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(72) Inventeurs/Inventors:  
 HILES, IAN D., GB;  
 DHAND, RITU, GB;  
 WATERFIELD, MICHAEL D., GB;  
 PARKER, PETER J., GB;  
 OTSU, MASAYUKI, GB;

(73) Propriétaire/Owner:

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(57) **Abrégé/Abstract:**

This invention relates to new polypeptides which exhibit kinase activity or, more specifically, which show phosphoinositide (PI)3-kinase activity. Such polypeptides are involved in pathways responsible for cellular growth and differentiation. An isolated polypeptide which possesses PI3-kinase activity when produced by recombinant production in insect cells is disclosed.

(72) **Inventeurs(suite)/Inventors(continued)**: PANAYOTOU, GEORGE, GB; VOLINIA, STEFANO, GB; GOUT, IVAN, GB; FRY, MICHAEL J., GB

(73) **Propriétaires(suite)/Owners(continued)**: LUDWIG INSTITUTE FOR CANCER RESEARCH LTD, CH

(74) **Agent**: OGILVY RENAULT LLP/S.E.N.C.R.L., S.R.L.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/GB93/00761</p> <p>(22) International Filing Date: 13 April 1993 (13.04.93)</p> <p>(30) Priority data: 9208135.5                      13 April 1992 (13.04.92)                      GB</p> <p>(71) Applicant (for all designated States except US): LUDWIG INSTITUTE FOR CANCER RESEARCH [CH/GB]; St. Mary's Hospital Medical School, Norfolk Place, Paddington, London WG 1PG (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : HILES, Ian, D. [GB/GB]; FRY, Michael, J. [GB/GB]; DHAND, Ritu [GB/GB]; WATERFIELD, Michael, D. [GB/GB]; Courtauld Building, 91 Riding House Street, London W1P 8BT (GB). PARKER, Peter, J. [GB/GB]; ICFR, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX (GB). OTSU, Masayuki [GB/GB]; PANAYOTOU, George [GB/GB]; VOLINIA, Stefano [GB/GB]; GOUT, Ivan [GB/GB]; Courtauld Building, 91 Riding House Street, London W1P 8BT (GB).</p>	<p>(74) Agents: BIZLEY, Richard, Edward et al.; Hepworth, Lawrence, Bryer &amp; Bizley, 2nd Floor, Gate House South, West Gate, Harlow, Essex CM20 1JN (GB).</p> <p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: POLYPEPTIDES HAVING KINASE ACTIVITY, THEIR PREPARATION AND USE</p> <p>(57) Abstract</p> <p>This invention relates to new polypeptides which exhibit kinase activity or, more specifically, which show phosphoinositide (PI) 3-kinase activity. Such polypeptides are involved in pathways responsible for cellular growth and differentiation. An isolated polypeptide which possesses PI3-kinase activity when produced by recombinant production in insect cells is disclosed.</p>		

Polypeptides Having Kinase Activity, Their  
Preparation and Use

5 This invention relates to new polypeptides which exhibit kinase activity. More specifically, the invention is concerned with polypeptides which show phosphoinositide (hereinafter "PI") 3-kinase activity, particularly molecules involved in pathways responsible for cellular growth and differentiation.

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Major advances have taken place in our knowledge of the structure and function of the signal transducing molecules and second messenger systems coupled to cell surface receptors. Thus, a subset of polypeptide growth factor receptors belong to the family of protein-tyrosine kinases (hereinafter "PTK" and activation of these receptors following ligand binding involves autophosphorylation of the receptor as well as phosphorylation of a number of intracellular substrate proteins (reviewed in Ullrich, A et al., 1990). The importance of receptor autophosphorylation had been unclear until recently, when evidence from several laboratories has suggested that this event may mediate the formation of complexes between receptor proteins and putative growth regulatory proteins such as phospholipase C $\gamma$ (PLC $\gamma$ ) (Meisenhelder et al, 1989), phosphatidylinositol PI3-kinase (Coughlin, S R et al, 1989). GTPase-activating protein (GAP) (Kaplan et al, 1990), the serine/threonine kinase Raf (Morrison et al, 1989), and members of the src-family of protein-tyrosine kinases (Kypta, R M et al., 1990) (reviewed in Cantley, L C et al., 1991).

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The association of PI kinase activity with activated receptors is of particular interest since increased turnover of PI and its phosphorylated derivatives has been implicated in the action of hormones, growth factors and transformation of cells by DNA and RNA viruses

(reviewed in Whitman, M et al., 1988; Cantley et al., 1991). Several species of PI kinase are known to exist, but up to now none of these enzymes have been characterised by cloning and expression and the demonstration of PI kinase activity. Fibroblasts contain at least two PI kinase activities which are distinguishable on the basis of their detergent sensitivity and kinetic properties (Whitman, M et al., 1987). These two activities were classified as Type I (inhibited by non-ionic detergents) and Type II (stimulated by non-ionic detergents and inhibited by adenosine). A third distinct species (Type III) has been identified in bovine brain but remains poorly characterised (Enderman, G et al., 1987). One species of PI kinase activity in particular has become of major interest in the search for second messenger systems linked to protein-tyrosine kinases because this activity was shown to co-immunoprecipitate with activated platelet-derived growth factor (PDGF) receptors (Kaplan, D R et al., 1987; Coughlin, S R et al., 1989) and with the polyoma middle T antigen/pp60<sup>c-src</sup> (mT:pp60<sup>c-src</sup>) complex (Whitman, M et al., 1985). This activity has been shown to be due to a Type I PI kinase which produces novel inositol lipids phosphorylated at the D-3 position of the inositol ring (Whitman, M et al., 1988). More recently this enzyme has also been shown to associate with the CSF-1 receptor (Varticovski, L et al., 1989) kit (Lev et al, 1991), the epidermal growth factor (EGF) receptor (Bjorge et al, 1990), the PDGF  $\alpha$ -receptor (Yu et al, 1991), the insulin receptor (Ruderman et al, 1990), the hepatocyte growth factor receptor, Met (Graziani et al, 1991), and with activated non-receptor protein-tyrosine kinases (Fukui & Hanafusa, 1989; Chan et al, 1990; Varticovski et al, 1991).

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PI3 kinase activity has been closely linked to the presence of 81/85 kD proteins in these immunoprecipitates which can be phosphorylated on tyrosine residues by the

associated protein-tyrosine kinase both *in vitro* and *in vivo* (Kaplan, D R et al., 1987; Courtneidge, S A et al., 1987; Cohen et al., 1990). Recently a 650 fold purification of PI3-kinase from bovine brain was described which, among other proteins present in the purest preparation, contained an 85 kD protein which was shown to be an *in vitro* substrate for the PDGF and EGF receptors (Morgan, S J et al., 1990). Using sequence information from tryptic peptides derived from this protein, two homologous bovine p85 proteins, denoted p85 $\alpha$  and p85 $\beta$  (Otsu, M et al., 1991) have recently been cloned. Two other groups have independently cloned murine and human p85 $\alpha$  homologues using different strategies (Escobedo, J A et al., 1991b; Skolnik, E Y et al., 1991). Both of these p85 proteins can be demonstrated to bind directly to phosphorylated PDGF receptor *in vitro* (Otsu, M et al., 1991; Escobedo, J A et al., 1991b). These proteins may function as the receptor binding subunits of the PI3-kinase since neither of them can be shown to encode intrinsic PI3-kinase activity when expressed in a variety of cell systems (Otsu, M et al., 1991; Escobedo, J A et al., 1991b). However, immunoprecipitation of  $^{125}\text{I}$ -labelled bovine brain PI3-kinase with antibodies raised against p85 proteins precipitates an 85 kD protein together with a second protein of molecular weight 110 kD (Otsu, M et al., 1991).

