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(19) (CA) **CANADIAN PATENT** (12)

(54) **Primary Biliary Cirrhosis Autoantigen**

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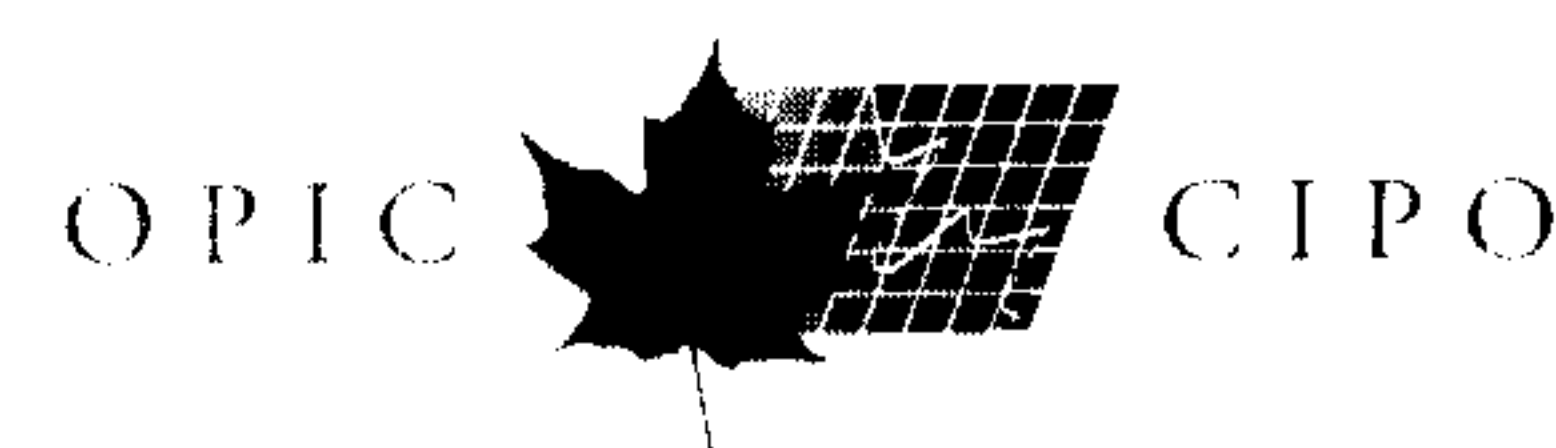
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1341231**ABSTRACT OF THE DISCLOSURE**

A synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of primary biliary cirrhosis, or an antigenically active fragment thereof, is produced by recombinant DNA techniques. The synthetic peptide or polypeptide, or fragment, may be used in the treatment of patients or in diagnostic tests, particularly for the detection of anti-mitochondrial antibody in a serum sample.

PRIMARY BILIARY CIRRHOSIS AUTOANTIGEN

This invention relates to the identification, cloning and expression of an auto-antigen which is recognised as a target in the characteristic autoantibody response in primary biliary cirrhosis (PBC), and to the use of this protein, fragments thereof or fused polypeptides containing the protein or fragments thereof in diagnostic tests for PBC, and in treatment of patients suffering from PBC.

Primary biliary cirrhosis (PBC) is a chronic disease characterised by progressive inflammatory obliteration of the intrahepatic bile ducts. The disease is marked by an autoantibody response to mitochondria¹⁻⁴, originally identified using immunofluorescence⁵. With the recent use of immunoblotting, specific proteins have been

recognized as targets of the anti-mitochondrial antibodies (AMA) of PBC^{2,6,7}. In particular, serum antibodies to a 70 kilodalton (kd) protein have been found in greater than 95% of patients with PBC but not in patients with other autoimmune liver diseases,^{2,8}; two other proteins of 45 and 39kd are less frequently detected in PBC sera^{1,2,9}. The identity of each of these autoantigens has been unknown, as is the relationship of these antigens to the pathogenesis of the disease. However, the 70kd antigen is highly conserved in evolution, being present in mammals, yeast and bacteria¹⁰ and it is therefore believed to have an important structural or biological function².

Despite the paucity of data on mechanisms of anti-mitochondrial antibody formation, by enzyme-linked immunosorbent assay (ELISA), clinically more than 95% of patients with PBC can be found to have such anti-mitochondrial antibodies^{2,6}. When crude mitochondrial antigen preparations are used, subjects with a variety of diseases, including patients with liver diseases other than PBC, certain connective tissue diseases, and drug reactions, and occasionally even healthy individuals, can also be demonstrated to have antibodies to mitochondria. Accordingly, assays using such crude preparations are unable to provide specific diagnosis of PBC. By way of example, Meyer zum Bushenfelde German Patent Publication No. 3,237,602 of October 11, 1982 discloses an ELISA for detection and determination of anti-mitochondrial antibodies in serum using a crude mitochondrial antigen preparation. The lack of specificity of the assay is evident from the suggested use of the assay in the specific

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diagnosis of disorders such as PBC as well as the cholestatic form of chronic active hepatitis, syphilis (II), drug-induced pseudo lupus erythematoses syndrome, certain primary non-hepatic

immunopathies, iproniazid-induced hepatitis and side effects of certain medicaments such as beta-receptor blockers. By more vigorous isolation of mitochondrial membranes, the problem of antigenic heterogeneity becomes clearer and has led to definitions of specific mitochondrial antigens based on trypsin sensitivity and location of antigens within inner vs outer mitochondrial membranes.

Notwithstanding this, however, the diagnosis of PBC still relies heavily on the demonstration of anti-mitochondrial
10 antibodies by the relatively insensitive procedure of immunofluorescence or by more sensitive, but still relatively nonspecific, methods, including complement fixation, ELISA, and immunoprecipitation²³⁻²⁸.

The present invention is based on the identification of a cDNA clone derived from a rat liver gene expression library that expresses the 70kd mitochondrial autoantigen of PBC, (called M2 by some groups of investigators^{1,9}) and on sequence determination thereof. The sequence is encoded by nuclear
20 and not mitochondrial DNA.

The present invention thus provides the basis of an extremely sensitive and specific diagnostic ELISA for anti-70kd antibodies found in PBC.

According to a first aspect of the present invention, there is provided a DNA molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the base sequence coding for the 70kd mitochondrial autoantigen of primary biliary cirrhosis (PBC), or an antigenically active fragment thereof.

30 The identification, cloning and expression of the 70kd mitochondrial autoantigen of PBC in accordance with the present invention, and its use in an ELISA, will now be described in detail, with reference to the accompanying drawings in which:

Figure 1 shows specificity of the fused polypeptide. In lanes A and B, two different PBC sera at a dilution of 1/1000 were probed against lysates of pRMIT transformed JM101 cells. Both sera reacted with a polypeptide at 160kd. In contrast, in lanes C and D, the same sera were nonreactive when probed against lysates of control cells containing an irrelevant insert that is also fused to β -galactosidase. The reactive bands in lanes C and D correspond to E.coli proteins. Duplicate blots probed with normal sera at 1/100 and 1/1000 failed to detect the fused polypeptide and are not shown. There is some breakdown of the fusion protein with reactivity at 36kd.

Figure 2 shows identification of the pRMIT fused polypeptide. The reactivity of absorbed and unabsorbed PBC serum against human placental mitochondrial proteins after PAGE was determined. In lane A, the probe was an unabsorbed PBC serum at a final dilution of 1/2000. In lane B, the probe was the same serum at a final dilution of 1/2000 after extensive absorption for 72hr against cells transformed with non-recombinant pBTA224 and passage over a solid support to which had been bound a lysate of cells transformed with non-recombinant pBTA224. In lane C, the probe was the same serum at a final dilution of 1/2000 after absorption for 72hr against cells transformed with non-recombinant pBTA224 and passage over a solid support to which had been bound a lysate of cells transformed with expressing pRMIT. The serum was also studied at 1/200 and 1/20,000 (Table II).

Figure 3 shows specificity of affinity-purified antibody. In lane A, an unabsorbed PBC serum at 1,2000 was probed against placental mitochondria, reacting with both the 70 and the 45kd protein. In lane B, the column eluate was probed against the same mitochondrial preparation. Note the reactivity was only to the 70kd protein, and the reduction in signal correspond to the expected recovery for such elution. Even on a very long

autoradiographic exposure time of 1wk, there remained activity only to the 70kd protein (data not shown). In lane C, the eluate was probed against a sonicate of induced JM101 transformed with pRMIT. The intensity of the 160kd fused polypeptide was due to the large quantity of fused polypeptide expressed. In lane D, the eluate was probed against a sonicate of induced JM101 transformed with an irrelevant plasmid that encodes an abundant fused polypeptide.

