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SCREENING OF 2 EXPRESSION LIBRARIES WITH AN ANTIBODY TO A CALCIUM BINDING ATPase INHIBITOR PROTEIN AND SEQUENCING OF THE ISOLATED CLONES

by

GAIL PENNER

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BY

GAIL PENNER

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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Abstract

The protein of interest for this project is a calcium binding ATPase inhibitor (CaBI) found in bovine cardiac and rat skeletal muscle. The protein prevents ATP hydrolysis by mitochondrial A particles in vitro below 10^{-7} and above 10^{-5} M Ca^{2+}. The calcium binding inhibitor is thought to play a role in Ca^{2+} homeostasis within mitochondria. It has been shown that Ca^{2+} levels are important to the activity of 2 dehydrogenases of the Krebs cycle and the activity of pyruvate dehydrogenase, which contributes acetyl CoA to the Krebs cycle. These enzymes catalyse irreversible decarboxylation steps and are rate limiting.

The amino acid sequence of CaBI is not known, although the composition is known. The purpose of this research was to isolate a clone for CaBI from a cDNA lambda gt11 expression library with CaBI antibody and to sequence it. From the nucleotide sequence the protein sequence was to be elucidated. A T47D human breast cancer expression library and a rat heart expression library were immunoscreened. A clone was isolated from the human library and 3 from the rat. The human clone was sequenced as was one of the rat clones. The 2 sequences were identical, and from hybridization experiments all of the clones were shown to have strong homology.

The sequence did not code for CaBI. The only open reading frame of 50 amino acids or more coded for a protein that was too large. The coding region was basic and CaBI has a pI of 4.4. The overall amino acid composition was wrong. The sequence compared to known sequences stored in GenBank. The clone was 99% homologous to nucleotides 160 to 788, and 187 to 815 of 2 reported sequences for a human ribosomal protein (S6) of the 40S ribosomal subunit.

A structural or functional similarity between S6 and CaBI had never been shown, therefore, further experiments were done. The chimeric protein coded for by the phage of S6 sequence together with ß-galactosidase protein was utilized for this study. From competitive

inhibition studies it was shown that the chimera interacted specifically with CaBI antibody. Research since then has shown that antibody to the C-terminal portion of S6 also interacts with CaBI.

The interaction of CaBI antibody with the chimera indicates an epitopic similarity between the 2 proteins. Since CaBI is a Ca^{2+} binding protein research was done since this project to see if S6 binds Ca^{2+}. It had been shown that $[Ca^{2+}]$ plays a role in activation of ribosomes for protein synthesis. There are however, 70-80 ribosomal proteins within a eukaryotic cell, and the interaction of Ca^{2+} with any one of these proteins could occur. It was also not known if the $[Ca^{2+}]$ effect was mediated through another protein. For example it is known that S6 is phosphorylated, in order for it to be more active. An enzyme responsible for phosphorylation of S6 could be activated by $[Ca^{2+}]$. Since this research was completed, it has been shown that the S6 portion of the chimeric protein does bind Ca^{2+}. This research provides some direct evidence that Ca^{2+} induced protein synthesis may be mediated by S6 at the translational level.

This research also, in part pointed to the possibility that Ca^{2+} interaction by CaBI may be through gamma-carboxy glutamate residues. It had been known that CaBI anti-serum does not interact with such calcium binding proteins as calmodulin and oncomodulin. The sequence that was present in the insert did not have the EF hands characteristic of these proteins. Prothrombin, which binds Ca^{2+} through gla residues has been shown to compete for CaBI antibody in a competition assay. The next step will be to do an alkaline hydrolysis of CaBI to see if it does indeed have gla residues. Gla residues had also been reported for prokaryotic and eukaryotic ribosomal proteins. The possibility that the epitopic similarity between S6 and CaBI is through gla residues should be considered, although this is speculative from these few data.

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Abbreviations

A..... *Absorbance*
BSA..... *bovine serum albumin*
CaBI..... *calcium binding ATPase inhibitor protein*
cDNA..... *copy DNA*
cpm..... *counts per minute*
dd water..... *deionized distilled water*
DNP..... *dinitrophenol*
DTT..... *dithiothreitol*
E. coli..... *Escherichia coli*
gla..... *gamma-carboxy glutamic acid*
GRB..... *gel running buffer*
IPB..... *immunoprecipitating buffer*
IPTG..... *isopropyl thio ß D-galactoside*
IP3..... *inositol trisphosphate*
kD..... *kilodalton*
PBS..... *Phosphate buffered saline*
PEG..... *polyethylene glycol*
PMI..... *Pullman-Monroy ATPase Inhibitor protein*
RF DNA..... *replicative form DNA*
SDS..... *sodium dodecyl sulphate*
SM..... *suspension media (used for lambda gt11)*
SSC..... *standard sodium citrate buffer*
ssDNA..... *single stranded DNA*
SSPE..... *standard sodium phosphate EDTA buffer*
TBE..... *Tris borate EDTA buffer*
TBST..... *Tris buffered Saline with Tween 20*
TE..... *10 mM Tris 1 mM EDTA pH 8.0 buffer*
TPEG..... *Para-aminophenyl ß-D thiogalactoside*

Introduction

Overview

The protein of interest in this research is a calcium binding ATPase inhibitor protein which is found in bovine heart and rat skeletal muscle mitochondria. Because the protein is believed to help control levels of Ca^{2+} within the mitochondria the role of Ca^{2+} within mitochondria will be discussed, as will the transport systems for influx and efflux of Ca^{2+} from mitochondria. The available data on the Ca^{2+} binding protein will be presented. Since $[Ca^{2+}]$ appears to be important for control of mitochondrial enzymes, this role will be discussed in a physiological context. The evidence for an effect of hormones on mitochondrial Ca^{2+} levels and on enzymic activities will be described.

The Calcium Uniport

It was shown that mitochondria were able to accumulate large amounts of Ca^{2+} under the appropriate conditions (Vasington and Murphy, 1961; Vasington and Murphy, 1962; De Luca and Engstrom, 1961). The driving force for Ca^{2+} accumulation is respiration (Lehninger et al., 1967). The Ca^{2+} influx is limited by the ability of mitochondria to support an H^+ efflux (Heaton and Nicholls, 1976; Bragadin et al., 1979). From the chemiosmotic theory it is known that the electron transport system pumps protons out of the cell to generate a proton electrochemical gradient which can then be used for ATP synthesis and the transport of various ions. Rossi and Lehninger (1963, 1964) showed that 2 H^+ had to be driven out per Ca^{2+} transported into mitochondria.

The electrochemical gradient (delta μ_{H+}) is composed of 2 factors; one determined by the change in pH (delta pH) across the membrane, the other (delta psi) is the mitochondrial inner membrane potential and is the electrical factor in the equation (equation 1). Delta psi is the driving

force for the influx of cations (Caroni et al., 1978; Fry and Green, 1979; Wikstrom and Sari, 1977 Krab and Wikstrom, 1978), since it is the major component of the equation (Mitchell and Moyle 1969; Padon and Rottenberg, 1973; Nicholls, 1974). For Ca^{2+} the magnitude of delta psi (equation 2) means that Ca^{2+} would attain electrochemical equilibrium with a distribution ratio of the free cation $[Ca^{2+}]i/[Ca^{2+}]e$ equal to 10^{5} to $10^{6.9}$ (Crompton, 1985). It is believed that a passive uniport system uses this gradient for the uptake of Ca^{2+} and that Ca^{2+} uptake is not coupled to H^{+} efflux (Schejter et al., 1979).

Ca^{2+} uptake requires energy metabolism

The uptake of Ca^{2+} requires energy metabolism (Tew, 1977; Vasington and Murphy, 1961; Vasington and Murphy, 1962; DeLuca and Enstrom, 1961; Lehninger et al., 1963) and can be blocked by metabolic inhibitors such as CN- (Izzard and Tedeschi, 1973) and CN- in the presence of oligomycin (Rottenberg, 1973), generally by uncouplers such as dinitrophenol (Izzard and Tedeschi, 1973) or by the onset of phosphorylation initiated by the addition of ADP and Pi (Izzard and Tedeschi, 1973).

Evidence that Ca^{2+} uptake is via a carrier

It is believed that Ca^{2+} uptake is via a carrier. Ruthenium red is a specific inhibitor of Ca^{2+} uptake. The kinetics of Ca^{2+} influx point to a carrier. Ca^{2+} uptake shows a hyperbolic relationship (in the absence of Mg^{2+}) (Crompton et al., 1976). Ca^{2+} uptake also shows cooperativity with a Hill number for Ca^{2+} ranging from 1.6 to 2.0 (Hutson et al., 1976; Bygrave et al., 1971, Vinogradov and Scarpa, 1973). Metabolites such as phosphoenolpyruvate inhibit Ca^{2+} uptake (Roos et al., 1978; Chudapongse and Haugaard, 1973), as does physiological concentrations of Mg^{2+} which shift the affinity of the carrier to much lower values (K_a in the order of 30 μM) (Hutson et al., 1976; Knauf et al., 1977).

Despite this evidence for a carrier, surface charges on mitochondria

Electrochemical Gradient Equation

1) $\Delta \mu_{H^+} = 2.3\ RT\ \Delta pH + F\ \Delta \psi$

2) $\Delta \mu_{Ca^{2+}} = 2.3\ RT\ \Delta [Ca^{2+}]_e / [Ca^{2+}]_i + 2F\ \Delta \psi$

where

$\Delta \mu$ *is the electrochemical gradient*

ΔpH *is the pH gradient across the inner membrane of the mitochondria*

e is extramitochondrial $[Ca^{2+}]$

i is intramitochondrial $[Ca^{2+}]$

$\Delta \psi$ *is the electrical component of the equation*

From Crompton, 1985

might mimic carrier-mediated transport cooperative effects (Thenvenet and Borst-Pauwels, 1976; Saris and Akerman, 1980). For example, the quadravalent polyamine spermine produces a change in the kinetics (Akerman, 1977), similar to the effect of Mg^{2+}, suggesting a less specific mechanism. Bound ions can interfere with the transport without exhibiting affinity for the carrier molecule (Thenvenet and Borst-Pauwels, 1976; Saris and Akerman, 1980).

Sequence of events in transport

Chance (1963, 1965) visualizes the interaction of Ca^{2+} with mitochondria as a three-step process. First, a surface binding occurs involving both high-affinity (K_d about 0.025 μM) and low-affinity (K_d about 100 μM) sites (Reynafarje and Lehninger, 1969). This binding is followed by an energy-dependent uptake into the membrane phase. A calcium binding inhibitor of ATP hydrolysis is thought to regulate this step (Yamada et al., 1988). The last step is the completed uptake into the mitochondrial matrix. This scheme has also been followed by Reed and Bygrave (1974a, 1974b); Schuster and Olson (1974); and by Saris and Akerman (1980).

The low affinity binding of Ca^{2+} does not depend on energy metabolism (Scarpa and Azzi, 1968; Scarpa and Azzone, 1968; Scarpa and Azzone, 1969), but does depend on the presence of phospholipid, and occurs at anionic sites (Tedeschi, 1981). At the Ca^{2+} concentrations presumed to exist in the cytosol, the low affinity system operates at a fraction of its maximal capacity (Carafoli, 1987). Nonspecific surface charges have been proposed for this step by Akerman (1977a, 1977b), however, Ca^{2+} and monovalent cations compete for the binding (Scarpa and Azzi, 1968) and K^+ inhibits the Ca^{2+} uptake only slightly if at all (Vaino et al., 1970; Hutson et al., 1976, Akerman, 1977a). Tedeschi (1981) does not exclude effects which involve interactions of the ions on the structure of the membrane itself and not directly on the transport of another ion.

The possibility of passage of bound Ca^{2+} from low-affinity to high-

affinity sites has been proposed (Scarpa and Azzone, 1968). Studies with mitochondria from various sources showed a correlation between the presence of the high-affinity Ca^{2+} binding (which does not occur in the presence of uncouplers) by mitochondria and their ability to transport Ca^{2+}. (Carafoli and Lehninger, 1971). The transport of bound Ca^{2+} during respiration seems to involve the total Ca^{2+}, including Ca^{2+} bound in the absence of energy metabolism (Scarpa and Azzone, 1968).

Upon leaving the carrier, the cations could be held by anionic charges of the membrane phospholipids (e. g. cardiolipin) at the inner interface of the internal membrane, which is referred to as limited loading (Schuster and Olson, 1973; Schuster and Olson, 1974, Hackenbrock and Caplam, 1969). In the presence of Pi, the Ca^{2+} complexes with phosphate, which if large amounts of Ca^{2+} enter the mitochondria leads to matrix loading (Rossi and Lehninger, 1963; Greenawalt et al., 1964). The matrix-free Ca^{2+} concentration also increases, since the Ca^{2+}-dependent Krebs cycle dehydrogenases also become more active (McCormack and Denton, 1984).

Role of a Channel in Ca^{2+} uptake

A nonspecific channel is hypothesized to transport Ca^{2+} (Patlak, 1957; Jardetsky, 1966; Lauger, 1980; Klingenberg, 1981) which is proposed to be subunit I of cytochrome c oxidase (Fry and Green, 1979). A nonspecific channel has been supported by evidence from several researchers (Akerman, 1977; Crompton et al., 1979, Tsokos et al., 1980; Reed and Bygrave, 1974b; Reed and Bygrave, 1974a).

The nonspecific channel may be preceded by a selector portion acting as a filter or gate (Tedeschi, 1981). This protein could be a 30,000 dalton glycoprotein implicated in Ca^{2+} transport or another protein such as calciphorin (discussed later). Tedeschi (1981) suggests that this filter would be analogous to the channels proposed by Saris and Akerman (1980). La^{3+} and ruthenium red are thought to complex with this protein

(Niggli et al., 1978). The idea that a channel may be responsible for Ca^{2+} transport is supported by a correspondence between the Stokes radius (Robinson and Stokes, 1959; Gorman and Hermann, 1979) of divalent ions (relative to that of Ca^{2+}) and the selectivity of the transport system (Selwyn et al., 1970; Vaino et al., 1970; Drahota et al., 1969). The exclusion of larger probes would be due to steric hindrance and the exclusion of smaller ions would be expected if the ion needs a minimal size to be able to bind to the sites of the selector channel.

Other observations suggest channels. The effect of temperature on the influx of Ca^{2+} in exchange for K^{+} provides Arrhenius plots without the characteristic break at about 14° C. which characterizes many mitochondrial membrane processes (Tedeschi, 1981). The activation enthalpy is relatively low and the turnover ratio high (Bragadin et al., 1979). The temperature dependence of Ca^{2+} transport is reduced by the presence of Mg^{2+} or spermine (Akerman, 1977b), also indicating that under these conditions the process involves a channel.

There have been some reports that the uniport can be reversed to release Ca^{2+} (Haworth et al., 1980; Nedergaard and Cannon, 1980; Goldstone and Crompton, 1982; Bernadi and Azzone, 1982). Carafoli (1987) argues that the magnitude of delta psi would preclude this.

Na^{+}-dependent efflux of Ca^{2+}

The capacity of $[Na^{+}]$ to depress the accumulation of Ca^{2+} by mitochondria (muscle) was first reported by Dransfield et al. (1969) and by Crompton et al. (1976). Further studies showed that $[Na^{+}]$ also induces the release of pre-accumulated Ca^{2+} in both the presence (Carafoli and Sottocasa, 1974); Carafoli et al., 1975; Carafoli et al., 1974) and absence (Thorn et al., 1975) of ruthenium red to inhibit re-uptake of Ca^{2+} via the uniporter. The use of intramitochondrial Ca2+-sensitive dehydrogenases as an index of matrix free Ca^{2+} concentration indicates that the presence of Na^{+} decreases matrix $[Ca^{2+}]$ during steady-state recycling

(Denton and McCormack, 1980), and also accelerates the net release of matrix Ca^{2+} when re-uptake is prevented by EGTA (Hansford, 1981; Hansford and Castro, 1981).

Today the nature of this path has been studied more fully (Crompton et al., 1976; Crompton et al., 1977; Crompton et al., 1978; Crompton and Heid, 1978; Nicholls, 1978b; Nicholls, 1978a; Hayat and Crompton, 1982). It is known that a Na^+/Ca^{2+} exchanger exists that operates electroneutrally (Crompton et al., 1976; Crompton, 1985) with the net driving force for Ca^{2+} extrusion given by the difference between the electrochemical potential gradients of Na^+ and Ca^{2+}, taking into account the stoichiometry of the exchange. The exchanger has a different inhibitor sensitivity with respect to the uptake pathway (Crompton et al., 1978). The exchanger is active in excitable tissue and is much less active in the liver. Values for adrenal cortex and brain at 10 mM Na^+ obtained were V_{max}=12-27 nmol Ca^{2+}/mg protein.min^{-1} at 25° C. (Crompton et al., 1976a; Crompton et al., 1978; Nicholls, 1978b; Hayat and Crompton, 1982). Intermediate activities have been reported in mitochondria isolated from brown fat, parotid gland, and red and white skeletal muscle, whereas relatively low activities have been detected in liver, kidney cortex, and ileum muscle (Crompton et al., 1978; Nedergaard and Cannon, 1980; Haworth et al., 1980; Heffron and Harris, 1981; Goldstone and Crompton, 1982). A slow, sodium-dependent calcium efflux mechanism was identified for liver mitochondria by Gunter et al. (1988) with a V_{max} of 2.6 nmol of Ca^{2+}/mg protein/minute. The K_m for internal Ca^{2+} was found to be 8.1 mM and for 9.4 nM for external $[Na^+]$ (Gunter et al. 1988).

Kinetics of Na^+-dependent Ca^{2+} efflux

The relationship linking external Na^+ concentration to rate of Ca^{2+} efflux is strongly sigmoidal, with half-maximal activity being seen at 4-5 mM Na^+ (Crompton et al., 1976). The values expected for the $[Na^+]$ in

vivo indicate that [Na^+] are saturating. The affinity of the exchanger for Ca^{2+} has been estimated at the external face of the inner membrane to be about 10 μM (Crompton et al., 1976). At the internal face values of 6 μM (Coll et al., 1982) and 3-4 μM (Hansford and Castro, 1981) have been obtained. K^+ activates this mechanism with a K_a of 17-19 mM (Crompton et al., 1980). Another regulatory feature in liver is that there are indications that there is an increase in internal affinity for Ca^{2+} after glucagon or ß-adrenergic treatment (Goldstone and Crompton, 1982; Goldstone et al., 1983).

At the outer face of the inner membrane, Na^+ decreases the binding affinity of the exchanger for Ca^{2+} (Hayat and Crompton, 1982). Crompton (1985) speculates that the exchanger operates asymmetrically in energized mitochondria, with a higher Ca^{2+} affinity at the matrix than at the external side and/or the converse for Na^+. This would allow Ca^{2+} to be exported from mitochondria in the presence of increased [Na^+] (Coll et al., 1982). In addition, Na^+ binding would need to affect the translocation step since, although Li^+ may substitute for Na^+ in inducing Ca^{2+} efflux, the V_{max} of Li+-induced Ca^{2+} efflux is only 30% of that observed with Na^+ (Crompton et al., 1977). It is not known if Na^+ is exchanged for Ca^{2+} or simply activates the carrier (Crompton, 1985). The matrix ATP may also be important for asymmetry of the carrier (Crompton, 1985).

It is believed that this antiport may contain regulatory sites that permit the exchanger to respond to physiological changes in extramitochondrial [Ca^{2+}] (Hayat and Crompton, 1982). These researchers have shown that external [Ca^{2+}] inhibits Na^+-dependent Ca^{2+} efflux by cooperative binding to sites that are half-saturated by 0.7 to 0.8 μM free [Ca^{2+}].

