piece of DNA inserted to the right of pR could interrupt the pattern of transcription necessary to replicate and package the vector. The other end of the replaceable region is defined by the end of gene J, the last essential gene for the "heads & tails" operon. This is known to be close to but less than 39.5% of the wild-type genome length to the right of the cohesive end. Gene cI codes for the repressor that keeps both major phage promoters pR

and pL shut off when the phage DNA is integrated in the E.coli chromosome. Promoter pL drives transcription to the left across gene N and the phage recombination systems gamma and red (these are dispensable for growth if the host supplies the recA {recombinant A} product), then across the phage gene for integration (int) and excision (xis) of the phage into and out of its E.coli host. The attachment site (att) of the phage provides the specific sequence to direct the recombination event that integrates the lambda genome into the E.coli chromosome at a corresponding bacterial attachment site. In short, the working part of a lambda vector is the fusion of a right end that must include pR and a left end that must include the head and tail genes 'A-J', fused together through the cohesive end (cos).

The requirement for a DNA molecule to be efficiently packaged in vivo is the presence of both the left and right cohesive ends of lambda separated by 78% to 105% of the wild type genome length. There is a precipitous decrease in viability in phages containing less than 78% of the genome.

Most strategies used to isolate genes from recombinant DNA libraries have employed nucleic acid probes (23,24). Genes can also be identified and isolated through the use of antibody probes of the antigen(s) expressed by specific genes (25).

A systematic examination of foreign polypeptide antigens in E.coli should be possible if the factors which influence production of detectable levels of each of many different kinds of proteins are adequately considered. There are three major problems associated with obtaining expression of foreign DNA as a stable antigen. The first problem is that most foreign DNA does not contain the transcription and translation control signals required for expression in E.coli. Thus, the foreign gene must generally be placed under the control of an E.coli promoter that is efficiently recognized by E.coli RNA polymerase, and an E.coli ribosome binding site that can be used by the bacterial translation machinery.

The second problem in expressing foreign DNA is that unusual polypeptides are efficiently degraded in E.coli (26-29). This problem is minimized by using host cells producing large quantities of temperature sensitive lac operon repressor, the product of lacI gene. Prevention of the lacZ-directed synthesis of the protein continues until the number of infecting cells surrounding the plaque is sufficiently large. The lacZ gene is then derepressed with isopropyl β-D-thiogalactopyranoside (IPTG), inducing lacZ-directed expression of the protein. The position within the β-galactosidase gene is near the carboxy terminus of the protein, which appears to aid in the stability of the fusion protein. To further increase the stability of the foreign protein, lon protease deficient mutants are used as hosts. Lon protease is one of the proteases in E.coli which is responsible for the low stability of foreign proteins in E.coli (30,31).

The lambda gt-11 library will be screened with antibody made to SDS denatured protein in the rabbit host. Since the protein of interest, GP-80, is a highly hydrophobic membrane protein, it may not fold correctly in the prokaryotic environment. Complete and native type folding may be necessary for the epitope presentation and in turn efficient screening of the cDNA library.

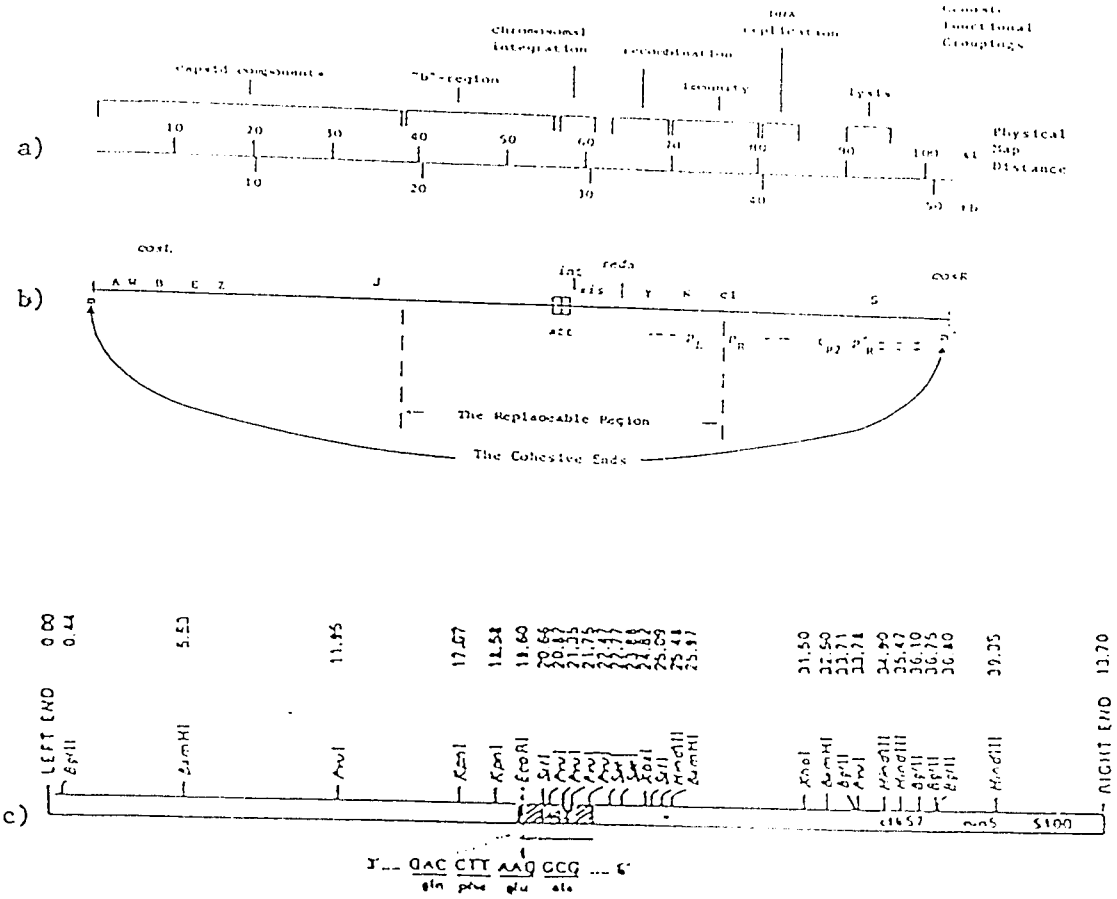


Figure 1: A simplified depiction of the phage lamda genome (31).

The physical map distance and regions of various genomic functions are dipicted in Figure 1a. Figure 1b shows the replaceable region of the λ genome starting from the end of the essential gene J to the begining of the promotor P_R. The cohesive ends m and m' are 12 base pair single stranded complimentary tails which hybridize to circularize the genome on infection. Figure 1c indicates the unique Eco RI site for cloning to λ gt-11 responsible for distributing the lacZ gene function as indicated in the restrictation map of λ gt-11.

Materials :

Tris base, tris-HCl, 4,4'-dicarboxy-2,2'-biquinoline (bicinchonic acid), phenyl methyl sulphonyl fluoride (PMSF), nitroblue tetrazolium, barbital buffer, sodium dodecyl sulfate (SDS), SDS-PAGE molecular weight standards, aprotinin, glycine (free base anhydrous), ampicillin, yeast extract, tryptone, sodium hydroxide, maltose, agarose, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), dimethyl formamide, Triton X-100, bovine serum albumin (BSA), sodium azide, diethyl pyrocarbonate (DEPC), NZ amineA (type-A hydrolysate of casein), polyethylene glycol 6000/8000 (PEG), Kodak X-OMat AR X-ray film, affinity purified goat anti-rabbit IgG horseradish peroxidase conjugate, isopropyl β-D-thiogalacto pyranoside (IPTG), and 3-[N-morphilino]propane sulfonic acid (MOPS) were obtained from Sigma Chemical Co. (ST. Louis, MO, U.S.A). Bis(N,N'-methylene-bis-acrylamide), affinity purified goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate, Coomassie brilliant blue R-250, and nitrocellulose membrane were purchased from Bio-Rad (Richmond, CA, U.S.A.). Methanol, sodium chloride, and N,N-dimethyl formamide were purchased from EM Science (Cherry Hill, NJ, U.S.A). Acrylamide was purchased from Spectrum Chemical MFG. Corp. (Gardena, CA, U.S.A). Magnesium chloride was purchased from Spectrum Chemical MFG. corp. (Redondo Beach, CA, U.S.A). Ponseau S was purchased from Helena Laboratories (Beaumont, TX, U.S.A). EDTA was obtained from Curtis Matheson Scientific, Inc. (Houston, TX, U.S.A). Centricon 30 micro-concentrators were purchased from Amicon Division, W.R.Grace & Co., (Danver, MA, U.S.A). Standard cellulose dialysis tubing with a molecular cut off at 12,000 to 14,000 daltons was purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA, U.S.A). Qiagene Tip 5 cartridge was purchased from Qiagen Inc. (Studio City, CA, U.S.A). Lambda gt-11 human mammary tissue c-DNA library ($9x10^9$ pfu/ml), ovalbumin recombinant ($2x10^9$ pfu/ml) and nonrecombinant lambda gt-11 clones, rabbit anti-ovalbumin antibody were purchased from Clontech (Palo Alto, CA, U.S.A). Lambda gt-11 bovine mammary tissue c-

DNA library ($4.8x10^9$ pfu/ml) was a gift from Dr. Deb (University of Washington WA, U.S.A). γ^{32}P-ATP was obtained from New England Nuclear (U.S.A). Gene Screen Plus membrane and circular nitrocellulose filters were pruchased from Dupont (Boston, MA, U.S.A). Purified human and bovine GP-80 was provided by Dr. Dale Greenwalt (San Jose State University, CA, U.S.A).

