THE PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO ASPARTATE TRANSCARBAMOYLASE FROM ESCHERICHIA COLI

by

Andrew L. deJong

Department of Biochemistry & Biophysics

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Approved by:

James R. Wild

Dr. James R. Wild

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Abstract

This research project focused on the production of monoclonal antibodies to the holoenzyme aspartate transcarbamoylase (ATCase) and to its component catalytic and regulatory subunits. The hybridoma cell lines were prepared using immunized splenocytes taken from BALB/c mice and a murine non-immunoglobulin secretor myeloma cell line Sp2/0-Ag/4. In addition to monoclonal antibody production this project also initiated the determination of the specific antigenic determinants for each hybridoma cell line. Elucidation of the epitopes utilized ATCase (*pyrBI*)-αβ-Galactosidase (*lacZ*) gene fusion products. By 3' sequential deletion of portions of *pyrBI*, various sized resulting polypeptides, each containing a constant α-complementing *lacZ* domain, were produced. The fusion sites in the *pyrBI* bicistron were located using agarose gel electrophoresis and dideoxy nucleotide sequencing. Resulting fusion protein-products were purified using an anti-β-Galactosidase immunoaffinity minicolumn. Once sequenced and purified, the fusion proteins can be tested with each monoclonal antibody sera to determine antigenic specificity.

Acknowledgements

I would like to thank Dr. J. R. Wild for his guidance and for giving me the opportunity to participate in his laboratory. I would also like to thank all members of Dr. Wild's laboratory. It has been a pleasure working with them all. I would like to extend a warm thank you to my parents for their support throughout the year. Finally, I would like to say a very special thanks to Dr. Melinda Wales. Her leadership, knowledge, and friendship have been invaluable. She has made this project both possible and enjoyable.

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Introduction

An intriguing problem currently being addressed by molecular biochemists involves determining how the structural organization of an enzyme correlates with its function as a biological catalyst. A variety of questions serve to direct this research: What are the inherent "rules" of the structure-function relationship between genes and their polypeptide products? Is it possible to identify specific guidelines which apply to all proteins? The answers to such questions could ultimately lead to the ability to design and manufacture synthetic proteins for specific catalytic functions.

One powerful technique which can be used to address these questions involves utilizing specific antibodies as structural probes for the catalytic proteins. Conventional polyclonal antibodies provide complex, varied structural probes which are impractical for intricate structural studies as a consequence of the "polyclonality" (multiple site recognition property) of the immune response (13). Literally hundreds of different immunoglobulins can be produced in response to a challenge by a complex antigen. This results in the recognition of many quite varied antigenic determinants with varying affinity. Furthermore, since polyclonal antiserum are raised in individual immune competent animal systems, they are available in limited quantities and can never be reproduced once the original supply has been exhausted. In order to overcome these limitations, it is necessary to provide specific immunoglobulin serum directed toward a single antigenic determinant, or epitope from a

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complex antigen like an enzyme. Furthermore, these monospecific sera must be available in large amounts.

In 1975, G. Kōhler and C. Milstein (8) developed a series of techniques that made it possible to generate unlimited amounts of homogeneous (monoclonal) antibody to a specific antigen (8). Animal tissue culture systems were defined in which hardy mouse myeloma tumor cells can be fused with fragile antibody producing B cells from the spleens of immunized mice. Thus it became possible to obtain an "immortal" cell line that continued to secrete specific immunoglobulins characteristic of the immunized lymphocytes (each B cell produces a single antibody). Each of these fused cells, called hybridomas, is capable of providing an unlimited source of individual monoclonal antibodies.

The experimental importance of monoclonal antibodies is inherent in their very distinct specificity; often defined by four or five amino acids (1). The application of these specific probes involved the development of immunochemical techniques directed toward selective proteins. A monoclonal antibody antagonistically reacts with a single epitope on an antigen or hapten. Because of their intense specificity, these immunoglobulins are an excellent tool for the elucidation of subtle enzymatic structural and conformational properties of a given protein in various active states.

This research involves the production and characterization of monoclonal antibodies to ATCase (aspartate transcarbamoylase) of Escherichia coli, and the application of these immunoglobulins as discrete structural probes toward that enzyme in various conformation states and in altered genetic constructions.

ATCase is an excellent model for the study of complex oligomeric proteins since it is composed of two types of protein subunits. In the Enterobacteriacae, ATCase is encoded by a bicistronic operon with a single control region (Figure 1). The pyrB cistron specifies a catalytic subunit (310 amino acids) which is functionally active as a trimer (7), and pyrI cistron produces a regulatory subunit (153 amino acids) which is functional as a dimer (15). These functional subunits spontaneously aggregate to form a dodecameric holoenzyme (310,000 d) consisting of two catalytic trimers, $2(c_3)$, and three regulatory dimers, $3(r_2)$ (2). The holoenzyme catalyzes the first step unique to the de novo pyrimidine biosynthetic pathway (Figure 2), but its structural and regulatory properties show extensive diversity between species (15). ATCase catalyzes the condensation of carbamoyl phosphate and aspartate to produce carbamoyl-L-aspartate while releasing orthophosphate (Figure 3). The enzyme is typically subject to allosteric regulation involving feedback inhibition by the biosynthetic endproduct of pyrimidine biosynthesis, CTP(2). In contrast, ATP is a heterotropic activator of ATCase and competes with CTP for specific binding sites on the regulatory subunit (2) thus potentially serving to balance purine and pyrimidine biosyntheses. Monoclonal antibody probes to ATCase permit investigation of antigenic relationships between various native and hybrid enzymes, as well as conformational changes dictated by ligand binding.

This research project can be divided into two phases. The first stage involved the development of immunologically productive hybridomas against catalytic and regulatory subunits of ATCase, as well as to the holoenzyme, using the basic methods of Galfre et al (3). The second

Figure 1. A schematic representation of the pyrBI operon. The pyrB gene encodes a catalytic polypeptide which trimerizes, and the pyrI gene produces a regulatory polypeptide which dimerizes. The holoenzyme consists of two catalytic trimers and three regulatory dimers.