Na^+-independent Ca^{2+} Efflux

In all types of mitochondria studied, it has been found that Ca^{2+} is released in the absence of Na^+ at a slow rate in the presence of ruthenium

red to inhibit reuptake of Ca^{2+} (Puskin et al., 1976; Azzone et al., 1976; Nicholls, 1978a). This was taken as evidence of a Na^{+}-independent Ca^{2+} release mechanism (Akerman, 1978; Fiskum and Cockrell, 1978; Fiskum and Lehninger, 1979; Tsokos et al., 1980).

This ruthenium red insensitive mechanism is most active in tissues where the Na^{+}-dependent efflux is least active. In heart, 0.1-0.2 nmol Ca^{2+}/mg protein is released at 25°C. (at physiological [Ca^{2+}]) (Crompton et al., 1976b; Hayat and Crompton, 1982). In liver and kidney the process is typically an order of magnitude faster (Crompton et al., 1978). In liver mitochondria, a sodium-independent calcium efflux mechanism has been identified, which shows second order kinetics. The V_{max} of the system is 1.2 nmol Ca^{2+}/mg protein/minute and the K_m is 8.4 nmol Ca^{2+}/mg protein/minute (Gunter et al., 1988).

Several investigators have proposed that the Na^{+}-independent process mediates a direct exchange between Ca^{2+} and H^{+} (Akerman, 1978; Tsokos et al., 1980; Fiskum and Cockrell, 1978; Fiskum and Lehninger, 1979). Today this is not believed to be the case (Gunter et al., 1983; Crompton, 1985; Crompton et al., 1979). This has caused researchers to speculate that Na^{+}-independent release of Ca^{2+} may not involve a specific carrier.

The Na^{+}-independent efflux may be due to alterations of the inner membrane which are induced by accumulation of excess Ca^{2+}. It is known that Ca^{2+} induces swelling of mitochondria and loss of membrane potential (Carafoli, 1987). There is some evidence that phospholipase A_2 may be responsible for activation of a nonspecific (damage linked) Ca^{2+} release pathway (Beatrice et al., 1980; Palmer and Pfeiffer, 1981; Beatrice et al., 1982). Recently Hoek et al. (1988) have reported that phospholipase A2 does not play a role in release of Ca^{2+}.

Proteins that appear to be involved in Ca^{2+} transport

A 30,000 dalton (Sottocasa et al., 1979a; Sottocasa et al., 1972; Carafoli et al., 1972; Carafoli, 1975) glycoprotein was isolated by

several investigators (Lehninger, 1971; Sottocasa et al., 1971; Sottocasa et al., 1972; Gomez-Puyou et al., 1972). It has a high affinity (2-3 mol/mol protein, a K_d of about 0.1 M (Sottocasa et al., 1979)) for Ca^{2+}. The Ca^{2+} binding is inhibited by La^{3+} and partially by ruthenium red (for reviews see Carafoli, 1975 and Sottocasa et al., 1977). The glycoprotein appears to be absent in mitochondria unable to transport Ca^{2+} (Panfili et al., 1974). The protein has been linked to a ruthenium red sensitive Ca^{2+} electrical conductance of lecithin bilayers (Prestipino et al., 1974), or bilayers prepared from phospholipid mixtures (Lukyanenko et al., 1980), Ca^{2+} conductance into osmotically shocked mitochondria (mitoplasts) (Carafoli and Sottocasa, 1974), or into extracted mitochondria by a glycoprotein fraction (Sandri et al., 1979). Inhibition of Ca^{2+} uptake is seen in mitochondria and mitoplasts by antibodies raised against this glycoprotein (Panfili et al., 1976; Sottocasa et al., 1979) or by extraction of mitochondria (Sandri et al., 1979). The glycoprotein shows a capacity to respond to effectors such as NAD^+ which lowers the affinity of the glycoprotein for Ca^{2+} (Panfili et al., 1980).

The protein is however, widely distributed in mitochondrial subfractions (Sottocasa et al., 1979, Sottocasa et al., 1971, Carafoli, 1975, Carafoli and Sottocasa, 1974) and seems to represent about 5-6% of the total rat liver mitochondrial protein, the binding was found to be 0.001 nmol/mg protein for La^{3+} and 0.08 nmol/mg protein for purified ruthenium red and in rat liver mitochondria (Reed and Bygrave, 1974). This suggests that most of the glycoprotein is inactive.

If this protein were involved in Ca^{2+} transport and internal to the phospholipid it should not be as easily extractable from the membrane as it is (Teschi, 1981). However, it might be able to function as a carrier through the lipid layer and at the same time as an extrinsic protein, since the conformation of the glycoprotein changes drastically when it binds Ca^{2+} (Presptipino et al., 1974) as does the association of the glycoprotein to mitochondria (Sandri et al., 1979).

Jeng et al. (1978) and Jeng and Shamoo (1980a; 1980b) isolated a ruthenium red and La^{2+} sensitive protein of approximately 3000 daltons from heart and 6000 daltons from liver (Ambudkar et al., 1984). The purified protein binds close to 1 mol Ca^{2+}/mole with an apparent affinity (K_d approx. 10-14 μM) similar to that of the Ca^{2+} uniporter in intact mitochondria. The ionophore (named calciphorin by Jeng et al., 1978) was able to incorporate divalent ions into an organic phase (Shamoo et al., 1984).

A tryptic digest of a 5000 dalton mitochondrial protein isolated by Blondin et al. (Blondin, 1974, Blondin et al., 1977) generated a peptide of about 1600 daltons which was shown to transport Ca^{2+} or K^+ into an organic phase and to stimulate K^+ transport in mitochondria.

A ruthenium red insensitive, chymotrypsin-sensitive polypeptide of about 8800 daltons has been reported to be a contaminant of cytochrome oxidase preparations. Cytochrome oxidase reconstituted vesicles have a decreased Ca^{2+} uptake when treated with chymotrypsin (Saltzgaber-Muller et al., 1980).

A glycopeptide (40K) and peptide (2K), purified from heart mitochondria have been reconstituted into black lipid membranes (Miranova et al., 1982). These membranes developed near-Nernstian potentials with applied Ca^{2+} gradients and their permeability to Ca^{2+} was blocked by ruthenium red.

The relationship between these various proteins if any is not clear. The interactions of the subunits of a possible carrier may be complex and involve several of these molecules. The kinetics of ruthenium red inhibition of Ca^{2+} transport suggest the involvement of four subunits (Bragadin et al., 1979). Further elucidation of the nature and roles of proteins involved in Ca^{2+} transport should help to determine the mechanism of Ca^{2+} transport.

Calcium Levels in Mitochondria

Mitochondrial [Ca^{2+}] in heart is normally maintained at relatively

low levels. Of the total cellular Ca^{2+} 13-16%, has been reported to be present in the mitochondrial matrix of rat heart (Kessar and Crompton, 1983; Patriarca and Carafoli, 1968). Similarly, low levels of intramitochondrial Ca^{2+} may be maintained in smooth muscle (James-Kracke et al., 1980).

Values reported for the Ca^{2+} content of mitochondria isolated from nonperfused hearts lie in the range 6.2-7.4 nmol Ca^{2+}/mg protein (Henry et al., 1977; Wrogemann and Nylen, 1978; Soetersdal et al., 1981). Lower Ca^{2+} contents of 1.8 nmol Ca^{2+}/mg protein for mitochondria from spontaneously beating perfused rat heart were obtained (Crompton et al., 1983). Crompton (1985) applied the relation between free and bound matrix Ca^{2+} derived from studies with mitochondria (0.6 μM/nmol Ca^{2+}.mg protein), and determined that contents of 2-7 nmol total Ca^{2+}/mg protein correspond to about 1-4 μM matrix free [Ca^{2+}]. Other researchers have estimated levels of 0.1 to 0.5 μM (Becker, 1981). These values are within the effective range for control of Ca^{2+}-sensitive dehydrogenases of the Krebs cycle (0.1-10 μM).

Ca^{2+} Cycle

The finding that the routes for Ca^{2+} uptake and Ca^{2+} release are independent form the basis for the proposal of the mitochondrial "Ca^{2+} cycle" (Carafoli, 1979), in which Ca^{2+} influx into the matrix from the cytoplasm is driven by the transmembrane potential, and efflux is electroneutral.

In vivo mitochondria take up Ca^{2+} at a marginal rate only (Sordahl, 1974; Crompton et al., 1976a; Crompton et al., 1976b; Parr and Harris 1976; Akerman, 1977a), and thus energy dissipation by the cycle will be very limited. It has been estimated to account for less than 0.2% of the total oxygen consumption of heart tissue (Crompton, 1984).

The overall rate of Ca^{2+} cycling will be determined by the rate-limiting step of the cycle, which contains, in addition to the Ca^{2+} uptake

and release legs, a Na^+/H^+ antiporter (Mitchell and Moyle, 1967) that is responsible for the efflux of the excess Na^+ that has penetrated into the mitochondria (Crompton et al., 1978; Crompton and Heid, 1978). The fact that this antiporter uses the proton gradient to rid the mitochondria of excess Na^+ means that both Ca^{2+} uptake and release are dependent on the proton gradient. Nicholls (1978a, 1978b) has proposed a kinetic regulation of the cycle based on an essentially constant Na^+-induced release rate (until about 60 μM free-matrix $[Ca^{2+}]$) and a rate of the uptake by the uniporter that will be dependent on the free Ca^{2+} concentration in the extramitochondrial medium. The rates of influx and efflux will be roughly equivalent at 1 μM external Ca^{2+}, which will then be the "set point" of the cycle. At higher external Ca^{2+}, concentration the uptake pathway would predominate, leading to temporary Ca^{2+} loading of mitochondria, whereas at lower external Ca^{2+} concentrations the efflux pathway would take over, returning the external $[Ca^{2+}]$ to the set point (and depleting mitochondria of Ca^{2+} in the process). Today it is believed that the internal "set point" is the most important (Crompton, 1985), and is responsible for modulating Ca^{2+}-dependent matrix proteins, especially dehydrogenases of the Krebs cycle (discussed later). One μM internal Ca^{2+} is estimated to exist in isolated respiring heart mitochondria in the presence of physiological Mg^{2+}, 0.5 μM external Ca^{2+}, and amounts of Na+ that saturate the efflux pathway (Coll et al., 1982; Hansford and Castro, 1981). The value is in the optimal range for the modulation of the Ca^{2+}-controlled matrix dehydrogenases both in the isolated state and in vivo. Denton et al. (1980) and Hansford (1981) have observed that coupled heart mitochondria suspended in media containing physiological concentrations of Na^+ and Mg^{2+} maintain the Ca^{2+}-dependent matrix dehydrogenases half-maximally activated at 0.1-0.5 μM external Ca^{2+}. The Ca^{2+} cycle, then, will modulate the matrix-free $[Ca^{2+}]$ based on the external (cytosolic) $[Ca^{2+}]$, affording precise control of the activity of the Ca^{2+}-dependent matrix dehydrogenases.

Another regulatory mechanism may be through a membrane bound hexokinase of brain mitochondria. Glucose phosphorylation via hexokinase activated the active Ca^{2+} uptake system and inhibited the passive efflux, resulting in an increase in brain intramitochondrial [Ca^{2+}] (Kottke et al., 1988)

Role of Calcium in Mitochondria

Mitochondria as a sink for Cytoplasmic Calcium?

Initially it was thought that mitochondria were important for taking up excess calcium within the cytoplasm (Nicholls and Akerman, 1982; Fiskum and Lehninger, 1982). This was because the matrix can retain more than 1000 nmol of Ca/mg and external free calcium can be reduced to below 1 μM if matrix is induced to take up free calcium (Nicholls and Akerman, 1982). The entire respiratory capacity of mitochondria can be harnessed to the uptake of the cation.

The evidence against mitochondria as a modulator of cytoplasmic calcium is that early experiments were done under nonphysiological conditions and now it is known that in vivo mitochondria do not contain large amounts of Ca^{2+} (discussed above) and the role for endoplasmic reticulum and sarcoplasmic reticulum has been established to be for control of cytoplasmic [Ca^{2+}]. The data obtained in studies on isolated mitochondria suggests that the gradient of Ca^{2+} ions across the mitochondrial inner membrane (in:out) would normally only be about 2-3 and that the calcium uniporter only has a low affinity (apparent K_a 10 -20 μM) for Ca^{2+} (Scarpa and Graziotti, 1973) and therefore does not have the capacity to buffer Ca^{2+}. Hence, mitochondria are unlikely to play any significant role in buffering the cytoplasmic Ca^{2+} concentration at normal physiological levels (Denton and McCormack, 1985; Malmstrom and Carafoli, 1977; Somlyo et al., 1985; Somlyo, 1985).

It now appears that buffering behaviour will only be exhibited at concentrations of Ca^{2+} where Ca^{2+}-sensitive processes in both the cytoplasm and the mitochondrial matrix would be saturated (Denton and McCormack, 1985). Under excessive Ca^{2+} influx into cells, e.g. after ischaemia and reperfusion in the heart (Bourdillon and Poole-Wilson, 1981), the Ca^{2+} buffering behaviour of mitochondria may play an important protective role to restrict increases in cytoplasmic $[Ca^{2+}]$. However, it is important to realise that derangement of mitochondrial function can also result at very large loads of excessive amounts of Ca^{2+} (McCormack 1985). Matrix loading occurs when Pi is present. Then a lot of Ca^{2+} is taken up, is precipitated with Pi and becomes visible as granular deposits in the electron microscope (Greenawalt et al., 1964).

Calcium as an activator of Mitochondrial Enzymes

Mitochondria are the most important energy producers within the cell and as such need to be able to respond rapidly to changing energy demands. There are a number of mechanisms by which $[Ca^{2+}]$ may help to effect this control on mitochondria. There is evidence that $[Ca^{2+}]$ causes volume changes within mitochondria which affects the activities of certain enzymes. Alternatively $[Ca^{2+}]$ interacts directly with enzymes to cause the activity to be changed. Of special importance in this context is the effect of $[Ca^{2+}]$ on the Krebs cycle.

Calcium effect on mitochondrial volume changes and the effect on enzyme activity

Increases in the intramitochondrial concentration of Ca^{2+} may be able to influence oxidative metabolism through mitochondrial volume changes. Net fluxes of Ca^{2+} are paralleled by mitochondrial volume changes as shown in rat liver mitochondria (Izzard and Tedeschi, 1970; Azzi and Azzone, 1967; Tupper and Tedeschi, 1967; Rottenberg and Solomaon, 1969; Massari

et al., 1972; Chavez et al., 1977). In the liver, glucagon and the Ca^{2+} mobilizing hormones increase respiratory chain activity (Yamazake, 1975; Halestrap et al., 1983). Halestrap et al. (1983) have shown that the increase occurs with an increase in mitochondrial volume (Quinlan et al.,1983; Halestrap et al., 1986). They have also found that the rate of fatty acid oxidation is sensitive to changes in mitochondrial volume over the range likely to occur physiologically (Halestrap and Dunlop, 1986; Halestrap, 1987) suggesting that $[Ca^{2+}]$ could influence fatty acid oxidation.

Krebs Cycle

There are two key rate limiting enzymes within the Krebs cycle and pyruvate dehydrogenase, which contributes acetyl CoA to the cycle, that catalyse irreversible decarboxylation steps. The enzymes are pyruvate dehydrogenase, NAD-dependent isocitrate dehydrogenase and oxoglutarate dehydrogenase. The hypothesis that calcium ions stimulate intramitochondrial oxidative metabolism including the citric acid cycle and thus actively promote the synthesis of ATP was first put forward in 1980 (Denton and McCormack) and now this has been shown to be the case for dehydrogenases from a wide range of vertebrate sources including various organs of rat, man, pig, pigeon, frog and trout (McCormack and Denton, 1981; Denton and McCormack, 1980).

All three dehydrogenases are activated by increases in NAD+/NADH and ADP/ATP concentration ratios (McCormack and Denton, 1979; Denton and McCormack, 1980). The effects of $[Ca^{2+}]$ and adenine nucleotides are not independent since the sensitivity of these enzymes to $[Ca^{2+}]$ becomes less as the ADP/ATP ratio diminishes (Denton et al., 1987). The physiological importance of $[Ca^{2+}]$ to these enzymes is that the effects occur within the range of $[Ca^{2+}]$ expected to occur within the mitochondria. By increasing the activities of the enzymes without a need for NAD^+/NADH or ATP/ADP

ratios to change and thus affect the activities of the enzymes, the mitochondria can respond to increases in energy demands rapidly.

Pyruvate Dehydrogenase Complex

The first calcium sensitive dehydrogenase to be recognized was the pyruvate dehydrogenase complex (Denton et al., 1972; Cooper et al., 1974). This enzyme complex is regulated both by endproduct inhibition and by reversible phosphorylation of its α-subunits. The active, non-phosphorylated form and the inactive, phosphorylated form are interconverted by a specific ATP-linked kinase and a Mg^{2+}-requiring phosphatase. $[Ca^{2+}]$ increases pyruvate dehydrogenase activity by both stimulating the phosphatase (Denton et al., 1972) and probably also by inhibiting the kinase (Cooper et. al.,1974). In experiments it was demonstrated that $[Ca^{2+}]$ inhibits pyruvate dehydrogenase kinase in permeabilized rat heart mitochondria by up to 50% at low concentrations of Mg^{2+} (Rutter et al., 1987). The concentration of Ca^{2+} which gives half-maximal effect ($K_{0.5}$) on the phosphatase was found to be about 0.4 μM at subsaturating levels of Mg^{2+} within permeabilized mitochondria. One of the actions of Ca^{2+} seems to be to decrease the apparent K_m for phosphorylated pyruvate dehydrogenase. The major effect of $[Ca^{2+}]$ however, is to decrease the phosphatase K_m for Mg^{2+} (pig heart mitochondria: Thomas et al., 1986).

The effect of $[Ca^{2+}]$ or saturating Mg^{2+} seems to be to overcome the inhibitory effects of 2 out of the 3 possible phosphorylated sites on the alpha subunits of pyruvate dehydrogenase. A likely explanation is that the dephosphorylation of site 1 is inhibited until the dephosphorylation of sites 2 and 3 is completed. The effect of $[Ca^{2+}]$ or high concentrations of Mg^{2+} may be to specifically increase the dephosphorylation of sites 2 and 3 (Thomas and Denton, 1986; Midgley et al., 1987).

NAD+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase

Both NAD+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase were also found to be activated by $[Ca^{2+}]$. (Denton et al.,1978; McCormack

and Denton, 1979). The effects of [Ca^{2+}] are directly on the dehydrogenases themselves and the principal effect is to lower the K_m values for their respective substrates, isocitrate and oxoglutarate. As with the pyruvate dehydrogenase system, the activating effects of [Ca^{2+}] were found in the 0.1-10 μM range (Rutter et al., 1987).

Studies on the Krebs Ca^{2+}-sensitive Dehydrogenases using isolated Mitochondria

Studies have been carried out to explore the calcium sensitivity of the dehydrogenases when they are located within mitochondria using rat heart (Denton et al., 1980) and adipose tissue mitochondria (McCormack and Denton 1980; Marshall et al., 1984) and a wide range of other rat tissues including liver (McCormack, 1985), brain (Hansford and Castro, 1985), skeletal muscle (Fuller and Randle, 1984), and kidney (McCormack and Bromidge (1987). In all cases, it has been found that, in coupled mitochondria incubated in media containing physiological (saturating: Denton et. al. 1980) concentrations of Na^+ and Mg^{2+} (around 10 mM NaCl and 1 mM $MgCl_2$) which promote Ca^{2+} egress from mitochondria and inhibit Ca^{2+} uptake respectively, increases in the extramitochondrial concentration of Ca^{2+} of 0.05-3 μM, cause activations of, at least, pyruvate dehydrogenase and oxoglutarate dehydrogenase. Half-maximal effects are seen at 0.3 to 0.6 μM Ca^{2+}.