PI3-kinase is one of a growing number of potential signalling proteins which associate with protein-tyrosine kinases activated either by ligand stimulation or as a consequence of cell transformation. A common feature of all these proteins (apart from Raf), is that they contain one or more SH2 domains (src homology) (Koch, C A et al., 1991). Both p85 $\alpha$  and p85 $\beta$  proteins contain two SH2 domains. Experiments from a number of laboratories have suggested that these domains may function by binding to

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peptide sequences usually phosphorylated on tyrosine residues, and thus mediate the complex formation which follows activation of protein-tyrosine kinases (Anderson et al, 1990; Meyer & Hanafusa, 1990; Moran et al, 1990; Matsuda et al, 1991; Meyer et al, 1991; reviewed in Koch, C A et al., 1991). In support of this, several studies suggest that tyrosine phosphorylation of the PDGF receptor or polyoma mT is essential for its association with proteins such as the PI3-kinase (Kazlauskas, A et al., 1989; Talmage, D A et al., 1989) GAP (Kaplan et al, 1990; Kazlauskas, A et al., 1990) and PLC $\gamma$  (Anderson et al, 1990; Margolis et al, 1990). The precise tyrosine residue required for binding of the PI3-kinase activity (and an 85 kD phosphoprotein) to the human PDGF receptor has been mapped to tyrosine 751 which lies within the kinase insert region of the protein-tyrosine kinase domain (Kazlauskas & Cooper, 1989, 1990; Kazlauskas et al, 1991). The binding sites for other proteins to this receptor (eg., PLC $\gamma$ , GAP and src-family kinases) have yet to be mapped, but these proteins may associate via other phosphorylated tyrosine residues.

This invention has been facilitated by the finding that certain synthesized peptides from the human PDGF  $\beta$ -receptor, namely peptides derived from the sequence around tyrosine 751 of the PDGF receptor, can be used to bind and isolate bovine brain PI3-kinase, making it possible to purify further partially purified bovine brain PI3-kinase (as described by Morgan et al, 1990) to apparent homogeneity and to obtain reasonably pure p110 protein. As will be described hereinafter, the PI3-kinase requires a phosphopeptide column containing a YXXM motif for its isolation by such a technique, the tyrosine being phosphorylated. Only if a column of this type is used are both the 85 kD and 110 kD proteins secured whereas 85 kD subunit binds to all phosphopeptide affinity columns tested and only fails to bind to non-

phosphorylated peptides. Moreover, the relatively small size of the phosphopeptides used for such columns gives good specificity and a high density of affinity groups per unit volume of column.

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This purification has allowed amino acid sequence information to be provided, and cDNA cloning to be performed. Such cloning has revealed some interesting facts. Thus, p110 is a 1068 amino acid protein having an unexpectedly high (compared to SDS-PAGE Figures) calculated molecular weight of about 124 kD (124247). The protein is related to Vps34p, a *Saccharomyces cerevisiae* protein involved in the sorting of proteins to the vacuole. Surprisingly, p110 when expressed in COS-1 cells was inactive and activity was only seen when complexed with p85. However, when expressed in insect cells, p110 could be shown to possess intrinsic kinase activity. The novel p110 polypeptide can be associated with p85 $\alpha$  into an active p85 $\alpha$ /p110 complex which binds the activated colony stimulating factor-1 receptor. The invention is also based upon these discoveries and unpredictable findings.

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Thus, in one aspect the present invention provides an isolated polypeptide of calculated molecular weight approximately 124 kD which possesses PI3-kinase activity when produced by recombinant production in insect cells, or a polypeptide derivable therefrom which has PI3-kinase activity and binds, when associated with a p85 mammalian PI3 kinase subunit, to a phosphopeptide which includes the YXXM motif, the tyrosine being phosphorylated. Such polypeptides are preferably those capable of association with p85 subunits of mammalian PI3-kinases to produce active p85/p110 complexes. Preferably, the polypeptides have either the amino acid sequence of Figure 9 hereof or exhibit significant sequence homology therewith. Preferred are polypeptides having at least amino acids 272 to 1068 of the sequence of Figure 9 hereof.



As used herein, the term "PI3-kinase activity" means phosphoinositide-3 kinase activity.

5 The invention embraces polypeptides as defined and exhibiting sequence homology with any chosen mammalian species of PI3-kinase. A human sequence is given in Figure 16 hereof. Amino acids 272-1069 (stop codon) (see Figure 16) are >99% conserved with the bovine p110 cDNA sequence and correspond to amino acids 272-1069  
10 (stop codon) of the sequence of Figure 9. Upstream of amino acid 37 (human sequence) there is no sequence similarity between the p110 cDNA sequences from the two species.

15 The invention includes antibodies, monoclonal or otherwise, against the polypeptides of the invention.

In another aspect the invention includes a DNA sequence comprising either: (a) a sequence set out in Figure 9  
20 hereof; (b) any one of the subsequences A to N of Figure 9 hereof; (c) the sequence represented by bases 816 to 3204 of Figure 9 hereof; (d) a sequence set out in Figure 16 hereof; or (e) a DNA sequence hybridizable to (a), (b), (c) or (d); which sequence (a), (b), (c), (d) or (e)  
25 encodes a polypeptide which has PI3-kinase activity if expressed in insect cells or can complex with a p85 mammalian PI3-kinase subunit to produce such activity. Subsequences A to N, referred to above, are themselves part of the present invention.

30 Hybridization conditions which may be used to find active sequences include, but are not limited to, 1 M NaCl/10 x Denhardt's solution/50 mM Tri-HCl (pH 7.4)/ 10 mM EDTA/ 0.1% SDS/100 µg/ml denatured herring sperm DNA (Sigma) at  
35 65°C for 16 h, with the following washing conditions, i.e. 2 x SSC/0.1% SDS, 42°C---->0.5 x SSC/0.1% SDS, 50°C---->0.1 x SSC/0.1% SDS, 65°C---->0.1 x SSC/0.1% SDS, 68°C.

The invention further provides a DNA construct comprising a DNA sequence as defined above under the control of a control sequence and in proper reading frame in an expression vector.

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The control sequence may include a regulatable promoter (e.g. Trp). Selected host cells which have been genetically altered to permit expression of the encoded polypeptide by the incorporation of such a construct are another aspect of the invention, and the invention also includes both a method of making such a polypeptide by cultivating such host cells and, of course, the resulting polypeptides.

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In general, new polypeptides of the invention can be used to provide PI3-kinase activity, either directly or after complexing with a mammalian p85 subunit. Enzymatically active complexes involving the above-defined polypeptides are part of the invention.

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The invention envisages a method of prophylaxis or therapy which involves the encouragement or discouragement of cell proliferation by the action of an agonist or antagonist, respectively, for the PI3-kinase activity of a polypeptide of the invention or complex including the same, wherein said cell proliferation is mediated through a cell surface receptor interactive with said activity. The present invention opens up for the first time, by providing pure sequenced active protein, the opportunity to screen (using standard techniques) for such agonists or antagonists.

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Another aspect of the invention is a pharmaceutical or veterinary formulation comprising an agonist or antagonist as defined above formulated for pharmaceutical or veterinary use, respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. Conventional pharmaceutical or

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veterinary practice may be employed to provide suitable formulations or compositions.

Thus, the formulations of this invention can be applied  
5 to parenteral administration, for example, intravenous,  
subcutaneous, intramuscular, intraorbital, ophthalmic,  
intraventricular, intracranial, intracapsular,  
intraspinal, intracisternal, intraperitoneal, topical,  
intranasal, aerosol, scarification, and also oral,  
10 buccal, rectal or vaginal administration.

Parenteral formulations may be in the form of liquid  
solutions or suspensions; for oral administration,  
formulations may be in the form of tablets or capsules;  
15 and for intranasal formulations, in the form of powders,  
nasal drops, or aerosols.

Methods well known in the art for making formulations are  
to be found in, for example, "Remington's Pharmaceutical  
20 Sciences". Formulations for parenteral administration  
may, for example, contain as excipients sterile water or  
saline, polyalkylene glycols such as polyethylene glycol,  
oils of vegetable origin, or hydrogenated naphthalenes.  
Biocompatible, biodegradable lactide polymers,  
25 lactide/glycolide copolymers, or polyoxyethylene-  
polyoxypropylene copolymers may be used to control the  
release of the present factors. Other potentially useful  
parenteral delivery systems for the factors include  
ethylene-vinyl acetate copolymer particles, osmotic  
30 pumps, implantable infusion systems, and liposomes.  
Formulations for inhalation may contain as excipients,  
for example, lactose, or may be aqueous solutions  
containing, for example, lactose or may be aqueous  
solutions containing, for example, polyoxyethylene-9-  
35 lauryl ether, glycocholate and deoxycholate, or may be  
oily solutions for administration in the form of nasal  
drops, or as a gel to be applied intranasally.  
Formulations for parenteral administration may also

include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

5 The concentration of PI3-kinase agonist or antagonist in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

10 In general terms, such agonists or antagonists may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1  
15  $\mu\text{g}/\text{kg}$  to about 1 g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of  
20 progression of the condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

The invention also includes the use of a polypeptide of the invention, or active complex containing the same, or  
25 an agonist or antagonist thereof in affecting the level of stimulation of platelets or neutrophils or in regulating blood glucose levels (the action of insulin may be mediated by PI3-kinase activity), and such use when employed for prophylactic or therapeutic purposes is  
30 envisaged.