Fig. 1

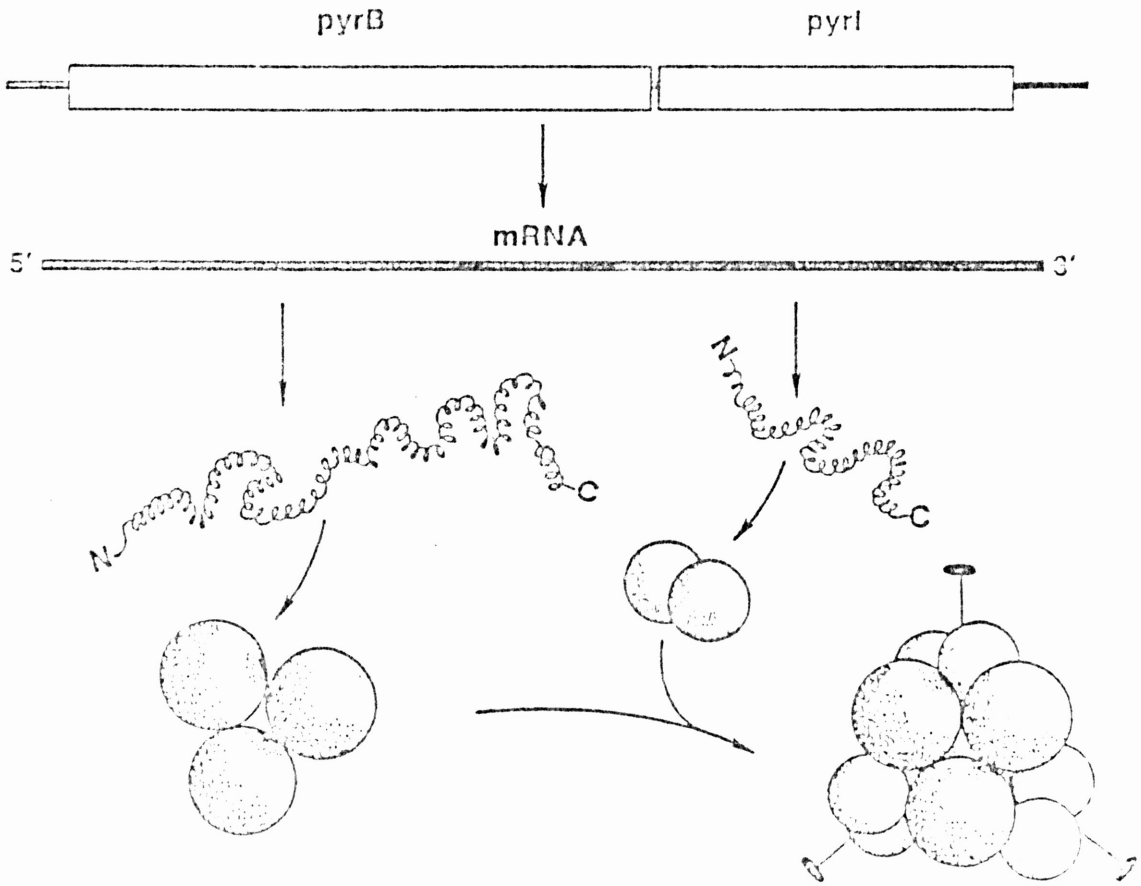


Figure 2. The pyrimidine biosynthetic pathway. ATCase, designated by its genetic construct pyrBI, catalyzes the condensation of carbamoyl phosphate (CP) and aspartate (ASP) to form carbamoyl aspartic acid (CAA). The ultimate endproducts of pyrimidine biosynthesis are uridine triphosphate (UTP) and cytidine triphosphate (CTP).

Fig. 2

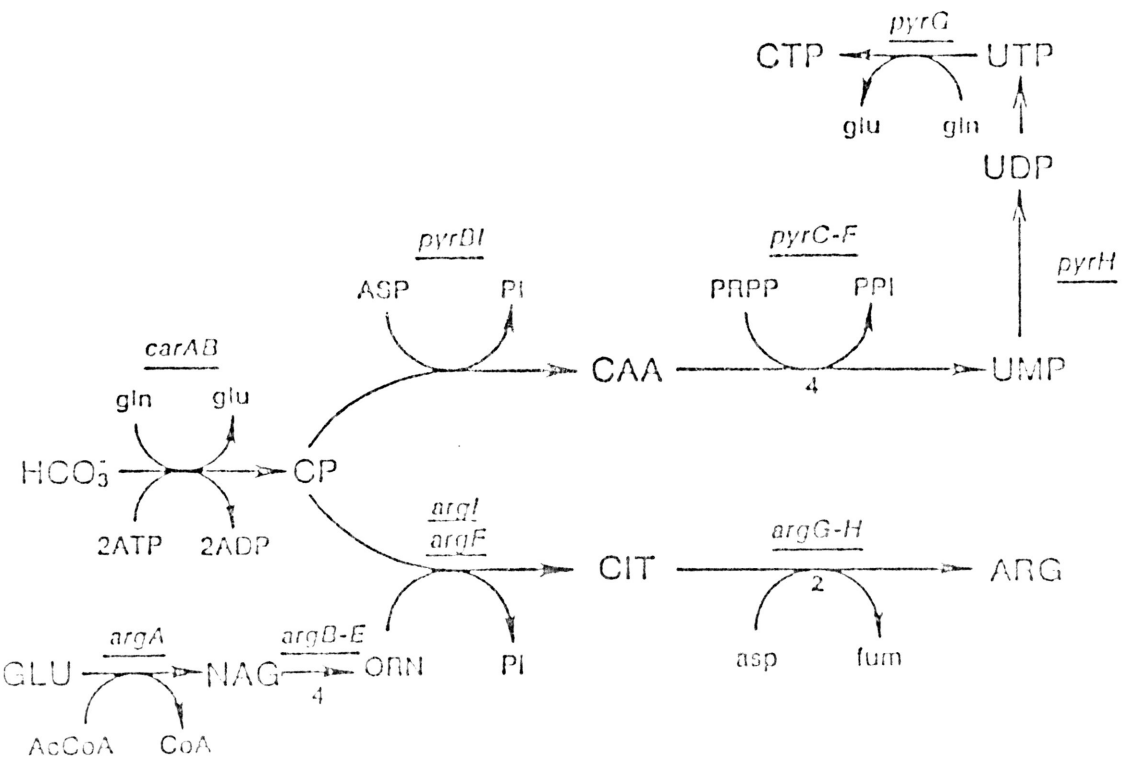
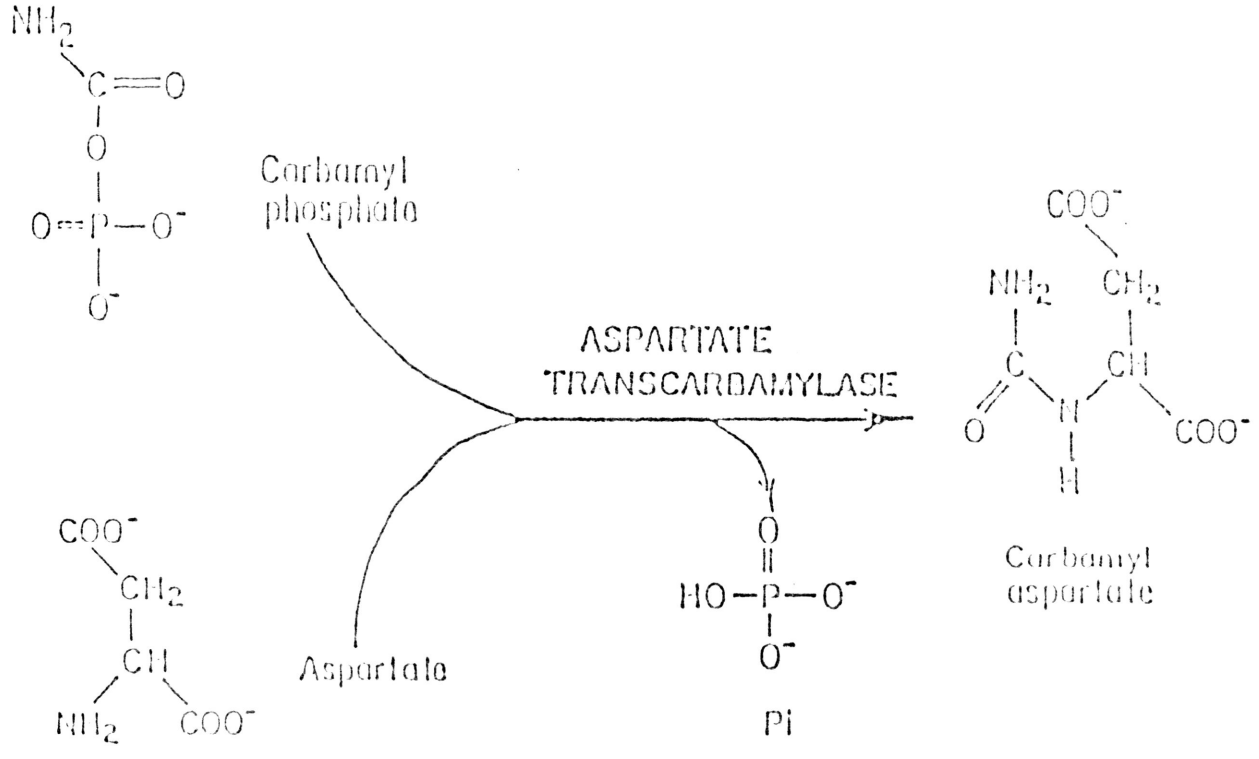

pyrG
CTP
UTP
glu
gln
UDP
pyrH
pyrBI
pyrC-F
ASP
PI
PRPP
PPI
carAB
gln
glu
CAA
UMP
4
HCO3
CP
2ATP
2ADP
argI
argF
argG-H
CIT
ARG
2
asp
fum
argA
argB-E
GLU
NAG
ORN
PI
4
AcCoA
CoA

Fig. 3

The Carbamoylation of Aspartate



phase involved the characterization of selected hybridoma cell lines using molecular gene-fusion techniques, immuno-chromatography, and radio-active immuno-assays. The determination of immunoglobulin specificity will involve the isolation of ATCase (pyrBI)-β-Galactosidase (lacZ) gene fusion protein products in collaboration with Timothy Hoover (Texas A&M Department of Biochemistry & Biophysics).