The study of the activity of NAD+-isocitrate dehydrogenase within intact mitochondria is more difficult than that of pyruvate dehydrogenase and oxoglutarate dehydrogenase (Denton et a., 1987). However, in adipose tissue (McCormack and Denton, 1980; Marshall et al., 1984) and liver (McCormack, 1985) it has proved possible to obtain evidence with intact mitochondrial preparations that NAD+-isocitrate dehydrogenase responses are similar to those of pyruvate dehydrogenase and oxoglutarate dehydrogenase. On the other hand there is evidence that NAD-isocitrate dehydrogenase may be less sensitive than the pyruvate dehydrogenase system

or oxoglutarate dehydrogenase (Denton et al., 1987).

Despite this evidence for a role for [Ca^{2+}] in activating Krebs cycle dehydrogenases within mitochondria, there is now some research which shows that the function of cytoplasmic [Ca^{2+}] may be to increase levels of Mg^{2+} within mitochondria. It has been shown that increases in cytoplasmic Ca^{2+} does not necessarily lead to increased [Ca^{2+}] and that Mg^{2+} levels are important to the control of pyruvate dehydrogenase Somlyo et al., 1988).

Other Ca^{2+}-Sensitive Intramitochondrial Enzymes

Branched-Chain α-ketoacid dehydrogenase complex

This dehydrogenase is regulated by phosphorylation (Patel and Olson, 1982; Paxton and Harris, 1982). Patel and Olson (1982) showed that [Ca^{2+}] and Mg^{2+} together result in active branched-chain α-ketoacid dehydrogenase in respiring rat liver mitochondria. From this study it is not known if the action is via a phosphatase or a kinase, nor is it known if these effects are within the physiological range since 2 mM $CaCl_2$ was used in the presence of the ionophore A23187. There is however other evidence that [Ca^{2+}] inhibits the phosphorylation of purified branched-chain α-ketoacid dehydrogenase from liver with a K_a for Ca^{2+} of 0.08-0.18 μM (Paxton and Harris, 1982).

Intramitochondrial Ca^{2+} changes by hormones and the effect on enzyme activity

For the hormones which act through a calcium dependent mechanism, there are a number of ways in which the hormones can act. The action may be to increase cytoplasmic Ca^{2+}, which will then be transported into the mitochondria. The effect may be directly on the Ca^{2+} transport system itself. That is the calcium influx is stimulated by the hormone, or that efflux is decreased by the hormones.

Adrenaline and other positive inotropic agents acting on rat heart

and α-adrenergic agonists, vasopressin and glucagon acting on rat liver are linked to the turnover of IP3 and to Ca^{2+} gating in the plasma membrane in many tissues (Benfey, 1982), oxygen uptake, flux through the citrate cycle, and the proportion of pyruvate dehydrogenase in its active, nonphosphorylated form (Marban et al., 1980; Randle et al., 1979; Exton, 1986; Quinlan and Halestrap, 1986). In the liver, the hormones cause activation of oxoglutarate dehydrogenase, without a decreases in ATP/ADP and NADH/NAD+ ratios.

It was found that α 1-adrenergic agonists did not affect respiration in heart mitochondria, suggesting that the effect of the hormones is on the uniporter, the activity of which was found to be increased by 40% (Kessar and Crompton, 1981; Crompton et al., 1983).

Adrenaline was shown to increase the rate of Ca^{2+} uptake by rat heart mitochondria (Friedman et al., 1980; Kessar and Crompton, 1981; Horn et al., 1971). Administration of adrenaline to perfused rat heart results in a 3- to 4-fold increase in the active form of pyruvate dehydrogenase, and this activation may be reproduced using methods that are known to increase Ca^{2+} concentrations in mitochondria, and throughout the cells (Hiraoka et al., 1980; McCormack and Denton, 1981). The effects of high extracellular [Ca^{2+}] on pyruvate dehydrogenase are prevented by ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake (McCormack and England, 1983). Na^+, which activates the efflux of Ca^{2+} by the Na^+/Ca^{2+} exchanger prevents the activation (McCormack and Denton, 1984). It was also found that $^{45}Ca^{2+}$ was incorporated into the mitochondria as a result of treatment with these hormones (McCormack and Denton, 1984). These data are interpreted to mean that dehydrogenase activation by α 1-adrenergic hormones is by raised matrix [Ca^{2+}].

Most researchers report that α 1-adrenergic effect is not on the Na^+-dependent and Na^+-independent rates of Ca^{2+} efflux from heart mitochondria (Crompton et al., 1983). Conflicting results on the effect of α 1-adrenergics on liver mitochondrial Na^+-Ca^{2+} carrier have been

reported (Goldstone et al., 1983; Coll et al., 1982; Taylor et al., 1980).

Contrary to the research by Altin and Bygrave (1986) and by Assimacopoulos-Jeannet et al. (1986), Somlyo et al. (1988) states that vasopressin and glucagon increases cytoplasmic [Ca^{2+}] but does not significantly affect levels of mitochondrial Ca^{2+} within rat hepatocytes (Bond et al., 1987). Somlyo et al. (1988) suggest that the previous results may have been due to contamination of the mitochondrial preparations with endoplasmic reticulum or have been a heterogenous mitochondrial population (Somlyo et al., 1978) or that the isolation of the perfused rat livers caused abnormal elevation of [Ca^{2+}]. These hormones were found to increase Mg^{2+} within mitochondria. This raised the possibility that physiologically pyruvate dehydrogenase may be activated by Mg^{2+}, at least in some cases, such as when hormonal induced cytoplasmic [Ca^{2+}] increases do not lead to increased mitochondrial [Ca^{2+}] (Somlyo et al., 1988). This evidence may suggest that [Ca^{2+}] may play a smaller role in Krebs cycle activation than once thought.

Calcium Binding ATPase Inhibitor Protein

The Ca^{2+} binding inhibitor (CaBI) is a protein that is found within mitochondria. The function of CaBI is to prevent ATP hydrolysis within the matrix of mitochondria (Yamada et al., 1980). CaBI inhibition of ATPase function is dependent on Ca^{2+} concentrations within the mitochondria. At low [Ca^{2+}] (10^{-9} to 10^{-7} M), in the presence of Mg^{2+}, CaBI acts as an inhibitor of ATPase, at intermediate concentrations of Ca^{2+} (10^{-7} to 10^{-5} M), the inhibition by CaBI is reversed and ATP hydrolysis occurs, whereas at higher levels of Ca^{2+} (10^{-5} to 10^{-3} M) inhibition is again seen (Yamada et al., 1981). These studies were done in vitro using an assay of submitochondrial particles, which were stripped of CaBI by treatment with Ca^{2+} (Yamada et al., 1980). From urea-SDS electrophoresis and kinetic studies, it is believed that CaBI has bound Ca^{2+}, is active as an inhibitor

of ATPase function, and is in a dimeric form, at low Ca^{2+} concentrations in the presence of Mg^{2+}; is an inactive monomer at slightly higher Ca^{2+} levels; and again is a dimer with 2 Ca^{2+} bound per dimer at even higher Ca^{2+} levels (Yamada and Huzel, 1985). The monomers are not covalently linked, nor is Mg^{2+} needed except at low Ca^{2+} concentrations. This hypothesis is reflected by SDS polyacrylamide electrophoresis of the purified protein when Ca^{2+} levels are varied. The protein has a molecular weight of 6300 daltons at 1 μM Ca^{2+} with 1 mM Mg^{2+}, and at 1 mM Ca^{2+} or 0.1 μM Ca^{2+} with 1 mM Mg^{2+} the molecular weight is 12,500 (Yamada et al., 1981). The protein has been shown to have three high affinity binding constants for Ca^{2+} with a maximum of 1 mol of Ca^{2+} bound/mol of protein monomer. Magnesium does not affect the first association constant but increases the second and third by about 1.2 and 1.5 respectively. The association constants are 4.87 +/- 0.24 μM, 16.9 +/- 0.42 μM and 62.2 +/- 2.8 μM (bovine) (Yamada and Huzel, 1985).

The model for the interaction of Ca^{2+} with CaBI is that at low concentrations of Ca^{2+} (10^{-7} M) in the presence of 1 mM Mg+, CaBI exists as a dimer with bound Mg^{+}, but without any bound Ca^{2+}, and is active as an inhibitor. As Ca^{2+} levels increase the CaBI monomerizes, which causes it to lose its activity and Ca^{2+} binds to the monomer. However this complex is in equilibrium with the monomer and as Ca^{2+} levels increase the monomer complexes with other monomers and Ca^{2+} to form a dimer with 1 Ca^{2+} bound to it. As Ca^{2+} levels increase even further, a second Ca^{2+} binds to the dimer allowing it to once more be active as an inhibitor of ATPase function (Yamada and Huzel, 1985).

It is believed that CaBI is important to regulating the use of ATP for the uptake of Ca^{2+} by mitochondria (Yamada and Huzel, 1988). [Ca^{2+}] has been shown to be important for control of the activity of several mitochondrial matrix enzymes (discussed in section on Krebs cycle enzymes). CaBI may be important for controlling [Ca^{2+}] within narrow limits so that activities of Ca^{2+}-dependent enzymes are regulated. The

levels of Mg^{2+} and Ca^{2+} that are required for dimerization of CaBI and the narrow range of Ca^{2+} levels under which CaBI undergoes changes from being active as an inhibitor to being inactive and back to being an inhibitor are within physiological levels. In fact there is good evidence that these changes in Ca^{2+} levels do occur within mitochondria (discussed under Krebs cycle).

It is not known if there is a human CaBI, however, it is expected that CaBI will be found in all mammalian tissues. It appears to be well conserved between bovine to rat. CaBI has been isolated from bovine cardiac tissue and from rat skeletal muscle and either protein has been shown to be cross reactive to Ca^{2+}-stripped A particles from either species (Yamada and Huzel, 1988). The function of CaBI as a regulator of $[Ca^{2+}]$ would also argue for conservation of the protein, since $[Ca^{2+}]$ has been shown to be important in all mammalian mitochondria studied to date.

The amino acid composition of CaBI is known from Yamada and Huzel (1988) and is shown in Table I. The protein is acidic with a pI of 4.6 (Yamada and Huzel, 1988). It was first isolated from submitochondrial particles by treatment with 0.085 nmol of Ca^{2+} per μg of protein (Yamada et al., 1979). The amino acid sequence of CaBI is not known due to a blocked amino-terminal.

Pullman-Monroy ATPase Inhibitor Protein (PMI)

PMI is the classical ATPase inhibitor of mitochondria. It was first isolated from bovine heart mitochondria by Pullman and Monroy (1963). It inhibits ATPase activity of soluble and membrane bound mitochondrial ATPase and is considered to be a regulatory component of the ATP synthesizing complex in mitochondria. It has been shown to be distinct from CaBI by Yamada and Huzel (1988). Bovine heart PMI is not affected by $[Ca^{2+}]$, has a different pI (8.1), is unreactive to CaBI antibody, is larger (9600 daltons), and has a different amino acid composition (Yamada and Huzel, 1988).

Aim of Research

It was the purpose of this experiment to isolate the gene for CaBI from a lambda gt11 cDNA library using CaBI antibody which was available. The gene was to be sequenced so that the amino acid could be elucidated from the codon sequence. The clone would also allow transcription levels to be determined for CaBI mRNA. This would lay the groundwork so that control of transcription could be examined.

This research in fact did not isolate the CaBI gene, although a ribosomal S6 cDNA clone was isolated and sequenced from 2 lambda gt11 libraries (human and rat heart). Since identification of the clones was based on antigenicity of the protein, this indicated an interaction of the S6 protein with the CaBI antibody. This fortuitous result was pursued further, by competition assays to find out if the interaction of S6 with CaBI antibody was indeed specific and also, research since this project was completed investigated possible reasons why the S6 clone would be epitopic to CaBI, for instance due to shared function, such as Ca^{2+} binding by S6.

Methods

Materials

Amersham

- L-[^{35}S] cysteine
- deoxyadenosine 5'-α [^{35}S] thiotriphosphate
- α-[^{32}P] deoxycytosine
- Nick translation kit for DNA
- [^{125}I]-Protein A labelled with Bolton and Hunter reagent

Beckman

- Quickseal polyallomer ultracentrifuge tubes

Bethesda Research Laboratories

- Agarose
- ^{14}C-labelled Protein Standards

Bio-Rad

- acrylamide
- CM Affi-gel Blue filtration media
- eppendorf pipette tips and tubes
- Klenow DNA sequencing kit
- 82 mm round nitrocellulose 0.45 μm filters
- 3 mm Whatman paper

Boehringer-Mannheim

- calf intestinal alkaline phosphatase
- chicken egg-white lysozyme
- EcoR I restriction enzyme
- Hae III digest of phi X 174 (DNA markers)
- HinD III digest of lambda gt11 DNA (DNA markers)
- proteinase K
- T4 DNA ligase

British Drug House

- agar
- tryptone

yeast extract

Fisher

Nitroplus 2000 transfer membrane

phenol

sterile 5 and 10 ml disposable pipettes

sterile petri plates

Miles Scientific

Trasylol

Pharmacia

gel filtration medium G-25

Promega

BamH I restriction enzyme

Hind III restriction enzyme

Pst I restriction enzyme

Sal I restriction enzyme

Sigma

ampicillin

chloramphenicol

chloroform

fetal bovine serum

IPTG

Para-aminophenyl ß-D thiogalactoside (TPEG) linked to 4% agarose

PEG

Terrochem

Pansorbin

All other materials were from Sigma.

United States Biochemical Corporation

Sequenase DNA sequencing kit

Growth media and Buffers used:

1x Denhardt's: 0.02% w/v each of bovine serum albumin (Pentax Fraction

V), Ficoll and polyvinylpyrrolidine (Sigma)

Gel Running Buffer: (GRB) 0.04 M MOPS pH 7, 0.01 M sodium acetate, 0.001 M EDTA

Luria-Bertani Medium (LB: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.5, sterile)

LB Medium with Agar/Agarose: 1.5% (w/v) Bacto-tryptone (plates) or 0.7% (w/v) Agarose (top agarose)

SM: 0.1 M NaCl, 0.8 mM $MgSO_4.7H_2O$, 50 mM Tris.Cl (pH 7.5), 0.01% gelatin

20 x SSC: 3 M NaCl, 0.3 M Na Citrate, pH 7.0 with HCl

1x SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA

20 X SSPE: 3 M NaCl, 0.02 M EDTA, 0.2 M $NaH_2PO_4.H_2O$ pH 7.4

TE buffer: 10 mM Tris.Cl, 1 mM EDTA

Distilled, deionized water was used throughout. For all culture and DNA or RNA experiments, autoclaved or filter sterilized media and buffers were used.

Methods

Ethanol Precipitation of DNA/RNA

To precipitate RNA or DNA, 3 M sodium acetate was added to the sample to make it 0.2 M. By increasing the salt concentration in this way a better precipitation occurred. Then 2 volumes of ethanol at -20° C. was added for a DNA precipitation or 2.2 volumes for RNA. The sample was precipitated overnight at -20° C and then pelleted by centrifuging at 11000 rpm for 30 minutes in an SS34 (Sorvall) rotor or if the sample was 1.5 ml or less, it was centrifuged in a microfuge for 10 minutes. The pellet was washed with cold 70% ethanol, respun and then briefly dried in a speed vacuum for 3-5 minutes.

Isopropanol Precipitation of DNA

Isopropanol (0.6 volumes) was added to the sample which was then vortexed and allowed to sit at room temperature for 15 minutes. The sample was centrifuged at 20° C. for 30 minutes at 6000 xg. The pellet was then washed with 70% ethanol and dried in a speed vacuum.

Phenol and Chloroform Extractions of Nucleic Acids

Phenol was buffered with TE buffer so that the pH of the aqueous overlay was greater than 7.5. Chloroform had 1/25 (v/v) of isoamyl alcohol added. The sample was extracted in sequence by phenol, chloroform/phenol (1/1) and by chloroform. An equal volume of the organic solvent was added to the sample. The mixture was vortexed and centrifuged to separate the organic from the aqueous phase.

Determination of DNA in sample

Sample (2 µl) was added to 1/2 ml of TE and the optical density at 260 nm was measured against TE as a blank (Bausch and Lomb Spectronic 2000). The A_{260} X 12.5 = DNA mg/ml (Ausubel et al.,1989).

Restriction Digests of DNA

The Promega restriction enzymes: Pst I, BamH I, Sal I and Hind III were supplied with restriction buffer, and these enzymes were used according to the instructions. EcoR I was used in high salt buffer (100 mM NaCl, 50 mM Tris.Cl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol). The amount of restriction enzyme used was typically 5-10 units with 1-5 µg of DNA.

DNA gel electrophoresis

DNA electrophoresis was done on a 1% agarose gel with TBE (0.09 M Tris, 0.09 M Borate, 2 mM EDTA) as the buffer, on a Bio-Rad mini-gel. High molecular weight standards were HinD III digest of lambda DNA (23,130; 9416; 6557; 4361; 2322; 2027 base pairs), low molecular weight

standards were Hae III digest of phi X 174 DNA (1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118; 72 base pairs). High and low molecular markers was applied at 1 μg each. The buffer of the standard was adjusted to that of the sample so that any changes in the mobility of the sample due to salt concentration would be the same for the standards. The DNA samples were loaded onto the gel in 5% glycerol (v/v) and 0.01% bromophenol blue (w/v) as the tracking dye. Electrophoresis was done at 100 volts for about 1 hour until the tracking dye was 2 cm from the lower edge of the gel.

Preparation of Antibody

CaBI was purified as described by Yamada and Huzel (1988). Preparation of antibody was as described earlier (Yamada and Huzel, 1988). CaBI (10 μg) in PBS and complete Freund's adjuvant was injected subcutaneously into 4 sites in a rabbit. A second immunization was done with incomplete Freund's adjuvant, and the third and fourth were done with CaBI only. These were all done at 2 week intervals. Antibody was collected 2 weeks after this. It was heat-inactivated at 50° C. for 30 minutes. Immunoglobulins were precipitated with ammonium sulphate and then 20% ethanol. The serum was dialysed against PBS and applied to a CM Affi-gel Blue (Bio-Rad) column (1.5 X 10 cm) at a flow rate of 0.5 ml/minute. The effluent was collected and the immunoglobulins were precipitated with ammonium sulphate, dissolved in PBS, and dialysed against PBS. Protein concentration at this time was 31 mg/ml. A 1/1000 dilution of this preparation of antibody (31 μg/ml of protein) was able to detect as little as 0.78 ng of CaBI, in the presence or absence of 1 mg/ml of E. coli lysate. The antibody was unreactive to calmodulin and oncomoculin (Yamada and Huzel, 1988).

Immunoscreening of Lambda gt11 libraries

Two lambda gt11 cDNA libraries were screened for clones

immunoreactive to the anti-CaBI serum. One library was prepared from the human breast cancer cell line T47D (Murphy et al., 1987). The other, a rat heart library was a commercial preparation obtained from Clontech. In the case of each library, the cDNA was cloned into the gene for ß-galactosidase. This gene is inducible with IPTG and since the cDNA was cloned into the coding region of the gene, the resultant protein was a chimeric protein of the ß-galactosidase and the library protein. Production of this chimeric protein can be induced in phage infected hosts with IPTG and is the basis for induction of expression of the inserted sequence.

Immunoscreening was done according to the method of Young and Davis (1985). Lambda gt11 (10^4 plaque forming units) was added in 100 µl of SM Buffer to 200 µl of an overnight culture of Y1090 in LB with 0.2% (w/v) maltose and 10 mM $MgSO_4$. The maltose induces the phage receptor in the culture, and the Mg^{++} is needed for attachment of the phage. Adsorption was accomplished by incubating for 20 minutes at 37° C. After this 3 ml of molten LB with 10 mM $MgSO_4$ at 60°C. was added to each tube and they were quickly poured and spread on 85 mm LB (with 0.2% glucose) plates.