The polypeptides of the invention (or complexes containing them) have a particular utility in the in  
35 vitro enzymatic production of 3-phosphorylated phosphoinositides eg PI(3)P, PI(3,4)P2, PI(3,4,5)P3). Such materials are of considerable biochemical interest, and are often very difficult to synthesize by conventional chemical techniques. This invention provides, for the first time, appreciable amounts of

purified and reliable enzymatic activity for such in vitro synthesis.

In general, the first step in the purification and cloning upon which the invention is based involved partial purification of PI3-kinase from bovine brain as previously described (Morgan et al, 1990) and then further purification by affinity chromatography on an immobilised 17 amino acid phosphotyrosine peptide whose sequence is based on that surrounding tyrosine 751 of the human PDGF- $\beta$  receptor. Following this final purification, p110 and p85 were eluted from the resin with SDS-containing buffers. The p85/p110 mixture was either digested directly with lysylendopeptidase, or p110 was further purified by SDS-agarose gel electrophoresis (see below) and digested following elution from the gel. Peptides were separated by reverse phase HPLC and sequenced using a modified Applied Biosystems 477A sequencer. Amino acid sequence analysis of 14 peptides (A to N, Figure 9) generated 235 residues which could be assigned with certainty (see Figure 9, attached).

It is important to note that the successful production of sequence information herein was dependent upon a novel SDS-agarose gel electrophoresis technique. Although, SDS-PAGE is widely used for high resolution protein separations, and is a method which resolves components primarily by their differences in molecular weight, as the polyacrylamide matrix is not readily disrupted, protein recovery following SDS-PAGE generally requires techniques involving electroelution from gel slices, electroblotting, or passive diffusion. Elution of proteins from polyacrylamide gels that have been previously stained using sensitive reagents (such as Coomassie Blue) is slow and recoveries are frequently low. Furthermore, these methods may concentrate impurities present in the polyacrylamide matrix and in the relatively large buffer volumes required for elution.

Preparative SDS-PAGE systems using continuous flow collection have also been developed, but these frequently exhibit decreased resolution and low recoveries.

5 The novel method employed herein uses SDS-agarose gel electrophoresis (SDS-AGE) and allows a combination of the high resolving capacity of slab gel electrophoresis and  
10 the detection of proteins using sensitive stains with a rapid recovery technique that isolates proteins in high yield and in small volumes. The recovered protein is highly purified and in a form that can be either readily precipitated or digested directly in SDS containing buffers. Peptides produced by this method can be  
15 fractionated by HPLC and then analysed by automated amino acid sequencing. The recovery of long hydrophobic peptides is particularly efficient using these digestion conditions. The following protocol guides the skilled reader.

20

### PROTOCOL

#### **Materials**

All chemicals should be of analytical or purer grades.  
25 Guanidinium hydrochloride was Aristar grade (BDH, UK). FMC Prosieve\* was purchased from Flowgen (UK) and ultrapure agarose was from BRL (USA). Other electrophoresis reagents were from Biorad (UK, Electrophoresis grade). Standard molecular weight  
30 proteins were from Bio-Rad (UK) and Amersham International (UK). Sequencing grade trypsin (porcine, EC 3.4.21.4) was from Boehringer Mannheim (UK) and lysylendopeptidase (Achromobacter lyticus, EC 3.4.21.50) was from Wako Chemicals GmbH (Germany). Glass  
35 capillaries were those supplied by Applied Biosystems Inc (USA) for use on the 430A HPEC system, but were frosted by abrasion with an aqueous carborundum suspension (C150 grade) and a steel rod. Frosted slab gel plates were

\* Trade-mark

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obtained from Hoefer (UK).

### Slab SDS-AGE

5 Slab Prosieve resolving gels of 0.75 or 1.5 mm thickness were poured essentially as described by the manufacturer using pairs of 16 x 18 cm glass plates, one of which was frosted in order to prevent the gel from slipping out of

10 the electrophoresis assembly. It is important to ensure that the gel plates be thoroughly warmed to 60°C prior to pouring the resolving gel. The inability to warm the gel plates prior to pouring an agarose stacking gel, the insertion of the comb into a rapidly cooling gel, and the removal of the comb from the fragile agarose stacking gel

15 initially caused severe problems. In order to remove these difficulties a 5%T, 2.6%C polyacrylamide stacking gel was used in place of agarose in later preparations.

20 Samples were denatured at 100°C in sample buffer (190 mM Tris/HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 10 mM DTT, 0.01% (w/v) bromophenol blue) and gels were run using Laemmli cathode buffer (0.192 M glycine, 0.025 M Tris, 0.1% (w/v) SDS) with a modified anode buffer (1M Tris/HCl, pH 8.3) at 200 v (approximately 50 mA for 1.5

25 mm and 25 mA for 0.75 mm gels) for about 4h using a SE400 gel apparatus (Hoefer, USA). Gels were stained using either Coomassie Blue G-250 (Bio-Rad, UK) with rapid destaining or 4M ammonium acetate solution. In the latter case proteins were identified within a few minutes

30 by optical contrast using incident light reflection observed against a dark background. Protein bands were immediately excised and gel slices stored at -20°C.

### HPEC Electroelution

35 Gel slices were thawed and washed twice in 1 ml of 62.5 mM Tris/HCl, pH 6.8 for 5 min each at 20°C. Slices containing Coomassie Blue were prewashed with 1 ml of 50% (v/v) methanol, 5% (v/v) acetic acid for 5 min at 20°C.

The volume of the gel slice was estimated, then 10% SDS and 20% DTT were added to final concentrations of 2% and 0.2% (w/v) respectively. The gel slice was melted and homogenized by immersion in boiling water for 5 min with occasional mixing. The sample volume was then measured and made up to the required amount (see Table 1 below) with prewarmed 62.5 mM Tris/HCl, pH 6.8. The diluted sample was heated for a further 5 min and loaded into a prewarmed glass HPEC capillary. It was important not to exceed 90% of the capillary volume at this stage. The capillary was incubated at 4°C for at least 10 min to allow the sample gel to solidify, before the slow addition of 0.8% agarose, 1 M Tris/HCl, pH 8.8 to overfill the capillary. After a further 10 min at 4°C, the ends of the gel were trimmed flush, sealed with Zytex discs, and applied to an Applied Biosystems 230A HPEC system. Electroelution was performed using an elution buffer pressure of 2.5 psi (generating a flow rate of approximately 1  $\mu$ l/min), an upper reservoir buffer pressure of 3.5 psi and a lower reservoir buffer pressure of 0.9 psi. These settings were changed from the manufacturer's recommendations in order to stop the gel from collapsing upwards during the run. The current settings were as described in the text and 3 min fractions were collected while monitoring the eluate at 280 nm. The fraction collector rack was cooled to 4°C and the gel compartment was cooled to 10°C.

30

Table 1  
HPEC Elution Gel Parameters

	<u>Capillary size (mm)</u>		<u>Gel volume (<math>\mu</math>l)<sup>a</sup></u>			<u>Current (mA)</u>
	<u>Length</u>	<u>i.d.<sup>a</sup></u>	<u>Total</u>	<u>Sample</u>	<u>Focussing</u>	
35	50	2.5	245	220	25	1.0-1.5
	50	3.5	480	432	48	1.5
	100	2.5	491	441	49	2.0-2.5
	100	3.5	960	864	96	2.5



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<sup>a</sup> These values are underestimated due to the variable increase in the internal diameter of the capillaries caused by the frosting procedure.