Cloned gene fragments in M13mp11 cloning vectors were subjected to 3'-exonuclease digestion and were used to select pyrBI fusions to a constant lacZ sequence. In frame gene-fusions contained a constant α-complementing lacZ domain with variable deletions of ATCase polypeptide chains. The resulting fusion proteins were purified with an anti-β-Galactosidase immuno-minicolumn utilizing the constant β-Galactosidase (β-gal) domain. In the future, the individual fusion proteins will be used to determine the antigenic specificity by radioactive immunoassays. Once produced and characterized, the immunoglobulins will be available for the investigation of structural properties and conformational change between the ATCases of different organisms, as well as providing a tool for evaluation of the properties of chimeric ATCase.

Materials and Methods

A. Production of monoclonal antibodies to ATCase.

The first phase of this research focused on the production of monoclonal antibodies to the catalytic subunits (c_3), regulatory subunits (r_2), and the holoenzyme ATCase from Escherichia coli. An overview of the production process followed is presented in Figure 4.

Monoclonal Antiboby Production Procedure

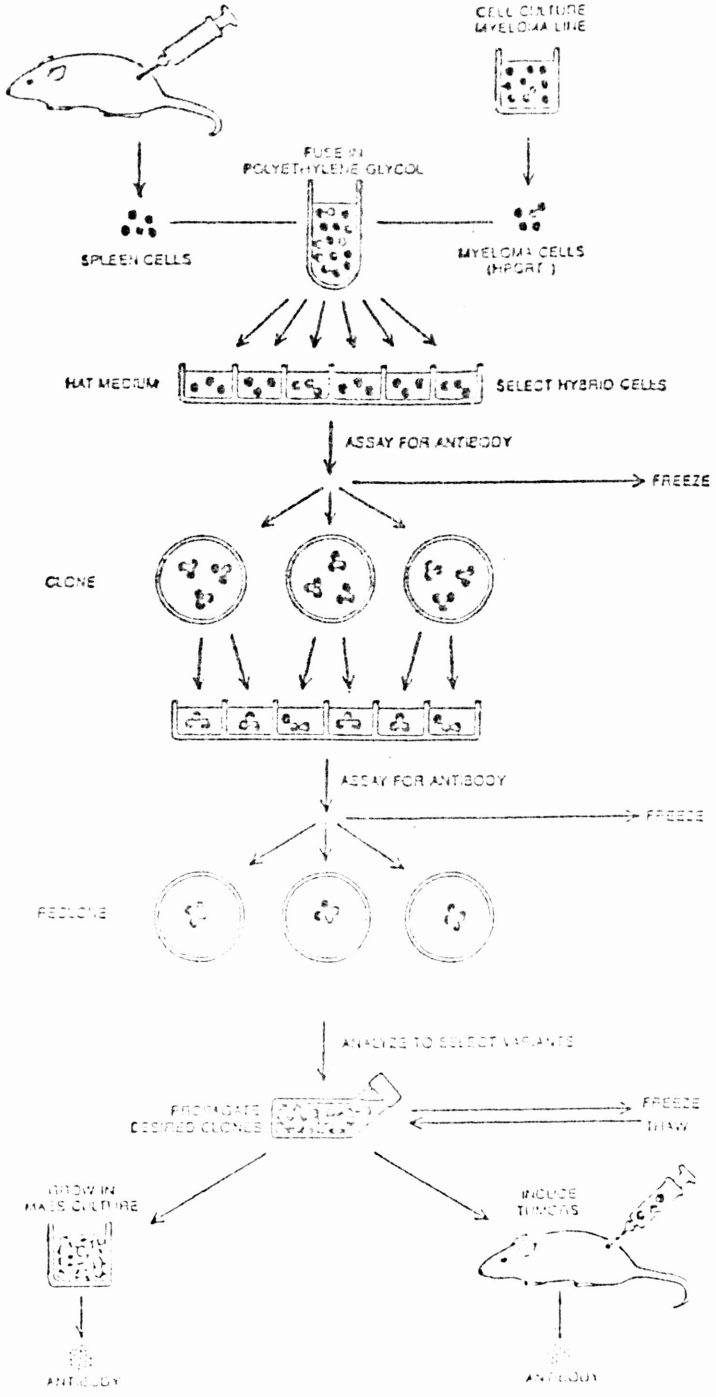


Fig. 4

Media. Tissue culture maintenance medium consisted of Dulbecco's Modified Eagle's medium (DMEM) with high glucose (4.5 g/liter) (K.C. Biological Inc., Lenexa, KS) supplemented with sodium bicarbonate (3.7 g/liter), 15% fetal bovine serum (FBS) (Sterile Systems Inc., Logan UT), and 50 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), pH=7.3. Hybridoma fusion medium contained equal volumes of polyethylene glycol 1000 (PEG) (Sigma Chemical Co., St. Louis, MO) and DMEM. Hybridoma medium (H-media) was composed of DMEM supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 100 μM minimum essential and non-essential amino acids, 100 u penicillin/ml, and 100 μg streptomycin/ml (K.C.Biological Inc., Lenexa, KS), 50 μM 2-mercaptoethanol, 19 mM HEPES (Sigma Chemical Co., St. Louis, MO), and 20% FCS. HAT medium consisted of H-media containing hypoxanthine (10^{-4}M), aminopterin (4×10^{-7}M), and thymidine (1.6×10^{-5}M) (9). HT medium is HAT medium minus the aminopterin.

Myeloma cell line. The murine non-immunoglobulin secretor myeloma cell line Sp2/0-Ag14 (14) was kindly provided by Dr. Gale Wagner (Texas A&M University). The cell line was cultured routinely in H-media at 37°C in an atmosphere of 7% CO_2. Prior to fusion with spleen B-cells, Sp 2/0-Ag14 was subcultured in H-media plus 15 μg 8-azaguanine/ml (Sigma Chemical Co., St. Louis, MO).

Immunized Splenocytes. The catalytic subunit, regulatory subunit, and holo ATCase were purified by the method of Gerhart and Holoubek (4). Three sets of 4 Sendai virus free 6-week old female BALB/c mice were injected intraperitoneally according to the following schedule:

Table 1

Immunization Schedule

Mouse Set	Immunization Date	Antigen	Innoculum conc.(μg)
1	11/05/84	c_3	100
	12/19/84	c_3	50
	2/25/85	c_3	50
2	11/05/84	holo	100
	12/19/84	holo	50
	4/23/85	holo	50
3	11/05/85	r_2	50*
	12/19/84	r_2	50

* Supplemented with Freund's incomplete adjuvant.

Three days after final antigenic boosts of the catalytic and holoenzyme sets, 2 mice from each set were killed by cervical dislocation, drenched in 70% ethanol, and the spleens were removed aseptically. The spleens were then gently pressed through a sterile, 100 mesh stainless steel screen into serum-free DMEM to make a single cell suspension. This suspension was washed twice in serum-free DMEM followed by mild aseptic centrifugation (100×g for 10 minutes at 25°C). After the second wash the splenocytes were resuspended in DMEM and adjusted to 10^{-8}cells/ml.

Feeder cells. Peritoneal exudate cells (PEC) were collected by peritoneal lavage from BALB/c mice and washed 3 times by centrifugation (100×g for 15 minutes at 25°C) in serum-free DMEM. After the final centrifugation the supernatants were removed, and the cells were resuspended in HAT medium at a concentration of 6×10^5PEC/ml. Fifty μl of this

suspension was introduced into each well of ten 96 well microtiter plates (Nunclon, Vanguard International, Neptune, NJ). The plates were incubated at 37°C in an atmosphere of 7% CO_2 for 24 hours prior to use.

Hybridization and hybridoma selection. Several slight modifications of the fusion method of Galfre et al (3) were used in forming the hybridoma fusion. Sp2/0-Ag14 myeloma cells ($5\text{-}8\times10^5$ cells/ml and >95% viability) were used for the fusion process. The Sp2 cells were washed twice as above and 10^7 myeloma cells were mixed with 10^8 immunized splenocytes. After centrifugation (100×g for 10 minutes) to pellet the cells, the supernatant was removed and the tube was gently tapped to loosen the pellet. Over a one minute time span, 0.8 ml of fusion medium was gradually added to the cell suspension with gentle mixing (37°C). The mixture was allowed to stand for one minute, and 1.0 ml of serum-free DMEM was added with gentle mixing over a one minute interval. Finally, 20 ml of serum-free DMEM was carefully added over a period of 5 minutes. The cells were then centrifuged as above, the supernatant removed, and the cells were resuspended gently in 10 ml HAT medium. This culture was transferred to a 75 cm^2 T-flask (Lux, Brandel, Gaithersburg, MD) containing an additional 10 ml HAT medium. The centrifuge tube was rinsed with 5 ml of HAT medium and added to the flask.