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Figure 4 shows immune response of BALB/c mice immunized with pRMIT induced fused polypeptide. Placental mitochondria were separated by PAGE on a 7.5% gel and blotted onto nitrocellulose, and the fractionated proteins were probed with sera at a dilution of 1/1000 (lane A) or with serum from a patient with PBC at 1/1000: immunized mice produced antibody against the 70kd but not the 45kd protein.

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Figure 5 shows immunofluorescence of HEp-2 cells. BALB/c mice were immunized with the purified fused polypeptide and sera incubated with HEp-2 cells. Note the typical mitochondrial pattern of reactivity.

Figure 6 shows nucleotide sequence of pRMIT and deduced amino acid sequence of the 70kd mitochondrial antigen of PBC.

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Figure 7 shows comparison of sensitivity between the ELISA (+) and immunofluorescence (□) in detection of AMA in PBC. PBC sera were tested at every 10 fold dilution starting from 1:1000 in the ELISA whereas in the immunofluorescence against Hep-2 cells every 2 fold dilution starting from 1:10 was used. The positives on ELISA were defined as 2 S.D. O.D. units above the mean for normal sera.

Figure 8 shows the nucleotide sequence and deduced amino acid sequence of a 2.2kb cDNA insert that encodes the human equivalent of the sequence depicted in

Figure 6, encompassing the human equivalent of the region of nucleotides 105-1065 in Figure 6. This human cDNA clone was obtained by probing a human placental library using pRMIT as a hybridization probe according to known techniques. The sequences are highly homologous and have comparable reactivity with auto-mitochondrial antibodies; accordingly either antigen sequence could be used as the basis of a diagnostic test to detect anti-mitochondrial antibodies or auto-reactive cells.

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Preferably, the DNA molecule in accordance with this aspect of the invention is characterised by at least a portion thereof comprising a base sequence substantially as shown in Figure 6, or a fragment thereof.

In another aspect, this invention provides a

recombinant DNA molecule comprising a nucleotide sequence as described above, operatively linked to an expression control sequence. Such a recombinant DNA molecule may for example comprise an expression vector such as a
5 bacteriophage or plasmid, or a host cell such as a bacterium or other microorganism transformed therewith.

In yet another aspect of this invention there is provided a synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial
10 autoantigen of primary biliary cirrhosis, or an antigenically active fragment thereof.

Preferably, the synthetic peptide or polypeptide of this aspect of the invention is characterised by at least a portion thereof comprising an amino acid sequence
15 substantially as shown in Figure 6 or Figure 8, or an antigenically active fragment thereof.

Such a synthetic peptide or polypeptide may, for example, be prepared by expression of a host cell transformed with a recombinant DNA molecule as broadly
20 described above, either as a fused polypeptide or directly. Alternatively, it may be prepared by chemical synthesis, such as by the well-known Merrifield solid-phase synthesis procedure.

The present invention extends to the synthetic
25 peptide corresponding to the entire 70kd autoantigen, and to nucleotide sequences coding for the entire autoantigen, as well as to fragments thereof. By way of example, one such fragment is the fragment encoded by nucleotides 76-679 of Figure 6. This fragment of approximately 200
30 residues is capable of adsorbing out of a patient serum all antibodies directed against the native autoantigen. Within this fragment is a 20 residue fragment of amino acid sequence:

A E I E T D K A T I G F E V Q E E G Y L

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which has been demonstrated to have substantial reactivity with autoantibodies. This fragment is common to the sequences of both Figure 6 and Figure 8. The present invention therefore extends to the use of antigenically
5 active fragments such as these, as well as to the use of the entire autoantigen, in diagnostic assays.

The present invention also extends to the use of the synthetic peptide or polypeptide, or fragment, of this invention as an antigen in a diagnostic test for PBC by
10 detection or determination of the titre of antimitochondrial antibody in a patient's serum, for example using ELISA or RIA technology or an agglutination assay using antigen-coated beads or the like. The invention also extends to use of the synthetic peptide or
15 polypeptide, or fragment, in the treatment of patients. In this latter aspect, such methods of treatment include the use of the synthetic antigen, as an adsorbent to remove PBC antibodies or reactive cells from a patient, as well as the use of these active components in direct
20 administration to a patient as a desensitizing agent to eliminate or diminish reactivity of the patient to the PBC autoantigen.

In addition to use of the synthetic autoantigen in the detection of anti-mitochondrial antibody in a serum
25 sample, the present invention extends to use of the synthetic peptide or polypeptide, or fragment, in the measurement of class-specific immunoglobulin titres using specific typing reagents. Applications also extend to the measurement of the affinity of either the whole
30 autoantibody, or the affinity of individual classes or subclasses of the autoantibody. Affinity may be measured by a number of procedures, for example, by replicate ELISA assays performed using different washes of guanidine thiocyanate⁴². A further extension of the diagnostic

assay is the measurement of the degree of interference of autoantibodies with the enzymic function of the 70kd autoantigen (now shown to be lipoate acetyl transferase, see later). The source of the enzyme may be derived from expression of full length clones as native polypeptides or fusion polypeptides, or from expression of enzymatically active fragments or purified protein from mitochondria. The enzyme assay is a standard assay well known in the art, but modified to include a step of incubation with sample serum or cells. In yet a further extension of the use of the synthetic peptide or polypeptide, or fragment, there is included the measurement of reactivity of patient cells to the autoantigen. The synthetic peptide or polypeptide, or fragment, may be added, in solution or bound to a solid support, to patient cells derived from peripheral blood or from tissue biopsies either unfractionated, fractionated or as a continuous cell line. Reactivity to the autoantigen may then be measured by standard proliferation assays such as incorporation of tritiated thymidine, standard cytotoxic assays such as release of marker radioactivity from target cells, or other standard assays of cellular reactivity which are well known in the art.

In one particularly important aspect of this invention, there is provided a diagnostic test for detection of antimitochondrial antibody in a serum sample, which comprises the steps of:

- (i) contacting said serum sample with a synthetic peptide or polypeptide displaying portion of the 70kd mitochondrial autoantigen of PBC, or an antigenically active fragment thereof, said synthetic peptide or polypeptide being immobilized on a support, and
- (ii) detecting the presence of anti-mitochondrial

antibody in said serum bound to said synthetic peptide or polypeptide.

In this aspect, the invention also provides a diagnostic test kit for detection of anti-mitochondrial antibody in a serum sample, which comprises:

- (i) a support having immobilised thereon a synthetic peptide or polypeptide displaying the antigenicity of all or a portion of the 70kd mitochondrial autoantigen of PBC, or an antigenically active fragment thereof, and
- (ii) means for detecting the presence of anti-mitochondrial antibody in said serum bound to said synthetic peptide or polypeptide.

Preferably the detection of the presence of bound AMA is by use of well known RIA or ELISA techniques.

As a result of the production of a recombinant fused polypeptide displaying the antigenicity of the 70kd mitochondrial autoantigen of PBC, this autoantigen has now been identified as lipoate acyltransferase. In addition, the immunoglobulin isotypes of the anti-mitochondrial antibodies has been determined, and IgG3 has been found to be the predominant isotope in a group of PBC patients, with IgM next most prevalent. Comparison of serum immunoglobulin isotype levels of PBC patients with healthy normal adults has shown that serum IgG3 and IgM were very elevated in PBC; IgG3 at 5.5 fold and IgM at 4.3 fold above normal.

In accordance with the present invention, expression of the cDNA insert encoding the mitochondrial autoantigen, or fragments thereof, may be achieved in a number of different ways. The detailed description herein provides examples of expression as β -galactosidase fusion proteins in the vectors λ gt11 and pBTA224, using as host cells E.coli strains such as JM101, JPA101 and

7118. Successful expression of the autoantigen as a fusion protein may also be achieved using the well-known PVC vectors, or using the pGEX series which give expression of glutathione S-transferase fusion proteins, again using E.coli as the host cells. Alternatively, the mitochondrial autoantigen may be expressed as a non-fused polypeptide, by using appropriate vector and host cell combinations. Other vector and host cell combinations which can be used in accordance with the present invention
10 including a number of well described yeast shuttle vectors growing in yeast cells, or eukaryotic vectors in continuous cell lines, or transgenic animals.

A. MATERIALS AND METHODS.

Screening cDNA library.