METHODS PART I:

I) Affinity purification of bovine GP-80 specific antibody :

Bovine GP-80-specific antibody was purified from the antisera obtained by injecting a rabbit host with purified SDS denatured bovine GP-80 protein, over a cyanogen bromide cross-linked GP-80 sepharose column containing a total column volume of 10ml (provided by Dr. Greenwalt). Two tenths of a percentage of Triton X-100 was added to 25ml of antiserum, dialyzed overnight against Tris buffered saline (50 mM Tris pH7.9, 150 mM NaCl) containing 0.2% Triton X-100 (TBST) and centrifuged for two hours at 26,000xg, at 4°C. The GP-80 affinity column was washed with three column volumes of TBST. The antiserum was loaded onto the column and 5ml fractions of the unbound material were collected during the subsequent TBST wash (5 column volumes). The GP-80 specific antibody was eluted in 3ml fractions with 3M NaSCN. These fractions were dialyzed twice against TBS. A bicinchonic acid (BCA) assay was done on the dialyzed fractions to determine the fraction(s) in which the GP-80 specific antibodies were eluted. The fractions containing the antibody were pooled and run on a reducing polyacrylamide gel to determine its purity. A western blot was conducted on the pooled sample to determine the dilution of antibody to be used in the screening of the library and also to determine the specificity of the antibody. The affinity column was washed with TBST and TBST containing 0.02% NaN_3, respectively, in order to preserve it.

II)Western blotting:

The specificity of the polyclonal antibody was examined by Western blotting (33). The protein samples were reduced by boiling in sample buffer (5%β-mercaptoethanol containing 0.005%bromophenol blue). Two identical samples were run on a reducing 7.5% polyacrylamide SDS mini-gel with pure bovine and human GP-80 next to the molecular weight standards. After electrophoresis the polyacrylamide gel was soaked in deionized water for 15 minutes and then in transfer buffer (75mM Tris buffer, 0.19M glycine, 20% methanol pH8.3) for 15 minutes. Proteins on the gel were transferred at 16V for 4 hours to a piece of

nitrocellulose filter paper pre-wetted in the transfer buffer. After transfer, the gel was discarded and the nitrocellulose stained with Ponceau S. Molecular weight markers consisting of carbonic anhydrase at 29Kda, egg albumin at 45Kda, phosphorylase B at 97.4Kda, E.coli β-galactosidase at 116Kda, and rabbit myosin at 205Kda were marked on the nitrocellulose paper after rinsing off the background color. The nitrocellulose paper was cut in half with one set of proteins on each piece. Both pieces were incubated in a 2% BSA/TBS solution overnight, and then washed in TBS. To the experimental set, the immune serum was added to a dilution of 1:100 in TBS containing 0.2% BSA and incubated for one hour at room temperature. The control set was incubated in the rabbit control serum at the same dilution for the same period of time. These two sets were then washed in 0.5% Triton X-100/TBS solution, 0.5M NaCl/TBS, and 0.1%BSA/TBS for a period of 20 minutes each. The filters were incubated next in goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate diluted one thousand fold with TBS/0.2% BSA for an hour. The wash steps were repeated with a final wash in TBS. The filters were incubated in the enzyme substrate (15ml TBS containing 3ml 4-chloronaphthol and 7ml 30% peroxide) for approximately 30 minutes. The filters were then washed in water to rinse off the excess substrate, wrapped in aluminum foil and preserved in the dark.

III) Determination of the log phase of E.coli Y1090 :

A loopful of E.coli Y1090 obtained from a stock agar stab was added to 30 ml of LB broth (24) containing 0.2% maltose and 50μg/ml ampicillin (growth media), and grown overnight in a 37°C water bath with aeration. One ml of the overnight culture was added to 100ml of the growth media in a nepheloflask and grown in a 37°C water bath. The growth was monitored at fixed time intervals until stationary phase was reached by taking O.D measurements at 600nm on a spec-20 spectrophotometer.

IV) Isolation of ovalbumin recombinant protein from a λgt-11 ovalbumin clone:

A lysate of a positive clone of ovalbumin was prepared according to Maniatis (24), and induced to synthesize the recombinant protein. Y-1090 was grown to log phase in 50ml of LB broth containing 0.2% maltose. A positive λgt-11 ovalbumin plaque picked up with a sterile pasteur pipette was resuspended in lambda diluent (24) for 2 hours at 4°C. One tenth of an ml of Y1090 was mixed with 0.1ml of λgt-11 ovalbumin extract and 0.1ml of 10mM $MgSO_4$/10mM $CaCl_2$, and incubated at 37°C for 15 minutes. The infected cells were added to 25ml of NZCYM media (24) and incubated in a 42°C water bath for 3 hours with aeration. Two hundred and fifty µls of 100mM IPTG, 1% aprotinin, and 0.5mM PMSF were added to the media and incubated for a further 6 hours in a 37°C water bath for the cell lysis to occur. The bacterial debris was then centrifuged at 500Xg for 10 minutes at room temperature. The supernatant was concentrated by passing through a centricon-30 filtration device at 6,000xg for 25 minutes at 4°C. A 20µl aliquot of the SDS denatured supernatant was loaded in duplicate on a reducing polyacrylamide gel. A western blot was done after transferring the samples onto nitrocellulose filter paper at 16V for 4hours.

V) Blocking the bovine anti-GP-80 antibody (primary antibody) with Y1090 lysate :

A culture of Y1090 was grown overnight at 37°C in LB broth containing 0.2% maltose and ampicillin(50µg/ml). The culture was centrifuged at 4,000xg for 10 minutes at room temperature. To the centrifuged cells 2ml of TE buffer was added, mixed well, and frozen. The cells were then thawed at room temperature and sonicated for 2 minutes. The cells were centrifuged at 6,000xg at 4°C for 5 minutes. A BCA assay was done on the lysate to determine the protein content in the supernatant. To block the primary antibody, 3mls of lysate was added to 150ml of a 1:500 dilution of the primary antibody.

VI) Screening of the human lactating mammary tissue lambda gt-11 library:

E.coli Y1090 was grown to log phase in the growth media. Two µl of the λgt-11 library was serially diluted to $8x10^5$ pfu/ml. Two µl of λgt-11 ovalbumin library was also serially diluted to 10^5 pfu/ml and used as a positive control in the screening. Twentry µl of this dilution was added to 200 µl of Y1090 and incubated at 37°C for 15 minutes. Three ml of LB-soft agar was added to the infected cells and overlayed on dry LB-agar plates containing ampicillin (50µg/ml). The plates were incubated at 42°C for 3.5 hours. Circular nitrocellulose filter paper discs were soaked in 10mM IPTG solution for 15 minutes and dried at room temperature for an hour. These fiters were layed on the plates and keyed to the plates with India Ink by a 16 gauage needle. The plates were incubated at 37°C for 3.5 hours.

The nitrocellulose discs were peeled off the plates and washed overnight with TBST containing 2% BSA and then washed with TBST. The filters were incubated with rabbit anti bovine GP-80 antibody at a dilution of 1:1000 for an hour at room temperature. The filters were washed thrice with TBST and incubated with goat anti-rabbit IgG horseradish peroxidase conjugate at a dilution of 1:1000 for 30 minutes at room temperature. The filters were washed with TBST for 3 minutes each. The filters were then incubated in the enzyme substrate (15ml TBS containing 3ml 4-chloronaphthol and 7µl of 30% hydrogen peroxide) for 15-30 minutes.

VII) Determination of cross reactivity of bovine anti GP-80 antibody:

To 200µl of an overnight culture of Y1090, 20µl of a 10^{-4} dilution of bovine mammary tissue λgt-11 was added and incubated for 15 minutes at 37°C. As a control, a 10^{-4} dilution of ovalbumin λgt-11 clone was incubated with 200µl of Y1090 for 15 minutes at 37°C. These infected cells were mixed with 5ml of soft agar at 55°C and overlayed on dry room temperature agar plates and incubated at 42°C for 3 hours. They were overlayed with nitrocellulose filter paper discs pre-soaked in 10mM isopropylthiogalactoside (IPTG) and incubated for a further 3 hours at 37°C.

The screening was done as follows:-

Each filter was treated with either (a)1° antibody/ 2° antibody/ substrate or (b) 1° antibody/ substrate or (c) 2° antibody/ substrate or (d) substrate alone. Subsequent steps were as described in the screening of the human λgt-11library.

VIII) Screening of bovine lambda gt-11 cDNA library :

Y1090 was grown to log phase in 250ml of LB broth (24) containing 50μg/ml ampicillin and 0.2% maltose. Twenty μl of a 10^{-4} dilution ($4.8x10^5$pfu/ml) of bovine λgt-11 cDNA library was incubated with 200μl of log phase culture of Y1090 at 37°C for 15 minutes. A 10^{-4} ($2x10^5$pfu/ml) dilution of ovalbumin λgt-11 was incubated similarly with Y1090 and used as a positive control. Five ml of 55°C LB soft agar was added to the tubes, mixed by inverting and overlayed onto LB agar plates containing 50μg/ml ampicillin. The plates were incubated inverted at 42°C for 3 hours. The plates were overlayed with nitrocellulose filter paper discs presoaked in 10mM IPTG and dried. The filters were keyed to the plates with India Ink and incubated at 37°C for 3.5 hours.

The screening of the filters was done at room temperature on a rotating platform. The filters were washed twice in TBST. The filters were incubated in TBST containing 2% BSA overnight. The filters were washed in TBST and incubated in 1:500 dilution of affinity purified rabbit anti GP-80 antibody for an hour. The filters were washed thrice in TBST, 3 minutes each. The filters were incubated in a 1:1,000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate or goat antirabbit IgG alkaline phosphatase conjugate. The filters were washed thrice in TBST, 3 minutes each with a final rinse in TBS.

The substrates added were;

For horseradish peroxidase:- 2ml 4-chloronaphthol (3mg/ml) containing 10ml TBS and 5μl of 30% H_2O_2.

For alkaline phosphatase:- 0.8ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate p-toludine salt) containing 160µl of 2M $MgCl_2$, 72ml barbital buffer pH 9.6, and 8ml 0.1% NBT (nitro blue tetrazolium).