Twenty-four hours after fusion, the cells were harvested by gently pipeting adherent cells from the flask surface. Thirty-five ml of HAT medium was added to this cell suspension and 50 µl aliquots were dispensed into each well of ten 96-well feeder cell conditioned plates. Seven days after fusion, 100 µl HT medium was added to each well in order to encourage vigorous cell growth. Between 12-20 days after fusion,

50-100 µl of supernatant from wells containing surviving hybridomas were tested against their respective immunogen (i.e., holoenzyme, catalytic or regulatory subunits).

Detection of anti-ATCase antibodies. Supernatants were assayed for anti-c_3, or anti-holoenzyme activity by solid phase enzyme-linked immunosorbant assay (ELISA), using a Streptavidin HyBRL Screening Kit (Bethesda Research Laboratory, Bethesda, MD). This kit provides a biotinylated goat anti-mouse antibody, a streptavidin biotinylated horseradish peroxidase complex, and ortho-phenylene diamine as a substrate providing colorimetric detection of the immunoglobulin-antigen reaction. The absorbance at 410 nm was determined using a Minireader II (DynaTech Laboratories, Alexandria, VA). Antigenic preparation for antibody detection consisted of attaching 25 ng/200 µl of antigen (catalytic subunit or holoenzyme) to the wells of 96-well polystyrene plates and incubating the plates for 24 hours at 4°C.

Supernatant aliquots of 50 µl from hybridoma culture were incubated in appropriate antigenically activated wells for 2 hours at 25°C. Untreated wells were used as negative controls and anti-c_3 and anti-holoenzyme sera were used as positive controls. The positive controls were allowed to reach an optical density of 1.00 (ELISA Minireader II) before the entire plate was evaluated.

Cloning. The hybridomas which were ELISA positive for three successive antigenic screenings were expanded into 24-well plates. The hybridomas were then cloned by an adaptation of the limiting dilution method of Oi and Herzenberg (1980) used as follows: (a) PEC were washed 3 times by centrifugation (100×g) and resuspended at $1\text{-}2\times10^5$ PEC/ml in H-medium,

(b) hybridoma cells were adjusted to 10^2 cells/ml in H-medium and 0.8 ml was added to 8 ml of PEC-medium, (c) 100 µl were added to each of 60 wells of a 96-well polystyrene plate (one hybridoma/well), and (d) the remaining PEC-hybridoma cell mixture was diluted with an equal volume of PEC suspension and 36 wells each received 100 µl (0.5 hybridoma cell/ wells). The wells were then examined microscopically for clonal growth prior to addition of 100 µl of H-medium on day 5. Antigenically positive clones showing antibody activity were expanded and allowed to grow for 3 days before each cloning.

B. Characterization of the hybridoma cell lines.

The determination of the antigenic specificity of each hybridoma cell line involved: (a) fusion protein production and analysis, (b) purification by immuno-affinity chromatography, and (c) immunoradioactive assay of antibody-fusion protein reactions.

Gene-fusion production and selection. The fusion proteins were obtained from gene-fusions created by Timothy Hoover (Texas A&M University, Department of Biochemistry & Biophysics). The logic employed to produce these gene-fusions, and consequently the fusion proteins, was as follows. A pyrBI insert from pPBh105, a derivative of pBR322 constructed in our laboratory (11) was cloned into M13mp11 using Pst 1 and Sal 1 restriction enzymes (Bethesda Research Laboratory, Bethesda, MD) (Figure 5). Replicative form (RF) recombinant M13 DNA was prepared and purified using standard plasmid isolation techniques (BRL Cloning/Dideoxy Sequencing Manual, 1980). The purified M13RF DNA was restricted with Sst and Bam H1 (BRL, Bethesda, MD). Various aliquots were 3'-unidirectionally

Figure 5. 3'-exonuclease sequentially deleted pyrBI fragments were inserted into the M13 cloning vector using SalI and PstI restriction enzymes.

Restriction Map M13

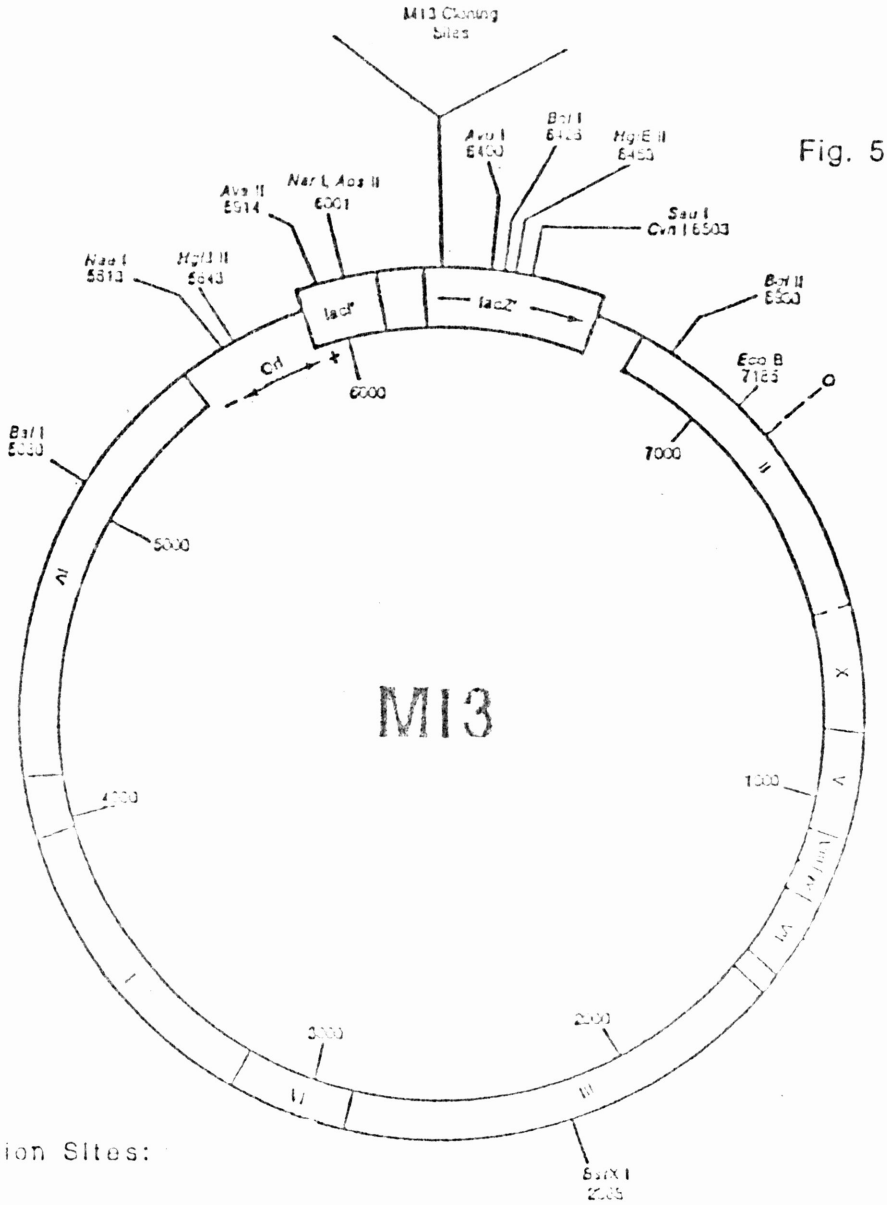


Fig. 5

Restriction Sites:

sal I

pst I

digested with exonuclease III (6). After a brief treatment with S1 nuclease (BRL, Bethesda, MD) and DNA polymerase I (Klenow enzyme) (BRL, Bethesda, MD) to produce 5' and 3' blunt-ended fragments, the M13 DNA timed aliquots were T4 ligated into the polyclonal site of M13mp11. Five µl of each sample was added to 0.3 ml of competent JM103 cells (40 kletts) and incubated on ice for 40 minutes. These mixtures were heat shocked for 2 minutes at 42°C and added directly to test tube containing 10 µl of 100 mM IPTG (isopropyl-β-D-thio-galactopyranoside), 50 µl 2% X-gal, 0.2 ml fresh, exponentially growing JM103, and 3 ml of soft agar at 45°C. The medium was mixed and plated onto Petri dishes, allowed to solidify, and then incubated at 37°C for 6-12 hours. Blue plaques indicated the presence of β-galactosidase activity and represented either inframe insertions (which meant fusion protein products) or M13mp11 phage containing no insert. These alternatives were distinguished by direct agarose gel eletrophoresis. Ninety to ninety-five percent represented gene fusions.