A rat liver cDNA library in λ gt 11-Amp3 consisting of 15,000 recombinants, of average length of 1.4kb, was probed by using sera from patients with PBC. The sera used for screening were from each of three patients with classical PBC who were shown to have antibodies to mitochondria by immunoblot analysis of electrophoretically separated proteins of human placental mitochondria². As some patients with PBC have high-titre antibodies to E.coli, the sera were extensively preabsorbed against E.coli infected with non-recombinant phage. The sera were used for probing at a final concentration of 1:1000^{11,12}. The λ -Amp3 library was incubated with E.coli, strain ST9, for 15min at 37°C and then was plated for 2hr at 42°C in LB agar. Thereafter, nitrocellulose filters that had been previously soaked in 10ml isopropyl-thiogalactosidase (IPTG) and allowed to air dry were overlayed on each plate. The plates were then incubated overnight at 37°C. The nitrocellulose was removed after alignment and was washed for 1hr in PBS with 5% milk powder, pH 7.4. The filters were then incubated for 45min with previously absorbed sera of patients with PBC, washed three times for 30min, and incubated with ¹²⁵I-protein A (300,000 cpm/ml) for 45min. Finally, the filters were washed three times, were allowed to air dry, and were placed on XRP-1 film with an intensifying screen for an overnight (12hr) exposure. All washings and dilutions of sera and ¹²⁵I-protein A were done with milk powder. Putative positive clones were picked and rescreened for plaque purification^{12,13}.

Subcloning.

Three clones gave positive signals, a frequency of approximately one in 50,000 clones. These positive clones were plaque purified. Each of these clones yielded an identical sized insert of approximately 1.4kd. The

inserts were subcloned in the plasmid vector pBTA224, which is a high copy plasmid expression vector with a site for insertion of foreign DNA identical to that of λ -Amp3. Therefore, 50% of the subclones should also give a positive signal on an immunoassay as the insert is in the same reading frame as λ -Amp3. Clones expressing an unrelated rat liver cDNA (the F alloantigen) were used as controls. Arrays of pBTA224 colonies were prepared to identify immunoreactive clones. Colonies were incubated for 16hr at 37°C, then were induced with 10mM IPTG for 4hr. The colonies were lysed and prepared for antibody probing as described¹¹. Filters were probed with either a 1/1000 dilution of absorbed PBC sera or a 1/100 dilution of normal serum. One positive clone, designated pRMIT, that expressed a fused polypeptide of 160kd was selected for further study.

Immunoblotting of mitochondrial proteins.

Mitochondria from human placenta were prepared as described^{2,14}. Polyacrylamide gel electrophoresis (PAGE) was performed on 1mm-thick slab gels in 0.1% SDS, using a 3.8% stacking gel and a 10% resolving gel. Before PAGE, the purified mitochondria were suspended at a concentration of 4mg protein/ml and were incubated for 30min with 10,000 U of bovine pancreatic DNase 1 at 37°C, and then were held with an equal volume of 3% aqueous octyl glucoside for 15min at 4°C. The final preparations were diluted with Tris-HCl, pH6.8, containing 4% SDS, 20% glycerol, and 5% 2-mercaptoethanol (sample buffer) and were boiled for 5min. Approximately 10 μ g protein were loaded in each gel lane².

Specificity of pRMIT fused polypeptide.

To demonstrate that pRMIT expressed an antigen specifically reactive with sera from patients with PBC, lysates of the expressing clone were probed with sera from

healthy persons or from patients suffering from different
autoimmune diseases. Briefly, a 100ml overnight culture
of JM101 cells transformed with pRMIT was diluted 1/10 in
L-broth containing 10mM IPTG. Four hours later the
5 cultures were spun at 5000xG for 10min and were snap
frozen after addition of 20ml of phosphate-buffered
saline. PAGE was performed on 1mm-thick slab gels with
0.1% SDS, using a 3.8% stacking gel and a 7.5% resolving
gel. Samples were diluted 1/100 in the above sample
10 buffer and were boiled for 5min. Each lane contained
approximately 5 to 10µg of protein. The samples were
probed with PBC sera diluted at 1/1000, and the reactivity
was determined as above, using ¹²⁵I-protein A and
exposure for 18hr. These same sera also were used to
15 probe immunoblots of lysates of non-recombinant control
clones or clones expressing a fused polypeptide coded by
an irrelevant DNA insert. The sera used were from
patients with PBC, systemic lupus erythematosus,
rheumatoid arthritis, Sjogren's syndrome, chronic active
20 hepatitis and from healthy normal volunteers. All control
sera were studied at a dilution of 1/100.

Identification of fused polypeptide.

The fused polypeptide expressed by pRMIT was
characterised to determine whether it was a mitochondrial
25 antigen recognised by PBC sera. The clone pRMIT was grown
in liquid culture overnight. It was thence put into log
phase and induced to give maximal expression of the fused
polypeptide with 10mM IPTG for 4hr. Bacterial lysates
were prepared as above and coupled to cyanogen
30 bromide-Sepharose^{*15}. This solid support was then used
as an affinity reagent to bind antibodies selectively from
seven different PBC sera. First, sera from seven patients
with PBC were absorbed extensively with sonicates of
E.coli transformed with non-recombinant pBTA224. Thence,

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the sera at dilutions of 1/200, 1/2000, and 1/20,000 were passed through the lysate of pRMIT-transformed bacteria bound to a solid support. The nonabsorbed antibodies were collected, compared with unmanipulated sera at the same
5 final dilution, and used to probe placental mitochondria, prepared as above.

Preparation of affinity-purified antibody.

Affinity-purified antibody was prepared by first extensively preabsorbing five different reactive sera with
10 sonicates of JM101, which had been transformed with non-recombinant pBTA224, and then passing this absorbed serum over a column of JM101 transformed with non-recombinant pBTA224¹⁵. Each serum was passed over a column of induced JM101 cells transformed with pRMIT, and
15 the column was washed for 24hr with 100-fold the bed volume of the column. Thence, lysine HCl was used to elute the bound antibodies¹⁵. The antibodies that had bound to the pRMIT absorbent were probed against fractionated placental mitochondria, a lysate of
20 expressing pRMIT, and a lysate of a control recombinant clone. They were also reacted by immunofluorescence with either HEp-2 cells or kidney tissue sections.

Isolation of mitochondrial antigen expressed as fused polypeptide.

25 Isolation of the fused polypeptide was performed by using gel filtration in the presence of SDS to fractionate the insoluble pellet and to obtain material suitable for immunization. A clone of pRMIT was incubated overnight at 37°C in L-broth containing 10µg/ml
30 ampicillin. Eighteen hours later it was diluted for log phase growth and was induced with 10mM IPTG for 4hr. The E.coli preparation was then harvested at 5000xG for 10min, and the pellets were resuspended in 40ml of 10mM Tris-HCl, pH8.0, containing 2mM EDTA. Lysozyme was then added to a

final concentration of 0.25mg/ml, and the mixture was rotated for 30min at room temperature. The solution was made up to 0.2% of Triton X-100* with continuous mixing for an additional 10min at room temperature. An equal volume of 10mM Tris-HCl with 2mM EDTA, 50mM NaCl, and 10mM MgCO₂ was added with a final concentration of 2mg/ml DNase. This was allowed to rotate for 15min at room temperature and then was spun at 1500xG for 5min. The pellet was discarded and the supernatant was spun for 30min at 10,000xG. This final pellet was then fractionated on a Sephacryl S-300* column in tandem with a Sephacryl S-400 column, after dispersion of the pellet in 0.1 M phosphate buffer, pH 6.0, with 2% SDS and 10mM dithiothreitol (DTT). The fractions were eluted at 50ml/hr, and 6-min fractions were collected for assay by analytical SDS-PAGE and immunoblotting. SDS was finally removed on a hydroxyapatite column after dilution with 0.5M phosphate buffer, pH6.8, and 1mM DTT. The purity of fractions was confirmed by SDS-PAGE and immunoblotting as above.

Immunization of mice.

Groups of six BALB/c female mice were immunized with 10µg of purified fused polypeptide in complete Freund's adjuvant (CFA). Three weeks later they were boosted with the same dose in CFA. Six weeks after the initial immunization, mice were bled and the sera were isolated. These sera were assayed at a dilution of 1/1000 and were probed against PAGE-separated placental mitochondria as above except that affinity-purified ¹²⁵I-goat anti-mouse Ig was used. The sera were also studied at 1/100 by immunofluorescence, using sections of HEp-2 cells and kidney tissue sections as described^{1,2,5}.

Nucleotide and amino acid sequence.

The cDNA insert of pRMIT was subcloned into M13,

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and the nucleotide sequence as determined^{16,17}. The correct frame and orientation of the insert was determined by double-stranded sequencing of an expressing clone¹⁷. The sequence was determined in both orientations, and use
5 was made of synthetic oligonucleotides to prime reactions¹⁸.