IX) BCA protein assay :

One tenth of an ml of each standard (BSA at 1mg/ml) and unknown protein was added to 0.2ml of working reagent (50), and mixed well. For a blank, 0.1ml of distilled water was added to 0.2ml of working standard solution (50). The tubes were incubated in a 37°C water bath for 30 minutes and then cooled under running water. The absorbance of each tube was read at 562nm vs a water blank. A standard curve was plotted of the absorbance at 526nm vs the standard protein concentration. The concentration of the unknown was extrapolated from the absorbance reading of the unknown.

RESULTS PART I:

I) Affinitiy purification of rabbit anti-bovine GP-80 antibody:

The antibodies specific for bovine GP-80 were separated by binding the antibody to a bovine GP-80 column provided by Dr. Greenwalt and eluting the antibody with NaSCN. Twenty-five ml of antisera was loaded onto the column. Twenty-five, 5ml fractions were collected on washing the column with TBST. A BCA assay was conducted on the first six fractions in order to determine if all of the unwanted proteins such as albumin, fibrinogen, thrombin and all the non-specific antibody were removed. There was an increase in the amount of unrelated proteins eluted in the first four fractions, after which a sharp decline in the concentration was observed. This indicated that the unrelated proteins had been eluted successfully. Thirteen, 3ml fractions were collected on elution with 3M NaSCN. These fractions were dialyzed against TBS and then a BCA assay was conducted on all the fractions to determine the protein concentration as shown in table 1, graph 1. Fractions 3,4 and 5 were pooled, as these fractions had the highest protein concentration indicating the elution of the antibody. These fractions were run on a reducing SDS polyacrylamide gel to determing the presence of contaminating albumin. The band corresponding to immunoglobulin was observed, whereas there were no bands corresponding to albumin, indicating the purity of the eluate (data not shown). The pooled rabbit anti-bovine GP-80 antibody fractions were titrated by western blot to determine the concentration of antibody to be used in screening the bovine lactating mammary gland λgt-11 library. Five duplicate lanes of bovine GP-80 were run with the molecular weight marker on a 7.5% reducing SDS polyacrylamide gel. The protein was blotted onto nitrocellulose filter paper and stained with Ponceau S. The nitrocellulose filter paper was cut into 5 strips, each strip consisting of a molecular weight marker and a bovine GP-80 lane. The strips were then treated with a series of washes, serial dilutions of primary

antibody, enzyme-conjugated secondary antibody and enzyme substrate with one of the strips being a control to which rabbit pre-immune sera was added instead of the primary antibody. The antibody was used at a five hundred fold dilution for screening bovine λgt-11 library, since a thousand fold dilution gave only a faint band on the western blot (data not shown).

II) Specificity/cross-reactivity of rabbit anti-bovine GP-80 antibody used in screening the human mammary gland λgt11 library :

One µg each of pure denatured human GP-80 and bovine GP-80 was electrophoresed in duplicate on a 7.5% reducing SDS polyacrylamide gel. The proteins were transfered onto nitrocellulose filter paper for blotting. After a series of washes, incubations in rabbit anti-bovine GP-80 antibody, enzyme-conjugated secondary antibody and the enzyme substrate, bovine GP-80 was visualized as a dark band with some nonspecific binding to molecular weight markers. There was no cross reaction with human GP-80 (data not shown). The positive results with the bovine GP-80 indicate a specificity of the antibody for that protein. Since there was very slight crossreactivity with human GP-80, the antibody was not used for screening human λgt-11 library.

iii) Log phase determination of E.coli Y1090:

E.coli Y1090 were grown to log phase in 250ml LB medium containing 0.2% maltose and 50µg/ml ampicillin from a single colony picked off a LB agar plate. As the density of the culture increases after log phase, the rate of division decreases, due to depletion of nutrients in the media, until it reaches a concentration where it can no longer divide but is viable, i.e. they have entered the stationary phase.

The rate of growth of bacterial cultures is monitored by reading the optical density at a wavelength of 600nm. As a rough estimate of the number of cells it is assumed that 1 O.D

$=8x10^8$ cells/ml (24). The $O.D_{600}$ of Y1090 was noted over a period of 3.25 hours as shown in table 2, graph 2. A semilog graph was plotted to determine the time interval required for the log phase growth of cells. The cells were then grown for the required time interval, i.e. two hours, in a 37°C water bath and assumed to be in log phase for the screening of the λgt-11 library.

IV) Screening bovine lactating mammary gland λgt-11 library:

A bovine λgt-11 library ($4.8x10^9$ pfu/ml) was screened for expression of GP-80 with affinity purified anti-bovine-GP-80 antibody. On developing the filters, using 4-chloronaphthol and H_2O_2 as the enzyme substrate many blue plaques were observed. Since GP-80 makes up iess than 0.1% of the total protein synthesized by lactating bovine mammary secretory cells (18), it would be highly unlikely that the plaques were true positives. On rescreening the plates the positive plaques were again observed. A possible explanation would be that the antibody was reacting with an E.coli protein since the cells are lysed by the phage. A crude lysate of E.coli was made as described in materials and methods and the antibody blocked with the E.coli proteins. On further screening, the background plaques still appeared. The library was then screened using goat anti-rabbit alkaline phosphatase conujgate as the secondary antibody and nitroblue tetrazoleum and BCIP as the substrate, since this substrate develops faster than 4-chloronaphthol and hence would require shorter development time, which in turn would prevent the background. The background still appeared very strong. The color development was only around the plaques mimicking a positive plaque. Thus after screening 10^4 pfu, the screening of λgt-11 bovine cDNA library was abandoned.

V) Determining the non-specific background obtained on screening bovine λgt-11 library with rabbit anti GP-80 antibody:

In order to determine the cause of the background obtained in screening the bovine λgt-11 library, the specificity of rabbit anti-bovine GP-80 antibody was determined against E.coli proteins. Y1090 were induced to lyse by infecting them with a positive λgt-11 ovalbumin clone. Aprotinin and phenyl methyl sulfonyl fluoride (PMSF) were added to inhibit proteases since lysis was taking place at 37°C. Concentrated E.coli proteins and the centricon filtrate fraction were loaded in triplicate onto a reducing polyacrylamide gel after reducing the sample by boiling in an equal volume of sample loading buffer for one minute. One part of the gel was stained with coomassie blue to determine the proteins fractionated in the E.coli lysate. The other two identical lanes were transferred onto nitrocellulose filter paper and stained with Ponceau S to visualize these same bands before blotting. The nitrocellulose filter paper was cut into two parts at the molecular weight marker lane. One piece was probed with rabbit anti-bovine GP-80 antibody and the other with anti-ovalbumin antibody. As seen in figure 2, anti-GP-80 antibody did not react with any of the E.coli lysate proteins. Slight reactivity with carbonic anhydrase in the molecular weight marker lane was observed. The anti-ovalbumin antibody reacted with most of the markers in the molecular weight marker besides the 45Kda ovalbumin protein present in the molecular weight marker lane but with none of the proteins in the E.coli lysate lane. Since the anti-ovalbumin antibody was obtained from Clontech (Palo Alto, Ca), the specificity of the antibody for ovalbumin was not known. The ovalbumin band expected at 90kd was not seen in the lysate. This may be due to several reasons: (1) insufficient production of the protein, (2) degradation of the protein due to insufficient addition of protease inhibitors, (3) failure to induce the derepression of the lacZ gene by IPTG.

VI) Blocking rabbit anti-bovine GP-80 antibody with E.coli proteins:

Since the rabbit anti-bovine GP-80 antibody was produced to SDS denatured protein, there is a possibility that the antibody might react with some E.coli protein epitopes in a non-specific manner. This could be the reason for the high nonspecific background obtained on screening the bovine mammary gland λgt-11 library. A lysate of Y1090 was obtained by lysing the cells on freezing, thawing and sonication. The supernatant obtained after centrifugation of the cell debris was subjected to a BCA assay in order to determine the total protein concentration in the lysate as shown in table 3, graph 3. For the standard curve, BSA at a concentration of 1mg/ml was used. The protein concentration from the graph was determined to be 8.8mg/ml by extrapolating the average O.D 562 reading of the lysate to the mg of the standard protein. Three ml of E.coli Y1090 was added to 150ml of a 1:500 dilution of rabbit anti-bovine GP-80 antibody, which was used for screening bovine λgt-11 library.

VII) Determining crossreactivity of rabbit anti bovine GP-80 antibody:

Nonspecific crossreactivity with plaques was observed on screening the bovine λgt-11 cDNA library with affinity purified anti-bovine-GP-80 antibody. In order to determine the cause of this crossreactivity, four plates containing plaques formed by bovine mammary tissue λgt-11, the ovalbumin λgt-11 clone (positive control) and unlysed Y1090 were overlayed with nitrocellulose filter paper discs saturated with IPTG as described in materials and methods. The filter paper discs of bovine λgt-11 and Y1090 were treated with either (1) 1° antibody/ 2° antibody/ substrate, (2) 1° antibody/ substrate, (3) 2° antibody/ substrate or (4) substrate alone. This was done so as to determine the cause of the non-specific binding. To the positive ovalbumin control, anti-ovalbumin antibody was added as the primary antibody, whereas to Y1090 and bovine λgt-11, rabbit anti-bovine GP-80 antibody, which had been either blocked or unblocked with E.coli lysate, was added. As shown in figure 3, there was no

cross reactivity observed to λgt-11 bovine mammary tissue cDNA or Y1090 with either the substrate or the secondary antibody. Cross-reactivity was observed on incubating the filters with the 1°antibody/2°antibody/substrate. This indicates that neither the substrate nor the precipitation of the enzyme product is responsible for the cross-reactivity obtained on screening the bovine λgt-11 c-DNA library.

Table 1: BCA assay of protein eluted from the affinity column.