Size determination of the M13mp11 pyrBI inserts. The sizes of the inframe gene-fusions (blue plaques) were estimated using direct agarose gel electrophoresis. Using 9 cm long sterile sticks, individual blue plaques were transferred to tubes containing 2 ml of trypton yeast extract (TYE) plus JM103 cells and incubated at 37°C. After 7 hours the culture was transferred into 1.5 ml Eppendorff tubes and centrifuged in a microfuge for 10 minutes. Twenty-eight µl was withdrawn from each supernatant and mixed with 8 µl of Miles Stop Reagent (5% SDS, 25% glycerol, and 0.025% bromophenol blue). Twenty-five µl from each solution was loaded onto a 0.5% agarose gel in Tris-boric acid-EDTA buffer (TBE). The

gel was subjected to electrophoresis at 75 volts fr 12-16 hours, stained with ethidium bromide, and evaluated with UV-transillumination.

The exact location of each fusion was determined by the dideoxy nucleotide sequencing method (12). The protocol followed is shown in Figure 6. Briefly, the method involved first adding 0.5 µg M13mp11 template DNA (i.e., single stranded) and 5 µg "universal" primer DNA (BRL, Gaithersburg, MD) to 400 µl microfuge tube containing 12.5 µl of water plus 1 µl of polymerase reaction buffer. This reaction was incubated at 85-90°C for 5 minutes. After the annealing reaction, 1 µl (5-10 µCi) [α-^{32}P] - dATP (ICN Pharmaceuticals INc., Plainview, NY), 1 µl 0.1 M dithiothreitol (Sigma Chemical Co., St. Louis, MO), 1 µl Klenow enzyme (1 µ/ml), and 2 µl (0.5 mM) dATP were added to the microfuge tube. Three µl aliquots were dispensed to each of four dideoxy (dd) reaction tubes:

Table 2

Dideoxy Reaction Mixtures

tube A: 1 µl A° mix and 1 µl dd A
tube C: 1 µl C° mix and 1 µl dd C
tube G: 1 µl G° mix and 1 µl dd G
tube T: 1 µl T° mix and 1 µl dd T
° mixtures lacking their respective deoxynucleotide.

and incubated for 15 minutes at room temperature. The reactions were stopped with 10 µl formamide-dye mix, and denatured by heating in boiling water for 3 minutes. Two to three µl of each sample were loaded onto a 8% polyacrylamide sequencing gel and electrophoresed for 2 hours. The gel was then autoradiographed using Kodak XR-5 film.

Figure 6. A flow chart outlining the basic steps followed during DNA sequencing with dideoxynucleotide triphosphate as chain terminators.

DNA Dideoxy Sequencing Flow Chart

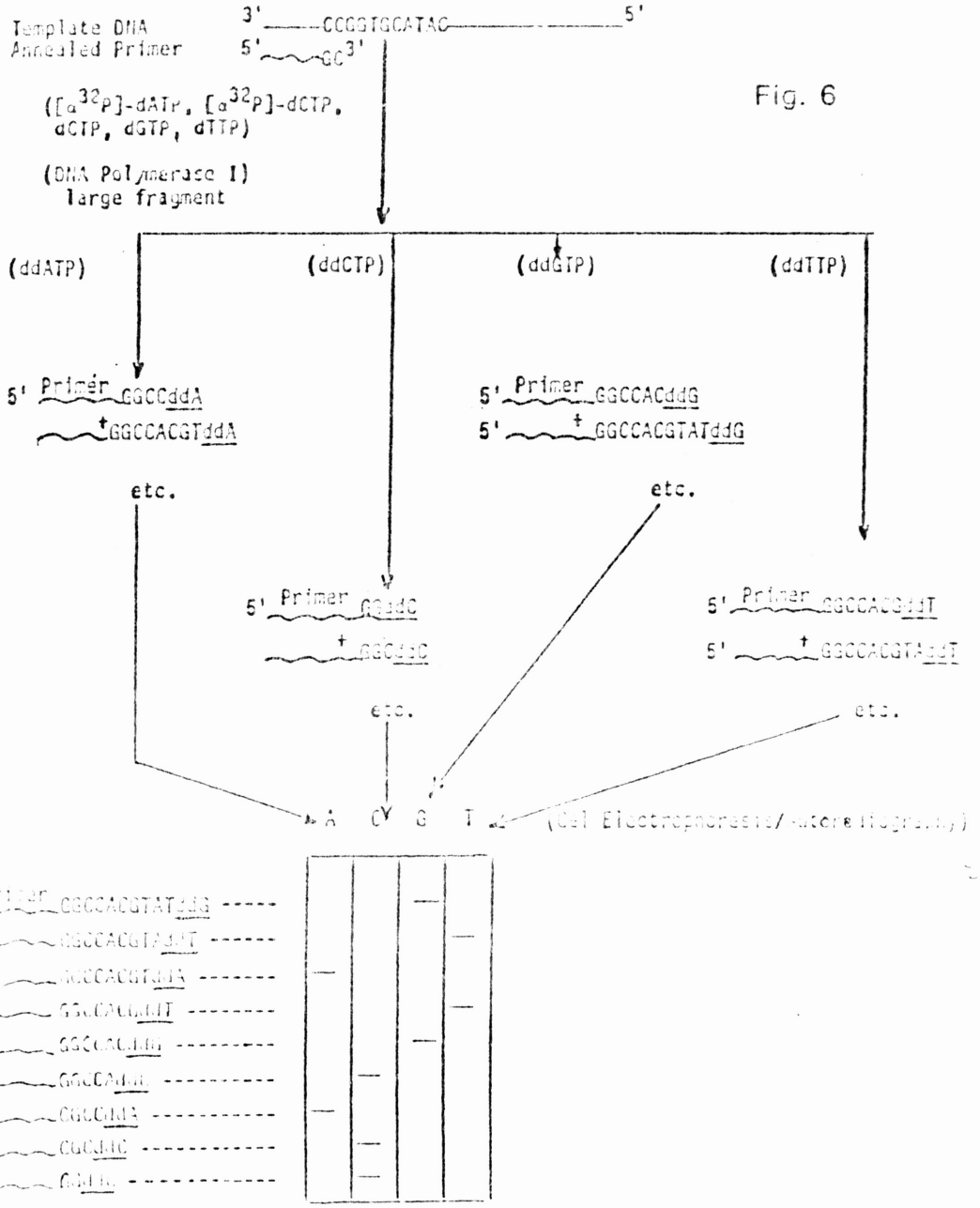


Fig. 6

Molecular Weight determination of the fusion protein products. Molecular weights of the fusion proteins were estimated using Sephadex G-200 ascending flow column chromatography. The Sephadex G-200 gel (2.6 cm × 80 cm) was equilibrated with 40 μM potassium phosphate. The calibration enzymes used were ribonuclease A (M.W. 13,700), chymotrypsinogen (M.W. 25,000), ovalbumin (M.W. 45,000), aldolase (M.W. 158,000), and ferritin (M.W. 400,000). Blue dextran 2000 was used to determine the void volume of the column. Five ml samples of cell free extract containing fusion protein were applied to the column. The column was eluted with 40 mM potassium phosphate (pH=7.0), and 5 ml fractions were collected at a flow rate of 15 to 18 ml/hr. The ATCase elution profile for each sample was determined by monitoring the enzymatic production of carbamoyl aspartate using modifications of the method of Gerhart and Pardee (5). A volume of 5-50 μl of each column fraction was assayed in a reaction containing 40 mM potassium phosphate (pH=7.0), 4 mM carbamoyl phosphate, and 5 mM aspartate. The assays were incubated at 28°C for 10-30 minutes and the reaction stopped by the addition of 1 ml of acid color mix (10). Color was developed at 60°C for 110 minutes. The tubes were cooled to room temperature and the absorbance at 466 nm was determined in a Gilford Model 300-N microsample spectrophotometer.