The plates were incubated at 42°C. for 3 1/2 hours and then overlaid with nitrocellulose filters which have previously been soaked in 10 mM IPTG and allowed to air dry. The plates were then incubated at 37° C. for a further 3 1/2 hours.

After this incubation the filters were marked so that they could be reoriented on the LB plates and then removed from the plates. Nonspecific binding of proteins was prevented by blocking for 30 minutes in 20% Fetal Bovine Serum (v/v) in TBST. The nitrocellulose filters were screened either with ^{125}I protein A labelled second antibody again using the method in Young and Davis (1985) or using Promega Goat-Anti-rabbit conjugated to alkaline phosphatase as the second antibody. With the ^{125}I labelling method, the first antibody was diluted to 1:100 in TBST (Tris-buffered Saline Tween 20: 50 mM Tris.Cl pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween

20). The nonspecific interactions with E. coli proteins was reduced by reusing the antibody up to 10 times. When the Promega kit was used the buffers were according to the Promega instructions. Promega TBST is 10 mM Tris.Cl pH 8.0, 150 mM NaCl, 0.05% Tween 20. The antibody was diluted to 1:1000 in protoblot TBST containing 1 mg/ml of E. coli BNN 97 lysate. This lysate was prepared according to Young and Davis (1985). The E. coli lysate was added to reduce nonspecific reactions by the antibody to E. coli proteins present.

The nitrocellulose filters were baked for 20 minutes at 80° C., in order to denature any alkaline phosphatase activity which might have been endogenous to the E. coli host. The first antibody in all cases was Rabbit anti-body against CaBI prepared as described. The ^{125}I method of detection was used to isolate 1 positive clone from the T47D library. The Promega kit was used for screening the rat heart library and 3 clones were isolated. This kit was more economical and gave faster results since colour development was done the same day as the screening and did not require the use of radioactivity.

Preparation of lambda gt11 DNA

Preparation of lambda gt11 DNA was done based on the method by Yamamoto et al. (1970). Plaque purified clones were plated out on 10 LB (with 0.2% glucose) plates at a density of 10^4 plaques per plate, and incubated overnight at 37° C. The phage were eluted into 5 ml SM buffer (with an additional 1 ml to wash the plates) by gentle shaking for 2 hours at room temperature. The eluent was collected and pooled. Cell debris was removed by centrifuging at 4° C. for 30 minutes, at 1500 xg in a SS34 (Sorvall) rotor.

The supernatant was incubated at 37° C. for 1 hour in the presence of 1 µg/ml of RNase and 1 µg/ml of DNase. The centrifugation was repeated. The supernatant was made 1 M NaCl and then 10% (w/v) polyethylene glycerol (PEG). It was incubated at 4° C. for 1 hour with

gentle shaking.

The sample was recentrifuged. The pellet was resuspended in 5 ml of SM, 0.1% SDS and 0.2 mg/ml Proteinase K. After a gentle vortex it was incubated for 1 hour at 65° C. Then 50 µl of 0.5 M EDTA was added and the incubation was continued for 30 minutes. This was followed by sequential extractions with phenol, phenol/chloroform and chloroform. The sample was then ethanol precipitated. Enough TE was added to the pellet to just dissolve it.

Ligation conditions

Ligation of an insert into a plasmid was done using 25 ng/µl of insert and 5 ng/µl cut dephosphorylated plasmid, in the presence of 1 X Ligation Buffer (0.05 M Tris.Cl pH 7.4, 0.01 M $MgCl_2$, 0.01 M Dithiothreitol, 1 mM ATP) and 0.1 units/µl T4 DNA ligase. This was then placed overnight at 15° C. The next day it was ready for transformation of a host. Dephosphorylation was used to remove 5' phosphate groups from restricted plasmids. This prevented the plasmids from religating to themselves without an insert being present since the ligation reaction requires the presence of a 5' phosphate group.

Preparation of Competent Cells

The strain of cells used was RR_1 or DH5-α for plasmid transformations. The preparation of competent cells was originally described by Mandel and Higa (1970). The method used for preparation of competent cells was based on Hanahan, (1983).

Cells grown to an A_{600} of 0.4-0.5 were chilled on ice for 10 minutes. The cells were centrifuged at 1500 xg in an SS34 (Sorvall) rotor for 10 minutes. The cell pellets were resuspended in 1/5 of the original volume in ice-cold 50 mM $CaCl_2$, 10 mM Tris.Cl pH 8. The cells were incubated on ice for 20 minutes and again pelleted. The cells were resuspended in 1/25 the original volume of 50 mM $CaCl_2$, 10 mM Tris.Cl pH 8. The cells were

aged on ice for 12-24 hours to cause them to be more competent.

Transformation of Competent Cells

Transformation of competent cells is described by Miller (1987). To 200 µl of competent cells 10-20 ng of DNA in Ligation Buffer was added. This was incubated on ice for 40 minutes. The cells were heat shocked for 3 minutes at 42° C. Then 0.2 ml of LB was added and the cells were incubated at 37° C. for 30-60 minutes. The cells were spread on agar plate with appropriate antibiotics and incubated overnight at 37° C.

Small scale preparation of plasmid DNA

An overnight culture of the strain containing the plasmid of interest was grown in LB containing the appropriate antibiotic. A 1.5 ml cell culture was pelleted by centrifugation for 1 minute in a microfuge. The pellet was resuspended in 100 µl ice cold buffer (0.9% (w/v) glucose, 10 mM EDTA, 25 mM Tris.Cl pH 8, 100% (w/v) lysozyme) and left at room temperature for 5 minutes. To this was added 200 µl freshly prepared 0.2 N NaOH, 1% SDS, and the sample was mixed by inverting 2-3 times, and stored on ice for 5 minutes. Then 150 µl of ice cold 3 M K acetate (pH 4.5) was added. The tube was capped and vortexed gently in an inverted position for 10 seconds, and stored on ice for 5 minutes. The sample was centrifuged for 5 minutes at 4° C. in a microfuge. The supernatant was extracted with phenol-chloroform (50/50, v/v), followed by a chloroform extraction. Ethanol precipitation was done at room temp for 2 minutes. The DNA was centrifuged at room temperature for 5 minutes and the pellet was washed with 1 ml of 70% ethanol. The sample was dried in a speed vacuum, dissolved in 15 µl of TE and 1 µl of 1 mg/ml DNase free RNase was added. This method was used when only small quantities of plasmid DNA were needed, for example when it was necessary to determine which colonies had been transformed by a plasmid of interest.

Large Scale Plasmid Preparation and Purification

A 500 ml culture of bacteria with the plasmid of interest was grown at 37° C. in LB with the appropriate antibiotic to an A_{260} greater than 0.6. Then 2.5 ml of chloramphenicol (4 mg/ml) was added, and incubation was continued at 200-250 rpm for approximately 16 hours. The next day the flask was placed on ice and the cells were harvested by centrifugation at 4° C., in a GSA (Sorvall) rotor at 10000 xg for 10 minutes.

The cell pellet was resuspended in 9.5 ml of ice cold lysis buffer (25 mM Tris.Cl pH 8, 10 mM EDTA, 0.9% glucose (w/v)). Ice cold (0.5 ml) lysozyme solution (20 mg/ml chicken egg-white lysozyme in lysis buffer) was added. The tubes were mixed by inversion, placed horizontically on ice and shaken gently for 30 minutes. The time of this step was increased if the suspension remained cloudy. Ten ml of freshly prepared NaOH/SDS (0.2 M NaOH, 4.0 ml 5 M NaOH, 0.2% SDS) was added and the tubes were mixed by inversion and incubated on ice for 10 minutes, with gentle shaking. 10 ml of 3 M NaAc was added, mixed by inversion and incubated a further 30 minutes on ice with gentle shaking.

Debris was removed by centrifugation at 15,000 rpm for 30 minutes at 4° C. The supernatant was precipitated with isopropanol. Each of the 2 pellets was dissolved in 3 ml of TE prewarmed to 65° C. Cesium Chloride (6.6 g) was added to the sample (6 ml). The sample was placed in a 13 ml polyallomer tube and 0.5 ml of 10 mg/ml ethidium bromide was layered on top. The tube was mixed by inversion after it had been placed in the rotor. Centrifugation was done in a Ti 70.1 (Beckman) rotor at 200000 xg at 22° C., 16 hours. Then the speed was reduced to 150000 xg for 45-60 minutes before stopping the rotor. The plasmid band was removed. The sample size at this point was approximately 1 ml. The ethidium bromide was extracted from the sample using up to 7 isoamyl alcohol extractions with additions of water to make up for the water lost to the isoamyl alcohol. The sample was made up to 4 ml using water, ethanol precipitated and dissolved in 100 µl of TE (pH 8). The DNA was stored at -20° C.

Preparation of DNA insert

The plasmid (80 μg) containing the insert of interest was digested with the appropriate restriction enzyme. The sample was electrophoresed to separate the insert from the plasmid. on a 1% agarose gel containing 5 μg/ml of ethidium bromide. Since ethidium bromide was present the DNA could be visualized with a UV lamp. When the insert was separated from the plasmid DNA, the band containing the insert was cut out of the gel and eluted. Elution was done electrophoretically. The band containing the insert of interest was cut from the gel and the DNA was eluted into a well containing TBE and lined by dialysis tubing which had been soaked in 1% (w/v) EDTA for 20 minutes, washed several times in dd water and then left in TBE until needed. The sample was extracted with equal volumes of isoamyl alcohol until the sample was reduced to 1 ml. The isoamyl alcohol both removes the aqueous phase and ethidium bromide which would cause strand breakage of the DNA. The DNA was ethanol precipitated and then resuspended in 100 μl TE.

Preparation of [^{32}P]-labelled DNA

The instructions from the Nick Translation kit were followed and the enzyme used was DNA polymerase I. Unless otherwise stated 200 ng of DNA insert was used. The source of radioactive [^{32}P] was 7 μl of α-[^{32}P]-dCTP (about 3000 μCi/mmol). Size fractionation of the [^{32}P]-labelled DNA was done by filtering through Sephadex G25 medium (TE was used as the buffer) to rid the sample of unincorporated nucleotides.

RNA Preparation

RNA preparation was done based on the method by Chirgwin et al. (1979). The tissue was harvested rapidly from the animal and frozen. The frozen tissue was pulverized and then 20 ml of 4 M GuSCN (4 M Guanidine Thiocyanate 0.5% (w/v) Sodium Lauroylsarcosine, 0.025 M Na Citrate, 0.3%

(v/v) ß-mercaptoethanol pH 7 with NaOH, sterile) was added. The tissue was homogenized in a polytron (PT-10 generator) for 1 minute and debris was removed by centrifuging in a table top centrifuge for 15 minutes. The supernatant was brought to 25 ml with 4 M GuSCN and DNA was sheared by drawing the sample into a syringe with a 16 gauge needle several times. The sample was layered onto 14 ml of CsCl (5.7 M Cesium Chloride pH 7.5, 0.05 M EDTA, sterile) in a Quick-Seal centrifuge tube. Centrifugation was done for 24 hours at 80000 xg, 22° C. At the end of this time the RNA pellet was dissolved in 2 ml of TE prewarmed to 65° C. The sample was made 0.2 M with sodium acetate and ethanol precipitated overnight at -22° C. with 2.2 volumes of ethanol. The RNA was pelleted by centrifugation at 15000 xg for 10 minutes in a SS34 (Sorvall) rotor. The pellet was redissolved in just enough TE to dissolve it (1-2 ml). This RNA was ready for preparing poly (A)+ RNA.

Selection of poly (A+) RNA was based on the method by Aviv and Leder (1972). Poly (A)+ RNA was prepared using an oligo-dT cellulose column which has been prewashed with NaOH/EDTA wash (0.1 M NaOH, 5 mM EDTA), water and 1 X loading buffer (20 mM Tris.Cl pH 7.5, 0.5 M NaCl, 1 mM EDTA). The denatured sample (3 minutes, 100° C.) in 1 X loading buffer was loaded and the column was washed with 1 X loading buffer to remove poly (A)- RNA until the A_{254} returns to 0. The poly (A)+ RNA was eluted using elution solution (1 mM EDTA pH 7.5, prewarmed to 65° C.) again until the A_{254} returns to 0. The RNA was ethanol precipitated. The sample was redissolved in 25 µl of TE.

DNA hybridization of RNA blots

RNA was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, 0.5 X GRB; and electrophoresed on a 1% (w/v) agarose gel, 2.2 M formaldehyde, 1 X GRB, 0.0005% (w/v) ethidium bromide to size fractionate the RNA species (Alwine et al., 1977). The samples were loaded onto the gel, which was placed in but not immersed by 1 X GRB and run into the gel at

120 volts for 25 minutes and then the gel was covered with 1 X GRB. The electrophoresis was done at 35 volts overnight. After this the RNA was blotted onto nitrocellulose (Thomas, 1980). The blotted filters were prehybridized in 7-10 ml of hybridization solution (50% (v/v) deionized formamide, 5X SSPE, 0.1% (w/v) SDS, 0.25 mg/ml denatured salmon sperm DNA, 5X Denhardt's solution) for at least 3 hours at 42° C. to block nonspecific binding sites. The filters were then hybridized with the [^{32}P]-labelled insert (5 X 10^5 cpm) by DNA polymerase I. Hybridizations were done for 2 days at 42° C. in 5-6 ml (0.2 ml/cm^2) of hybridization solution containing [^{32}P]-labelled DNA. At the end of the hybridization period the blots were washed twice in 500 ml of 2 x SSC, 0.1% SDS for 20 minutes at room temperature, followed by one wash in 0.1 X SSC, 0.1% SDS for 45-60 minutes at 65° C., until the background was reduced. Blots were then exposed to Kodak XAR film at - 70° C. with an intensifying screen.

In order to calculate the size in base pairs of the RNA species seen by the probe, the sizes of the 28 S and 18 S rRNA species were used. Despite the use of the oligo dT column to create a sample containing poly (A)+ RNA, enough rRNA was present so that it can be seen as distinct bands on the gel on visualization with ethidium bromide. The position of these bands were noted when the RNA was transferred to the nitrocellulose and since the size of these species are known, the size of the mRNA can be calculated. The size of the human rRNA species present on the gel were 4865 and 2000 base pairs for the 28 S and 18 S rRNAs respectively and of rat rRNA is known to be 4718 and 1874 base pairs respectively (Noller, 1984).

DNA probing of DNA transblots

For probing DNA transblots with [^{32}P]-labelled DNA the DNA was electrophoresed on a TBE, 1% agarose gel. The DNA applied per lane was 0.5 μg bacteriophage DNA or 0.2 μg plasmid DNA. After visualization of

the DNA by ethidium bromide staining, the gel was denatured in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 1 hour, at room temperature on a shaker. The gel was then neutralized in neutralization solution (1 M Tris.Cl pH 8, 1.5 M NaCl) for 1 hour at room temperature with shaking. The DNA was transferred to a nitrocellulose filter overnight using the Southern (1975) procedure.

Transfer of RNA or DNA to nitrocellulose

The buffer used for the transfer of RNA was 10 X SSC, and 20 X SSc for DNA. The paper used to wick the buffer from the reservoir to the gel was 3 mm CHR Whatman paper. The nitrocellulose membrane was prewet in water for RNA transfer or 2 X SSC for DNA transfer. The gel was laid upside down on the prewet Whatman paper wick followed by the Nitroplus 2000 nitrocellulose membrane, 3 layers of Whatman 3 mm CHR paper and a stack of paper towels. After the transfer, the membrane was soaked in 6 X SSC for 5 minutes for the DNA transfer. The nitrocellulose was air dried for 1 hour and then baked in a vacuum oven at 80° C. for 2 hours.

Hybridization of DNA transblots with ^{32}P-labelled DNA

The filter was prewet in 6 X SSC and then sealed into a plastic heat sealable bag. Prehybridization solution (6 X SSC, 0.5% SDS, 5 X Denhardt's Solution, 100 µg/ ml denatured salmon sperm DNA) prewarmed to 65° C. was added at 0.2 ml/cm^2 of nitrocellulose. The filter was preincubated at 65° C. for 2-4 hours in order to block any nonspecific binding sites. The filter was then hybridized with the [^{32}P]-labelled DNA of interest in hybridization solution (6 X SSC, 0.01 M EDTA, 5 X Denhardt's Solution, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA) and incubated at 68° C. The time of hybridization depended on the sample and is given separately for each experiment.

After hybridization was complete the filter was washed, first with 2 X SSC, 0.5% SDS at room temperature for 5 minutes, then with 2 X SSC,

0.9% SDS at room temperature for 15 minutes with agitation. The final wash was at 68° C. with 0.1 X SSC, 0.5% SDS. This wash was also with agitation and continues until the background radiation was reduced. Visualization was done by autoradiography.

Subcloning of insert into M13 phage DNA and isolation of ssDNA

The preparation of ssDNA takes place in two steps: a) the concentration of phage by PEG precipitation; and b) the removal of the viral coat protein by phenol extraction. This procedure was based on the PEG procedure described by Yamamoto et al. (1970). Subcloning of the DNA insert into M13 vectors, preparation of ssDNA and sequencing reactions using the Klenow fragment were done according to directions from Bio-Rad M13 Cloning and Sequencing Kit.

The phage was precipitated with 0.03% PEG/0.36 M NaCL, then 100 µl of TE and 50 µl of phenol was added to the sample and phenol extraction was carried out. The aqueous phase was ethanol precipitated. The sample was dissolved in 50 µl TE, and stored at -20° C. Recovery was 5-10 µg. 5 µl was used per sequencing reaction.

DNA sequencing

Sequencing was done by the dideoxy chain termination method (Sanger, et al., 1977. The enzyme used for synthesis of the DNA was either the Klenow fragment (Jacobsen et al., 1974) or the sequenase enzyme which was a modified bacteriophage T7 DNA polymerase, (Tabor and Richardson, 1987). Each of these enzymes was obtained as a sequencing kit (Materials) and the instructions with the kits were followed. It was found that the sequenase enzyme was better to work with. Sequence differences did not cause uneven labelling of the sample as readily for the sequenase as for the Klenow fragment. Other properties that are useful about the sequenase enzyme are that it is highly processive, has low 3'-5' exonuclease activity and works rapidly. The reaction typically takes 10 minutes.

The label used was deoxyadenosine 5'-α [^{35}S] thiotriphosphate. The DNA sequencing gel was a denaturing gel: 5.9% (w/v) acrylamide, 0.2% bis-acrylamide, 1 X Winter TBE (0.13 M Tris, 0.01 M borate, 0.005 M EDTA, pH 8.8), 0.05% (w/v) ammonium persulfate, 40 M urea, 0.0025% TEMED (v/v). A 24 well shark's tooth comb (Bio-Rad) was used. The gel was prerun in order to heat it to 50-55° C. Gel running conditions were about 1700 volts for 1.25-8 hours depending on the sequence which was to be elucidated. The sequencing apparatus was a Bio-Rad Sequi-Gen® Nucleic Acid Sequencing Cell and the power supply was a model 3000/300 Bio-Rad Power Pack. The gel was fixed for 20-30 minutes in 10% (v/v) methanol, 10 % Acetic Acid (v/v), dried and a film was exposed for 16-18 hours to create an autoradiogram.