Fraction #	Abs 562		Avg Abs 562	µg/20ul	mg/ml
1	-	0.013	0.013	0.440	0.022
2	0.030	0.035	0.033	0.440	0.022
3	0.229	0.240	0.235	8.010	0.400
4	0.118	0.143	0.129	4.400	0.200
5	0.067	0.070	0.069	2.400	0.120
6	0.071	0.060	0.066	2.300	0.120
7	0.077	0.053	0.065	2.200	0.110
8	0.056	0.060	0.058	2.000	0.100
9	0.057	0.050	0.054	1.800	0.090
10	0.053	0.047	0.050	1.700	0.085
11	0.053	0.052	0.053	1.800	0.090
12	0.050	0.050	0.050	1.700	0.085
µg BSA					
2	0.043	-	0.043		
5	0.146	0.157	0.152		
10	0.334	0.300	0.317		
15	0.440	0.440	0.440		
20	0.600	0.596	0.598		
25	0.737	0.711	0.724		

Graph 1: BCA assay of protein eluted from the affinity column.

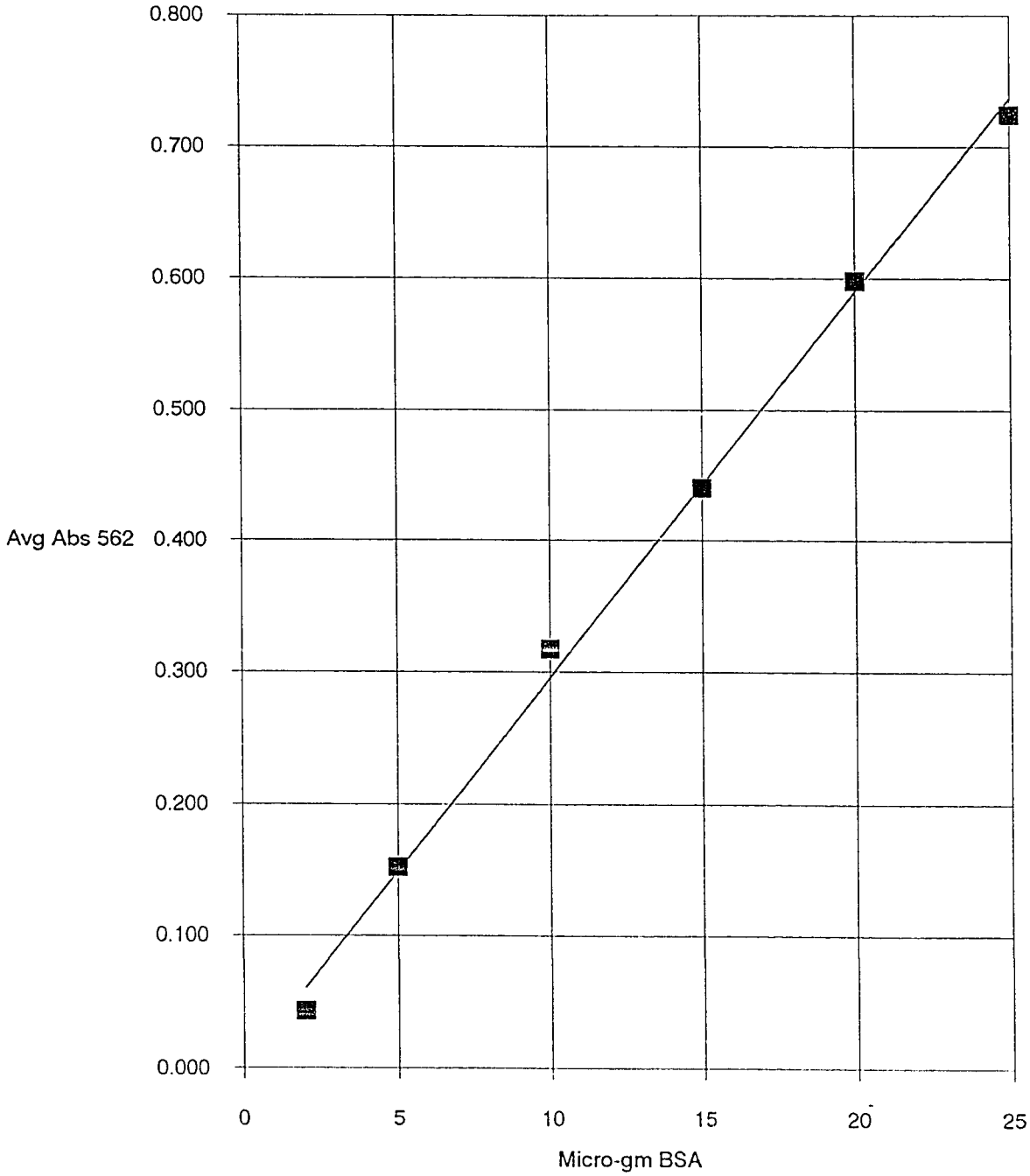
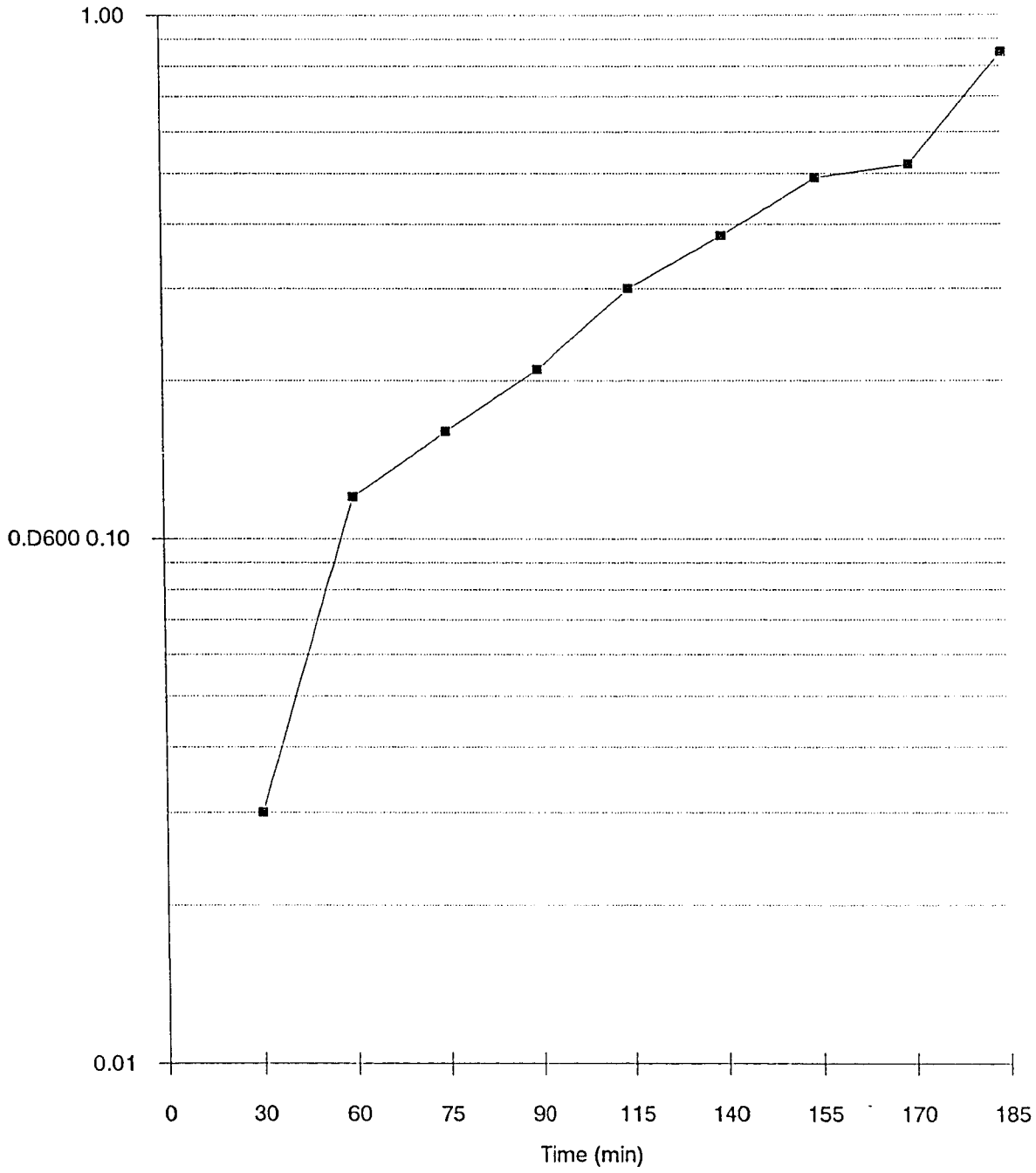


Table 2: Determination of the growth phase of E.coli Y1090

Time (min)	O.D 600	# of Cells/ml
0	0.00	0
30	0.03	2.40E+07
60	0.12	9.60E+07
75	0.16	1.28E+08
90	0.21	1.68E+08
115	0.30	2.40E+08
140	0.38	3.04E+08
155	0.49	3.92E+08
170	0.52	4.16E+08
185	0.85	6.80E+08

As seen from the graph an approximate generation time of two hours is required to obtain the cells in log phase of growth.