Purification of the fusion proteins. The fusion proteins were purified using an anti-β-gal, CNBr-activated Sepharose-4B immuno-affinity column. The affinity column was prepared by swelling 1 g of of CnBr-activated Sepharose-4B resin in ice-cold 1 mM hydrochloric acid (HCl) and then washing the resin with 200 ml of 1 mM HCl. The gel was washed with 5 ml of coupling buffer (0.1 M sodium bicarbonate, pH=8.8, containing

0.5 M sodium chloride) and immediately transferred to a 2 ml coupling buffer solution containing rabbit anti-β-gal (60 mg/ml) (Cooper Biomedical, Malvern, PA). The coupling solution was mixed gently for 2 hours at room temperature. Any remaining active groups were blocked by the addition of 5 ml of Tris-HCl (0.1 m, pH=8.0) and stirring for 1 hour. Excess protein was removed using successive washes of coupling buffer, 0.05 M Tris is 0.5 M NaCl (pH=8.0), and 0.05 M acetate in 0.5 M NaCl (pH=4.0). The gel was transferred into a Pharmacia minicolumn (23 mm × 10 mm). 2 ml portions of fusion protein extract were applied to the column 3 times and allowed to adhere to the column for 1 hour. The column was washed with 30-40 ml of 10 mM Tris, 0.1% triton x-100, and 50 µg 1 ml PMSF (TX buffer) to remove unbound protein. Non-specifically bound protein was removed by washing the column with 10 ml of 5 mM lithium 3,5-diiodosalycylic acid (LIS: Sigma Chemical Co., St. Louis, MO) in TX buffer. The fusion proteins were specifically eluted using 35 mM LIS in TX buffer. The majority of the purified protein was found in fractions 2-4 (1 ml fractions) of eluted volume.

Detection of monoclonal antibody specificity. Antigenic determinants for each hybridoma cell line were determined by radioactive immuno-assay (IRA). 200 µl of a 25 µg/200 µl solution of the antigen (fusion protein) was blot dotted onto a sheet of nitrocellulose and allowed to incubate for 16-24 hours using a 96-well polystyrene plate as a template. The filter was dried at 60°C for 1-2 hours and then washed 3 times for 30 minutes in a blocking solution consisting of PBS, 0.1% Tween 20, and 2.0% BSA. Upon removal of the blocking solution, the filters were soaked in an antibody solution (0.5-5 ml hybridoma supernatant in 50 ml of the

blocking solution). This was allowed to shake for 16-24 hours at 4°C. The antibody solution was removed and the filter was washed 6 times for 15 minutes each at 4°C in PBS. The filter was then washed 3 more times in blocking solution at 4°C. After the washings, the filter was incubated for 3 hours in 50 ml of blocking solution containing I^{125}-protein A at 4°C. The I^{125} solution was then removed, and the filter was washed 6 times for 15 minutes each at 4°C in PBS. The filter was then exposed to Kodak XR-5 film for 4-24 hours.

Results

The production procedure has been performed on both the catalytic subunit and holo-ATCase immunized BALB/c mice. The hybridization of the c_3 immunized B-cells produced 40 hybridomas out of a total of 960 inoculated wells (0.1% efficiency). Results for the screening of the 40 hybridomas against c_3 are shown in Figure 7. Row A was used as the control utilizing known ICR anti-R_2 positive and negative sera. Wells B1, B2, B11, and B12 tested c_3 whole serum obtained from the BALB/c mice used in the hybridization. The plate was read using a Micro-ELISA Minireader II when the anti-r_2 positive controls reached an optical density of 1.00. As the results indicate none of the 40 hybridomas were c_3 positive. The production procedure for monoclonal antibodies against holo-ATCase is in the screening stage. Preliminary results indicate that this fusion was more successful producing 750 initial hybrids (78% efficiency). Figure 8 shows the results of the first screening of 84 of the initial hybrids; again, row A was used for the positive and negative controls. Figure 8 indicates that there are 18 antigenically positive

Figure 7. ELISA results for the catalytic hybridization. Row A was used as a control: A1 and A2 were negative controls; A2, A3, A4 were ICR 1-1000 anti-r_2 positive controls; A5 and A6 were BALB/c 1-500 anti-r_2 positive controls; A7-A11 were BALB/c anti-r_2 negative controls. Wells B1, B2, B11 and B12 were anti-c_3 whole serum from BALB/c mice used in the fusion. The remaining wells were used to test the 40 possible anti-c_3 producing hybridomas.

Fig. 7

MINIREADER II

PLATE ID # Catalytic

DATE_____
TECH_____

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.00	1.20	1.30	0.89	0.04	0.00	0.09	0.06	0.15	0.10	0.06	0.08
B	1.46	1.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.98	1.56
C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
G	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Figure 8. ELISA results for the first screening of the holo-ATCase fusion. Row A was used for positive and negative controls. Wells A1, A2, and A12 are ICR 1:1000 anti-r_2 positive controls. Wells A4 and A6 are anti-holo ATCase whole serum (1:10 dilution) from BALB/c mice used in the fusion. Wells A9 and A10 are anti-holo ATCase whole serum in 1:1000 dilution. A positive reaction was an optical density of 0.20 or greater. Results indicate that there are 18 anti-holo ATCase positive hybridomas.

Fig. 8

MINIREADER II

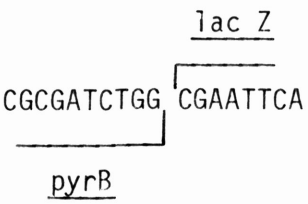
PLATE ID # Holo ATCase

DATE_____
TECH_____

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.66	1.22	0.13	1.90	0.00	1.34	0.00	0.00	0.58	0.26	0.00	1.18
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	1.38	1.63	1.48	1.44
C	1.67	0.89	0.91	0.98	0.00	0.00	0.00	0.00	0.13	0.03	0.00	1.19
D	1.21	0.63	0.32	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.28	0.00
E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
G	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.93	0.00	0.00
H	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.53	0.71	0.27	0.00	0.00

hybridomas against ATCase holoenzyme. Thirty-two other holo ATCase positive hybridomas have been found from the first comprehensive screening. It should be emphasized that the hybridomas must show positive results for three successive screenings before they are accepted as anti-ATCase antibody sources. At this time, 5 individual hybridomas have given 3 successive positive screenings, while 14 more have screened positive twice.

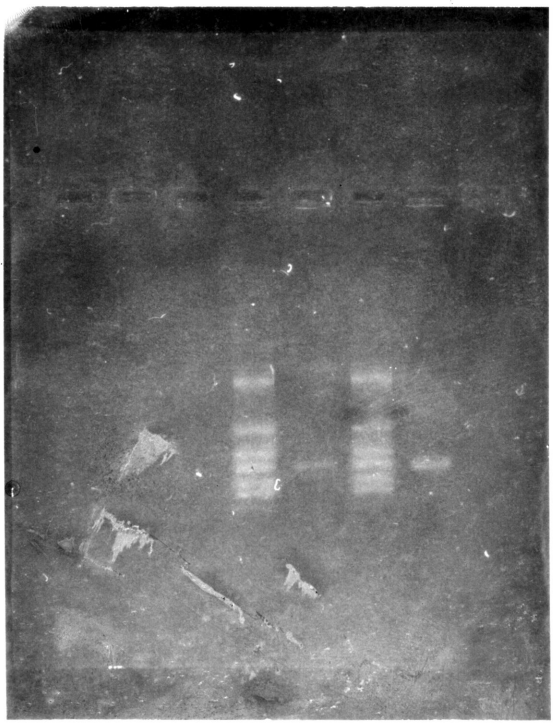
Characterization of the monoclonal antibodies can not begin until the hybridoma cell lines are produced. Significant progress has been made on the preparation of the "tools" for hybridoma characterization. Figure 9 demonstrates the electrophoretic analysis of the pyrB-lacZ fusion number 134 (lane 5). The size standards used were (smallest to largest) M13 (no insert), fusion 96 (start of pyrB), fusion 133 (start of pyrI), fusion 64 (start of pyrX), fusion 172 (end of pyrX), and fusion 22 (Pst/Sal: 2.9 kb insert). Electrophoretic analysis placed fusion 134 in the pyrB region. The locations of the fusions were determined by the Sanger Sequencing method. To illustrate, the DNA sequence of fusion 134 near the fusion site was as follows:



The vertical lines indicate the location of the fusion site. Sequential deletion from the carboxy terminus of pyrB placed the site of fusion 134 within the codon corresonding to amino acid 309 of the catalytic polypeptide. This was ligated to residues within the fourth codon of lacZ (com-

Figure 9. Electrophoretic analysis of fusion number 134 (lane 5). The standards (lanes 4 and 6) from smallest to largest were M13 (no insert), fusion 96 (start of *pyrB*), fusion 133 (start of *pyrI*), fusion 64 (start of *pyrX*), fusion 172 (end of *pyrX*), and fusion 22 (*Pst/Sal*: 2.9 Kb insert). Analysis indicates that fusion 134 is in the *pyrB* region as is fusion 317A (lane 7).