Generation of lambda gt11 Recombinant Lysogen in Y1089

The Y1089 strain of E. coli has a temperature sensitive repressor of the lytic cycle. At 32° C. this host, when infected with lambda gt11 will be in a lysogenic state, whereas at 42° C. the repressor is inactive and the phage will enter the lytic cycle. Host cells (Y1089) were grown in LB with 0.2% maltose and ampicillin (150 μg/ml at 37° C. overnight. The Y1089 cells were infected with the lambda gt11 clone isolated from the T47D library at a multiplicity of 5, for 20 minutes at 32° C. in LB medium with 10 mM $MgCl_2$. The phage had previously been titered, and the rule of thumb used for the host was that 1 O.D. 600 was about 8 X 10^8 cells/ml. The cells were plated on LB plates with ampicillin (150 μg/ml) at a density of approximately 200 per plate and incubated at 32° C. overnight. At this temperature, the temperature sensitive repressor was functional. Single colonies were tested for temperature sensitivity at 42° C. This was done by replica plating single colonies onto separate LB plates. One plate was incubated at 32° C. and the other at 42° C. Clones that grew at 32° C. and not at 42° C. were assumed to be lysogens. Lysogens arise at a frequency of 10 to 70%. (Huynh et al., 1986).

Preparation of crude lysate from lambda gt11 recombinant lysogen

The lysogen was incubated at 32° C. in LB until an A_{600} of 0.5 was reached. The temperature of the culture was increased rapidly to 42-45° C. for 20 minutes and then the culture was made 10 mM with IPTG to induce the ß-galactosidase gene. The temperature was reduced to 37° C. for 1 hour and the cells were harvested by centrifugation in a GSA (Sorvall) rotor at 4000 xg for 5 minutes. The cells were rapidly resuspended at 1 gram/ml of lysis buffer (10 mM Tris.Cl pH 7.5, 10 mM magnesium acetate, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT). The cells were frozen to -70° C. and thawed to induce lysis. The sample was sonicated 3 times for 1 minute each at 100 watts. Cell debris was removed by centrifuging at 40000 xg in a 65 Ti (Beckman) rotor for 1 hour.

The chimeric protein was purified further by passing over a 1 ml (1.3 cm height X 1 cm diameter) affinity column of TPEG covalently linked to 4% agarose, equilibrated with lysis buffer. Most of the cellular proteins were eluted with lysis buffer. The bound chimera was eluted with 100 mM Sodium Borate pH 10. This was dialysed against 50% glycerol, 40 mM Tris-Cl pH 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) for 5 hours and then stored at -20° C. This method was done according to the method by Koenen et al., (1986). It was found using this method that degradation of chimera was extensive. When this preparation was electrophoresed varying numbers and sizes of bands were seen, with some gels only having protein at the dye front. The stored sample also formed a precipitate. Later the method by Ullman (1984) was used. The bacterial extract from the above paragraph had NaCl added to make the sample 1.6 M NaCl, was applied to a 1 ml TPEG column which had been equilibrated in the same buffer and column was eluted again with the same buffer at 1.3 ml/minute to rid the sample of most of the nonchimeric proteins. The ß-galactosidase fusion protein was eluted in 100 mM sodium borate, 10 mM ß-mercaptoethanol, pH 10. Both of these methods are based on the method by Steers et al. (1971). The

major advantage of the second method is the use of high salt to increase the capacity of the column for ß-galactosidase and the speed with which the protein is passed over the column. Thus the final sample is purer and of better quality. Using this method the chimeric protein seen on gels was 115 kD (not shown, work done by Dr. E. Yamada and Norm Huzel).

Labelling of lysogenic proteins with [^{35}S]-Cysteine

The lysogen was prepared in the same way as for the preparation of the crude lysogen, until the harvesting step which was done in a SS34 (Sorvall) rotor at 4000 xg for 5 minutes. At this point the cells were washed in M9 salts (0.04 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.002 M NH_4Cl, 0.0084 M NaCl, 1 mM $MgSO_4.7H_2O$, 0.2% glucose) twice. The cells were then resuspended in 1/2 the original volume of M9 salts with 50 µCi/ml of [^{35}S]-Cysteine, and incubated a further 20 minutes at 37° C. to allow the cellular proteins to be labelled. The cells were again pelleted as above. The labelling was done according to Dr. F. Jay, (Dept. of Med. Micriobiol.). The lysis and removal of cell debris was done as for the preparation of the crude lysate. This preparation was used for immunoprecipitation of the ß-galactosidase chimeric protein.

Precipitation of antigenic proteins from a lysate.

Immunoprecipitation was done using Pansorbin, a trade name for Protein A staphylococci, which have been shown to interact with immunoglobulin G. Immunoprecipitation using Protein A Staphylococci was first described by Kessler (1975). The sample in this experiment was a radioactive lysate of labelled cells. To the cell pellet was added 0.5 ml of immunoprecipitating buffer (IPB: 50 mM Tris.HCl pH 7.8, 100 mM NaCl, 100 units/ml Trasylol, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.5% (v/v) NP 40) and 5 µl of pre-immune serum was added. The purpose of the pre-immune serum was to remove proteins with which the serum interacts nonspecifically. The sample was rotated overnight on an

Adams nutator at 4° C. The next day 50 µl of 10% pansorbin in IPB (10 µl of 10% pansorbin/µl of anti-serum) was added to the sample and incubated for 1 hour with rotation at 4° C. The pansorbin interacts specifically with the immunoglobulin G fraction of the pre-immune serum and this was then centrifuged for 5 minutes at 4° C. on the microfuge at setting 5. To the supernatant is 5 µl of pre-immune serum was added and the immunoprecipitation was repeated. After this the sample was divided into 2 lots, 1 of which was treated with 5 µl pre-immune serum and the other with 5 µl of immune serum. The samples were again immunoprecipitated as above except that the time of incubation was for 5-6 hours. After this incubation the immunoreactive protein was found in the pellet which was resuspended in 800 µl of IPB/4 M urea, spun down and washed 3 times with IPB. The pellets were then resuspended in SDS sample buffer for running on a SDS polyacrylamide gel. The samples were boiled for 5 minutes, and the Pansorbin was spun down and the supernatant applied to a Laemmli (1970) SDS gel. After the gel was run, it was put into fixative (7% acetic acid in water/methanol (7:3) for 30 minutes, followed by a soak in Dupont enlightening for another 30 minutes, dried and an autoradiogram was done. This procedure was used for both preparation of immunoreactive protein from the T47D cell line and for the immunoreactive protein from the lysogen of the lambda gt11 clone isolated from the T47D library.

Competitive Inhibition of ATPase by Chimeric Protein

The purpose of this experiment was to see if the chimeric protein produced by the T47D clone competed for the same antibody as CaBI. Yamada et al. (1988) describe a competitive inhibition experiment to determine the interaction of antibody to CaBI with inhibitor proteins. In the experiment CaBI antibody was preincubated with CaBI before the assay for ATPase inhibition. The experiment showed that CaBI antibody interacts with CaBI so as to prevent ATPase inhibition by CaBI. The chimeric protein was used to take this experiment one step further. The antibody

was preincubated with chimeric protein and CaBI before the assay of inhibition of ATPase activity. In this way the chimera competed for antibody which was then unavailable for interaction with CaBI. The CaBI was then free to act as an inhibitor of ATPase.

The experiment was done according to Yamada et al. (1988). The chimeric protein in this case had been passed over the TPEG column. For this method ATPase activity was assayed by the coupling of ATP hydrolysis to the oxidation of NADH and is based on the SDS termination method of Monk and Kellerman (1976). The reaction mixture, containing 0.32 M mannitol, 9 mM KH_2PO_4, 11 mM EDTA, 10 mM Tris/sulphate buffer (pH 7.7), 1.67 µg/ml of antimycin, 2 mM phosphonenolpyruvate, 8.5 units/ml of pyruvate kinase, 15 units/ml of lactate dehydrogenase, 2 mM Mg^{2+}ATP and 0.205 µmol of NADH, was equilibrated for 10 minutes at 30° C. The reaction was initiated by the addition of enzyme. Incubation was then for 10 minutes at 30° C. The reaction was terminated by SDS to a final concentration of 0.33% (w/v). Control tubes had enzyme added after the addition of SDS. Nonincubated blanks were prepared containing the complete reaction mixture but with NADH omitted. Samples were read against the blank at 340 nm on a dual beam spectrophotometer.

Results

The CaBI antibody had been shown to be highly specific to CaBI (Yamada and Huzel, 1988; Penner et al., 1989). This is also shown from Table I, in which small levels of antibody (0.1 μg) are able to compete for 0.2 μg of CaBI to prevent ATPase inhibition by CaBI. The antibody is unreactive with calmodulin (Table II), oncomodulin, and bovine serum albumin (Yamada and Huzel, 1988).

A protein had been shown to be precipitated from a lysate of labelled T47D cells. This is shown in Figure 1. Although both the pre-immune serum and the anti-serum precipitate a large number of proteins, there is a band unique to the anti-serum at 19.5 kD. The size of this band was too large to be CaBI, either as a monomer or as a dimer. Previous work had shown that there was a 6 kD protein immunoprecitable from T47D cells (unpublished data of work done by Dr. Leigh Murphy). From the presence of at least one immunoreactive protein in the T47D cell line; because of the specificity of the antibody to CaBI; and because CaBI is thought to be ubiquitous to mammalian species, it was decided to screen a T47D cDNA library for the CaBI clone.

The T47D clone in Figure 2 was isolated by plaque purification. This clone will be referred to as T47D-1. The method for plaque purification is to replate and screen positive clones. Three clones from the rat heart library were isolated. One of the purifications is shown in Figure 3. The rat heart clones will be referred to as rat heart-1, rat heart-2 and rat heart-3. The DNA from each of the rat heart clones was purified and digested with EcoR I. The results of restriction digests of both the T47D-1 and rat heart-1, -2 and -3 DNA are shown in Figures 4 and 5. Each of the insert lengths is 800 base pairs long. The gel from Figure 5 was transblotted to nitrocellulose and hybridized with [^{32}P]-labelled DNA isolated from T47D-1. From Figure 6 is can be seen that all of the inserts hybridize to T47D-1 DNA. Lane 3 is of a false positive, this phage clone was not purified, since there were 2 insert bands on

Figure 1: Immunoprecipitation of [^{35}S]-methionine labelled T47D cell lysate with CaBI antibody.

Lane 1: Standards: Bovine serum albumin 70,000, Ovalbumin 44,000, Carbonic anhydrase 30,000 and Lysozyme 12,300 daltons; Lanes 2 and 4: pre-immune serum was used to precipitate proteins; Lanes 3 and 5: anti-serum was used to precipitate proteins. Lanes 2 and 3 were of the same sample which was split into 2 and one treated with pre-immune serum and the other with anti-serum. Lanes 4 and 5 were similarly treated. The band unique to the anti-serum treated samples is 19,500 daltons. The film was exposed for 4 days. The gel was a 15% SDS polyacrylamide gel. The anti-body had not been treated with an E. coli extract.

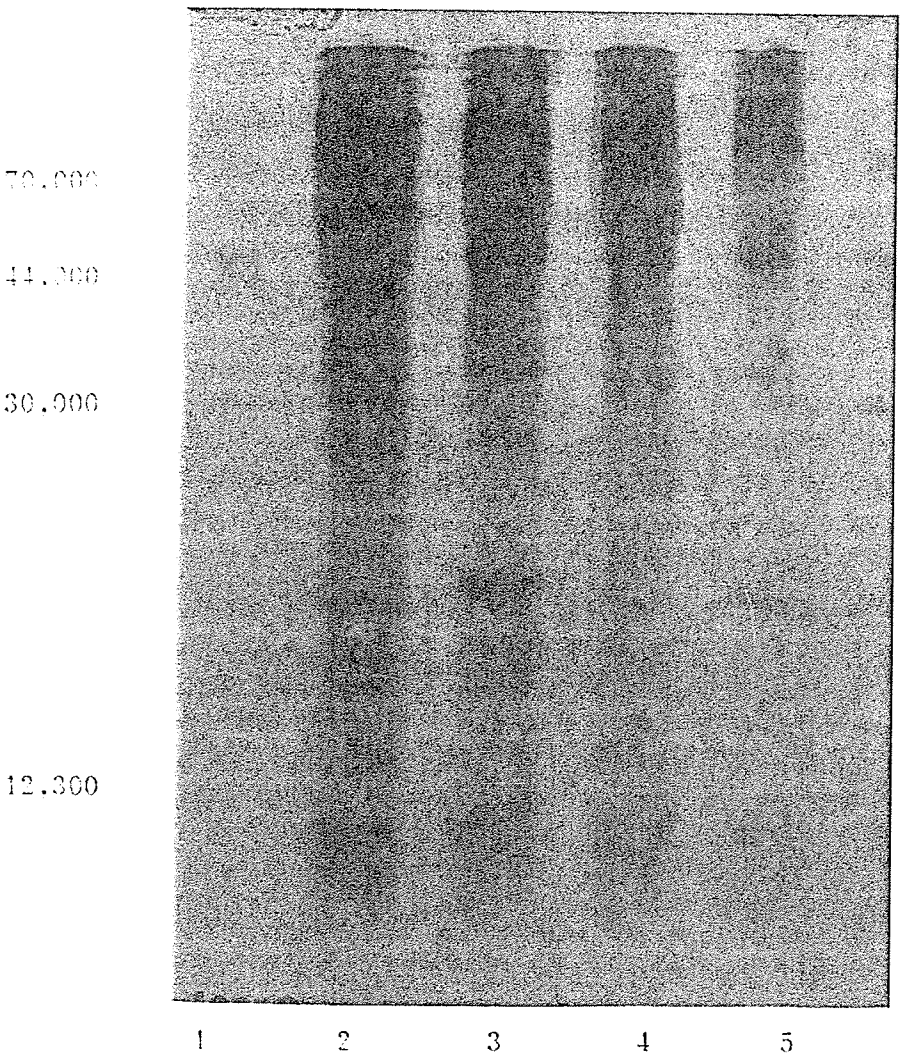

70.000
44.300
30.900
12.300
1
2
3
4
5

Table I: Competition of Chimeric Protein for antibody in ATPase assay of CaBI.

The proteins: CaBI, chimera or ß-galactosidase were preincubated with or without anti-CaBI-serum followed by the assay for ATPase activity. ß-galactosidase was included as a negative control since any nonspecific competition by ß-galactosidase could be subtracted from the value obtained for the fusion protein. ATPase activity is defined as the amount of protein that resulted in the oxidation of 1 µmol of NADH/min. Ab = CaBI antibody. This work was done by Dr. E. Yamada and Norm Huzel.

CaBI (ug)	CaBI Ab (ug)	chimera (ug)	ß-galactosidase (ug)	ATPase activity (units/mg protein)	% Inhibition
0	0	0	0	8.84	-
0.2	0	0	0	0.43	95.2
0.2	0.1	0	0	8.66	2.1
0.2	0.1	1.0	0	6.36	36.9
0.2	0.1	2.0	0	4.76	61.5
0.2	0.1	3.0	0	3.17	79.6
0.2	0.1	0	3.0	8.66	1.9
0	0.1	0	0	8.60	2.7
0	0.1	3.0	0	8.86	0
0	0.1	0	1.0	8.66	2.1
0	0	2.0	0	8.82	0.3
0	0	2.0	3.0	8.86	0

Table II: Competition of Prothrombin for antibody in ATPase assay of CaBI.

The proteins: CaBI, prothrombin or calmodulin were preincubated with or without anti-CaBI-serum followed by the assay for ATPase activity. Calmodulin was included as a negative control since it has been shown to be unreactive to CaBI antibody. ATPase activity is defined as the amount of protein that resulted in the oxidation of 1 μmol of NADH/min. Ab = CaBI antibody. This work was done by Dr. E. Yamada and Norm Huzel.

CaBI (ug)	CaBI Ab (ug)	pro-thrombin (ug)	calmodulin (ug)	ATPase activity (units/mg protein)	% Inhibition
0	0	0	0	8.84	-
0.2	0	0	0	0.43	95.2
0.2	0.1	0	0	8.66	2.1
0	0.1	0	0	8.60	2.7
0.2	0.1	5.0	0	7.15	19.2
0.2	0.1	7.0	0	6.75	23.7
0.2	0	7.0	0	0.47	94.7
0.2	0.1	0	2.0	8.67	1.9
0.2	0.1	0	3.0	8.66	2.1
0.2	0	0	3.0	0.37	95.8

Figure 2: Immunoscreening of T47D lambda gt11 library with [^{125}I]-Protein A used for immunodetection.

The autoradiograms are of 1: initial screening which produced the first positive clone; 2: second screening of the clone in which there is a higher proportion of positive plaques; 3: final screening in which all plaques are positive. (Clone T47D-1)

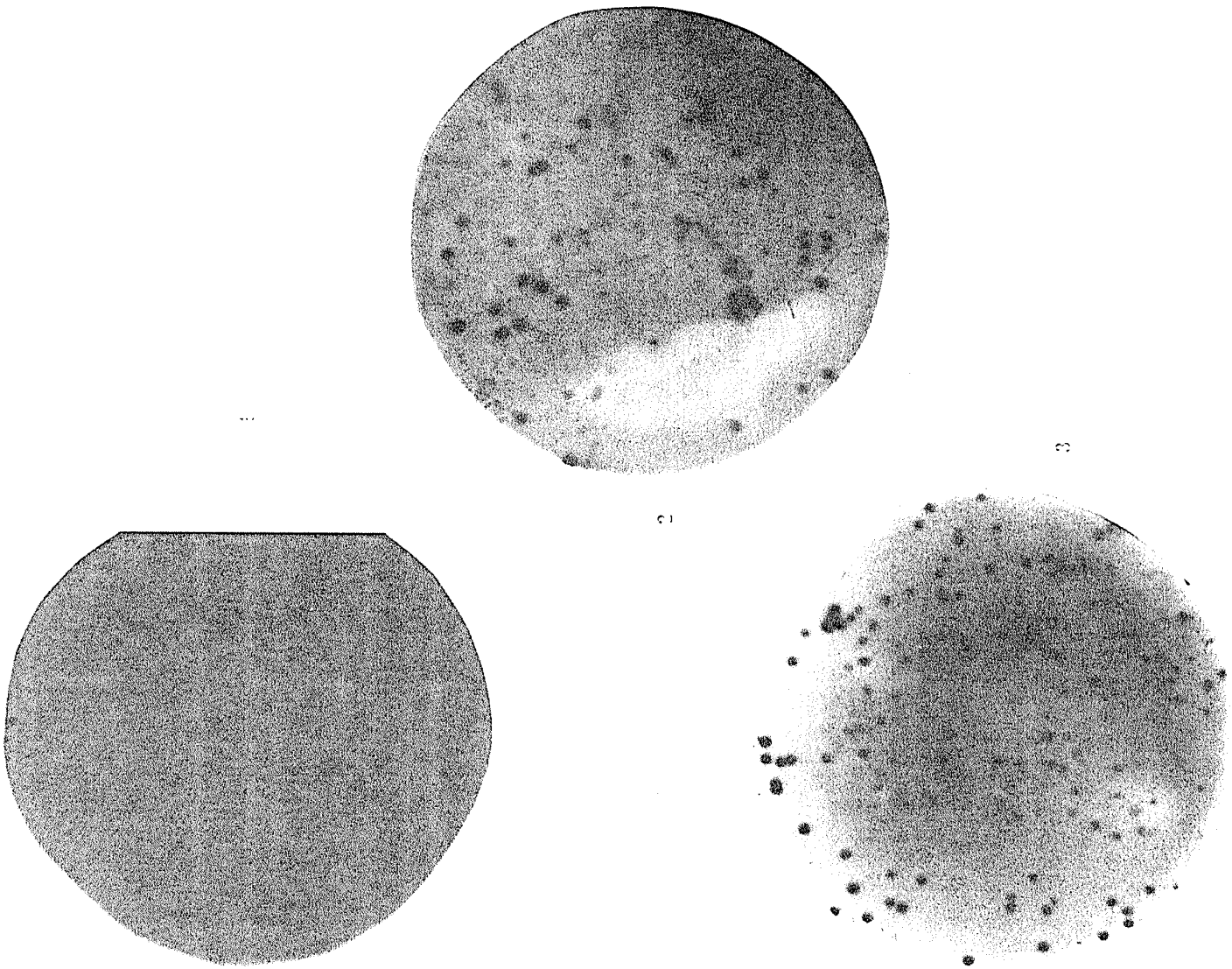
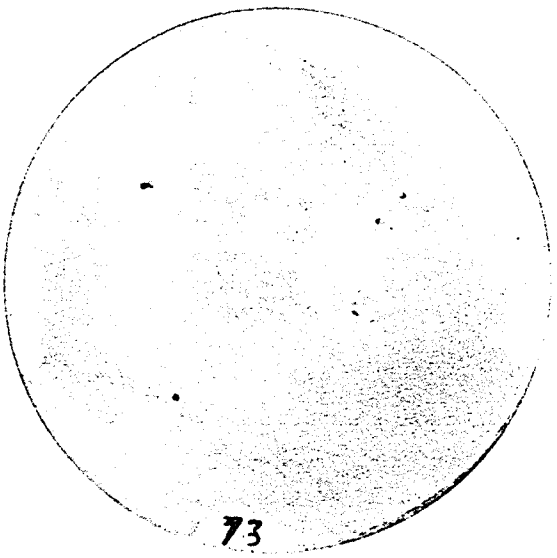
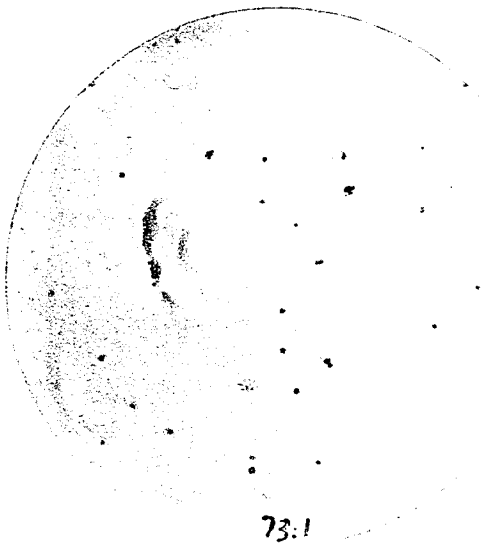


Figure 3: Immunoscreening of rat heart lambda gt11 library with alkaline phosphatase conjugated to goat anti-rabbit immunoglobulin as the second antibody.