Graph 2: Determination of the growth phase of E. coli Y1090



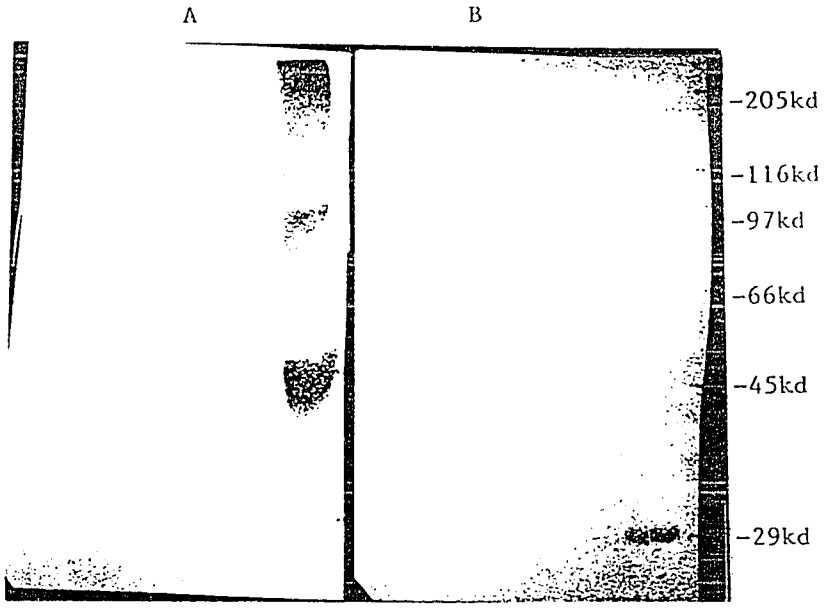


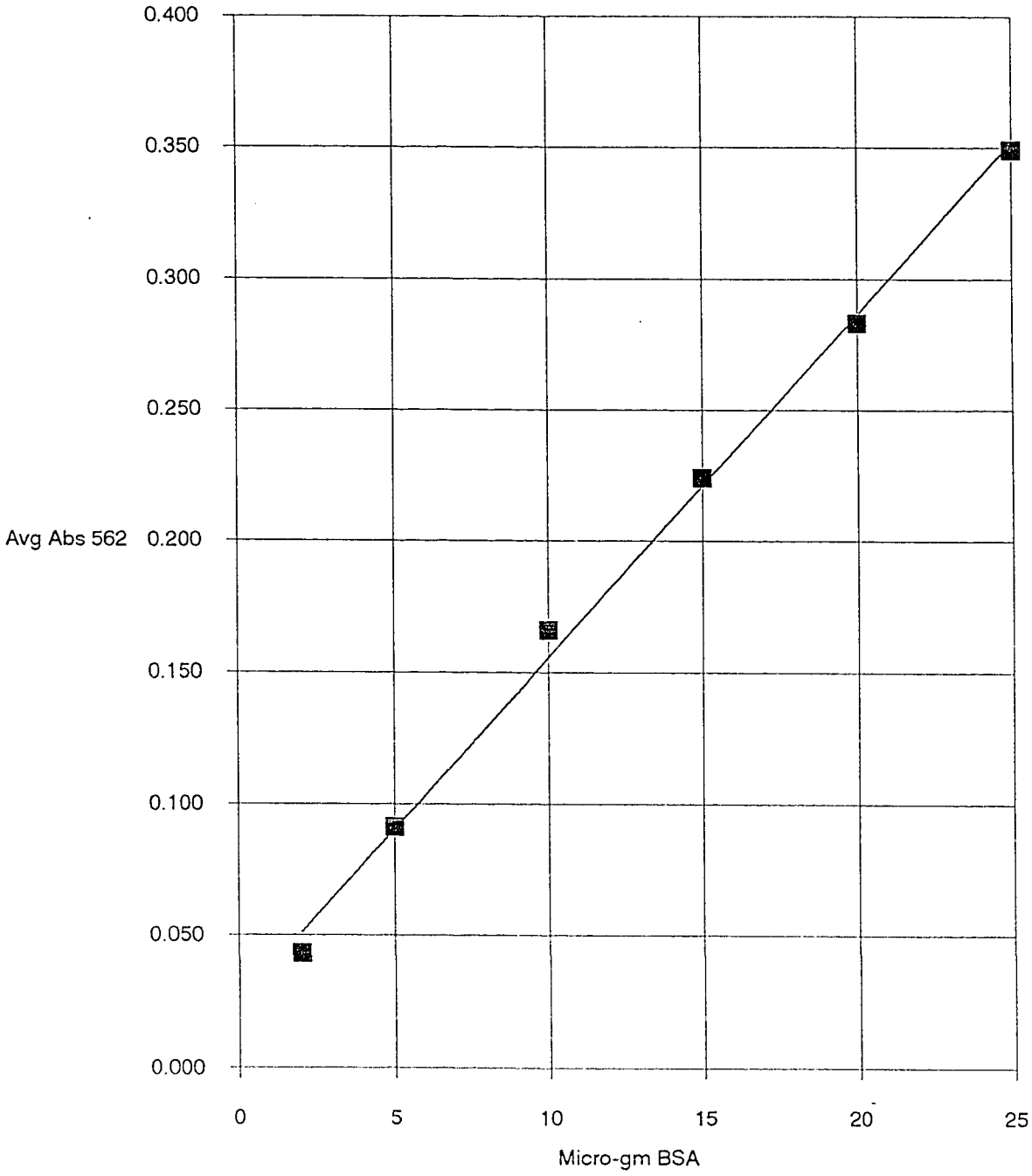
Figure 2: Cross-reactivity of rabbit anti-Gp-80 antibody to E. coli Y1090 proteins.

E. coli Y1090 lysate was transferred onto nitrocellulose filter paper and cut at the molecular weight marker. The order in which the lysate was loaded was : centricon filtrate, unconcentrated lysate, concentrated lysate and molecular weight marker. 'A' represents the filter probed with anti-ovalbumin antibody. 'B' represents the filter probed with rabbit anti-GP-80 antibody.

Table 3: BCA assay of the lysate of E.coli Y1090.

µg BSA	O.D 562		Avg 0.D 562
2	0.034	0.051	0.043
5	0.093	0.088	0.091
10	0.167	0.165	0.166
15	0.223	0.225	0.224
20	0.287	0.279	0.283
25	0.341	0.356	0.349
unknown µl			
2.5	0.330	0.360	0.345
5.0	0.558	0.567	0.563

Graph 3:BCA assay of the lysate of E.coli Y1090



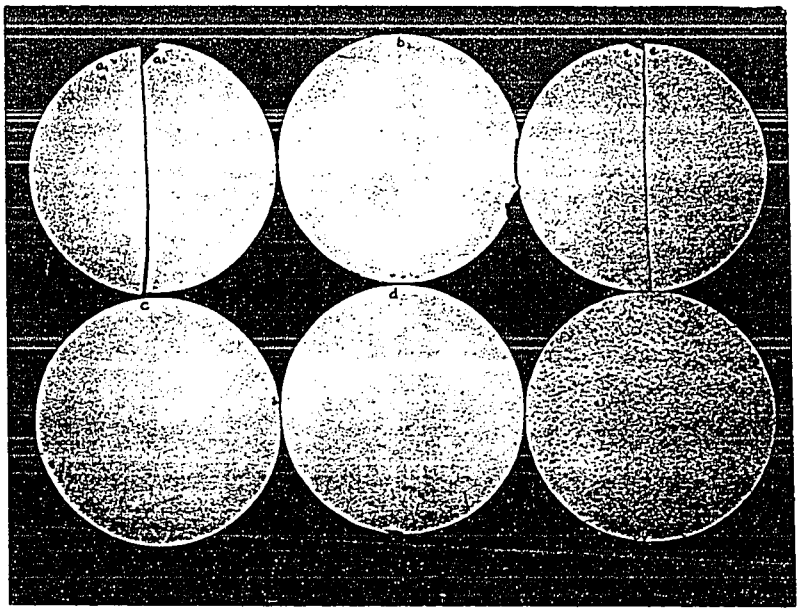


Figure 3: Cross-reactivity of rabbit anti-bovine GP-80 antibody.

Plaque lifts of bovine mammary tissue λgt-11 were hybridized to:

a_1	= unblocked 1° antibody / 2° antibody / subsatrate.
a2	= blocked 1° antibody / 2° antibody / substrate.
b	= blocked 1° antibody / substrate.
c	= 2° antibody / substrate.
d	= substrate.

Where the 1° antibody was blocked by incubating the antibody with E.coli lysate.

Filter 'e' represents the unlysed Y1090 incubated with the 1° antibody / 2° antibody substrate.

Ovalbumin λgt-11 clone was used as the positive control to which 1° antibody / 2° antibody / substrate was added (filter marked 'ov').

METHODS PART II:

(I) Synthesis of Probes:

Probes for the ovalbumin and bovine GP-80 gene sequence (table 4) were synthesized on an automated Biosearch DNA synthesizer according to the manufacturer's instructions using phoshoramidite chemistry (47). The ovalbumin probe was synthesized from the published mRNA sequence (51). The bovine GP-80 gene sequences were synthesized from the available protein sequence (unpublished data). Those fragments of the sequence containing the least degeneracy were chosen and the most frequently occurring codon were utilized in the synthesis of the probes (33,34). A single probe (probe #4) was made to the N-terminus of the protein in order to be able to detect the 5' end of the gene. A 0.25mM CPG column with the corresponding 3' base attached to it was primed twice for 1/2 hour each before initiating synthesis. After each nucleotide addition, the dimethyl trityl (DMT) group was removed automatically. These fractions were collected for the first probe synthesis, i.e. the ovalbumin probe, in order to determine the efficiency of the synthesis. Two and one half ml fractions of DMT were manually collected every cycle for the first 5 bases and then for every 5th base from then on. The DMT fractions were diluted to 25ml with 0.1M p-toluene sulfonic acid in acetonitrile (9.51gm p-toluene sulfonic acid to 500ml with acetonitrile), and its absorbance at 498nm was determined in a Beckman Du-2 spectrophotometer (slit width= 0.325). The 5' trityl group was detached from the synthesized probe on the column in order to obtain the free 5'-OH group required for radioactively end labelling the probe.

Elution of the probe from the X-CPG column: The metal ring and cap were removed from the column, and the glass beads squirted out with 1ml of fresh, full strength ammonium hydroxide into granulated glass vials. The vials were heated overnight at 55°C. in a Pierce Reactitherm heating block. The solution was then spun at 3,000 rpm for three minutes in an eppendorff centrifuge and the supernatant was evaporated to dryness under N_2. The dried

Table 4: Sequence of the oligonucleotides synthesized on the Biosearch DNA synthesizer.