ELISA

Briefly, the purified recombinant fused polypeptide at 2µg/ml, diluted in carbonate buffer, was
10 absorbed to Immulon* microtitre plates (Dynatech Laboratories, Alexandria, VA) overnight at 4°C. After blocking the non-specific sites with foetal calf serum (FCS) buffer (5% FCS, 1% BSA, 0.3% gelatin in PBS), PBC sera diluted in FCS buffer, were incubated for one hour.
15 The plates were washed three times with PBS/0.1% tween and then incubated with each of the following mouse monoclonal antibodies specific against human heavy chain isotypes: SG-11 for IgG1, GOM-1 for IgG2, SJ-33 for IgG3, SK-44 for IgG4, MB-11 for IgM and GA-1 for IgA (Miles Scientific,
20 Naperville, IL). The binding of mouse MoAbs were detected with peroxidase conjugated goat anti-mouse IgG (Tago, Burlingame, CA) for all except SJ-33 which was detected with peroxidase conjugated goat anti-mouse IgM (Tago, Burlingame, CA). ABTS was used as the colour substrate
25 for the peroxidase. For detection of all isotypes of AMA, HRP-G Hulg was used in the place of isotype specific monoclonals.

Human myeloma proteins were used to obtain the optimal dilutions of the isotype specific MoAbs.
30 Predetermined dilutions of myeloma proteins were coated onto microtitre plates and ELISA performed as before with serial dilutions of isotype specific MoAbs followed by the peroxidase conjugated reagents. The dilutions of isotype specific MoAbs which gave similar O.D. units at

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approximately equal serum isotype concentrations were used in the ELISA.

To obtain the optimal serum dilutions for screening, previously screened (by immunofluorescence) AMA-positive PBC, progressive sclerosing cholangitis and normal sera were titrated by the ELISA. It was found that a serum dilution of 1:1000 yielded the highest signal to noise ratio, and this dilution was used to obtain all results. The cut-off point for negatives were determined as 2 standard deviation above the mean O.D. of normal sera.

B. RESULTS

Arrays of pRMIT in BTA224.

Subclones of pRMIT in JM101 were very immunoreactive when probed with sera from patients with PBC, whereas control clones were nonreactive. In contrast, sera from normal volunteers reacted with neither pRMIT in JM101 nor control clones. Positive colonies from arrays were used in all subsequent studies.

Specificity of pRMIT fused polypeptide.

Sera at dilutions of 1/1000 from 25 of 25 patients with PBC reacted with a 160kd fused polypeptide made in pRMIT (Table I and Fig.1). This band also reacted with a rabbit antiserum to β -galactosidase (data not shown). A number of bands corresponding to components of lower m.w. also were recognised, including one at approximately 36kd, which was apparently a breakdown product of the 160kd molecule. These lower m.w. materials were only associated with pRMIT and were immunoreactive with PBC sera. The titre of reactivity for these 25 sera ranged from 1:1000 to 1:1,000,000. With the use of the same 25 sera, the fused polypeptide was not detected in lysates of bacteria produced by non-recombinant pBTA224 or bacteria transformed with an irrelevant insert and induced

to express an abundant fused polypeptide. None of the sera from patients with systemic lupus erythematosus, rheumatoid arthritis, or chronic active hepatitis reacted with the fused protein at dilutions of 1/100, even with
5 autoradiographic exposures of up to 4 days.

TABLE 1. Reactivity of human sera with the pRMIT fused polypeptide.

	Group ^a	No. positive ^b /Total
5	PBC	25/25
	Normal persons	0/25
	Systemic lupus erythematosus	0/21
	Rheumatoid arthritis	0/18
10	Chronic active hepatitis	0/32

a PBC sera were studied at 1/1000 sera dilution; other groups were studied at 1/100 sera dilution.

15 b A positive blot was one in which reactivity to a band of 160kd was readily visible after an autoradiographic exposure level of 12hr. See Figure 1.

Identification of fused polypeptide.

20 After absorption with the lysate of pRMIT, sera from all seven patients with PBC were shown to be depleted of antibodies reactive with the 70kd antigen (Table II). In contrast, such absorption did not change the reactivity to the 45kd or 39kd antigen. No such depletion was seen when
 25 PBC sera were absorbed against a lysate of a control clone bound to a solid support. The finding that the reaction of PBC sera with the pRMIT fused polypeptide appeared to remove detectable anti-70kd reactivity indicates that the cDNA encodes all determinants recognised by the
 30 autoantibodies to the 70kd antigen (Table II; Fig.2).

Affinity-purified antibodies.

The eluted antibodies of five different PBC sera reacted with the 70kd polypeptide of fractionated placental mitochondria and with the 160kd fused

polypeptide in pRMIT (Fig.3), further indicating that pRMIT encodes the 70kd antigen. The eluted antibodies did not react with a lysate of bacterial proteins from a clone expressing a control liver cDNA. The eluted antibodies
5 also gave a characteristic pattern of anti-mitochondrial staining by immunofluorescence with either HEp-2 cells or kidney tissue sections.

Immune response of mice.

BALB/c mice, after injection of the pRMIT fused
10 polypeptide, gave an antibody response to the 70kd placental mitochondrial protein. Control nonimmunized mouse sera was nonreactive (Fig.4). In addition, these sera produced a typical pattern of anti-mitochondrial immunofluorescence on both HEp-2 cells and kidney tissue
15 sections (Fig.5).

Nucleotide and amino acid sequence.

The insert is 1370 base pairs long and consists entirely of coding region (Fig.6). The 456 amino acids would code for a polypeptide of approximately 48kd,
20 consistent with the observed size of the fused polypeptide produced by the clone; it is thus not a full-length sequence of the antigen. The sequence contains 11% proline, and the proline is frequently found preceded by short stretches of hydrophobic amino acids such as alanine
25 and valine, e.g., from nucleotides 54 to 102. Comparison of the sequence of the 70kd mitochondrial autoantigen with known protein and DNA sequences did not reveal any closely homologous sequences. The sequence is not present in mitochondrial DNA (data not shown), and the 70kd protein
30 is therefore coded for by nuclear genes.

The sensitivity of ELISA was compared with immunofluorescence for 37 patients with PBC (Figure 7). ELISA was found to be approximately 250 fold more sensitive; the average titre detected by ELISA was
35 $10^{5.4}$ whereas by immunofluorescence it was only 10^3 .

TABLE II. Absorption of PBC sera with the pRMIT fused polypeptide

Patient	Preabsorption Titre ^a			Postabsorption Titre ^a		
	70kd	45kd	39kd	70kd	45kd	39kd
1	1:2,000	1:2,000	0	0	1:2,000	0
2	>1:20,000	1:20,000	0	1:200	1:2,000	0
3	>1:20,000	>1:20,000	1:2,000	1:200	>1:20,000	1:2,000
4	1:2,000	1:200	0	0	1:200	0
5	>1:20,000	>1:20,000	1:2,000	1:200	>1:20,000	1:2,000
6	1:2,000	0	0	0	0	0
7	>1:20,000	1:2,000	0	0	1:2,000	0

a Reactivity on immunoblots using placental mitochondria as described; absorption with control lysates does not influence the titre.

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CLAIMS:

1. An isolated DNA molecule consisting essentially of a nucleotide sequence selected from the group consisting of a nucleotide sequence which encodes the human 70kDa mitochondrial autoantigen of primary biliary cirrhosis (PBC), a nucleotide sequence which encodes an antigenic fragment of said human autoantigen, and a nucleotide sequence which hybridizes to the nucleotide sequence encoding said human autoantigen and which encodes a mitochondrial autoantigen of PBC.
2. A DNA molecule according to claim 1, characterised by comprising a base sequence as shown in Figure 6 of Figure 8, or a fragment thereof.
3. A DNA molecule according to claim 2, characterised by comprising a base sequence corresponding to nucleotides 76-679 as shown in Figure 6.
4. A DNA molecule according to claim 2, characterised by comprising a base sequence coding for the amino acid sequence:

A E I E T D K A T I G F E V Q E E G Y L.
5. A recombinant DNA molecule comprising a nucleotide sequence as claimed in any one of claims 1 to 4, operatively linked to an expression control sequence.
6. An expression vector containing a nucleotide sequence as claimed in any one of claims 1 to 4, operatively linked to an expression control sequence.
7. A transformed host cell containing a nucleotide sequence as claimed in any one of claims 1 to 4, operatively linked to an expression control sequence.
8. A isolated peptide or polypeptide, encoded by a DNA molecule consisting essentially of a nucleotide sequence

selected from the group consisting of a nucleotide sequence which encodes the human 70kDa mitochondrial autoantigen of primary biliary cirrhosis (PBC), a nucleotide sequence which encodes an antigenic fragment of said human autoantigen, and a
5 nucleotide sequence which hybridizes to the nucleotide sequence encoding said human autoantigen and which encodes a mitochondrial autoantigen of PBC, said isolated peptide of polypeptide being substantially free of other proteins with which it is naturally associated.