## 5 Preparation of Proteins for Sequence Analysis

Fractions were assayed for protein content and purity either by monitoring radioactivity or by SDS-PAGE and silver staining. Samples required for trypsin or  
10 lysylendopeptidase digestion and subsequent sequence analysis were separated from Coomassie Blue by sequential precipitation on ice using 10% (w/v) TCA and then 20% TCA with centrifugation for 10 min at 4°C. Pellets were  
15 washed with 1 ml of acetone at -20°C overnight and then washed again briefly in order to remove trace contamination by TCA and SDS before air drying and the addition of the required digestion buffer. Tryptic digestions were performed in 0.1 M Tris/HCl, pH 8.0 at 37°C and lysylendopeptidase digestions in 20 mM Tris/HCl,  
20 pH 8.8 containing 0.1% (w/v) SDS at 30°C. Solid guanidinium hydrochloride was added to tryptic digests (6M final concentration) and incubated for 1 h at 37°C. Products were applied directly to HPLC columns using a Hewlett-Packard 1090M system and the effluent was  
25 monitored with a 79880A diode array detector. Trypsin digests were fractionated using an Applied Biosystems RP-300 column (2.1 x 100 mm) while lysylendopeptidase products required an Applied Biosystems AX-300 (2.1 x 30 mm) and an OD-300 column (2.1 x 100 mm) connected in  
30 series essentially as described by Kawasaki and Suzuki (1990).

The following Examples are given to illustrate the present invention without limiting the same. The  
35 Examples refer to the accompanying drawings.

In the accompanying drawings:-

Figures 1 to 9 are concerned with Example 1, sections A and B.

**Figure 1. Phosphorylation and purification of Y751 phosphopeptide.**

Panel A. HPLC profile for separation of the phosphorylated from the non-phosphorylated Y751 peptide on a C<sub>18</sub> reverse phase column. The trace shows the spectra monitored at 214 nm during the course of the elution. The peaks corresponding to the phosphorylated and non-phosphorylated peptide are indicated by arrows. The small peaks observed are derived from the A431 membranes.

Panel B. Spectral analysis of the purified phosphorylated and non-phosphorylated Y751 peptides between 240 and 300 nm as measured by the diode-array detector. The absorption maximum for the peptide is observed to shift to a lower wavelength following tyrosine phosphorylation.

Panel C. Phosphoamino acid analysis of Y751 peptide phosphorylated by either purified EGF receptor (left panel) or A431 cell membranes (right panel). Following the phosphorylation reaction the phosphopeptide was purified by reverse phase HPLC. The peptide was subjected to acid hydrolysis and the phosphoamino acids separated by two-dimensional thin layer electrophoresis. Internal standards were stained with ninhydrin and the <sup>32</sup>P-labelled phosphoamino acids were detected by autoradiography. The positions of inorganic phosphate (P<sub>i</sub>), and phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) standards are indicated.

**Figure 2. Purification of PI 3-kinase complex on the Y751 phosphopeptide affinity column.**

Panel A. Peak 1 (P1) and peak 2 (P2) of PI 3-kinase

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fractions from the second MonoQ step were analysed on a 7.5% SDS-PAGE gel. Proteins in these two peak fractions were visualised by silver staining. The migration positions of molecular weight markers are indicated.

5

Panel B. Affinity purification of peak 1 (P1) and peak 2 (P2) PI 3-kinase using the Y751 phosphopeptide column. Silver stain of a 7.5% SDS-PAGE gel showing PI 3-kinase associated proteins from MonoQ P1 and P2 which bound to, and were eluted from, the Y751 phosphopeptide column with 0.1% SDS-containing phosphate buffer at 80°C. Lanes 1, 2 and 3 for both the P1 and P2 material indicates the proteins eluted by successive 50  $\mu$ l elutions.

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Figure 3. Characterisation of the binding of PI 3-kinase activity to Y751 derived peptide columns.

One microgram of partially purified peak 1 bovine brain PI 3-kinase was applied to 10  $\mu$ l of the Y751 derived peptide resins in 100  $\mu$ l of binding buffer. Bound proteins were assayed for PI 3-kinase activity. Lane 1, PI 3-kinase activity bound to non-phosphorylated Y751 column. Lane 2, PI 3-kinase activity bound to phosphorylated Y751 column. Lane 3, PI 3-kinase activity removed from supernatant of column in lane 2 by fresh phosphorylated Y751 column. Lane 4, PI 3-kinase activity remaining associated with the column from lane 2 following removal of the bound material using 0.1% SDS at 80°C. Lane 5, PI 3-kinase activity bound to recycled phosphorylated Y751 column as used in lane 2 following addition of a fresh aliquot of bovine brain PI 3-kinase in binding buffer. Lane 6, Equivalent amount of peak 1 soluble bovine brain PI 3-kinase activity as applied to columns in lane 2 or lane 5.

35

Figure 4. Identify of p85 species in peak 1 and 2 of bovine brain PI 3-kinase preparation.

Protein samples were separated on 7.5% SDS-PAGE gels and

transferred to nitrocellulose. The blots were then probed with antisera raised against the COOH-terminal peptide sequences of p85 $\alpha$  or p85 $\beta$ .

5 Panel A. Western blot probed with anti-p85 $\alpha$  COOH-terminal antisera.

10 Lane 1, peak 1 bovine brain PI 3-kinase; lane 2, peak 2 bovine brain PI 3-kinase; lane 3, Cos-1 cell lysate from pMT2 vector alone transfected cells; lane 4, Cos-1 cell lysate from pMT2p85 $\alpha$  transfected cells; lane 5, Cos-1 cell lysate from pMT2p85 $\beta$  transfected cells; lane 6 Sf9 cell lysate containing p85 $\alpha$ ; lane 7, Sf9 cell lysate  
15 containing p85 $\beta$  Panel B. Western blot probed with anti-p85 $\beta$  COOH-terminal antisera.

Lanes are as described for panel A.

20 Panel C. Competition of peptides with antibodies in Western blots. Samples in lanes 1 and 2 were probed with p85 $\alpha$  specific antiserum while samples in lanes 3 and 4 were probed with the p85 $\beta$  specific antiserum. Lanes 1 and 2. Sf9 cell lysate containing baculovirus expressed  
25 p85 $\alpha$ . Lanes 3 and 4, Sf9 cell lysate containing baculovirus expressed p85 $\beta$ . In the odd numbered lanes the nitrocellulose was probed with specific antiserum alone. In the even numbered lanes the antiserum was competed with 100  $\mu$ g/ml of p85 $\alpha$  (lane 2) and p85 $\beta$  (lane  
30 4) specific C-terminal peptides respectively.

Panel D. Anti p85 $\alpha$  western blot of bound and soluble PI 3-kinase material after chromatography using the Y751 phosphopeptide column.

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Peak 1 (P1) and peak 2 (P2) of bovine brain PI 3-kinase were immobilised on the Y751 phosphopeptide column. Material which did not bind was collected and then the

resin was washed extensively. Bound proteins were eluted from the column with SDS-PAGE sample buffer. Bound and unbound proteins were separated by SDS-PAGE on a 7.5% gel and then transferred to nitrocellulose. The filter was then probed with anti-p85 $\alpha$  COOH-terminal antisera and visualised with <sup>125</sup>I Protein A-Sepharose. Lane 1, P1 bound material; Lane 2, peak 1 material which did not bind to column; Lane 3, peak 2 bound material; Lane 4, peak 2 material which did not bind to column.

**Figure 5. Specificity of binding of PI 3-kinase complex to Y751 peptide column:-comparison with Y857 phosphopeptides.**

Sf9 cell lysates containing p85 $\alpha$  proteins or one microgram of partially purified bovine brain PI 3-kinase (P1 and P2 MonoQ) was allowed to bind to the columns for 4 h at 4°C as described. The columns were then washed repeatedly with binding buffer, bound proteins were eluted with SDS-containing buffers and then analysed by electrophoresis on 7.5% SDS-PAGE gels. Bound proteins were visualised by silver staining. Panel A. Proteins bound to Y751 phosphopeptide column. Panel B. Proteins bound to Y857 phosphopeptide column. The migration position of molecular weight markers are indicated.