Probe # 1107 degeneracy=2^4=16

Protein sequence:	Met	Phe	Gln	Thr
Probe sequence :	TAC	AAG	GTC	TGG
		A	T	T
				A

Probe # 1097 degeneracy=2^6=64

Protein sequence:	Gln	Val	Asn	Met	Tyr	Lys	Gly
Probe sequence :	GTC	GAC	TTG	TAC	ATG	TTC	CCG
	T	G	A		A	T	C

Probe # 1089 degeneracy= 2^7=128

Protein sequence:	Gln	Phe	Trp	Ile	Phe	Asp	Val
Probe sequence :	GTC	AAG	ACC	TAG	AAT	CTG	CTC
	T	A		A	A	A	G
						T	

Probe # 4 degeneracy=2^{11}=2048

Protein sequence:	Ile	Ala	Gly	Ala	Val	Ile	Gly	
Probe sequence :	TAG	CGA	CCG	CCG	CGA	CAC	TAG	CC
	A	G	A	A	G	G	A	
		T	T	T	T			

ovalbumin probe

probe sequence :	CAG	TGT	TGA	AGG	AAC	TGT	AT

residue was resuspended in 200µl of sterile distilled water. Probe #1089 for GP-80 was resynthesized as the yield was very poor. The ovalbumin probe and probe #1089 were ethanol precipitated (24) to rid the sample of smaller fragments of DNA and the oligonucleotides were evaporated to dryness under N_2. These oligonucleotides were suspended in 200µl of 2.5M ammonium acetate(pH7.0), 600µl of ice cold ethanol was added and the oligonucleotides were left to precipitate at -70°C for 15 minutes. The samples were spun for 10minutes at 4°C. The supernatant was discarded and the pellet rinsed with 1ml of 95% ethanol. The pellet was then air dried and resuspended in 200µl of sterile distilled water (dH_2O). Absorbance at 260nm was determined in a Beckman Model 25 spectrophotometer (ε=40cm²/mg) on diluting 5µl of oligonucleotide solution to 1ml with sterile dH_2O. The oligonucleotides were stored at -4°C.

(II) Extraction of total RNA from bovine heart and mammary tissue:

All glassware and equipment where possible was washed in a 0.1% solution of diethyl pyrocarbonate (DEPC) treated H_2O and autoclaved. All solutions were prepared in DEPC treated H_2O and autoclaved where possible.

The total RNA was extracted from the bovine heart and mammary tissue according to the method of Chirgwin et al. (48). Half a gram of liquid N_2 frozen tissue was pulverized in a mortar and pestle in the presence of liquid N_2. The ground tissue was added to 10ml of solution D (250gm guanidinium thiocynate, 293ml H_2O, 17.6ml 0.75M sodium citrate pH7.0, 26.4ml 10% sarcosyl at 65°C; to 50ml of this solution was added 0.36ml of β-mercaptoethanol) and homogenized at very low speed for approximately 2 minutes. One half ml of 2M sodium acetate (pH4.0) was added to the homogenized tissue and shaken well by inversion. Five ml of water saturated phenol and 1ml of chloroform:isoamylalcohol (49:1) was added and shaken vigorously for 10 seconds. It was cooled on ice for 15 minutes and centrifrged at 10,000xg at 4°C for 20 minutes. The aqueous phase was transferred to another

tube with a broad tipped pasteur pipette. Five ml of isopropanol was added to the aqueous phase and kept at -20°C for at least an hour to precipitate total RNA. Total RNA was recovered by centrifuging at 10K x g at 4°C for 20 minutes. The RNA pellet was dissolved in 5ml of solution D and reprecipitated with 1.5ml of isopropanol as above. The total RNA was washed once with 75% ethanol and resuspended by heating to 65°C for 10 minutes in 50µl of DEPC treated H_2O. The total yield was determined by reading the absorbance at 260nm of 1µl of total RNA diluted to 1ml against DEPC treated water blank, using an extinction coefficient of 40 cm^2/mg.

Denaturing gel electrophoresis of total RNA:

All solutions were made in DEPC treated H_2O. A 1.2% agarose/formaldehyde gel was prepared by heating 4.2gms agarose in 304.5ml H_2O. 35ml of 10X MOPS running buffer (41.8gms MOPS pH7.0, 16.6ml 3M sodium acetate, 20ml 0.5M EDTA pH8.0, 800ml H_2O) and 10.5ml 37% formaldehyde were added to the cooled agarose. The RNA was denatured before loading onto the gel by adding 15.75µl sample premix (5µl 10xMOPS running buffer, 8.75µl 37% formaldehyde, 2µl formamide) to 10µg RNA solution, and heated to 55°C for 15 minutes. Two µl of formaldehyde loading buffer (1mM EDTA pH8.0, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was added and the mixture was loaded onto the gel. The gel was run without any molecular weight markers. The gel was run at 100V for approximately 3 hours until the bromophenol blue had run halfway through the gel.

(III) **Isolation of DNA from λgt-11 recombinant and nonrecombinant ovalbumin clones**:

Isolated plaques of ovalbumin λgt-11 recombinant and nonrecombinant clones were picked from a plated lysate and suspended in 0.4ml of sterile lambda diluent (5.8gms NaCl, 2gms $MgSO_4$, 50ml 1M TrisCl, 5ml 2% gelatin/liter water) for 2 hours. One tenth ml of eluted phage was added to 0.1ml of an overnight culture of E.coli Y1090 and 0.1ml of 10mM

$MgCl_2$/10mM $CaCl_2$, and incubated for 15 minutes at 37°C. The infected E.coli were added to 50ml of NZCYM media (10gm NZ amine, 5g NaCl, 5g yeast extract, 1g casamino acids, 2g $MgSO_4.7H_2O$ to a liter with distilled water and autoclaved) and shaken vigorously at 37°C. On lysis of the cells (approximately 6 hours), 1ml of $CHCl_3$ was added to each flask and shaken vigorously at 37°C for 30 minutes. The cells were harvested by centrifuging at 18K xg at 4°C for 10 minutes.

One hundred ml of the liquid lysate was added to 100µl of DNase (1mg/ml) and 150µl of RNase (1mg/ml), and incubated at 37°C for 1 hour. Solid NaCl was added to a final concentration of 1M and the lysate was kept on ice for an hour. The cell debris was centrifuged at 11,000x g at 4°C for 10 minutes. Solid polyethylene glycol 6000 was added to the supernatant to a final concentration of 10% w/v and dissolved by very slow stirring at room temperature. The phage suspension was cooled on ice for an hour and centrifuged at 11,000x g at 4°C for 10 minutes in order to pellet the phage. The phage pellet was resuspended in 1.6ml of lambda diluent. One half molar EDTA pH8.0 was added to the supernatant to a final concentration of 20mM, proteinase K (50µg/µl) was added to a final concentration of 50µg/ml, 20% SDS was added to a final concentration of 0.5%, and the solution was incubated at 65°C for an hour. The lysed phage were extracted subsequently with equal volumes of phenol, phenol:$CHCl_3$, and $CHCl_3$ until the interface between the organic and aqueous phases was clear. The DNA was precipitated by addition of two volumes of ice cold ethanol and placed at - 70°C for 20 minutes. The phage DNA was pelleted by centrifugation in a microfuge and resuspended in 100µl of TE (10mM Tris-Cl pH8.0, 1mM EDTA) buffer. One µl of this DNA was dissolved in 1ml of TE, and the concentration determined by reading the absorbance at 260nm, using an extinction coefficient of 20cm^2/mg.

(IV) **Extraction of bovine heart and mammary tissue DNA:**

One and half grams of frozen bovine heart tissue and 2.4gms of frozen bovine lactating mammary tissue were ground in liquid N_2 and added to 20ml of sterile digestion buffer (100mM NaCl, 10mM Tris HCl pH8.0, 25mM EDTA pH8.0), and shaken to submerge the tissue. One ml of 20% SDS and 1ml of proteinase K (10mg/ml) were added to the tissue and incubated overnight at 37°C in a shaking water bath. An equal volume of phenol (saturated with TE) was added to the digest and the mixture was shaken by occasional inversion for 3 hours at room temperature. The organic phase was separated by centrifugation at 3,000 rpm for 10 minutes and discarded. The aqueous phase was extracted twice with phenol and once with phenol/chloroform/isoamyl alcohol (50:49:1), 30 minutes each. The clear aqueous layer was centrifuged at 9,000 rpm for 20 minutes at 25°C. The aqueous layer was dialyzed against TE at 4°C for 24 hours. The volume of the aqueous phase was measured and the DNA was spooled out by addition of sodium acetate pH6.5 to a final concentration of 0.3M, and 0.8 volumes of 2-propanol. The precipitated DNA was rinsed in ice cold ethanol, air dried, dissolved in 3ml of sterile dH_2O, and stored at 4°C. The amount of DNA was determined by absorbance measurements at 260nm, using an extinction coefficient of 20 cm^2/mg.

(V) **Southern Blot:**

A Southern blot was performed on HindIII digests of λgt-11 ovalbumin recombinant and non-recombinant DNA and on partially digested bovine mammary tissue DNA.

The oligonucleotides (table 4) used for probing the blots were radioactively labelled with γ^{32}P-ATP and purified on Qiagene tip 5 cartridges (Diagen), according to the manufacturer's instructions. Six pmole of oligonucleotide containing 5µl of phosphorylating buffer and 3 units of polynucleotide kinase were added to 32pmole of γ^{32}P-ATP and the volume made up to 50µl.

The reaction was incubated at 37°C for 30 minutes, and stopped with 400µl of reagent A (400mM NaCl, 50mM MOPS, 15% ethanol, at pH 7.0), and placed on ice until the Qiagene cartridge was primed for extraction of the oligonucleotide.