Fig. 9



mon to all fusions). Figure 10 shows a genetic map of the pyrBI bicistron indicating the locations of the pyrBI-lacZ fusions identified at present. There are 31 unique fusion sites shown on the map and fusion 134 occurs at the extreme 3'-end of the pyrB gene.

Molecular weight estimates of the corresponding fusion proteins have been run using a G-200 Sephadex calibrated column. As an example, an elution profile of fusion 134 is presented in figure 11. The estimated molecular weight is 152 Kd. This is significantly larger than expected trimer (100 Kd). The increase in molecular weight of the fusion protein can be attributed to the addition of the β-gal domains. The elution profile also provides evidence that the β-gal domains fused into the last five amino acids of the ATCase catalytic chain do not interfere with catalytic trimerization. The anti-β-gal immuno-affinity column for protein purification has been prepared and is functional. The column has been successfully utilized to capture pure β-gal for β-gal binding activity. The column has also proven capable of binding the fusion proteins. The only major problem with the column is detection of the fusion proteins once they have been eluted. This problem is especially critical for those fusions in the regulatory subunit and those in the catalytic region which are too small to maintain catalytic activity. We are currently developing immunological techniques with polyclonal antibodies and assays which detect β-galactosidase activity.

Discussion

The major difficulties of the early research involved our inability to select catalytic subunit hybridizations. There are several plausible

Figure 10. A genetic map of the pyrBI bicistron indicating the specific locations of the fusions analyzed. Fusion 134 occurs at the 3'-end of the pyrB gene. Fusion 131, 138, and 134 possessed both ATCase and α-complementing β-galactosidase activity.

FUSION LOCATIONS

Fig. 10

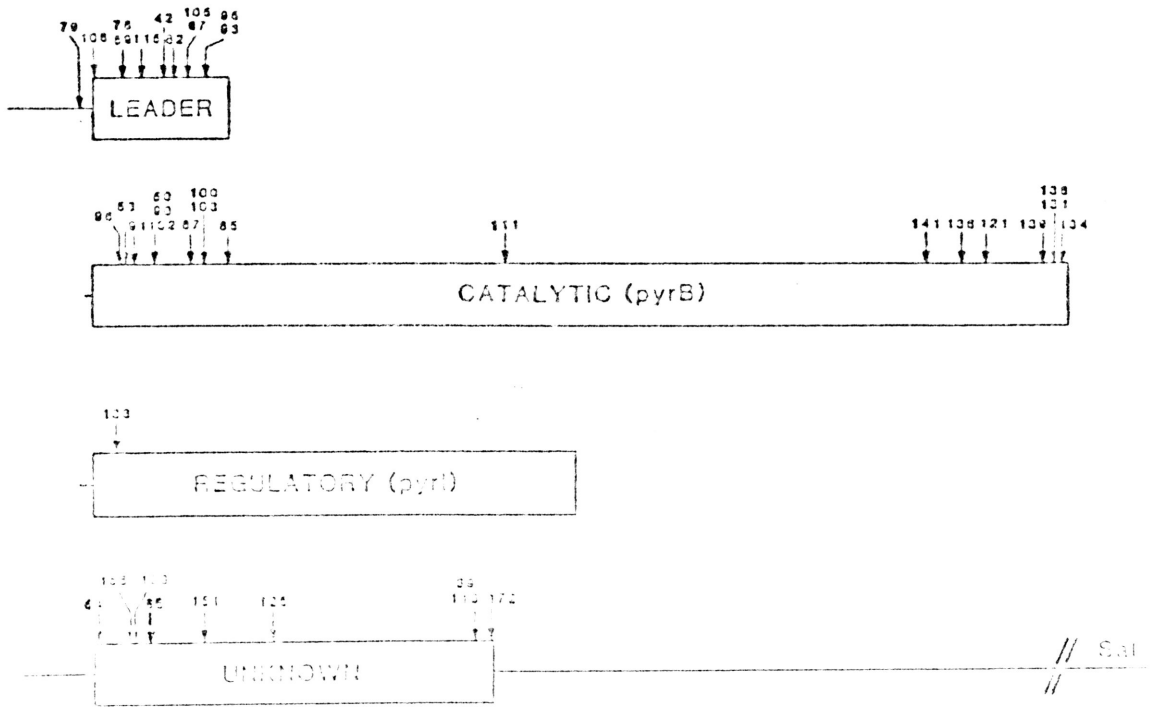


Figure 11. A G-200 Sephadex elution profile of fusion protein 134. V_0 indicates the void volume as determined by the elution of Blue dextran 2000.