10 9. An isolated peptide, or antigenically active fragment thereof according to claim 8, characterised by comprising an amino acid sequence as shown in Figure 6 or Figure 8, or an antigenically active fragment thereof.

10. An isolated peptide or polypeptide according to claim
15 9, characterised by comprising an amino acid sequence as coded by nucleotides 76-679 as shown in Figure 6.

11. An isolated peptide or polypeptide according to claim 9, characterised by comprising the amino acid sequence:

A E I E T D K A T I G F E V Q E E G Y L.

20 12. A process for preparing an isolated peptide, polypeptide or antigenically active fragment thereof, as claimed in claim 8, comprising the steps of:

(i) cultivating a transformed host cell, containing a nucleotide sequence as claimed in any one of claims 1 to 4,
25 operatively linked to an expression control sequence;

(ii) inducing the transformed host cell to express protein encoded by said nucleotide sequence; and

(iii) isolating and purifying the expressed peptide or polypeptide.

13. Use of an isolated peptide, polypeptide or antigenically active fragment thereof as claimed in any one of claims 8 to 11, as an antigen in a diagnostic test.

14. Use of an isolated peptide, polypeptide or
5 antigenically active fragment thereof as claimed in any one of claims 8 to 11, in treating patients.

15. Use of an isolated peptide, polypeptide or antigenically active fragment thereof as claimed in any one of claims 8 to 11 for the manufacture of a medicament for treating
10 patients.

16. A use according to claim 14 or 15 wherein said patients suffer from primary biliary cirrhosis (PBC).

17. A diagnostic test for detection of anti-mitochondrial antibody in a serum sample, which comprises the steps of;

15 (i) contacting said serum sample with an isolated peptide, polypeptide or antigenically active fragment thereof, as claimed in any one of claims 8 to 11, said isolated peptide, polypeptide or antigenically active fragment thereof being immobilised on a support, and

20 (ii) detecting the presence of anti-mitochondrial antibody in said serum bound to said isolated peptide, polypeptide or antigenically active fragment thereof.

18. A diagnostic test kit for detection of anti-mitochondrial antibody in a serum sample, which comprises;

25 (i) a support having immobilised thereon an isolated peptide, polypeptide or antigenically active fragment thereof, as claimed in claim 8, and

(ii) means for detecting the presence of

anti-mitochondrial antibody in said serum bound to said isolated peptide, polypeptide or antigenically active fragment thereof.

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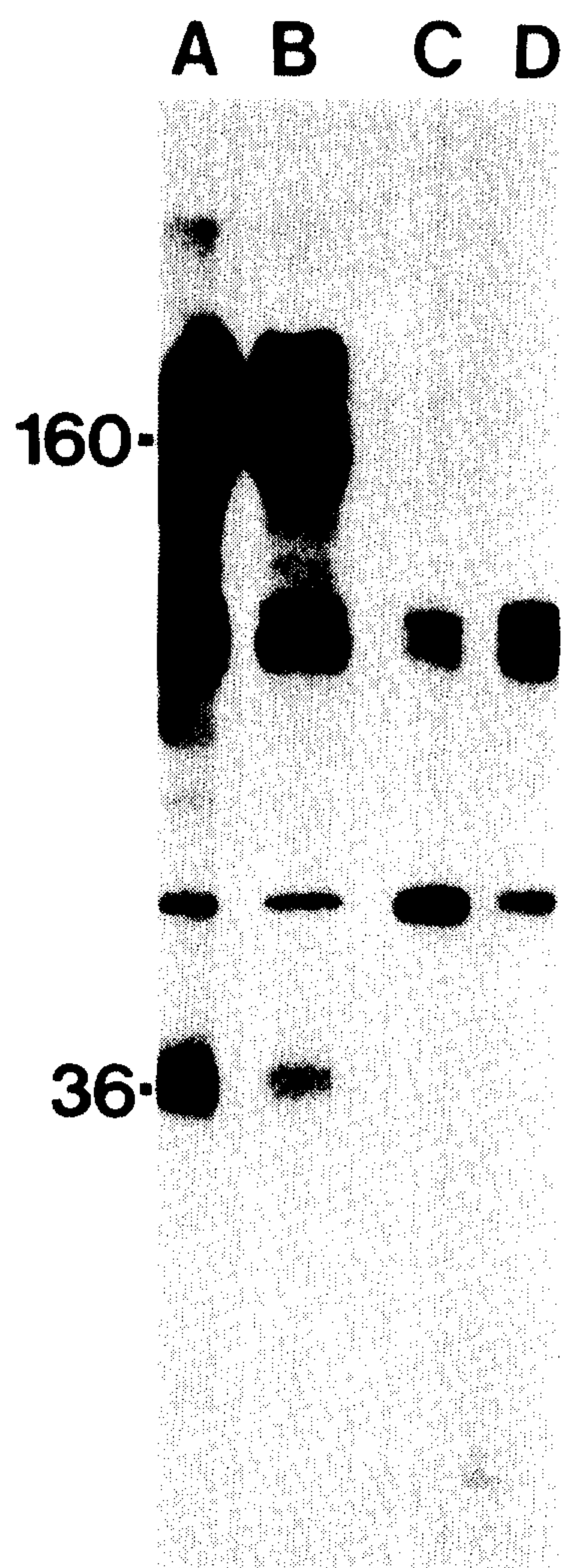


Fig. 1.

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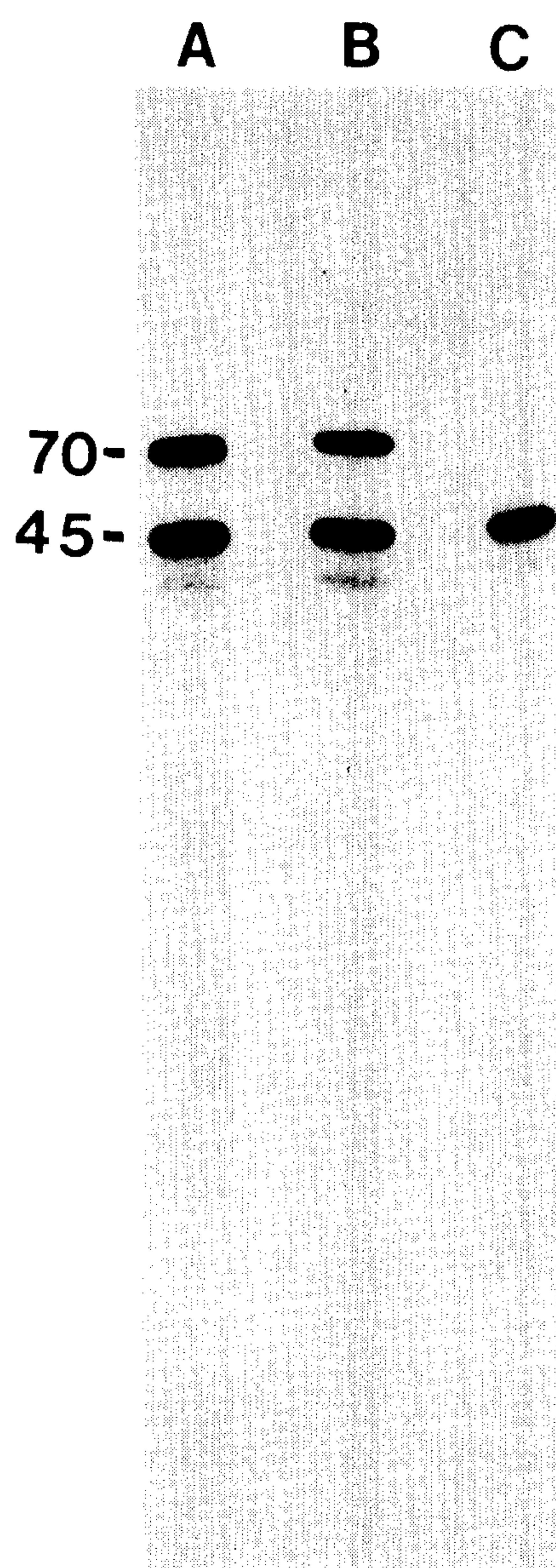


FIG. 2.