Ovalbumin λgt-11 recombinant and non-recombinant DNA were digested with HinDIII (24), and electrophoresed on a 0.8% agarose gel in Tris-borate EDTA (TBE) buffer (24). The gel was stained with ethidium bromide and transferred onto gene screen plus hybridization transfer membrane according to the manufacturer's protocol. The DNA in the gel was depurinated by soaking for 10 minutes in 0.25M HCl with agitation at room temperature. The gel was rinsed in water and DNA denatured with 1M NaCl/0.5M NaOH for 15 minutes with agitation at room temperature. The gel was neutralized by soaking in 0.5M Tris pH7.4/1.5M NaCl for 15 minutes with agitation. The gel was washed twice with TBE buffer and electro-transferred to the gene screen plus membrane at 5mAmp/cm^2 for one and half hours.

Hybridization:

The membrane was prehybridized in hybridization solution (5xSSC {sodium chloride/sodium citrate} [24], 50mM sodium phosphate pH7.0, 10xDenhardts solution [24], 1% SDS), at 45°C for 30 minutes in a shaking water bath. Ten pmole of radiolabeled probe was added to 5ml of hybridization solution and hybridized onto the membrane overnight at 45°C. The membrane was washed with wash solution (5xSSC, 1%SDS) at room temperature for 5 minutes and rinsed repeatedly with wash solution at room temperature. The filter was wrapped in saran wrap and exposed to Kodak X-OMat AR X-ray film for 24 hours and developed.

Bovine mammary tissue DNA was digested with HinDIII, EcoRI, and BamHI and run on a 0.8% agarose gel. It was transferred onto the gene screen plus membrane and probed as stated above with the exception that 3M TMACl (tetra methyl ammonium chloride) was used instead of 5xSSC for the hybridization and wash solutions.

RESULTS PART II:

I) Efficiency of synthesis of oligonucleotide probes:

The collected dimethyl trityl fractions after dilution with 0.1M p-toluene sulfonic acid in acetonitrile and the absorbance read at 498nm (table 5, graph 4). The efficiency of synthesis of the oligonucleotide during each step was determined to be 46.5%, by dividing the absorbance obtained for the last base by the absorbance obtained for the second base. The overall efficiency was calculated by the formula; $e^{[\ln Y/(n-1)]}$ (Y= efficiency during each step and n= # of nucleotides) and was determined to be 96.06%. These figures indicate that the synthesis was not very efficient. The inefficiency of the synthesis could be due to the use of a larger pore size column instead of a smaller pore size column as suggested by the manufacturer (personal communication). On synthesizing the ovalbumin probe and probe #1089 on the smaller pore size column significantly higher yields were obtained, as indicated by the absorbance readings of the oligonucleotides (the DMT fractions were not collected during this synthesis).

II) Quantitation of the synthesized probes:

The absorbances at 260nm of the oligonucleotides were determined after removal from the CPG column (table 6). The oligonucleotides were synthesized in two individual batches indicated as 1 and 2 in the table. The low yield of the oligonucleotides is thought to be due to the use of a larger pore size column than was required for the synthesis as explained above. Probe #1089-3 and the ovalbumin probe were synthesized with the right pore size column and hence their yield was significantly higher (326 times higher).

III) Quantitation and visualization of total RNA:

The total RNA extracted from 0.5gms of heart tissue was resuspended in 50µls of 0.5% DEPC treated SDS, whereas that from 0.5gms of mammary tissue was resuspended in 150µls of 0.5% DEPC treated SDS as much more RNA was extracted from mammary tissue than from heart tissue. Table 7 shows the absorbance readings at 240, 260, and 280nms of 1µl of RNA made up to 1ml with sterile dH_2O. The ratio of O.D 260/280 for the heart tissue and the lactating mammary tissue were 1.5 and 1.89, respectively, indicating the purity of the preparation (24).

On electrophoresing the total RNA on denaturing formaldehyde gel a smear was observed with 2 distinct bands at 28S and 18S rRNA indicating that the RNA preparation was not degraded (data not shown).

IV) Isolation and quantation of DNA from recombinant and non-recombinant λgt-11 ovalbumin clone:

DNA was extracted by lysing E.coli Y1090 infected by λgt-11 clone, at 37°C and dissolved in TE. The concentration of the DNA obtained was measured by diluting the DNA in TE and reading the absorbance at 260 and 280nm (Table 8). Recombinant λgt-11 ovalbumin was obtained at a total amount of 3.6µgs, whereas the non-recombinant ovalbumin clone was obtained at a total amount of 0.396µgs. The ratios of the O.D 260/280 were 2.1 and 2.86, respectively, indicating the presence of RNA in the preparation (24). This could be due to insufficient RNase in the digest. Since a clear band of DNA could be observed on a 0.8% agarose gel (data not shown), the preparations were not purified further to remove the contaminating RNA.

V) Extraction and quantitation of DNA from bovine heart and mammary tissue :

DNA was extracted from bovine heart and lactating mammary tissue by proteinase K digestion followed by phenol and phenol/chlorform extractions. The DNA solution was dialyzed against TE to equilibrate the solution and remove any residual organic solvents which may have interfered with the enzyme digestion. The DNA was then spooled out of the dialyzed solution and dissolved in sterile H_2O. The concentration of the DNA was dermined from the absorbance readings at 260nm to be 1.7mg and 1mg for mammary and heart tissue, respectively. The purity of the extraction was determined from the ratio of $O.D_{260}/O.D_{280}$ to be 1.8 for the mammary and heart tissue, respectively, as shown in table 9 indicating that the preparation was free of contaminating protein (24).

VI) Southern blot:

Six pmole of ovalbumin probe and the probes for bovine GP-80 were radiolabelled with γ^{32}P-ATP and the unincorporated γ^{32}P-ATP was removed by passing the reaction mix through a Qiagene column. Table 10 shows the elution profile of the radiolabelled probe.

The λgt-11 ovalbumin recombinant and nonrecombinant clone DNA were digested with HinDIII and electroblotted onto the gene screen plus membrane. The membrane was probed with a radioactively labelled ovalbumin probe (2,013cpm) and exposed to X-ray film for 24 hours. On developing the film a very faint band was observed at 7.3kb in the recombinant DNA lane but re-exposure of the blot for a further 2 days did not result in an increase in intensity of the band. The faint band could be attributed to background (data not shown).

The bovine lactating mammary tissue was partially digested with three frequently cutting enzymes HinDIII, BamHI, and EcoRI, transferred onto nylon membrane and probed with radioactively labelled probes #1089, 1097 and 4 at 21,636, 17,280 and 7,362 cpm,

respectively. The membrane was exposed to X-ray film for 2 days and developed. No bands were observed with either of the probes.

Table 5:Efficiency of synthesis of oligonucleotide probes.

Sample #	Base #	Abs 498	μmoles DMT
1	1	1.250	0.446
2	2	0.805	0.288
3	3	0.750	0.268
4	4	0.755	0.270
5	5	0.656	0.234
6	9	0.590	0.211
7	14	0.525	0.188
8	19	0.460	0.164
9	20	0.375	0.134

μmoles DMT = (Abs 498) (volume)/ε volume = 25ml, ε = 70ml/μmol

Efficiency during each step = last base 0.D498 / second base 0.D 498

= 46.6%

Overall efficiency = $e^{[\ln y/(n-1)]}$ Where n = # of nucleotides

and y = Efficiency during each step.

Overall efficiency = 96.06%

Graph 4: Efficiency of synthesis of oligonucleotide probes

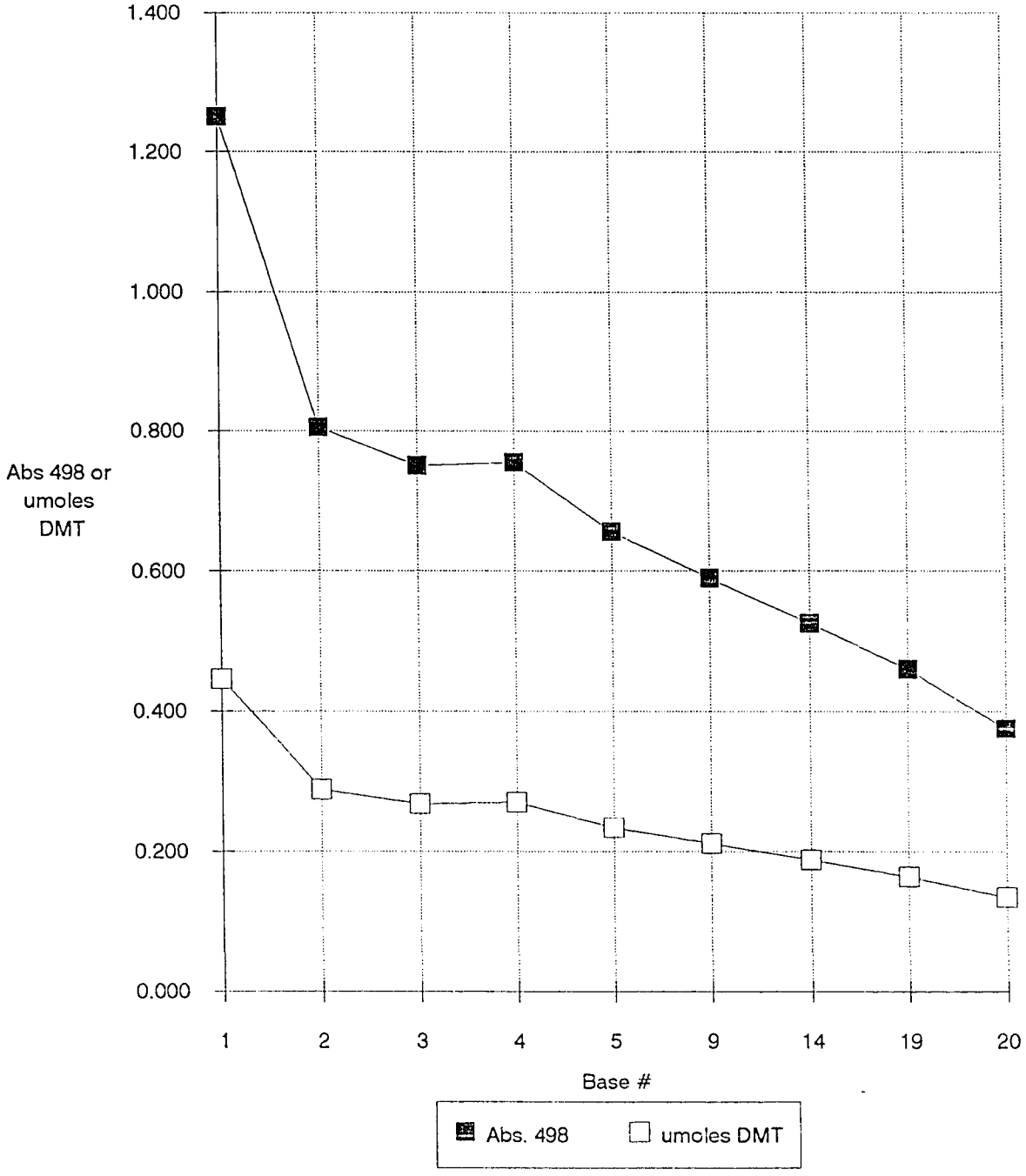


Table 6:Quantitation of synthetic Oligonucleotide Probes.