The nitrocellulose filters are of 1: initial screening which produced the first positive clone; 2: second screening of the clone in which there is a higher proportion of positive plaques; 3: final immunoscreening in which all plaques are positive.



1



2



3

Figure 4: EcoR I digest of T47D-1 DNA.

The T47D-1 DNA was digested with the restriction enzyme EcoR I, and electrophoresed. Lane 1: Standards: Hind III digest of lambda DNA (23130, 9416, 6557, 4361, 2322, 2027 base pairs) and Hae III digest of phi X 174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 base pairs); Lane 2: T47D-1. The size of the insert is 808 base pairs.

2
1

Figure 5: EcoR I digest of lambda gt11 DNA.

Lane 1: Standards: Hind III digest of lambda DNA (23130, 9416, 6557, 4361, 2322, 2027 base pairs) and Hae III digest of phi X 174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 base pairs); Lane 2: Rat heart-1; Lane 3: a false positive clone; Lane 4: Rat heart-2; Lane #5: Rat heart-3; Lane #6: T47D-1. The DNA from this gel was transferred to nitrocellulose and probed with ^{32}P-labelled total T47D-1 DNA. The autoradiogram is seen in Figure 6.

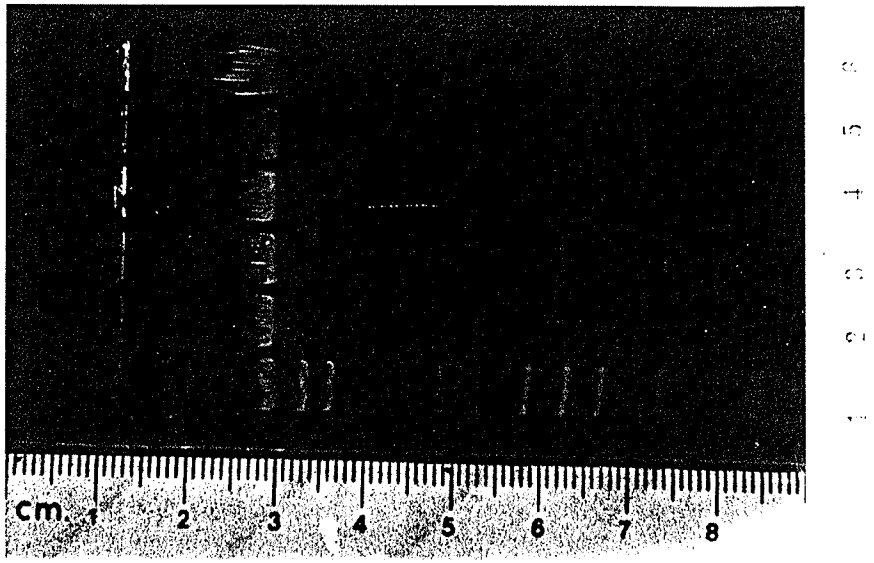

cm.
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7
8

Figure 6: DNA transblot of Figure 5 probed with [^{32}P]-labelled T47D-1 insert.

Lane 1: Standards: Hind III digest of lambda DNA (23130, 9416, 6557, 4361, 2322, 2027 base pairs) and Hae III digest of phi X 174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 base pairs); Lane 2: Rat heart-1; Lane 3: a false positive clone; Lane 4: Rat heart-2; Lane #5: Rat heart-3; Lane #6: T47D-1.

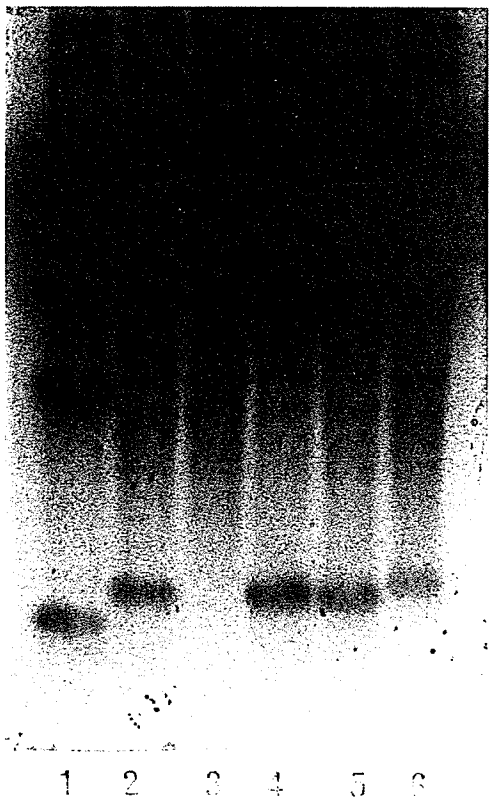

1 2 3 4 5 6

Figure 5 (Lane 3). The phage mixture was immunoscreened again and found to not have a clone reactive to the antibody. The cDNA of this clone also did not hybridize with the DNA of T47D-1, indicating that nonspecific hybridization was not occurring. For this DNA transblot, the probe was [^{32}P]-labelled T47D-1 DNA, and therefore not only does the insert hybridize, but also the lambda gt11 DNA present in the upper part of the gel and the standards which have Hae III digested lambda gt11 DNA.

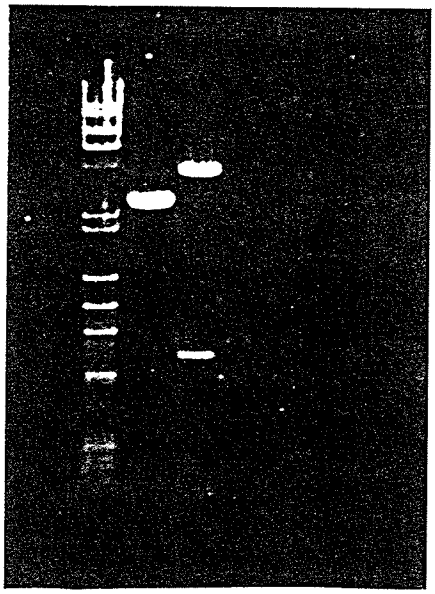
The insert DNA from T47D-1 was isolated and subcloned into pBR 322 plasmid in RR1 cells. After this a culture of the transformed RR_1 cells were grown, the plasmid DNA was purified from the culture, restriction digested with EcoR I to excise the cDNA insert and a sample was electrophoresed on an agarose gel. (Figure 7). The excised insert was purified from the plasmid DNA by electroelution and electrophoresed (Figure 8).

The purified insert DNA was hybridized to both a blot of T47D poly (A)+ RNA (Figure 9) and to rat tissue poly (A)+ RNA (Figures 10 and 11). In both cases it was shown that the size of the RNA species recognized was around 940 nucleotides. It was difficult to determine the correct size of the rat RNA that the T47D-1 insert hybridized to due to curvature in the gel, however the results from this membrane hybridization appear to agree quite closely with that of the T47D preparation.

The T47D-1 insert was subcloned into M13mp18 and a number of these clones were sequenced. It was found that the 3' end of the insert had a long polyadenylated (more than 100 Adenine bases are present) region. None of the sequencing kits available were able to sequence 3' of this length of polyadenylation, therefore the insert was digested with a variety of restriction enzymes in order to subclone smaller fragments of the insert. The results of this restriction digest are shown in Figure 12. BamH I, Sal I and HinD III did not cleave the insert. Pst I did cut the insert into lengths of about 400 and 340 base pairs respectively. These fragments were subcloned into both M13mp18 and M13mp19. The

Figure 7: Subclone of T47D-1 cDNA into plasmid pBR 322.

DNA insert isolated from T47D-1 was subcloned into plasmid pBR 322. The plasmid was used to transform RR1 strain. A culture was grown, the plasmid was isolated and digested with EcoR I. Lane 1: Standards: Hind III digest of lambda DNA (23130, 9416, 6557, 4361, 2322, 2027 base pairs) and Hae III digest of phi X 174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 base pairs), Lane 2 is of a pGEM plasmid which did not have the insert. This was a plasmid which had been used to transform RR1, and had been expected to contain the insert. Lane 3: pBR 322 and insert.



1 2 3

1
2
3
4
5
6
7
8
9
1
1
2

Figure 8: DNA insert isolated from pBR 322 containing T47D-1 insert.

The insert was separated from the pBr 322 seen in Figure 7. After purification the insert was electrophoresed. Lane 1: DNA low molecular weight standards: Hae III digest of phi X 174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 base pairs); Lane 2: 100 ng of insert; Lane 3: 250 ng of insert.

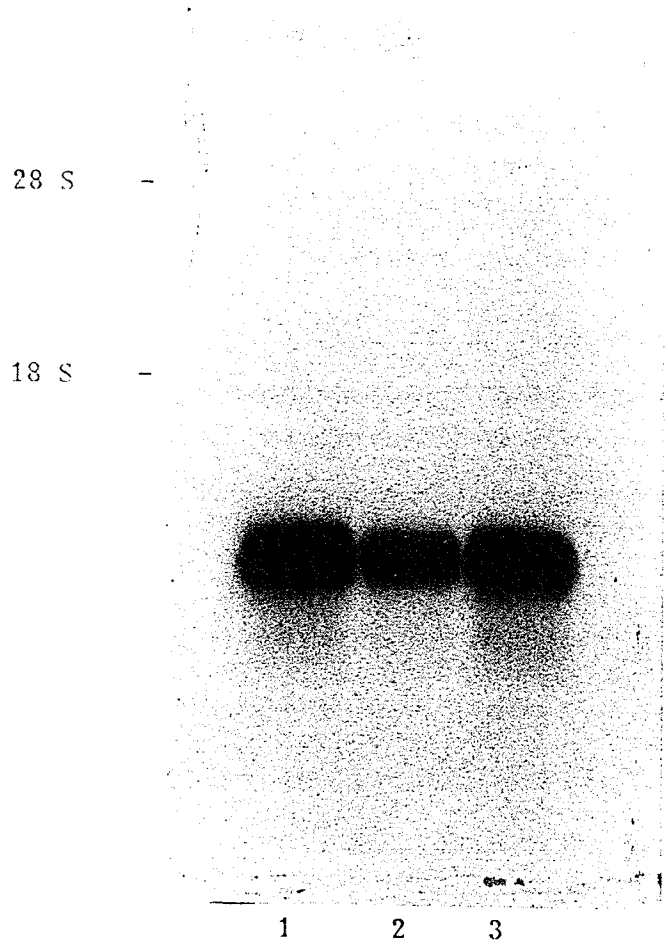

28 S
18 S
1
2
3

Figure 9: Probe of T47D poly (A)$^+$ RNA with T47D-1 DNA.

The probe was 250 ng of [^{32}P]-labelled T47D-1 insert DNA (5 X 10^6 cpm/μg DNA). A transblot (supplied by Dr. Leigh Murphy) of RNA prepared from T47D cells was probed with the insert. The size of the RNA recognized by the probe is 940 base pairs. Lanes 1, 2 and 3: 15 μg of T47D poly (A)+ RNA. Exposure was for 1 hour with an enhancing screen.

Figure 10: Agarose gel electrophoresis of poly (A)+ RNA prepared from rat tissues.

All lanes had 10 μg of RNA applied except Lane 1 from which part of the sample was lost during loading of the gel and Lane 7 for which only 7 μg was available. Lane 1: Brain; 2: Lung; 3: Liver; 4: Testes; 5: Heart; 6: Kidney; 7: Skeletal Muscle; 8: Spleen. This gel was used to transblot for Figure 11.

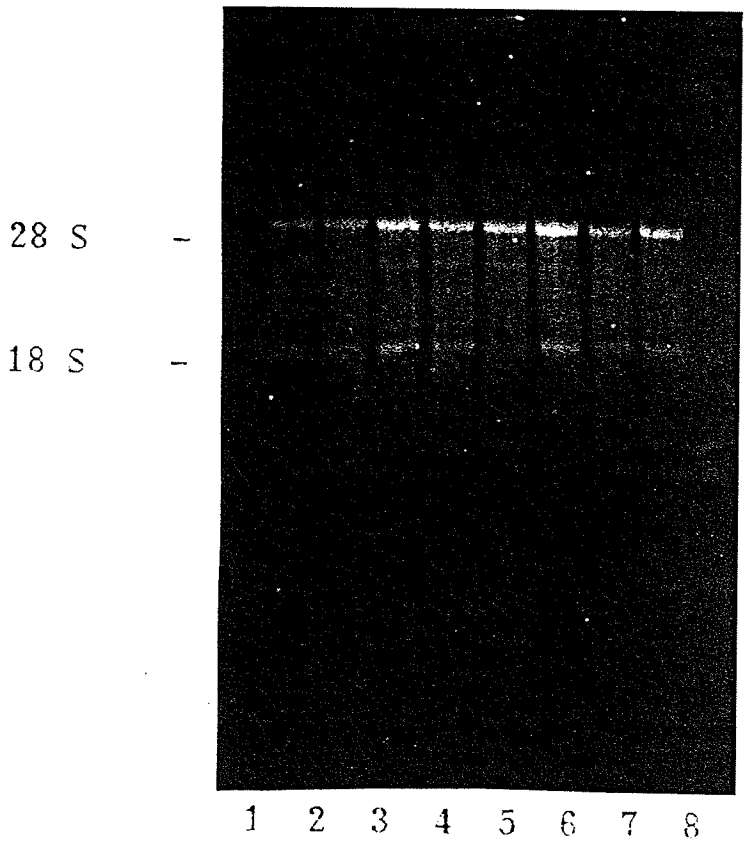

28 S
18 S
1 2 3 4 5 6 7 8

Figure 11: Probe of rat poly (A)$^+$ RNA with T47D-1 DNA.

The probe was 250 ng of [^{32}P]-labelled T47D-1 insert (5 X 10^6 cpm/μg DNA). A transblot of poly (A)+ RNA from rat tissues (Figure 10) was prepared and hybridized with the labelled insert. All lanes had 10 μg of RNA applied except Lane 1 from which part of the sample was lost during loading of the gel and Lane 7 for which only 7 μg was available. The tissue source and approximate size in bases of the RNA is given: Lane 1: Brain (920); 2: Lung (910); 3: Liver (980); 4: Testes (980); 5: Heart (980); 6: Kidney (1010); 7: Skeletal Muscle (990); 8: Spleen (970). The autoradiogram was exposed at -70° C overnight with an enhancing screen.

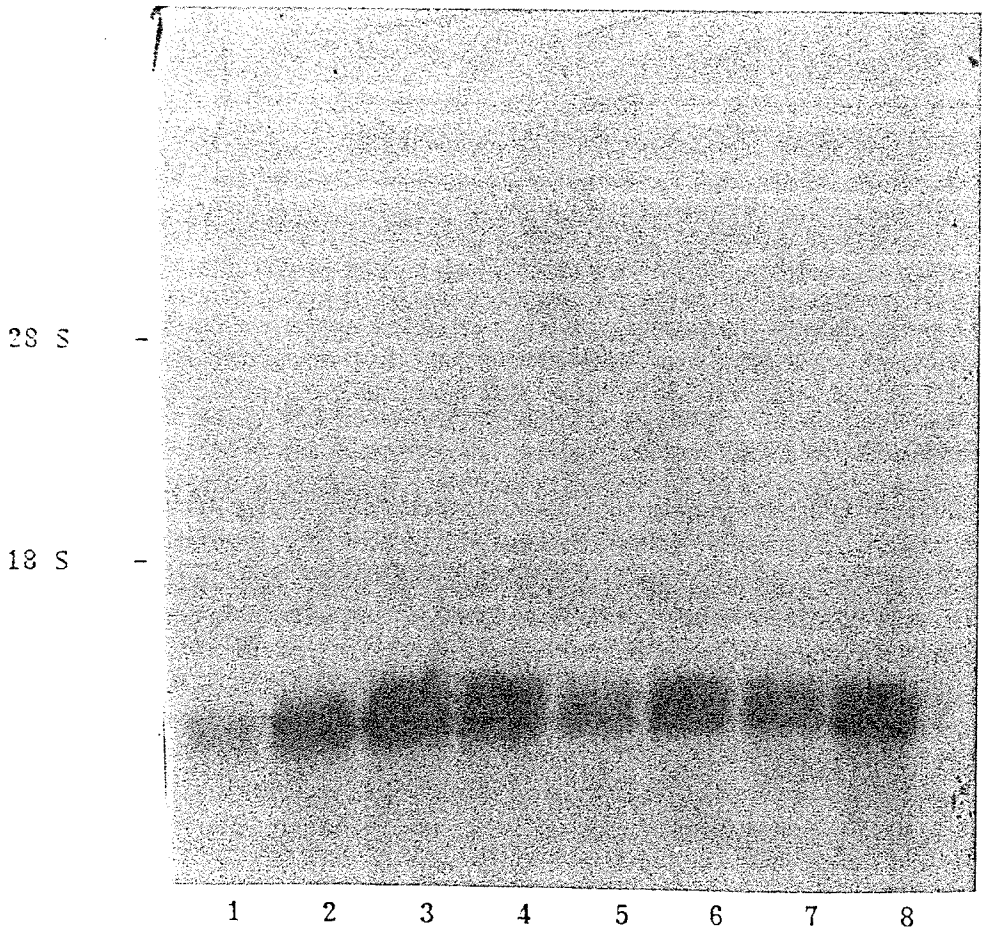
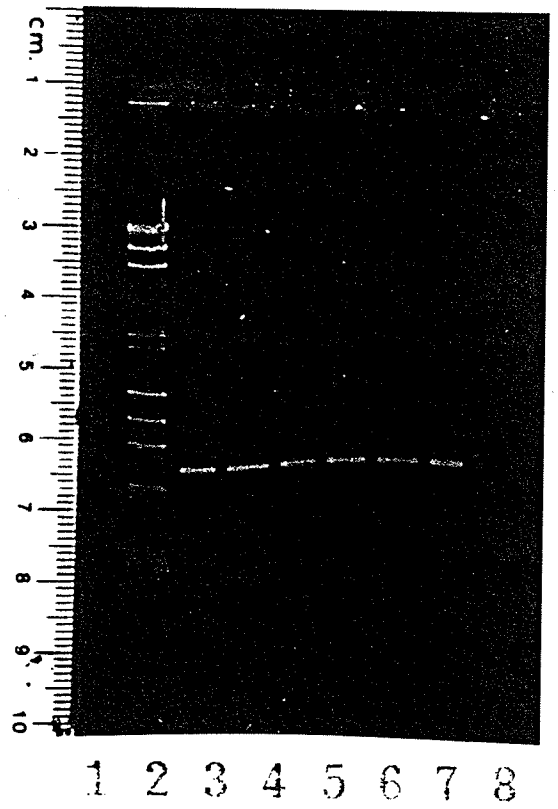

28 S
18 S
1
2
3
4
5
6
7
8

Figure 12: Restriction digests of T47D-1 cDNA insert.