**Figure 6. Binding of recombinant baculovirus expressed p85 proteins to a panel of phosphopeptide columns.**

P85 proteins in SF9 cell lysates were tested for their ability to bind to the various peptide column. After extensive washing, bound proteins were eluted from the columns, separated on 7.5% SDS-PAGE gels and the visualised by staining with Coomassie Blue. Panel A. Bound p85 $\alpha$ . Panel B. Bound p85 $\beta$ . CON, 17 amino acid non-phosphorylated Y751 column; Y751, 17 amino acid phosphopeptide from the kinase insert region of the PDGF  $\beta$ -receptor; Y751.S, 11 amino acid version of Y751 phosphopeptide; Y857, 17 amino acid phosphopeptide

derived from the sequence around the second major tyrosine phosphorylation site in the PDGF  $\beta$ -receptor; pGAT, poly Glu:Ala:Tyr phosphopeptide; Y416 and Y527, 13 and 16 amino acid phosphopeptides derived respectively  
5 from the two major tyrosine phosphorylation sites of pp60<sup>c-src</sup>.

Figure 7. The p85/100 complex and PI 3-kinase activity show specificity in the range of phosphopeptides to which  
10 they will bind.

One microgram of partially purified bovine brain PI 3-kinase (P1 MonoQ) was allowed to bind to peptide affinity columns for 4 h at 4°C as described. The columns were then washed repeatedly with binding buffer. Bound  
15 proteins were then either eluted with SDS-containing buffers and then analysed by electrophoresis on 7.5% SDS-PAGE gels or assayed for PI 3-kinase activity bound to the column.

20 Panel A. Bound proteins were visualised by silver staining. The migration of molecular weight markers is indicated.

Panel B. PI 3-kinase activity bound to various  
25 phosphopeptide columns. The <sup>32</sup>P-labelled lipid products were separated by TLC and the visualised by autoradiography. PI3P indicates the migration position of a P13P standard. Ori indicates the origin of the TLC plate.

30

Figure 8. Binding of PI 3-kinase activity of phosphopeptides containing the YXXM motif.

Panel A. One microgram of partially purified peak 1 bovine brain PI 3-kinase was bound to 10  $\mu$ l of the  
35 indicated peptide columns. Following extensive washing the columns were assayed for bound PI 3-kinase activity. Lane 1, PI 3-kinase activity bound to non-phosphorylated Y751 column; Lane 2, PI 3-kinase activity bound to

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phosphorylated Y751 column; Lane 3, PI 3-kinase activity bound to phosphorylated Y751.S column; Lane 4 PI 3-kinase activity bound to phosphorylated Y857 column; Lane 5, PI 3-kinase activity bound to phosphorylated Y740 column;

5

Lane 6, PI 3-kinase activity bound to phosphorylated Met Y1313 column. PIP indicates the migration position of a P14P standard. Ori indicates the origin of the TLC plate.

10

Panel B. Comparison of identified PI 3-kinase binding sites in the peptides tested. The proposed consensus sequence for binding is also shown for comparison (Cantley et al., 1991).

15

Figures 9 to 15 are concerned with Example 1, sections C and D, and Figures 16 to 25 relate to Example 2.

**Figure 9. Nucleotide Sequence and Deduced Amino Acid Sequence of p110.**

20

(Top Panel) The nucleotide sequence of the coding region and the deduced amino acid sequence in one letter code are shown. Peptide sequences (lettered from A-N) obtained by protein sequencing are highlighted.

25

(Lower Panel) Schematic representation of the p110 cDNA. The bold line indicates coding sequence. (p2.1): extent of clone p2.1, (Race Product): region amplified by RACE PCR, (a): probe used in Southern blot analysis, (b): probe used in northern blot analysis, (S): Sau3AI site changed to BamHI site for expression in Sf9 cells.

30

**Figure 10. Comparison of p110 and Vps34p Protein Sequences**

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(A) Dot plot comparison of Vps34p (875 amino acids: horizontal axis) and p110 (1068 amino acids: vertical axis) using the Compare program (UWGCG package; Devereux et al., 1984).

(B) The optimal alignment of p110 (upper sequence) and Vps34p (lower sequence) over the region of homology, using the Gap program (UWGCG package: Devereux et al.,

5

1984). Identical residues are indicated by (I), conserved residues are indicated by (:). Residues proposed to be involved in ATP binding are marked with (\*).

10

#### Figure 11. Genomic Southern Analysis of p110

High molecular weight DNAs (3  $\mu$ g) of bovine (lanes 1, 2, 3), human (lanes 4, 5, 6) and rat (lanes 7, 8, 9) origin were digested with EcoRI (lanes 1, 4, 7), BamHI (lanes 2, 5, 8) or HindIII (lanes 3, 6, 9), fractionated through a 0.5% agarose gel and transferred to a nitrocellulose membrane as described in Example 1. The filter was probed with a  $^{32}$ P-labelled XbaI-PstI fragment (probe a in Figure 9, lower panel). The filter was washed in 0.5 x SSC, 0.1% SDS at 50°C and exposed overnight (Panel A). The filter was then washed in 0.1 x SSC, 0.1% SDS at 68°C and exposed for seven days (Panel B). The marker track shows the positions of lambda HindIII markers.

25

#### Figure 12. Analysis of Tissue Distribution of p110 Message

##### (A) Northern Blot Analysis of p110

5  $\mu$ g of poly(A)<sup>+</sup>RNA isolated from total bovine brain (lane 1) or the SGBAF-1 cell line (lane 2) were fractionated on a 0.9% agarose gel and immobilised on membranes as described in Example 1. The filter was probed with a  $^{32}$ P labelled antisense RNA probe (probe b in Figure 9, lower panel). After washing in 0.1 x SSC, 0.1% SDS at 60°C, the filter was treated with 1  $\mu$ g ml<sup>-1</sup> RNAase A and autoradiographed overnight.

35

##### (B) PCR Analysis to Detect p110 Transcripts

Poly(A)<sup>+</sup> RNA was isolated from various sources and PCR



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performed as described in Example 1. Bands of 218 bp and 212 bp indicate the specific amplification of human and bovine transcripts, respectively. Lane 1; Human T-cell blasts, lane 2; Human peripheral blood acute lymphocytic leukaemia cells, lane 3; A431 cells (Human), lane 4; COS-1 cells (Simian), lane 5; bovine brain, lane 6; SGBAF-1 cells (Bovine), lane 7; ZNR cells (Porcine).

(C) PCR Analysis to Detect p85 $\alpha$  Transcripts

Poly (A)<sup>+</sup> RNA was isolated from various sources and PCR performed. Specific amplification of p85 $\alpha$  message gives a band of 190 bp. Lanes are the same as indicated for (B).

**Figure 13. Expression of p85 $\alpha$  and p110 in Sf9 Cells Using Baculovirus Vectors**

(A) Sf9 cells were infected with a wild type baculovirus (lanes 1 and 2) or with baculoviruses expressing p85 $\alpha$  (lane 3), p110 (lane 4) or p85 $\alpha$  and p110 (lanes 5 and 6). Immunoprecipitates were prepared with either anti-p85 $\alpha$  (lanes 1, 3, and 5) or anti-p110 antisera (lanes 2, 4 and 6), samples fractionated on a 7.5% SDS-PAGE gel and visualised by staining with Coomassie blue.

(B) PI3-kinase assays were performed on immunoprecipitates of p85 $\alpha$  and p110 expressed in Sf9 cells. lanes 1-6 the same as Panel (A); lane 7: pI3-kinase activity from 1  $\mu$ l of the partially purified bovine brain PI3-kinase preparation.

**Figure 14. In Vitro Association of PI3-Kinase Activity with the CSF-1 Receptor**

An in vitro PI3-kinase assay was performed on anti-CSF-1 receptor immunocomplexes prepared from Sf9 cells infected with a baculovirus expressing the CSF-1 receptor and treated as follows; lane 1: anti-CSF-1 receptor immunoprecipitates, untreated; lane 2; anti-CSF receptor immunoprecipitate, pre-treated with ATP and incubated

with a p85 $\alpha$ /p110 containing Sf9 cell lysate; lane 3: anti-CSF-1 receptor immunoprecipitate, treated in the absence of ATP and incubated with a p85 $\alpha$ /p110 containing

5 Sf9 cell lysate; lane 4: anti-CSF-1 receptor immunoprecipitate, pre-treated with ATP and incubated with a p85 $\alpha$  containing Sf9 cell lysate; lane 5; anti-CSF-1 receptor immunoprecipitate, pre-treated with ATP and incubated with a p110 containing Sf9 cell lysate.