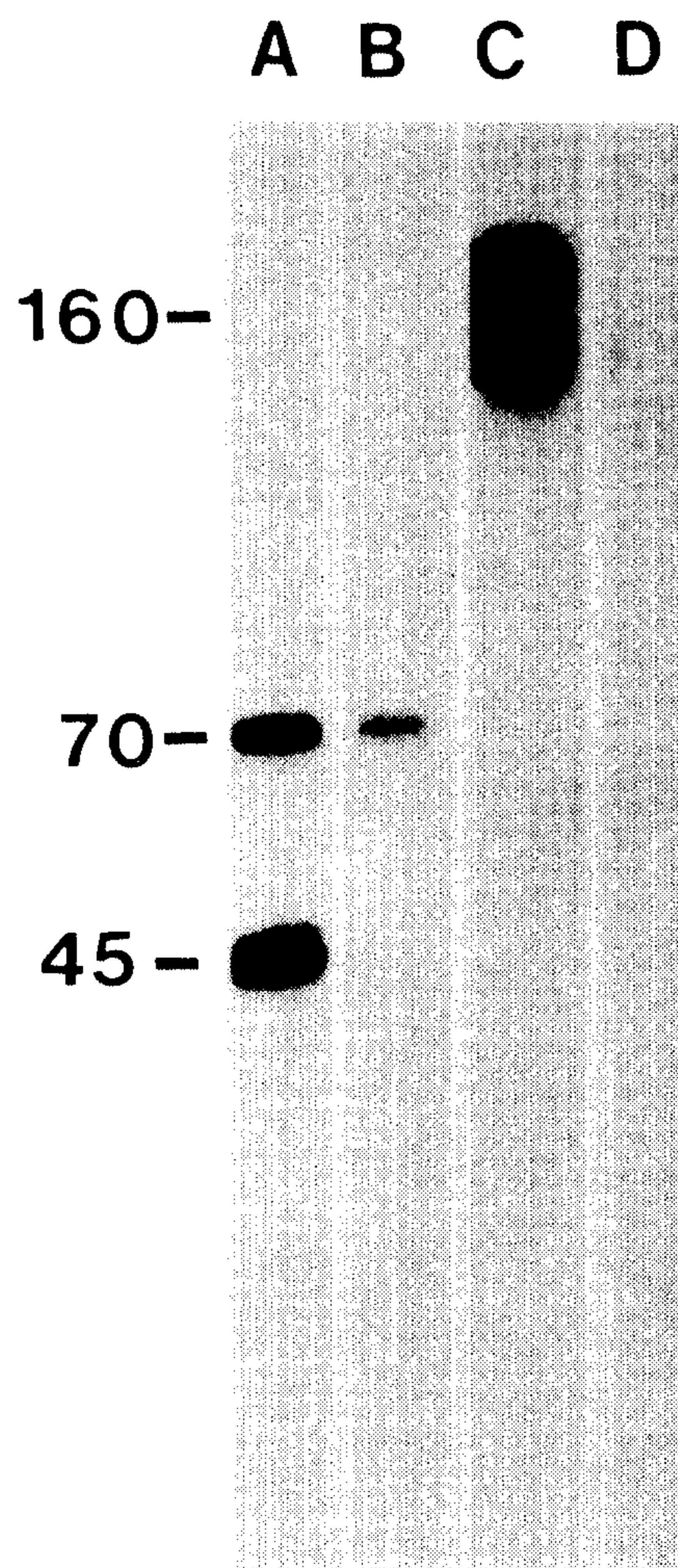


FIG. 3.

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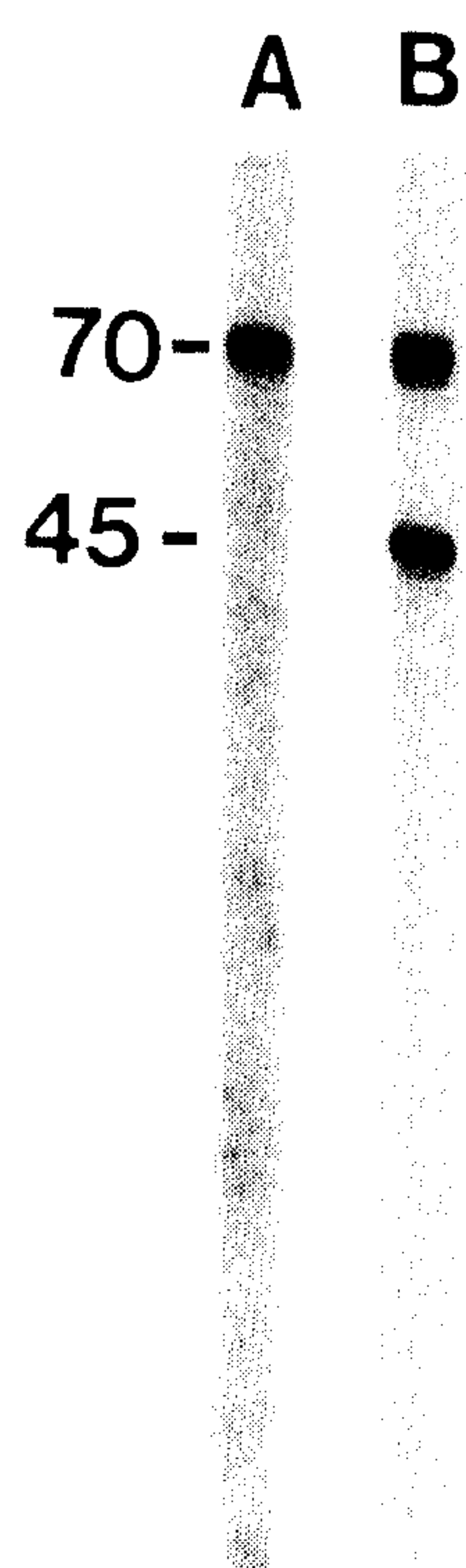


FIG. 4.

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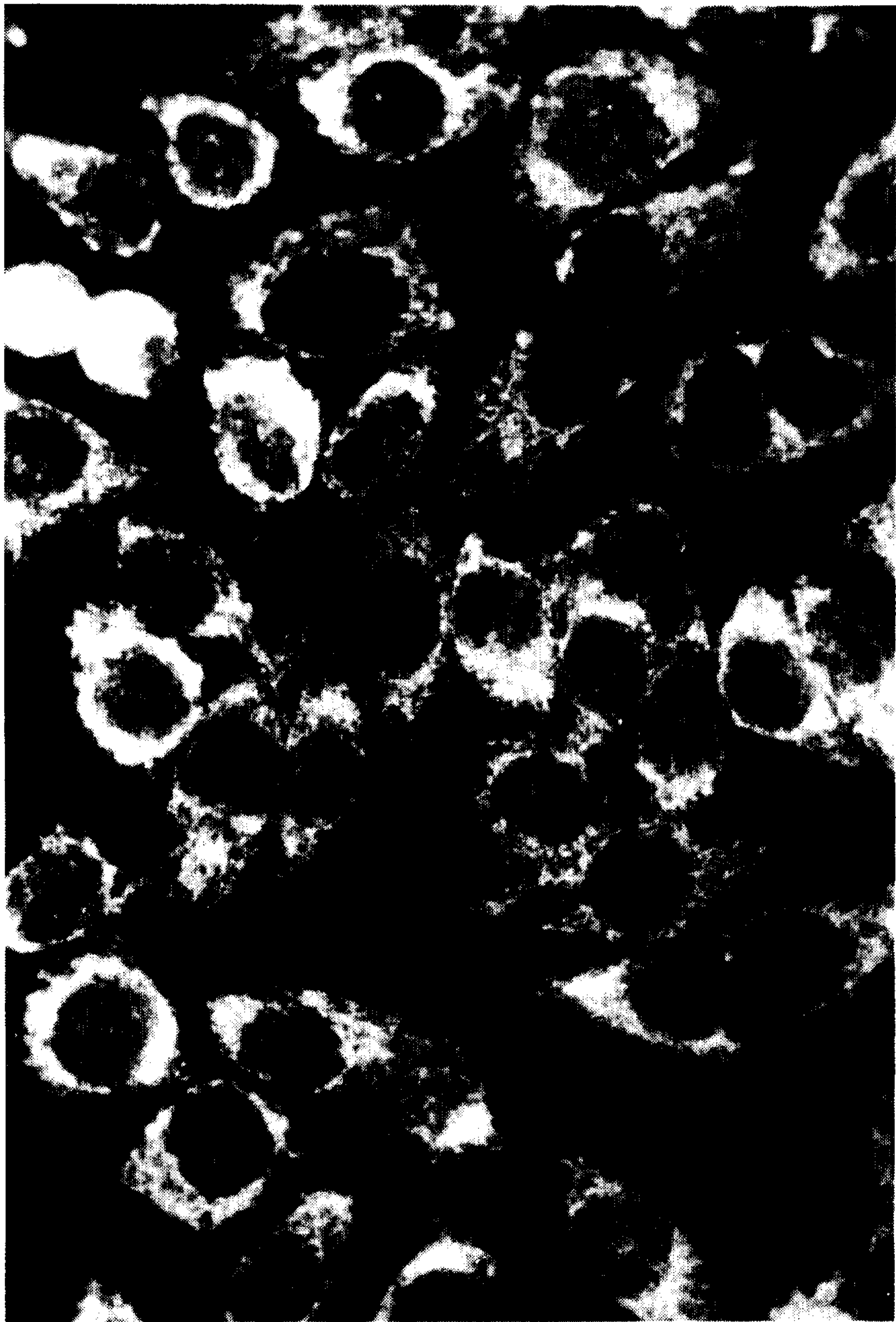


FIG. 5.