Lane 1: Standards: Hind III digest of lambda DNA (23130, 9416, 6557, 4361, 2322, 2027 base pairs) and Hae III digest of phi X 174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 base pairs); Lane 2: undigested insert DNA with BamH I restriction enzyme buffer; Lane 3: BamH I digested insert DNA; Lane 4: Sal I digested insert DNA; Lane 5: undigested insert DNA electrophoresed in Sal I/HinD III restriction enzyme buffer; Lane 6: HinD III digested insert DNA; Lane 7: undigested insert DNA in Pst I restriction enzyme buffer; Lane 8: Pst I digested insert DNA. The 2 fragments in this lane are at 400 and 340 base pairs.


cm.
1
2
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10
1 2 3 4 5 6 7 8

difference between M13mp18 and M13mp19 is that the cloning cassettes in each of the phage are in opposite orientations. This allowed the fragments to be subcloned in a known orientation. These fragments were then sequenced. The sequence information obtained from these portions combined with the sequence from the 5' end of the full length clone showed that a small portion of the insert was still missing since there was a gap between the two fragments of the insert, but for which there was sequence from the full length clone. A Pst I digest of the insert was subcloned into M13mp18 and M13mp19 DNA which had only been digested with Pst I. Since the full length insert had EcoR I restricted ends only a fragment with 2 Pst I sites would subclone into the M13 vectors. After subcloning and sequencing of the inserts it was shown that there were indeed two Pst I sites in the full length clone 54 base pairs apart that corresponded with the DNA sequence which was present in the full length clone but not in either of the EcoR I - Pst I fragments. The sequencing strategy is shown is Figure 13.

A similar approach was taken to sequence Rat heart-1. Portions of sequencing gel from both rat heart-1 and T47D-1 are shown in Figure 14. Both of the sequences were the same. The DNA sequence of the cDNA insert and the corresponding derived amino acid sequence is shown is Figure 15. The amino acid sequence was determined using the DNA Inspector II software package, which also showed that this was the only open reading frame in either direction which coded for 50 or more amino acids. The derived amino acid composition of this DNA sequence was dissimilar to that of CaBI, since the amino acid composition for CaBI is known. CaBI is an acidic protein (pI 4.6). The amino acid residues for the sequence of these clones was basic. The amino acid composition of CaBI and the clone are given in Table III. As can be seen from this Table the two amino acid compositions are quite different. The length of the protein for the clone (196 amino acids from the first met residue) is also much larger than the known CaBI size (62 amino acids).

Figure 13: Cloning Strategy for sequencing cDNA.

The length of DNA isolated from the lambda gt11 clones is depicted in G with the 5' end of the coding strand on the left edge. B-F depicts each of the fragments which were subcloned into M13mp18 or M13mp19 and were then sequenced. Line A represents the portion of the full length clone which was sequenced. The asterisks (*) represent the end from which each portion was sequenced. The dA represent the start of the polyadenylated region.

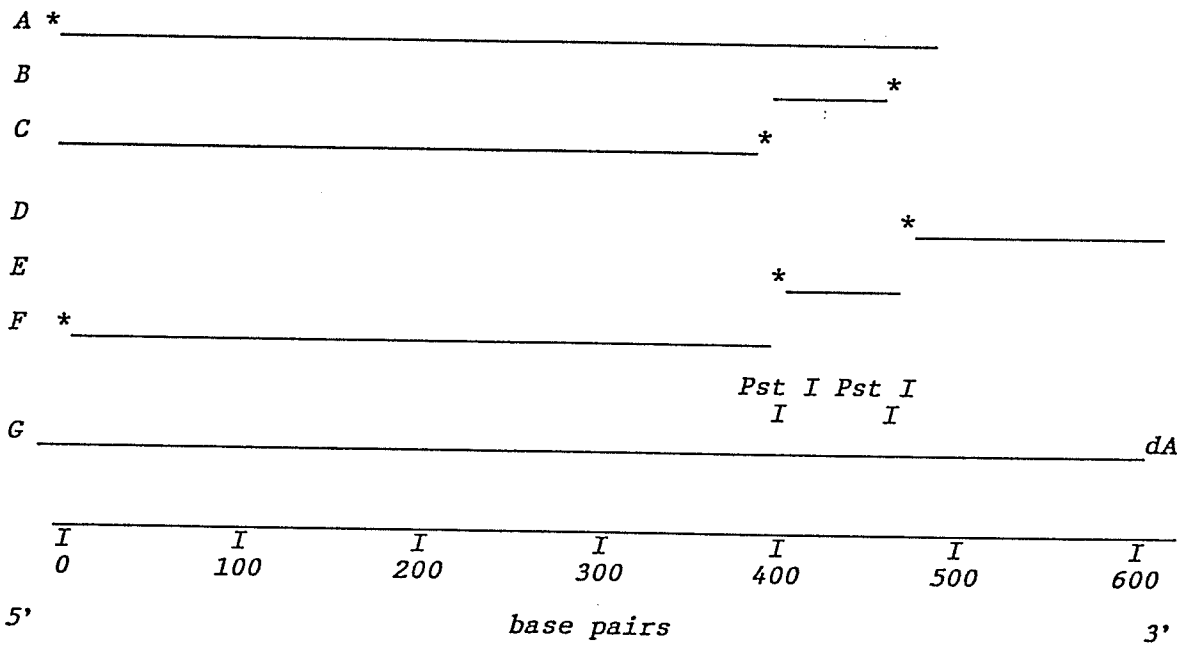

A
B
C
D
E
F
G
Pst I Pst I
I I
dA
I I I I I I I
0 100 200 300 400 500 600
5'
base pairs
3'

Figure 14: Autoradiogram of Sequencing Gel.

1: The sequence from nucleotide #1 of the insert to #77 is present. The enzyme used for the sequencing reaction is the Klenow fragment. 2: The sequence from nucleotide #395 to #464 is shown. This is the sequence of DNA which was located between the 2 sites recognized by Pst I restriction enzyme. The enzyme used for the sequencing reaction for this autoradiogram was sequenase. 3: A portion of the poly A region is shown. Since this part of the DNA is from the 3' end of the coding region toward the 5' end, the opposite strand from the coding strand is read, and therefore the poly A region is read as Ts.

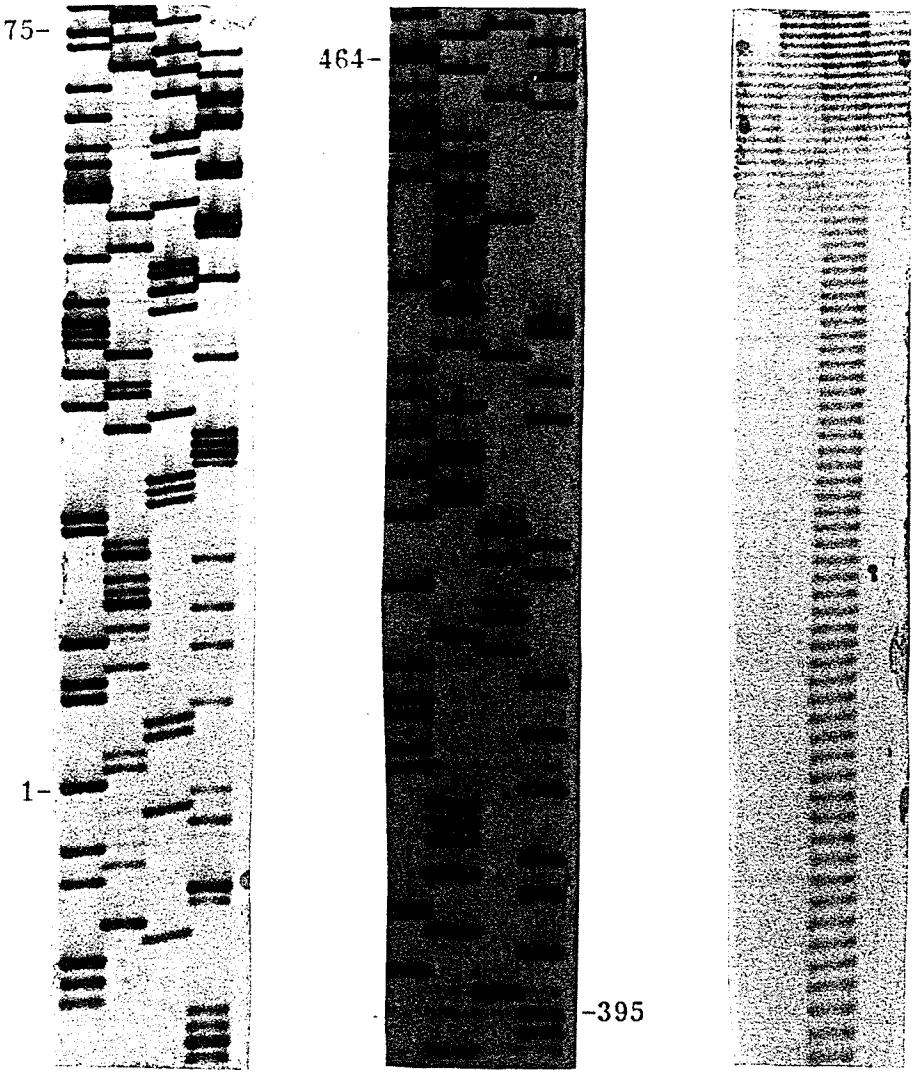

75-
1-
G A T C
1
464-
-395
G A T C
2
G A T C
3

Table III: Amino acid composition comparisons between CaBI and S6

*A comparison of the amino acid composition of CaBI to that of the portion of the S6 protein that was sequenced and to the 62 amino acids of the C-terminal end of S6 protein (Lott and Mackie, 1988). *Note that for CaBI glu residues include both glu and gln, asp includes asp and asn residues.*

Amino Acid	CaBI		Sequenced S6		62 amino acids of C-terminal	
	#	%	#	%	#	%
Asp	6	10	7	4	0	0
Thr	6	10	9	5	2	3
Ser	6	10	14	7	7	11
Glu	4	6	11	6	7	11
Pro	3	5	9	5	0	0
Gly	6	10	10	5	0	0
Ala	6	10	11	6	8	11
Cys	2	3	2	1	0	0
Val	7	11	13	7	0	0
Met	0	0	2	1	1	2
Ile	3	5	7	4	2	3
Leu	5	8	19	10	5	8
Tyr	1	2	3	2	1	2
Phe	2	3	2	1	0	0
His	0	0	3	2	0	0
Lys	4	6	31	16	14	23
Arg	1	2	6	3	10	16
Asn*			6	3	1	2
Gln*			9	5	4	6
total		62		174		62

The DNA sequence, which was identical for Rat heart-1 and T47D-1 was compared to reported sequences at Genbank. It was shown to have 99% similarity to a 623 nucleotide overlap for the coding region for the human lymphoblast ribosomal protein S6 (Heinze et al., 1988). The sequence of these two is compared in Figure 15. There is also 99% sequence homology to a human fibroblast S6 protein clone reported by Lott and Mackie (1988). It should be kept in mind that the first two amino acids are coded for by an EcoR I digestible region of DNA. EcoR I sites are created for cloning cDNA into the lambda gt11 arms and thus these two amino acids may not reflect the actual sequence of the gene. The DNA sequence was 99% homologous to the one reported by Chan and Wool (1988), but it coded for the same amino acid sequence. These DNA sequences may be variants of each other.

A lysogen of T47D-1 in Y1089 was prepared. Pictures of replica plates incubated at 32° and 42° C. (see methods) are shown in Figure 17. In order to determine whether the clones isolated did indeed produce a protein antigenic to CaBI antibody, the chimeric protein was immunoprecipitated from a lysogen. Initially this was done by radiolabelling a growing cell culture of the lysogen, followed by an immunoprecipitation of the lysate and SDS polyacrylamide gel electrophoresis. The autoradiogram is shown in Figure 18. A protein was specifically precipitated from the lysate. The size of the protein is not known, however later purification of the chimeric protein and SDS polyacrylamide gel electrophoresis (done by Dr. E. Yamada and Norm Huzel) showed the chimera to be 115 kD.

A culture of the lysogen was grown and the chimera was purified using an affinity TPEG column for ß-galactosidase. The protein was used in competitive inhibition experiments to see if it interacted with the same antibody as CaBI does. The results are shown in Table I. CaBI (0.2 μg) inhibits 95% of the ATPase activity of submitochondrial particles. When 0.1 μg of antibody is added to this assay the inhibition is reduced

Figure 15: DNA sequence and derived corresponding amino acid sequence of both T47D-1 and Rat heart-1.

The upper line represents the nucleotide sequence and the lower line represents the corresponding amino acids. The number of the nucleotide and of the amino acid from the start of the insert is shown above and below the lines respectively.

```
1          10          20          30          40          50
G AAT TCG GAC GAC AAA CAA GGT TTC CCC ATG AAG CAG GGT GTC TTG ACC CAT
  asn ser asp asp lys gln gly phe pro met lys gln gly val leu thr his
   1                                  10

         60          70          80          90          100
  GGC CGT GTC CGC CTG CTA CTG AGT AAG GGG CAT TCC TGT TAC AGA CCA AGG
  gly arg val arg leu leu leu ser lys gly his ser cys tyr arg pro arg
         20                                  30

        110         120         130         140         150
  AGA ACT GGA GAA AGA AAG AGA AAA TCA GTT CGT GGT TGC ATT GTG GAT GCA
  arg thr gly glu arg lys arg lys ser val arg gly cys ile val asp ala
                     40                                  50

      160         170         180         190         200
  AAT CTG AGC GTT CTC AAC TTG GTT ATT GTA AAA AAA GGA GAG AAG GAT ATT
  asn leu ser val leu asn leu val ile val lys lys gly glu lys asp ile
                                 60

     210         220         230         240         250
  CCT GGA CTG ACT GAT ACT ACA GTG CCT CGC CGC CTG GGC CCC AAA AGA GCT
  pro gly leu thr asp thr thr val pro arg arg leu gly pro lys arg ala
     70                                  80

    260         270         280         290         300
  AGC AGA ATC CGC AAA CTT TTC AAT CTC TCT AAA GAA GAT GAT GTC CGC CAG
  ser arg ile arg lys leu phe asn leu ser lys glu asp asp val arg gln
                 90                                  100

  310         320         330         340         350
  TAT GTT GTA AGA AAG CCC TTA AAT AAA GAA GGT AAG AAA CCT AGG ACC AAA
  tyr val val arg lys pro leu asn lys glu gly lys lys pro arg thr lys
                             110

  360         370         380         390         400
  GCA CCC AAG ATT CAG CGT CTT GTT ACT CCA CGT GTC CTG CAG CAC AAA CGG
  ala pro lys ile gln arg leu val thr pro arg val leu gln his lys arg
  120                                 130

 410         420         430         440         450         460
  CGG CGT ATT GCT CTG AAG AAG CAG CGT ACC AAG AAA AAT AAA GAA GAG GCT
  arg arg ile ala leu lys lys gln arg thr lys lys asn lys glu glu ala
             140                                 150

           470         480         490         500         510
  GCA GAA TAT GCT AAA CTT TTG GCC AAG AGA ATG AAG GAG GCT AAG GAG AAG
  ala glu tyr ala lys leu leu ala lys arg met lys glu ala lys glu lys
                        160                                 170

          520         530         540         550         560
  CGC CAG GAA CAA ATT GCG AAG AGA CGC AGA CTT TCC TCT CTG CGA GCT TCT
  arg gln glu gln ile ala lys arg arg arg leu ser ser leu arg ala ser
                                 180

        570         580         590         600         610
  ACT TCT AAG TCT GAA TCC AGT CAG AAA TAA GAT TTT TTG AGT AAC AAA TAA
  thr ser lys ser glu ser ser gln lys STOP
         190

       620
  ATA AGA TCA GAC TCTG
```

Figure 16: Comparison of T47D-1 and rat heart-1 to S6

The insert sequences of both Rat heart-1 or T47D-1 (the two were identical) to that of the S6 ribosomal protein were compared. The insert sequence is listed above the S6 sequence. Only that part of the S6 sequence is listed which corresponds to insert sequence. The S6 protein sequence is from Heinze (1988). The number of the nucleotide from the start of the insert is listed above the rows.

```
1          10          20          30          40          50
G AAT TCG GAC GAC AAA CAA GGT TTC CCC ATG AAG CAG GGT GTC TTG ACC CAT
T GGT GGG AAC GAC AAA NAA GGT TTC CCC ATG AAG CAG GGT GTC TTG ACC CAT

          60          70          80          90          100
  GGC CGT GTC CGC CTG CTA CTG AGT AAG GGG CAT TCC TGT TAC AGA CCA AGG
  GGC CGT GTC CGC CTG CTA CTG AGT AAG GGG CAG GCC TGT TAC AGA CCA AGG

        110         120         130         140         150
  AGA ACT GGA GAA AGA AAG AGA AAA TCA GTT CGT GGT TGC ATT GTG GAT GCA
  AGA ACT GGA GAA AGA AAG AGA AAA TCA GTT CGT GGT TGC ATT GTG GAT GCA

      160         170         180         190         200
  AAT CTG AGC GTT CTC AAC TTG GTT ATT GTA AAA AAA GGA GAG AAG GAT ATT
  AAT CTG AGC GTT CTC AAC TTG GTT ATT GTA AAA AAA GGA GAG AAG GAT ATT

    210         220         230         240         250
  CCT GGA CTG ACT GAT ACT ACA GTG CCT CGC CGC CTG GGC CCC AAA AGA GCT
  CCT GGA CTG ACT GAT ACT ACA GTG CCT CGC CGC CTG GGC CCA AAA AGA GCT

   260         270         280         290         300
  AGC AGA ATC CGC AAA CTT TTC AAT CTC TCT AAA GAA GAT GAT GTC CGC CAG
  AGC AGA ATC CGC AAA CTT TTT AAT CTC TCT AAA GAA GAT GAT GTC CGC GAG

  310         320         330         340         350
  TAT GTT GTA AGA AAG CCC TTA AAT AAA GAA GGT AAG AAA CCT AGG ACC AAA
  TGT GTT GTA AGA AAG CCC TTA AAT AAA GAA GGT AAG AGA CCT AGG ACC AAA

  360         370         380         390         400
  GCA CCC AAG ATT CAG CGT CTT GTT ACT CCA CGT GTC CTG CAG CAC AAA CGG
  GCA CCC AAG ATT CAG CGT CTT GTT ACT CCA CGT GTC CTG CAG CAC AAA CGG

410         420         430         440         450         460
  CGG CGT ATT GCT CTG AAG AAG CAG CGT ACC AAG AAA AAT AAA GAA GAG GCT
  CGG CGT ATT GCT CTG AAG AAG CAG CTA CCA AGA AAA AAT AAA GAA GAG GCT

            470         480         490         500         510
  GCA GAA TAT GCT AAA CTT TTG GCC AAG AGA ATG AAG GAG GCT AAG GAG AAG
  GCA GAA TAT GCT AAA CTT TTG GCC AAG AGA ATG AAG CAG GCT AAG GAG AAG

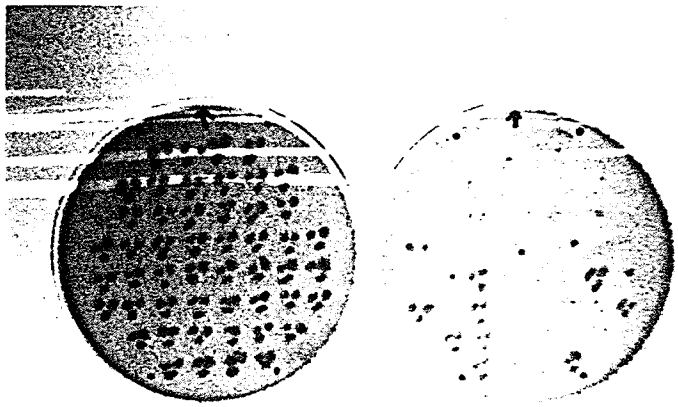
          520         530         540         550         560
  CGC CAG GAA CAA ATT GCG AAG AGA CGC AGA CTT TCC TCT CTG CGA GCT TCT
  GCG CAG GAA CAA ATT GCG AAG AGA CGC AGA CTT TCC TCT CTG CGA GCT TCT

        570         580         590         600         610
  ACT TCT AAG TCT GAA TCC AGT CAG AAA TAA GAT TTT TTG AGT AAC AAA TAA
  ACT TCT AAG TCT GAA TCC AGT CAG AAA TAA GAT TTT TTG AGT AAC AAA TAA

       620
  ATA AGA TCA GAC TCTG
  ATA AGA TCA GAC TCTG
```

Figure 17: Lysogeny of T47D-1.