10

**Figure 15. Expression of p85 $\alpha$  and p110 in COS-1 Cells**

COS-1 cells were transfected with 5  $\mu$ g of the respective DNAs and harvested 48 h later. Transfected cells were labelled with 100  $\mu$ Ci ml<sup>-1</sup> of <sup>35</sup>S-methionine for the last 4 h of this period. Immunoprecipitations were performed with either an p85 $\alpha$  polyclonal antiserum or a p110 C-terminal peptide antiserum. After washing, the pellet was divided in two and half was then analysed on a 10% SDS-PAGE gel while the other half was subjected to P13-kinase assay.

20

(A) <sup>35</sup>S-labelled proteins immunoprecipitated with anti-p85 $\alpha$  antiserum.

25

(B) PI3-kinase activity immunoprecipitated with anti-p85 $\alpha$  antiserum.

(C) <sup>35</sup>S-labelled proteins immunoprecipitated with 110 C-terminal peptide antiserum.

30

(D) p13-kinase activity immunoprecipitated with 110 C-terminal peptide antiserum.

35

Lanes contain results from COS-1 cells transfected with the following DNAs; lane 1: vector DNA, lane 2: pMT2-p85 $\alpha$ , lane 3: pSG5-p110, lane 4: pMT2-p85 $\alpha$  and pSG5-110, lane 5 in panels B and D show the PI3-kinase activity immunoprecipitated with the two antisera from 1  $\mu$ l of the

partially purified bovine brain p13-kinase preparation. The exposure times for panels A and C, and B and D are identical.

5 **Figure 16. cDNA for human p110**

The figure shows the sequence of human p110 cDNA, together with the corresponding amino acid sequence.

10 **Figure 17. A comparison of the human p110 sequence and bovine p110 sequence at the DNA level.**

**Figure 18. A comparison of the human p110 sequence and bovine p110 sequence at the protein level.**

15 **Figure 19. The protein sequence of human p110.**

**Figure 20. The sequence of a cDNA related to p110, P1TR-c.**

20 **Figure 21. The sequence of a cDNA related to p110, P1TR-f.**

**Figure 22. The alignment of human p110, P1TR-c, P1TR-f and the yeast PI3-kinase VPS34.**

25

**Figure 23A. SDS PAGE analysis of proteins able to bind to various domains of human p110.**

30 **Figure 23B. Schematic representation of the domains of p110 analysed for their ability to bind p85.**

**Figure 24. Various deletion mutants and PCR fragments of p110 fragment p110-N.**

35 **Figure 25. The ability of the various deletion mutants and PCR fragments of p110-N to bind the p85 subunits.**

Example 1PROTEIN PURIFICATION5 A. Methods and Materials**Cells**

10 A431 cells were maintained in Dulbecco's modified Eagle's medium containing 10% foetal calf serum. Maintenance of insect cell culture and infection of *Spodoptera frugiperda* (Sf9) cells were carried out as described in Summers and Smith (1987).

**Preparation of A431 Membranes**

15 This preparation was modified from that described by Thom et al (1977). Harvesting solution (0.05 M boric acid (pH 7.2), 0.15 M NaCl), extraction solution (0.02 M boric acid (pH 10.2), 0.2 mM EDTA) and borate solution 0.5 M Boric acid (pH 10.2) were all prepared fresh. Cells were  
20 washed once with ice-cold harvesting solution and then scraped into fresh harvesting solution. Cells were pelleted by low speed centrifugation at 200g, and then resuspended by pipetting in 2 pellet volumes of harvesting solution. This was added slowly, with  
25 stirring, to 100 pellet volumes of extraction solution. After 10 min, 8 pellet volumes of borate solution was added and stirring continued for a further 5 min. This solution was filtered through nylon gauze (Av. mesh size 900  $\mu$ m), and spun at 500g for 10 min at 2°C to pellet any  
30 nuclei/whole cells. Finally, the supernatant was centrifuged at 12,000g in a ultracentrifuge SW28 rotor at 4°C for 30 min. The membrane pellet was resuspended in a minimum volume of 50 mM Hepes (pH 7.5) and stored at -70°C.

35

**Synthesis of Peptides**

Peptides described in Table 2 below were synthesized on an Applied Biosystems 430A peptide synthesizer using FMOC

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chemistry and an appropriate amino acid addition program according to ABI's recommendations. Peptides were then purified by preparative reverse-phase HPLC. Composition of the peptides was checked by analytical HPLC, amino acid analysis and protein sequencing on an 477A automated pulse-liquid sequencer.

Table 2

<u>Peptide</u>	<u>Sequence</u>
Y740	G E S D G G Y M D M S K
Y751	D M S K D E S V D Y V P M L D M K
Y751.S	C D E S V D Y V P M L
Y857	A R D I M R D S N Y I S K G S T F
Y1313	E F C P D P L Y E V M L K
Y527	R R F T S T E P Q Y Q P G E N L
Y416 <sup>a</sup>	R R L I E D N E Y T A R G

<sup>a</sup> This peptide was purchased from Sigma Chemical Co Ltd rather than synthesized.

#### Phosphorylation of Peptides

Peptides were lyophilised to dryness to remove any contaminating chemicals remaining from synthesis/purification and then dissolved in HPLC grade water at a concentration of ~4 mg/ml.

For small scale phosphorylation: 20 µg of peptide, 10 µl 5x kinase buffer (250 mM Hepes (pH 7.4), 750 mM NaCl, 0.1% Triton X-100, 10 nM MnCl<sub>2</sub>, 60 mM MgCl<sub>2</sub>, 50% glycerol, 500 mM sodium orthovanadate), 5 µl A431 membrane preparation and ATP/[γ-<sup>32</sup>P]ATP (relative amounts depends on aim of phosphorylation). Water was added to adjust the volume to 50 µl.

For preparative phosphorylation, 2-3 mg of peptide was dissolved in 1.5 ml of water and added to 450 µl 5x kinase buffer. The pH was adjusted to 7.0. 250 µl of

0.1 M ATP and 500  $\mu$ l of A431 plasma membranes (~2 mg/ml) was added and then the reaction was allowed to proceed for 18 h at room temperature with continual mixing.

5 **Isolation of Phosphorylated Peptides by Reverse Phase HPLC**

One millilitre of buffer A (Buffer A: 0.08% trifluoroacetic acid, 1% acetonitrile in water; Buffer B: 0.08% trifluoroacetic acid, 90% acetonitrile) was added to  
10 the kinase reaction and mixed. This solution was then spun for 20 min at 10,000g to pellet the membranes. The supernatant containing the phosphopeptide was then loaded onto a Sep-Pak\* column ( $C_{18}$ ) equilibrated with buffer A. The column was washed with 20 ml buffer A to elute ATP  
15 and then the peptide was eluted with 3 x 1 ml of 40% buffer B. The OD of the fractions was monitored at 268 nm and fractions containing peptide were pooled and then lyophilised to dryness (note that the phosphorylated Y751 peptide has essentially no absorption at 280 nm). The  
20 phosphopeptide was then separated from non-phosphorylated peptide using a 1090 HPLC system. For preparative separation a  $C_{18}$  column (Aquapore OD-300, 250 x 7 mm) equilibrated with 100% buffer A (214 nm (sen. 50 mV)/280 nm (sen. 200 mV) was used with a 2 ml/min flow rate. The  
25 peptide was dissolved in 200  $\mu$ l HPLC grade water and then loaded via a 500  $\mu$ l loop. The column was then washed for 10 min with 100% buffer A before eluting the peptide and phosphopeptide with a 30 min linear gradient 0 to 45%  
buffer B followed by 5 min linear gradient to 100% buffer  
30 B. Peak fractions were collected manually. The pool fractions were diluted with water, lyophilised and then stored at  $-20^{\circ}\text{C}$ .

**Phosphoamino Acid Analysis of Phosphorylated Peptides**

35 Peptides phosphorylated in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  using either purified EGF receptor or A431 cell membranes were purified by  $C_{18}$  Sep-Pak column and HPLC as described above. This material was then hydrolysed at  $110^{\circ}\text{C}$  for 1

\* Trade-mark

h in 1 ml of 6 M HCl. One millilitre of HPLC grade water was added and the sample was centrifuged at 10,000g for 10 min to removed debris. The remaining supernatant was frozen and lyophilised to dryness. The pellet was  
5 resuspended in 2 ml of water, frozen and then lyophilised once more. This material was analysed by two dimensional thin-layer electrophoresis (essentially as described by Cooper et al, 1983).