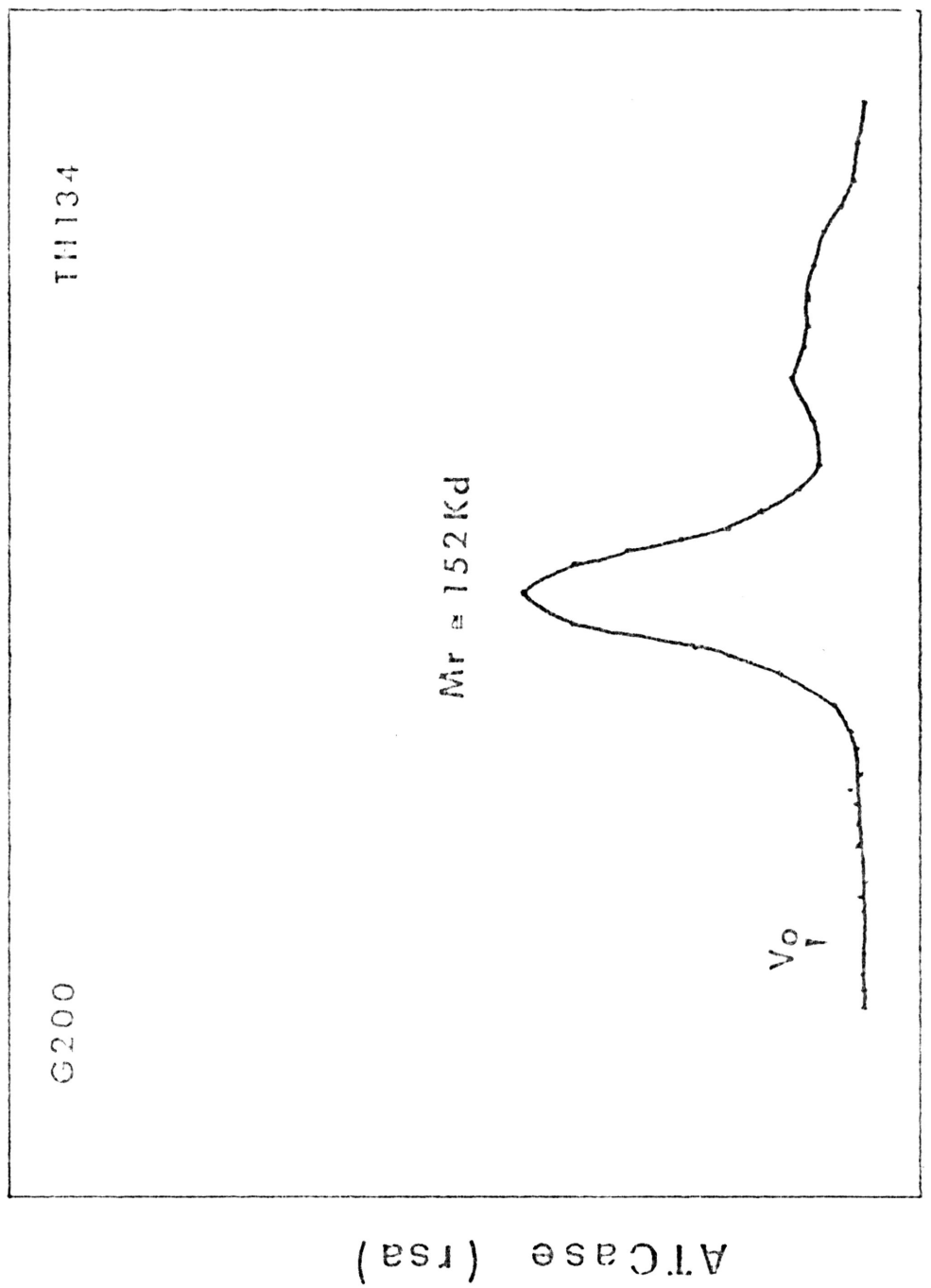


Fig. 11

explanations. The first consideration might be simply technique. The fusion protocol is very exacting, with proper feeder cell concentrations, cell ratios and viability, and PEG exposure being very critical. Experience and repeated effort will reduce the problems in this area. A second critical point was the low number of fusion cells originally obtained. It is generally accepted that following the fusion and the plating of the fusion cell mixture into 10 96-well plates, the initial number of hybrids is approximately one out of every 2 or 3 wells (14). This corresponds to roughly 50% while our initial results with the catalytic fusion only showed 0.1% hybridization. Of these possible hybridomas, it is also generally expected that only 10-20% will survive producing the desired antibodies. Thus, chances for success were greatly reduced from the beginning, due to the low hybridization frequency. The question arises why were only 40 hybrids produced in the initial study? The main factor influencing such a low yield may have been the type of 96-well plates used. For best results flat-bottom 96-well plates are recommended. The c_3 fusions were performed with U-bottom 96-well plates. The curvature of the wells apparently affected hybridoma viability. The U-shape allowed all cells (hybridomas, macrophages, and non-fused myeloma cells) to converge in a small proximity at the bottom of the wells. The majority of the more sensitive hybridomas may have been over-crowded and consequently died. Flat bottom 96-well plates were used in the subsequent holo-ATCase immunization fusions. The holo-ATCase fusion produced 750 initial hybrids which is approximately 20 fold better than the c_3 fusion. This is a strong indication that U-bottom shape of the plates in the c_3 fusions greatly diminished hybrid cell viability.

Initial screening results of the holo-ATCase fusions are encouraging but must be viewed objectively. Only two screenings have occurred at this time and one more screening must be performed to verify these results. In addition, it is likely that several hybridoma cell lines will be lost because of contamination (yeast or fungi), or loss of antibody producing ability due to stress or more rapid growth of non-antibody producing hybrids which may be in some wells.

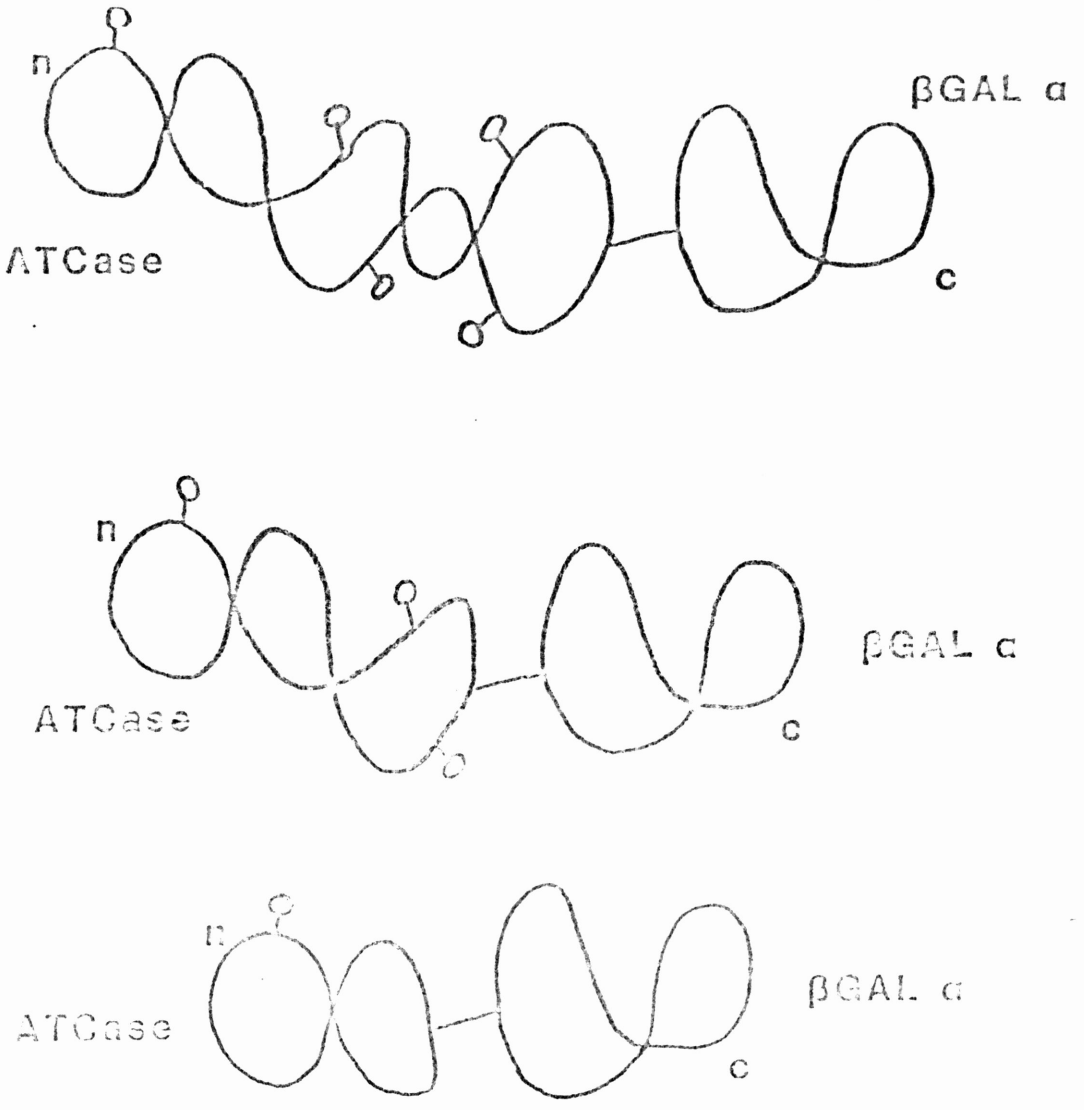
Once the anti-ATCase monoclonal antibodies are produced, each hybridoma serum will be reacted with selected ATCase-β-gal fusion proteins. To illustrate, three possible fusion proteins are shown in Figure 12. Each fusion protein contains a different length of ATCase, and consequently, epitopes present on some of the proteins will not be found on others. Reacting the immunoglobulins with each separate fusion protein and visualizing the antagonistic reactions by RIA, we expect results similar to those shown in Figure 13. Knowing the exact size of each fusion protein, a simple positive or negative reaction will determine the region of specificity of the monoclonal antibodies. For example, using the results in Figure 13, it is evident that monoclonal antibody number 6 is specific for the last 10 amino acids of the catalytic subunit. In this way it becomes possible to determine the region of specificity for each hybridoma cell line.

This project is not complete and much work is yet to be done. It is important to point out that once the monoclonal antibodies are produced and characterized, we will have an excellent tool to explore discrete structural and conformational domains of ATCase. We will be able to explore antigenic similarities between ATCase of different organisms, and

Figure 12. Schematic representation of fusion proteins with their respective antigenic determinants. Variation in fusion protein size will consequently lead to variation in the number of epitopes present on each fusion protein.

Fusion Proteins

Fig. 12



♀ : antigenic determinant

Figure 13. A model monoclonal antibody-fusion protein reaction chart indicating epitope recognition (+) and nonrecognition (-). With this approach existing epitopes may be detected by positive recognition.

Fig. 13

	Monoclonal A.Bs					
	1	2	3	4	5	6
aa. CAT (100)	+	−	−	−	−	−
CAT (150)	+	−	−	−	−	−
CAT (200)	+	+	−	−	−	−
CAT (250)	+	+	+	−	−	−
CAT (300)	+	+	+	+	−	−
CAT (310)	+	+	+	+	+	+

hybrid enzymes relative to native E. coli ATCase. Once the hybridomas are defined the list of questions and applications that can be addressed with monoclonal antibodies will expand.

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