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Gly	Pro	Glu	Ala	Phe	Lys	Asn	Tyr	Thr	Leu	Asp	Ser	Ala	Thr
GGG	CCT	GAG	GCT	TTT	TAAA	AAT	TAT	ACAT	TGG	ATT	CAG	CAACA	
10					20				30			40	
Pro	Ala	Ala	Pro	Ser	Ala	Ser	Ala	Pro	Gly	Ser	Ser	Tyr	Pro
CCAG	CTG	CAC	CTT	CTG	CAAG	TGCT	CCAG	GTA	GCT	CCT	TAT	CCC	
100					110				120			130	
Gly	Thr	Val	Gln	Arg	Trp	Glu	Lys	Lys	Val	Gly	Glu	Lys	Leu
GGC	ACCG	TCC	CAG	AGG	TGG	GAAA	AGAA	AGT	GGG	GAG	AGA	AAG	CTG
190					200				210			220	
Gly	Phe	Glu	Val	Gln	Glu	Glu	Gly	Tyr	Leu	Ala	Lys	Ile	Leu
GGC	TTT	GAA	GTACA	AGA	AGA	AGG	TTAT	CTG	GCA	AAA	AAT	CCT	G
280					290				300			310	
Ile	Val	Glu	Lys	Gln	Glu	Asp	Ile	Ala	Ala	Phe	Ala	Asp	Tyr
ATA	GTA	GAAAA	ACAG	GAA	GAT	TAT	AGC	AGC	ATTT	GCAG	ACT	AC	
370					380				390			400	
Pro	Pro	Pro	Val	Ala	Ala	Val	Pro	Pro	Ile	Pro	Gln	Pro	Leu
CCAC	CCCC	CAG	TGG	CAG	CTG	TTCC	TCCC	ATC	CCCC	CAG	CCT	TTTA	
460					470				480			490	
Val	Ser	Pro	Leu	Ala	Lys	Lys	Leu	Ala	Ala	Glu	Lys	Gly	Ile
GTT	AGC	CTC	TTG	CAA	AGAA	ATT	TGG	CAG	CAG	AGAA	AGG	GATT	
550					560				570			580	
Lys	Asp	Ile	Asp	Ser	Phe	Val	Pro	Thr	Lys	Ala	Ala	Pro	Ala
AAG	GAC	ATT	GACT	CTTT	TGT	GCCT	ACTA	AGG	CTG	CTC	CTG	CC	
640					650				660			670	

Fig. 6a.

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AlaAlaThrGlnAlaAlaProAlaProAlaAlaAlaProAlaAlaAla				
GCTGCCACACAGGCAGCCCCAGCCCCAGCTGCAGCTCCAGCTGCTGCC	50	60	70	80 90
ValHisMetGlnIleValLeuProAlaLeuSerProThrMetThrMet				
G TTCACATGCAGATTG TTCCTTCCTGCCCTCTCCCCAACCATGACCATG	140	150	160	170 180
SerGluGlyAspLeuLeuAlaGluIleGluThrAspLysAlaThrIle				
AGTGAAGGAGACTTGCTGGCAGAGATAGAGACCGACAAGGCCACCATA	230	240	250	260 270
ValProGluGlyThrArgAspValProLeuGlyThrProLeuCysIle				
GTCCCTGAAGGCACAAGGGATG TTCCTCTGGGAACCCCGCTATGTATC	320	330	340	350 360
ArgProThrGluValThrSerLeuLysProGlnAlaProProProVal				
AGGCCAACAGAAGTGACCAGCTTAAAGCCACAGGCACCACCACCTGTC	410	420	430	440 450
AlaProThrProSerAlaAlaProAlaGlyProLysGlyArgValPhe				
GCACCTACCCCTCAGCCGCTCCTGCTGGACCAAAGGGAAGGGTGTTTC	500	510	520	530 540
AspLeuThrGlnValLysGlyThrGlyProGluGlyArgIleIleLys				
GACCTCACCCAAGTTAAAGGGACGGGACCAGAAGGCAGAATCATCAAG	590	600	610	620 630
AlaAlaAlaAlaAlaProProGlyProArgValAlaProThrProAla				
GCTGCAGCAGCTGCTCCCCCGGGTCCAAGAGTGGCACCAACTCCTGCA	680	690	700	710 720

Fig. 6b.

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GlyValPheIleAspIleProIleSerAsnIleArgArgVal			
GGTGTCTTCATAGACATCCCCATCAGCAACATTCGTCGAGTG	730	740	750
			760
LeuSerValAspValAsnMetGlyGluValLeuLeuValArg			
CTTTCTGTTGATGTAAATATGGGAGAGGTGCTGTTGGTGCGG	820	830	840
			850
AspPheIleIleLysAlaSerAlaLeuAlaCysLeuLysVal			
GACTTCATCATAAAAGCTTCAGCTTTGGCCTGCCTGAAAGTT	910	920	930
			940
ValValAspValSerValAlaValSerThrProAlaGlyLeu			
GTGGTTGATGTCAGCGTTGCTGTCAGTACCCCTGCAGGACTT	1000	1010	1020
			1030
AlaSerAspValValSerLeuAlaSerLysAlaArgGluGly			
GCTAGTGATGTTGTTTCTTTAGCCTCCAAAGCAAGAGAGGGT	1090	1100	1110
			1120
LeuGlyMetPheGlyIleLysAsnPheSerAlaIleIleAsn			
TTAGGGATGTTTCGGAATTAAGAATTTCTCTGCGATTATTAAC	1180	1190	1200
			1210
IleProAlaAspAsnGluLysGlyPheAspValAlaSerVal			
ATCCCTGCAGATAATGAGAAAGGCTTTGACGTGGCTAGTGTG	1270	1280	1290
			1300
LeuGluProSerGlyLeuLeu			
TTGGAGCCCAGTGGCTTGCT	1360	1370	

FIG. 6c.

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IleAlaGlnArgLeuMetGlnSerLysGlnThrIleProHisTyrTyr				
ATTGCGCAGAGGCTCATGCAGTCGAAGCAGACTATACCTCATTATTAC	770	780	790	800
				810
LysGluLeuAsnLysMetLeuGluGlyLysGlyLysIleSerValAsn				
AAGGAAC TTAATAAGATGCTTGAAGGTAAAGGAAAAATCTCCGTCAAT	860	870	880	890
				900
ProGluAlaAsnSerSerTrpMetAspThrValIleArgGlnAsnHis				
CCTGAAGCAAAC TCATCTTGGATGGACACAGTTATACGACAAAATCAT	950	960	970	980
				990
IleThrProIleValPheAsnAlaHisIleLysGlyLeuGluThrIle				
ATCACCCCTATTGTGTTTAATGCACACATAAAAGGACTGGAAACCATT	1040	1050	1060	1070
				1080
LysLeuGlnProHisGluPheGlnGlyGlyThrPheThrIleSerAsn				
AACTTCAGCCTCACGAGTTCCAGGGTGGGACATTTACAATCTCCAAC	1130	1140	1150	1160
				1170
ProProGlnAlaCysIleLeuAlaIleGlyAlaSerGluAspLysLeu				
CCACCTCAGGCATGTATTTTGGCAATTGGTGCTTCCGAGGATAAACTG	1220	1230	1240	1250
				1260
MetSerValThrHisSerAlaValIleIleGluLeuTrpMetGluGln				
ATGTCTGTCACACACTCAGCTGTGATCATCGAGTTGTGGATGGAGCAG	1310	1320	1330	1340
				1350

Fig. 6d.