### 10 **Coupling of Peptides to Actigel Resin**

Peptides were coupled to the matrix essentially as described by the manufacturers. Briefly, 500  $\mu$ l (packed volume) of Actigel-ALD Superflow resin (Sterogene, CA, USA) was washed five times with 100 mM phosphate buffer  
15 (pH 7.8) (coupling buffer). Phosphorylated or non-phosphorylated peptide (1 mg) was dissolved in 200  $\mu$ l of coupling buffer and added to the resin. NaCNBH<sub>3</sub> (coupling solution) was added to a final concentration of 100 mM and this was then mixed at 4°C for 6 h. The resin  
20 was washed with 10 column volumes of 500 mM NaCl and then incubated with 100 mM Tris-HCl (pH 8.0) for 1 h in the presence of coupling solution to block any unreacted sites on the resin. The resin was washed with 500 mM NaCl and finally with coupling buffer plus 500  $\mu$ M  
25 vanadate and 0.02% NaN<sub>3</sub> and then stored at 4°C. Phosphopeptides bound to the Actigel matrix were stable for several months under these conditions.

### **Binding of Proteins to the Phosphopeptide Columns**

30 Proteins were diluted in binding buffer (50 mM phosphate buffer (pH 7.2), 150 mM NaCl, 0.02% Triton X-100\*, 2mM EDTA and 200  $\mu$ M sodium orthovanadate), mixed with the appropriate peptide affinity resin and then allowed to  
35 bind for 2h at 4°C with rotation. The column material was washed repeatedly (>6x) with 50 column volumes of the same buffer and then with various elution buffers containing NaCl, urea or detergents. Bound proteins were

\* Trade-mark

either assayed for PI3-kinase activity or were removed from the column by boiling in SDS-PAGE sample buffer and then analysed by SDS-PAGE.

#### 5 **PI3-kinase Assay**

PI3-kinase assays were carried out essentially as described in Whitman et al, (1987) in 50  $\mu$ l containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 5 mM MgCl<sub>2</sub>, 100  $\mu$ M ATP (plus 0.5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP/assay), 1 mM PI plus soluble or column immobilised bovine brain PI3-kinase. Incubation was for 5 min at room temperature. The reaction was terminated by the addition of 100  $\mu$ l of 0.1N HCl and 200  $\mu$ l chloroform:methanol (1:1). The mixture was vortexed and then centrifuged to separate the phases. The upper phase was discarded and the lower organic phase washed with 80  $\mu$ l of methanol: 1N HCl (1:1). After centrifugation the upper phase was again discarded and the lower phase evaporated to dryness. Reaction products were spotted on thin layer Silica gel 60 plates (pretreated with 1% oxalic acid, 1 mM EDTA in water:methanol (6:4)) and developed in chloroform:methanol:4 N ammonia (9:7:4).

#### 25 **Preparation of C-terminal Specific Antisera for p85 $\alpha$ and p85 $\beta$**

C-terminal peptide antisera were prepared against the bovine C-terminal sequences determined by cDNA cloning (Otsu et al, 1991). The peptides TLAYPVYAQQRR for p85 $\alpha$  and TLAHPVRAPGPGPPAAR for p85 $\beta$  were synthesized by Fmoc chemistry and purified by HPLC. The peptides were coupled using gluteraldehyde to KLH and then injected into the lymph nodes of rabbits using methods described in Kypta et al, (1988). Positive antisera as determined by enzyme-linked immunoassay were affinity purified on specific peptide-Actigel affinity columns.

#### 35 **B. Procedure and Results of Purification**



### Preparation of Y751 Phosphopeptide Column

A 17 amino acid peptide which contains Y751 of the human PDGF- $\beta$  receptor was chosen for synthesis in an attempt to include all necessary sequence determinants following a survey of the known binding sites for the PI3-kinase (see Table 2 above; reviewed in Cantley et al, 1991). In addition to the peptide context of Y751 of the PDGF  $\beta$ -receptor, the sequences around Y315 of polyoma middle T (Talmage et al, 1989) and Y721 of the human CSF-1 receptor (Shurtleff et al, 1990) were also considered. Using the phosphorylation protocol described above, greater than 50% phosphorylation of the Y751 peptide was achieved using either purified human EGF receptor or A431 membranes as a source of protein-tyrosine kinase. The phosphorylated Y751 peptide could be clearly identified during reverse phase HPLC analysis, where it eluted approximately one minute earlier than the nonphosphorylated peptide, since it produced a strong 214 nm absorbance, but little or no 280 nm signal (Figure 1, panel A). Analysis of the absorption properties showed that phosphorylation of the Y751 peptide led to a shift in the absorption maximum from 280 to 267 nm (Figure 1, panel B). For large scale phosphorylations A431 membranes were the preferred source of protein-tyrosine kinase activity since they could be more easily generated. However, as the Y751 peptide contains two serines, as well as a single tyrosine residue, it was thought important to demonstrate that peptide was phosphorylated exclusively at the tyrosine residue. This was established by two separate methodologies; analysis of HPLC purified phosphopeptide by phosphoamino acid analysis or by protein microsequencing. Phosphoamino acid analysis of the Y751 peptide, phosphorylated by either purified EGF receptor or A431 membranes, demonstrated that phosphorylation of the Y751 peptide was occurring exclusively at the tyrosine residue (Figure 1, panel C). Sequence analysis of the phosphorylated and non-phosphorylated peptides also

confirmed that both these peptides were 17 amino acids in length and that their sequences were identical except at cycle 10 where as expected no phenylthiohydantoin-Tyr derivative was observed for the phosphorylated peptide due to its modification.

#### **Extended Purification of Bovine Brain PI3-kinase Using a Y751 Phosphopeptide Affinity Column**

A 650-fold purification of PI3-kinase from bovine brain has recently been described (Morgan et al, 1990), and this same method was used except that the gradient for the second Mono Q column was extended to give two distinct peaks containing PI3-kinase activity (Figure 2, panel A). Both of these peaks (referred to hereafter as peak 1 (P1) and peak 2 (P2)) contained no PI kinase activity other than PI3-kinase activity as determined by HPLC analysis of deacylated product lipids (data not shown). However, both of these fractions still contained greater than 20 peptides detectable after SDS-PAGE gel analysis by silver staining (see Figure 2, panel A). The precise subunit composition of the active PI3-kinase complex was still a point of some contention, so an attempt was made to address this question by affinity purifying the PI3-kinase activity from these two Mono Q pools. The bovine brain PI3-kinase preparation was diluted 10-fold in binding buffer and allowed to bind batchwise to the Y751 phosphopeptide affinity resin for 4 h at 4°C. After washing the column extensively with binding buffer, those proteins which remained bound were eluted with SDS-containing buffers and examined by SDS-PAGE. Two major polypeptide species of approximate molecular weights 85 and 110 kD, which bound specifically to the phosphopeptide column, but not to an identical column prepared with unphosphorylated Y751 peptide, were identified in both Mono Q peaks and were observed to be quantitatively depleted from the bovine brain PI3-kinase preparation (Figure 2, panel B). Assaying the bound material, the presence of these two proteins appeared to

be sufficient to generate full PI3-kinase activity (Figure 3, lane 2). With fresh preparations of bovine brain PI3-kinase this column routinely removed >90% of the PI3-kinase activity present in Mono Q peaks 1 or peak 2 (c.f., Figure 3, lanes 2 and 3) following a single incubation. Neither the 85 and 110 kD proteins, nor PI3-kinase activity bound to a column with an equivalent concentration of non-phosphorylated Y751 peptide (Figure 3, lane 1) or to a column prepared with phosphotyramine, a phosphotyrosine analogue (data not shown). It should also be noted that binding of the PI3-kinase complex to the phosphopeptide column did not result in any apparent increase in the total enzyme activity present (Figure 3, c.f., lanes 2 and 6). In fact a slight decrease in activity was often observed, but this was judged to be due to the unstable nature of the highly purified enzyme which was found to be inhibited by traces of metal ions and reversibly inhibited by oxidation. It is estimated that this affinity purification step results in a 7-8,000-fold purification of PI3-kinase from bovine brain relative to the DEAE load (the overall purification achieved from tissue is in fact much greater).