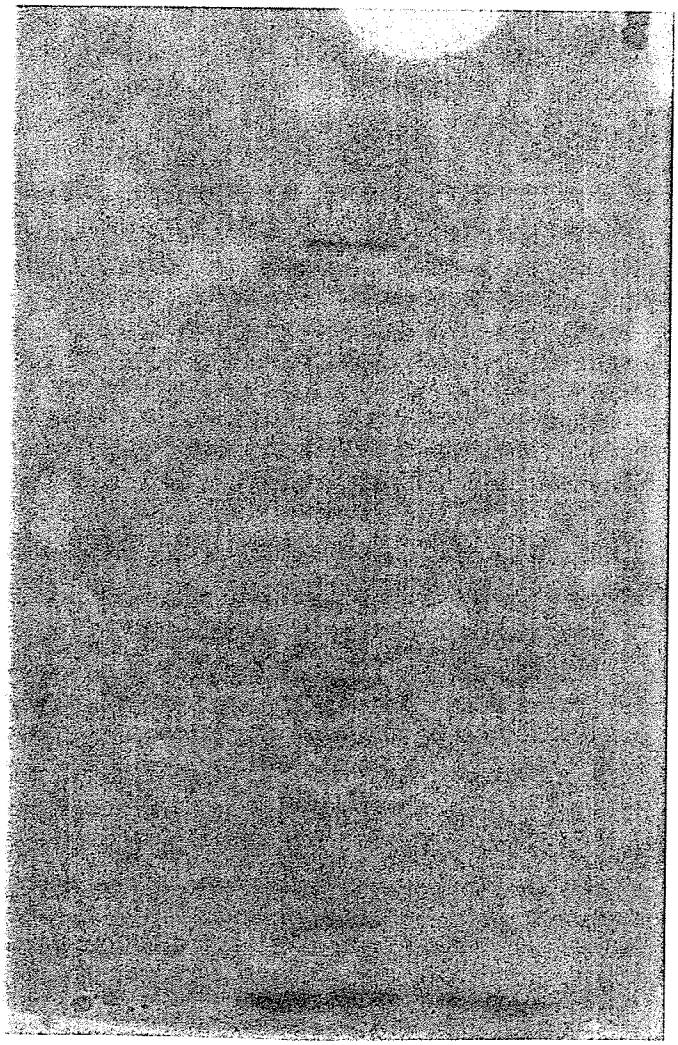
T47D-1 (lambda gt11 phage) was lysogenized into Y1089. Several colonies were picked and replica plated onto 2 LB plates. One was incubated at 32° C. and the other at 42° C. Plate 1: 32° C. Plate 2: 42° C. Lysogeny for this sample is 76%.



1 2

Figure 18: SDS polyacrylamide gel of immunoprecipitation of T47D-1 lysogen lysate with CaBI antibody.

T47D-1 was lysogenized into Y1089. One of the lysogens was grown and labelled with [^{35}S]-cysteine. The cell lysate was immunoprecipitated with CaBI antibody. Lane 1: The lysate was precipitated with anti-serum (10110 cpm were applied to the gel); Lane 2: the lysate was immunoprecipitated with pre-immune serum (8019 cpm were applied to the gel). Electrophoresis was on a 7.5% polyacrylamide SDS gel.



1 2

to 2%. This indicates that the antibody binds CaBI so that CaBI cannot interact with submitochondrial A particles. The negative control of the chimeric protein by itself does not cause inhibition of ATPase activity (0.3% at 2 μg chimera), indicating that the chimera does not interact with submitochondrial A particles. In the presence of chimera (1, 2 and 3 μg) and antibody (0.1 μg), CaBI (0.2 μg) inhibits ATPase activity (37, 62 and 80% respectively). This means that the chimera competes for CaBI antibody, so that CaBI antibody is not free to bind CaBI. The unbound CaBI is then able to inhibit ATPase activity. The importance of this result is the indication that CaBI and the chimera share a common epitope. The negative control for ß-galactosidase, which forms part of the chimeric protein does not interact with the antibody as evidenced by the fact that in the presence of ß-galactosidase (3.0 μg), CaBI (0.2 μg) and antibody (0.1 μg) there is little inhibition (2%). This shows that in the presence of ß-galactosidase, CaBI antibody is free to interact with CaBI. This negative control was done since ß-galactosidase forms part of the chimera. Since ß-galactosidase does not interact with CaBI antibody, the S6 portion of the chimera must. The other negative controls: 0.1 μg of antibody; antibody (0.1 μg) with chimera (3.0 μg) and with ß-galactosidase (1.0 μg); and chimera (2.0 μg) with ß-galactosidase (3.0 μg) confirm that none of these proteins affect the inhibition of ATPase by CaBI, since in each case inhibition is less than 3%.

For reasons which will be expanded on in the discussion, prothrombin was thought to have similarity to CaBI in the way each of them bound Ca^{2+}, and therefore the competition assay was done in the presence of this protein (Table II). The effect of prothrombin (5.0, 7.0 μg) was to increase the inhibition of ATPase function (19 and 24% versus 2%) by CaBI (0.2 μg) in the presence of antibody (0.1 μg). This would indicate that prothrombin competes for CaBI antibody and therefore is also epitopic to CaBI. Larger amounts of prothrombin were needed than of the chimera to produce less effect. Seven μg of prothrombin caused inhibition to be

increased to 23% whereas only 1 μg of chimera was needed to increase inhibition to 37%. Calmodulin (3.0 μg) had no effect. In other experiments it has been shown that the CaBI antibody does not interact with calmodulin on dot-blots (Yamada and Huzel, 1988) and it does interact with prothrombin on dot-blots (Penner et al., 1989). These results are important when the nature of Ca^{2+} binding by CaBI is considered (this is expanded on in the discussion).

Discussion

The CaBI antibody used in this research was of high quality. This is shown by the lack of interaction of the antibody with calmodulin, oncomodulin, bovine serum antibody (Yamada and Huzel, 1987), Protein C and Factor X (Penner et al., 1989), through immunodetection of the proteins spotted onto nitrocellulose membranes. A less pure antibody would have interacted positively with a larger number of the lambda gt11 library clones which were screened. This antibody only recognized the S6 clone in each instance. The quality of the antibody was important to this research, since it was responsible for identifying positive clones from the library. CaBI antibody detected as little as 0.78 ng of CaBI, 12.5 ng of purified, partially phosphorylated S6 and 6.25 ng of the chimeric protein spotted onto nitrocellulose membranes, at 31 μg/ml of antibody (before the addition of E. coli lysate) in the binding buffer (Penner et al., 1989). This is a very low level of antigen that the antibody was able to detect. Because of the high specificity of CaBI antibody for CaBI, any of the clones screened which had the CaBI cDNA, should have been recognized. This raises the possibility that for some reason neither rat heart nor T47D lambda gt11 library had a CaBI clone. A possible reason that neither library might have the CaBI cDNA, is that the CaBI cDNA may be relatively short and may not have been present in the library. It is hoped that a new library will be made which is enriched in shorter inserts, since it is expected that CaBI will be coded for by a short mRNA. An attempt is being made to get the amino acid sequence for a fragment of CaBI. This would allow a new library to be screened with oligonucleotides which code for the known amino acid sequence. Prothrombin cDNA was not isolated from either library even though CaBI antibody (31 μg antibody protein/ml in the binding buffer before the addition of E. coli lysate) recognizes as little as 50 ng of prothrombin protein spotted onto nitrocellulose membrane. This is because prothrombin is normally expressed in the liver. The mRNA for prothrombin would not be found in

rat heart and is not expected to be seen in T47D cells, which are a human mammary tumour cell line.

The protein that was precipitated from the lysate of labelled T47D cells is about 19.5 kD (Figure 1). This size is to large to be a monomer or a dimer of CaBI. The unmodified rat S6 protein has a calculated molecular weight of 28.7 kD and on Laemmli gels it migrates at about 31 kD (Chan and Wool, 1988). It is unlikely that the protein that was immunoprecipitated was S6, unless the protein was a breakdown product of S6. The large number of proteins seen in all of the lanes both those precipitated with anti-body and those precipitated with pre-immune serum is not uncommon with this experiment (Kessler, 1981).

The hybridization of T47D-1 cDNA with the cDNA of the rat heart clones that were isolated was done at high stringency. This indicates that there is a strong homology between all of the clones isolated. In fact when Rat heart-1 was sequenced, it was found to have the same sequence as the T47D-1.

The length of the cDNA insert (800 base pairs) is rather long for the size of CaBI. Bovine CaBI is known to have 62 amino acids (Yamada and Huzel, 1988). Rat CaBI is close to the same size as bovine CaBI at 60 amino acids. For 62 amino acids, the coding region should be 186 nucleotides long. Within the cDNA of CaBI it would be expected that there would be more than 186 nucleotides. Since CaBI is a mitochondrial matrix protein, it is expected to have a leader sequence which destines the protein for the matrix (Reid, 1985; Hurt and Van Loon, 1986). This leader sequence is usually about 20-30 amino acids long. mRNA also has other coding regions, including the poly A tail, however it was not expected that the cDNA for CaBI would be as large as the ones isolated.

The size of the mRNA species recognized by the insert in both the rat tissue and the T47D blots was close to the same length. It was difficult to determine the exact length of the mRNA in the rat tissue because of the curvature of the gel, however on the T47D gel the length

was 940 nucleotides. This meant that the complete mRNA was not coded for by the insert which was isolated, since the insert was only 800 base pairs long. This turned out to be the case. The cDNA does not account for the first 160 base pairs of the coding region for S6. Heinze et al. (1988) also found that hybridization was to a 1000 base pair mRNA species for the S6 protein in human lymphoblast. The comparison between the lengths of the mRNA species recognized on both the T47D and the rat tissue blots indicates that the mRNA species are similar in size. The fact that the cDNA hybridized at high stringency with both species shows homology between the species. The strength of the signal from both blots is not unexpected, since ribosomal proteins are abundant in the cell and thus there should be a large population of the mRNA for ribosomal proteins in cell.

Prothrombin competitively inhibits the ATPase assay. This is important because prothrombin is a Ca^{2+} binding protein. CaBI is known to be dissimilar to the Ca^{2+} binding proteins calmodulin and oncomodulin which have EF hands (Yamada and Huzel, 1988) because these proteins do not react to CaBI antibody. CaBI antibody does not interact with Factor X or Protein C (Penner et al., 1989) which are characterized by Ca^{2+} binding via ß-hydroxy aspartate residues. Prothrombin binds Ca^{2+} via the anionic carboxyl oxygens of gamma-carboxy glutamate (gla) residues (Esnouf and Prowse, 1977; Friedman et al., 1977). The interaction of the CaBI antibody both as seen from ATPase inhibition in the presence of prothrombin (Table II) and as seen by the interaction of CaBI antibody with prothrombin spotted onto nitrocellulose membranes (Penner et al., 1989) indicates that CaBI may also have gla residues. CaBI does have glutamic acid. Alkaline hydrolysis of proteins does not destroy gla residues. This method will be used to see if CaBI does indeed have gla residues. If it does this may prove to be the way in which CaBI interacts with Ca^{2+}, although the presence of gla does not necessarily mean that CaBI binds Ca^{2+} in through gla residues.

The lambda gt11 rat heart and T47D clones code for ribosomal protein S6

The DNA sequence that was found in this project showed 99% homology to the recorded sequence for human lymphoblast and fibroblast S6 ribosomal protein (Heinze et al., 1988; Lott and Mackie, 1988). It also showed 70% similarity over 611 nucleotides to mouse S6 protein. There is also some similarity to the Saccharomyces yeast ribosomal protein S10 (62% over 567 nucleotides), which had been reported earlier to be similar to S6 (Chan and Wool, 1988; Heinze et al., 1988). There is little doubt that the DNA that was sequenced codes for the S6 ribosomal protein. The extent of the sequence similarity indicates this. The human genome has been shown to have several copies (Heinze et al., 1988) and the rat to have 10-15 copies of S6 protein (Chan and Wool, 1988). The sequence that was found in this experiment is a variant of the literature sequences of both rat and human S6, which may not be surprising given the number of copies of the gene in both these genomes. Most of the copies are expected to be pseudogenes (Heinze et al., 1988).

Similarity of S6 to CaBI

The S6 chimeric protein was shown to competitively inhibit the ATPase assay. Other recent experiments (Penner et al., 1989) have also shown that antibody to the C-terminal portion of S6 competitively inhibit the ATPase assay, indicating a cross reactivity of this antibody with CaBI. The C-terminal S6-antibody also recognizes CaBI specifically (Penner et al., 1989), indicating that the epitopic similarity between the two proteins may be as a result of the carboxy terminus of the S6 protein. The region of epitopic similarity in CaBI is not known. The epitopic similarity between CaBI and S6 does not mean that the 2 proteins have an amino acid sequence in common. From the research done to date this cannot be said. The experiment which could have pointed towards a similarity between the two proteins was the hybridization of the cDNA isolated from

T47D-1 to the RNA which had been transferred to nitrocellulose. If a second band had been seen, other than the one at 1000 bases, this could have indicated hybridization to CaBI cDNA. This was not seen, although very low levels of hybridization may have been occurring. Some of the reasons that a second band was not seen are that the strength of the signal from S6 may have been much greater than that for CaBI, since S6 is more abundant than CaBI. Binding of S6 cDNA to CaBI mRNA would probably occur at lower stringency, since even if the 2 proteins had a conserved sequence, it would not need to be identical to the S6 sequence. The important finding of this project is that the 2 proteins S6 and CaBI are epitopic since antibodies to either protein cross react with the heterologous protein. This result may indicate a function which is common to both proteins. The Ca^{2+} binding by both proteins, with a known effect of Ca^{2+} on CaBI and on translation suggest that the epitopic similarity may be as a result of Ca^{2+} binding by both proteins.

S6 has 6 serine residues which can be phosphorylated in order for the protein to be more active in translation (Duncan and McConkey, 1982b). S6 is known to have at least 5 serine residues which may be phosphorylated in the carboxy terminus of the protein (Gresser and Wool, 1974). It is known that CaBI has 6 serines (Yamada and Huzel, 1988). This raises the possibility that CaBI may be phosphorylated and dephosphorylated. The 32 amino acid sequence from amino acid 45 to 78 of the insert is acidic and falls within the amino acid composition of CaBI. The significance of this is not known and may turn out to be irrelevant. This is probably the case because of the evidence that the epitopic similarity is with the C-terminal portion of S6. There is a possibility that CaBI may be produced by alternative splicing of the RNA from S6. This probably does not occur. The 32 amino acid length from S6 amino acid 45 to 78 is the only one of any length in any of the 3 possible reading frames which falls within the amino acid composition of CaBI. The possibility of S6 protein being cleaved after translation to produce CaBI also would not occur since 62

amino acids of CaBI form a single polypeptide from gel electrophoresis in the presence of reducing agents.

Role of [Ca^{2+}] in translation of Proteins

Because of the specificity of the interaction of CaBI antibody with S6, it was suspected that S6 may bind Ca^{2+}. Since this research, this has been shown to be the case by using $^{45}Ca^{2+}$ stained gels of S6 chimeric protein and of purified S6 (Penner et al., 1989). It is known that Ca^{2+} levels affect ribosomal activity in vivo (Brostrom et al., 1986; Chin et al., 1987;). Chin et al. (1987) provides evidence that [Ca^{2+}] affects mRNA translation at the initiation step. S6 is known to be part of the initiation complex (Krieg et al., 1988). S6 forms part of the mRNA binding domain and phosphorylation favours the attachment of certain classes of mRNA or messenger ribonucleoproteins (Chan and Wool, 1988). This result may indicate that S6 may in fact be a protein which directly transduces the effect of [Ca^{2+}], which is to increase transcription within the cell. There are 70-80 proteins involved in translation and therefore it was difficult to narrow down which protein or proteins were responsible for this interaction. The evidence from this work points to S6 being important as a transducer of the effect of [Ca^{2+}] to increase translation of protein.

The mode of interaction of S6 with Ca^{2+} may be through gla residues. The carboxy-terminus of S6 has 7 glutamic acid residues in the last 42 amino acids. It is possible that S6 is modified by gamma-carboxylation. Gla has been reported to be present in purified ribosomes of prokaryotic and eukaryotic cells (Van Buskirk et al., 1980; Van Buskirk and Kirsch, 1978a, b; Olson et al., 1978; Scheinbuks, 1980). S6 does not however, have sequence similarity to the Ca^{2+} binding sequences of gla containing Ca^{2+} proteins (Van Eldik et al., 1982).

Despite this evidence, there is still a possibility that the effect of [Ca^{2+}] on protein synthesis is through phosphorylation and dephosphorylation of protein. This has been suggested by Chin et al.

(1987), and [Ca^{2+}] frequently functions as a second messenger through action of protein kinases. S6 has been shown to be preferentially incorporated into ribosomes in the phosphorylated state. Thus this may also form a part of the control of translation, either alone or in conjunction with Ca^{2+} interaction with the protein.

Conclusion

This experiment did not accomplish what it should have which was to isolate and sequence the cDNA for CaBI. In the course of this research 4 clones were isolated from the T47D and the rat heart libraries, which were immunoreactive to CaBI antibody. Each of the clones coded for the ribosomal protein S6. These results together with more recent work (Penner et al., 1989) indicate that S6 is epitopic to CaBI.

The question that this research has raised is whether CaBI is related to S6. There are numerous examples of proteins belonging to gene families. Whether or not CaBI and S6 have a sequence which is preserved between them is speculative at this point.

A fortuitous result of this experiment is that it provides some evidence that S6 may be a Ca^{2+} binding protein and that Ca^{2+} may interact directly with S6 to cause activation of S6.

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