Sample #	Abs 260	ng/µl
1089-1	0.002	8.0
1089-2	0.002	8.0
1107-1	0.004	16.0
4-1	0.013	52.0
4-2	0.005	20.0
1097-1	0.010	40.0
1097-2	0.016	64.0
1089-3	0.652	2608.0
ovalbumin	0.645	2580.0

Table 7: Quantitation of total RNA from bovine heart and mammary tissue.

Heart tissue RNA		Mammary tissue RNA	
nm	Absorbance	nm	Absorbance
260	0.180	260.0	0.388
240	2.470	240.0	2.461
280	0.115	280.0	0.205

For heart tissue:

Abs 260/280 = 1.5. Total amount = 72 μg.

For mammary tissue:

Abs 260/280 = 1.89. Total amount = 155 μg.

Table 8: Quantitation of DNA from non-recombinant and recombinant λgt-11.

Recombinant λgt-11		Non-recombinant λgt-11	
nm	Absorbance	nm	Absorbance
260	0.733	260.0	0.396
280	0.350	280.0	0.138

For recombinant λgt-11:

Abs 260/280 = 2.1. Total amount = 3.67mg.

For non-recombinant λgt-11:

Abs 260/280 = 2.86. Total amount = 396 μg.

Table 9: Quantitation of DNA from bovine heart and mammary tissue.

Bovine nm	heart tissue Absorbance	Bovine nm	mammary tissue Absorbance
260	0.060	260.0	0.100
280	0.032	280.0	0.055

For bovine heart tissue.

Abs 260/280 = 1.78. Total amount = 1 mg.

For bovine mammary tissue.

Abs 260/280 = 1.8. Total amount = 1.7 mg.

Table 10: CPM of radiolabelled probes.

Sample	Fraction #	cpm
blank	-	25
control	-	13,649
ovalbumin	1	1,640
	2	373
	3	173
	4	94
	5	255
#1089	1	14,148
	2	7,488
	3	734
	4	348
	5	242
	6	295
#1097	1	16,242
	2	1,038
	3	295
	4	205
	5	84
	6	198
#4	1	7,362
	2	295
	3	96
	4	66
	5	71

DISCUSSION:

The study of eukaryotic gene structure and expression relies on the availability of cloned genes as probes. The successful strategy toward isolating a particular eukaryotic gene has been first to isolate a DNA copy of the messenger RNA encoded by that gene, i.e. a cDNA clone. A cDNA library representative of the mRNA population is constructed using polyadenylated RNA extracted from the appropriate tissue or cell type. The cDNA clone of interest is then identified within the cDNA clone population by screening the library with either (1) synthetic oligonucleotide probes, or (2) an antibody probe, or (3) differential cDNA probes. Double stranded cDNAs prepared from reverse transcription of mRNA in vitro are ligated into the lambda vector and screened with any one of the above probes. The use of a lambda vector instead of a plasmid vector makes it possible to take advantage of the high efficiency and reproducibility of in vitro packaging of lambda DNA as a method of introducing DNA sequences into E.coli. The high efficiency of cloning cDNAs into lambda vectors is useful when cDNA clones of rare mRNAs are sought or when mRNA for preparing cDNAs is limited in quantity.

The site used for insertion of foreign DNA into lambda gt-11, the expression vector used to screen for the intrinsic membrane protein GP-80, is a unique EcoRI cleavage site located within the lacZ gene, 53 base pairs upstream from the β-galactosidase translation termination codon. λgt-11 can accommodate up to 7.2kb of insert DNA, assuming a maximum packageable phage DNA of 105% wild type length. Because the site of insertion of foreign DNA in λgt-11 is within the structural gene for β-galactosidase, foreign DNA sequences in this vector have the potential to be expressed as fusion proteins within β-galactosidase since they are under the transcriptional control of the β-galactosidase gene. Depending on the

availability of the type of probe, recombinant genomic DNA or cDNA libraries constructed in λgt-11 can be screened with antibody probes for antigen produced by specific recombinant clones. Alternatively, these libraries can be screened with nucleic acid probes.

Polyclonal antibody against bovine GP-80 was produced in a rabbit host. The serum was enriched for antibody against GP-80 by purification on a GP-80 affinity column. A western blot of GP-80 was done to confirm the specificity and affinity of the antibody. The antibody to bovine GP-80 was found to be specific to the bovine protein only and not with the corresponding human protein. On screening a bovine λgt-11 expression library with the antibody against GP-80, background plaques were observed. This could be due to the cross-reactivity of the antibody to the E.coli proteins. The antibody was blocked by E.coli proteins and used for screening the library but the background was still apparent. The reason behind this is not apparent since a western blot of E.coli proteins probed with the antibody did not show any cross-reactivity.

GP-80 is an integral membrane protein with at least one membrane spanning region as postulated from the amino acid sequence of the N-terminus of the protein (35). The antibody was produced against SDS denatured protein which may have no or reduced affinity to the native protein produced in the E.coli. On the other hand, since GP-80 is a highly hydrophobic protein, it may fold differently in the prokaryotic environment and its epitope(s) may not be available for the antibody or it may be highly unstable in the prokaryotic environment. The protein is highly glycosylated (18) but when produced in E.coli the protein will not be glycosylated, hence antibody against these glycosylated sites will not be available for screening. The insertion of the foreign genome in lambda gt-11 should be in the right reading frame or else the antibody will fail to recognize the protein produced.

Degenerate oligonucleotides were made to the amino acid sequence available for bovine GP-80 selecting the most frequent third base (33,34) whenever possible to screen the bovine λgt-11 library. The probes were electrophoresed on a denaturing polyacrylamide gel to determine the quality of the synthesis. As seen from the gel, most of the probe was full length. This degenerate probe mix was used to probe a Southern blot of the enzyme digest of DNA prepared from lactating bovine mammary tissue and bovine heart muscle, the tissues in which GP-80 is abundant. A non-degenerate ovalbumin probe was used as a control to probe a Southern blot of DNA extracted from an ovalbumin clone. Negative results were obtained in probing the bovine Southern blot. This could be due to the high degeneracy and low concentration of the probe. A very faint band was observed in the ovalbumin blot but the experiment was not repeated due to the unavailability of radiolabeled ATP and lack of space for working with radioactive material.

Future prospects:

Of the 18 N-terminal amino acids identified in bovine GP-80, 17 were identical to the human GP-80 isolated from milk (35). Human GP-80 is similar to endothelial cell and platelet membrane glycoprotein CD36 (35). CD36 has been identified as a receptor for erythrocytes infected with the malarial parasite Plasmodium falciparum (36,37,38). Bovine GP-80, unlike the human protein, does not bind infected erythrocytes, suggesting that there exist specific structural features in the human protein which are responsible for recognition of the erythrocyte ligand. Human CD36 is also a receptor for thrombospondin, a large adhesive protein which participates in the secretion-dependent phase of platelet aggregation (39). The relationships between the ability of GP-80 to bind both thrombospondin and infected RBCs (red blood cells) and the ability of thrombospondin to mediate infected RBC binding remains unclear. With few exceptions (e.g., glomerular capillary endothelial cells), GP-80 has been localized to the microvasculature of most human tissues examined, whereas microvasculature of major bovine organs such as the lung and brain lack GP-80. The microvasculature of the brain is a preferential site of infected RBC sequestration in human malaria (40,41,42) and occlusion of brain capillaries with infected erythrocytes is thought to contribute significantly to the pathology of cerebral malaria (43). Demonstration of infected RBC binding and nonbinding forms of GP-80 is important in that studies of the structural differences between the two proteins may reveal the molecular basis of infected RBC binding. The differences between the two proteins could be determined by screening a cDNA library and consequently obtaining a full length clone of the gene for the protein. Sequencing the gene would determine the difference between the two proteins. Alternatively, the epitope(s) responsible for the differences in the function of the two proteins could be deduced by site directed mutagenesis of the gene or by preparation of chimeric proteins.

Bovine GP-80 immunologically cross-reacts with human GP-80 (46). A bovine mammary tissue cDNA library could be screened with the cDNA clone available for human CD36 (44).

On the other hand, PCR (polymerase chain reaction) technology could be used to screen the library (45), this being a much quicker alternative than screening with a cDNA clone. Primers could be made to the N and C-terminus of the gene for amplification, as it's possible to resolve a kb band by PCR. The predicted size of the cDNA would be approximately 1.5kb (44). It is also possible to amplify the specific mRNA species by PCR, although this would be more cumbersome since it is difficult to isolate undegraded mRNA. Once the sequence of the bovine protein is known it would be possible to mutate the gene and/or synthesize chimeric proteins in order to determine the domain(s) responsible for nonbinding of the infected RBCs. Determination of factors responsible for non-binding of the protein to infected RBC would be important in developing therapeutic measures for combating infection by the most dangerous of the malarial parasites Plasmodium falciparum.

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