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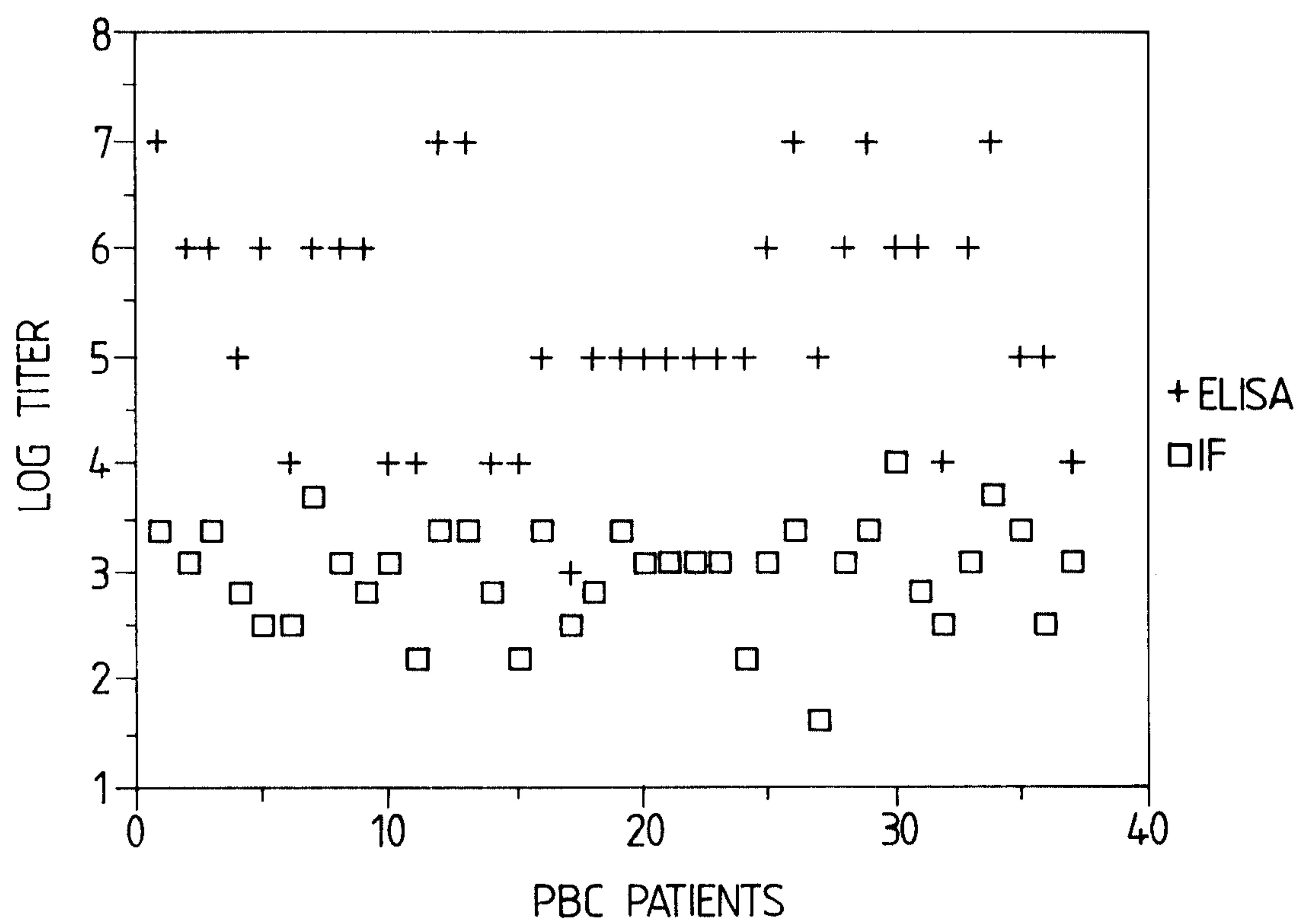


Fig. 7.

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1 3 4 1 2 3 1

ProGlySerSerTyrProProHisMetGlnValLeuLeuProAla
CCTGGTAGCTCATATCCCCCTCACATGCAGGTACTTCTTCCTGCC
10 20 30 40

LysValGlyGluLysLeuSerGluGlyAspLeuLeuAlaGluIle
AAAGTGGGTGAGAAGCTAAGTGAAGGAGACTTACTGGCAGAGATA
100 110 120 130

TyrLeuAlaLysIleLeuValProGluGlyThrArgAspValPro
TATCTGGCAAAAATCCTGGTCCCTGAAGGCACAAGAGATGTCCCT
190 200 210 220

SerAlaPheAlaAspTyrArgProThrGluValThrAspLeuLys
TCAGCATTGCTGACTATAGGCCAACCGAAGTAACAGATTAAAA
280 290 300 310

ProThrProGlnProLeuAlaProThrProSerAlaProCysPro
CCAACCTCCCGAGCCTTTAGCTCCTACACCTTCAGCACCTTGCCCA
370 380 390 400

AlaLysLysLeuAlaValGluLysGlyIleAspLeuThrGlnVal
GCAAAGAAGTTGGCAGTAGAGAAAGGGATTGATCTTACACAAGTA
460 470 480 490

SerPheValProSerLysValAlaProAlaProAlaAlaValVal
TCTTTTGTGCCTAGTAAAGTTGCTCCTGCTCCGGCAGCTGTTGTG
550 560 570 580

ThrAspIleProIleSerAsnIleArgArgValIleAlaGlnArg
ACAGATATCCCAATCAGCAACATTCGTGGGGTTATTGCACAGCGA
640 650 660 670

AspValAsnMetGlyGluValLeuLeuValArgLysGluLeuAsn
GATGTAAATATGGGAGAAGTTTTGTTGGTACGGAAAGAACTTAAT
730 740 750 760

IleLysAlaSerAlaLeuAlaCysLeuLysValProGluAlaAsn
ATAAAAGCTTCAGCTTTGGCATGTTTAAAAGTTCCCGAAGCAAAT
820 830 840 850

ValSerValAlaValSerThrProAlaGlyLeuIleThrProIle
GTCAGTGTTGCGGTCAGTACTCCTGCAGGACTCATCACACCTATT
910 920 930 940

Fig. 8a.

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Leu	Ser	Pro	Thr	Met	Thr	Met	Gly	Thr	Val	Gln	Arg	Trp	Glu	Lys
CTCTCT	CCCACC	ATGACC	ATGGGG	CACAGT	TCAGAG	ATGGG	AAAAA							
50		60		70		80	90							
Glu	Thr	Asp	Lys	Ala	Thr	Ile	Gly	Phe	Glu	Val	Gln	Glu	Glu	Gly
GAAACT	GACAAAG	CCACTA	TAGGTT	TTTGA	AGTAC	AGGAAG	AAGGT							
140		150		160		170	180							
Leu	Gly	Thr	Pro	Leu	Cys	Ile	Ile	Val	Glu	Lys	Glu	Ala	Asp	Ile
CTAGGA	ACCCCA	CTCTGT	ATCATT	GTAGAA	AAAGAG	GCAGAT	ATA							
230		240		250		260	270							
Pro	Gln	Val	Pro	Pro	Pro	Thr	Pro	Pro	Pro	Val	Ala	Ala	Val	Pro
CCACAAG	TGCCACC	ACCTAC	CCCCAC	CCCCCG	GTGGCC	GCTGTT	CCT							
320		330		340		350	360							
Ala	Thr	Pro	Ala	Gly	Pro	Lys	Gly	Arg	Val	Phe	Val	Ser	Pro	Leu
GCTACT	CCTGCT	GGACCA	AAGGGA	AGGGT	GTTTGT	TAGCCCT	CCT							
410		420		430		440	450							
Lys	Gly	Thr	Gly	Pro	Asp	Gly	Arg	Ile	Thr	Lys	Lys	Asp	Ile	Asp
AAAGGG	ACAGGAC	CAGATG	GTAGAA	TACCA	AAGAAG	GATATC	GAC							
500		510		520		530	540							
Pro	Pro	Thr	Gly	Pro	Gly	Met	Ala	Pro	Val	Pro	Thr	Gly	Val	Phe
CCTCCC	ACAGGT	CCTGGA	ATGGCA	CCAGTT	CCTAC	AGGTGT	CCTC							
590		600		610		620	630							
Leu	Met	Gln	Ser	Lys	Gln	Thr	Ile	Pro	His	Tyr	Tyr	Leu	Ser	Ile
TTAATG	CAATCA	AAGCAA	ACCATA	CTCATT	TATTAC	CTTTCT	ATC							
680		690		700		710	720							
Lys	Ile	Leu	Glu	Gly	Arg	Ser	Lys	Ile	Ser	Val	Asn	Asp	Phe	Ile
AAGATA	TAGAA	GGGAGA	AGCAAA	ATTCTG	TCAATG	ACTTCAT	C							
770		780		790		800	810							
Ser	Ser	Trp	Met	Asp	Thr	Val	Ile	Arg	Gln	Asn	His	Val	Val	Asp
TCTTCT	TGGATG	GACAC	AGTTAT	AAGACA	AAATCAT	GTTGTT	GAT							
860		870		880		890	900							
Val	Phe	Asn	Ala	His	Ile	Lys								
GTGTTT	AATGCA	CATATA	AAAA											
950		960												

Fig. 8b.

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