#### Elution of p85, p110 and PI3-kinase Activity from the Phosphopeptide Column

Elution of the above PI3-kinase complex from the phosphopeptide column proved to be difficult to achieve due to the high affinity of the interaction. Kazlauskas and Cooper (1990) have previously noted that the binding of cellular p85 proteins to phosphorylated PDGF-receptor was stable to treatment with solutions containing ionic detergents, 2 M NaCl, 1 M urea or 0.2% SDS. The p85 subunits and PI3-kinase complex were also found to bind tightly to the Y751 phosphopeptide matrix, and were likewise not eluted under any of the above conditions. At 20°C the 85 and 110 kD proteins remained bound in the presence of either 2 M NaCl plus 0.5% Triton X-100, 5 M

NaCl, 6 M Urea, 50 mM phosphotyrosine or up to 1 mg/ml free Y751 phosphopeptide. Several alternative elution protocols were investigated without success. An elution medium supplied with the Actigel resin was able to remove both proteins but led to a complete loss of activity. Interestingly no suitable conditions could be established whereby the 110 kD, but not the 85 kD, subunit was released from the column suggesting that the interaction between the 110 and 85 kD subunits is of high affinity. Elution of bound proteins was routinely carried out by heating the resin to 80°C for 3 min in the presence of 5 mM phosphate buffer (pH 7.0), 0.1% SDS, 0.1 mM DTT, 10% glycerol. The phosphopeptide column could be simply regenerated following elution by extensive washing in binding buffer (Figure 3, lanes 4 and 5) and could be successfully used at least ten times before any deterioration in binding was observed.

#### **Analysis of the p85 and 110 kD Proteins Bound to the Phosphopeptide Column**

The relationship of the 85 kD proteins observed to bind to the Y751 phosphopeptide column to the recently cloned p85 $\alpha$  and p85 $\beta$  proteins was investigated using the polyclonal antisera generated against the predicted C-terminal 12 and 18 amino acids of p85 $\alpha$  and p85 $\beta$ , respectively. Despite the high degree of overall sequence similarity between p85 $\alpha$  and p85 $\beta$ , the amino acid sequence over this segment is significantly different and thus p85 $\alpha$  or p85 $\beta$  specific antisera were expected to be produced. Furthermore the amino acid sequence corresponding to this peptide in p85 $\alpha$  is completely conserved between human, bovine and murine cDNAs suggesting that antibodies generated against this sequence might be useful for studying the expression of different p85 proteins in species other than bovine (Escobedo et al, 1991b; Otsu et al, 1991; Skolnik et al, 1991). The corresponding region of p85 $\beta$  in species other than bovine is currently unknown.

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The p85 antisera generated against these peptides could specifically immunoprecipitate the appropriate species of expressed recombinant p85 from either COS-1 or Sf9 cells but were not very efficient at immunoprecipitating PI3-kinase activity from either cell lines or from the partially purified bovine brain PI3-kinase preparation. However, these antisera were found to work well in Western blots. The data presented in Figure 4 shows that these two antisera specifically recognized expressed p85 proteins present in either COS cells or in Sf9 cells. Longer exposures also revealed the endogenous COS p85 protein(s), but no such proteins were detected in Sf9 cells with these antisera. No cross reactivity was observed even at high concentrations of the recombinant proteins suggesting that they are specific for p85 $\alpha$  (Figure 4, panel A) and p85 $\beta$  (Figure 4, panel B) respectively. The ability of these antisera to interact with the appropriate p85 species was demonstrated to be completely blocked in the presence of the appropriate peptide used to raise the antisera (Figure 4, panel C). The p85 species in the two peaks of bovine brain PI3-kinase activity which bound to the Y751 phosphopeptide column was found to react exclusively with the anti C-terminal antisera raised against the p85 $\alpha$  specific sequence (Figure 4, panel A). Following immobilisation of the bovine brain PI3-kinase material on the Y751 phosphopeptide column, all the p85 $\alpha$  immunoreactive material was bound to the column with none detectable by either silver staining or Western blot analysis in the supernatant (Figure 4, panel D).

For sequence analysis of the PI3-kinase complex, the 110 and 85 kD subunits were eluted from the column, following extensive stringent washing, by briefly boiling the resin in 5 mM phosphate buffer (pH 7.0), 0.1% SDS, 0.1 mM DTT, 10% glycerol. Preparation of both 85 and 110 kD proteins for digestion with lysylendopeptidase and subsequent sequence analysis were performed in accordance with the

protocol given hereinbefore. Amino acid sequence analysis of a lysylendopeptidase C digest of the p85 protein bound to be Y751 phosphopeptide column confirmed that the p85 protein present in both peak 1 and peak 2 from the mono Q column were identical to the previously cloned p85 $\alpha$  (Otsu et al, 1991). No peptides corresponding to p85 $\beta$  were found in either peak. Extensive sequencing of the 110 kD protein affinity purified from both mono Q peak 1 and peak 2 material enabled the isolation of a novel cDNA (see below).

#### **Specificity of Binding of the Purified Bovine Brain PI3-kinase**

In order to evaluate the specificity of the Y751 phosphopeptide column for purifying the PI3-kinase, other phosphopeptide columns were prepared using peptides based on the amino acid sequences which surround known protein-tyrosine kinase phosphorylation sites. Tyrosine 857 is the other major autophosphorylation site in the human PDGF  $\beta$ -receptor and has been shown to be required for the binding of GAP, but not for association with the PI3-kinase (Kazlauskas & Cooper, 1989, 1990; Kazlauskas et al, 1991). For a direct comparison with the Y751 peptide a 17 amino acid peptide centred around tyrosine residue 857 was synthesized (see Table 2 above). A comparison the proteins from baculovirus expressing p85 $\alpha$  Sf9 cell lysate or from bovine brain PI3-kinase fractions from mono Q peak 1 (P1) and peak 2 (P2) binding to either the Y751 (panel A) or Y587 (panel B) phosphopeptide columns is shown in Figure 5. Whereas the baculovirus expressed p85 $\alpha$  is observed to bind both columns to a similar extent, the 85 and 110 kD proteins from both peaks of activity are seen only to bind to the Y751 phosphopeptide column. Similarly, PI3-kinase activity is only found associated with the Y751 phosphopeptide column (Figure 7, panel B).

To determine whether this binding specificity could be

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extended several other peptides were synthesized based on known tyrosine autophosphorylation sites (see Table 2 above). A shorter, 11 amino acid version of the Y751 peptide, termed Y751S, was also synthesized in an attempt to further refine the minimal SH2 recognition domain required. Two other peptides containing the YXXM motif were prepared, one based on the sequence around tyrosine 740 of the PDGF- $\beta$  receptor, a second residue within the PDGF receptor kinase insert which may play a role in PI3-kinase binding (Escobedo et al, 1991a), and the second based around tyrosine Y1313 of Met, the hepatocyte growth factor receptor. To introduce a totally random sequence the synthetic peptide poly Glu:Ala:Tyr (6:3:1) was also phosphorylated and coupled to the Actigel matrix. Finally the peptides surrounding the two major phosphorylation sites from pp60<sup>c-src</sup>, Y416 and Y527, were purchased and synthesized respectively. All peptides efficiently phosphorylated specifically on tyrosine residues using the EGF receptor and then were purified by HPLC as described above for the Y751 phosphopeptide.

Baculovirus expressed bovine p85 $\alpha$  and p85 $\beta$  were chosen to test these columns (Otsu et al, 1991). Binding analysis was carried out under identical conditions to those previously established for the Y751 phosphopeptide column. Somewhat unexpectedly the baculovirus expressed p85 subunits bound to all phosphopeptide columns tested (see Figure 7, panels A and B). They did not however bind to identical columns containing non-phosphorylated peptides (Figure 6, panels A and B, lane 1 and data not shown). However when partially purified bovine brain PI3-kinase was applied to these columns it was found to bind exclusively to the phosphopeptide columns containing a YXXM motif (see Figure 7 and Figure 8, panel A).

That the Y751S phosphopeptide column appears to be as efficient at binding the active PI3-kinase complex as the longer Y751 phosphopeptide column suggests that the

consensus sequence recently proposed by Cantley et al, (1991) does indeed contain all the sequence data necessary for correct recognition by the PI3-kinase SH2 domain (Figure 8, panel B).

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### CLONING OF p110

#### C. Experimental Procedures

##### 10 **Materials**

Restriction enzymes and DNA modification enzymes were obtained from standard commercial sources and used according to the manufacturer's recommendations. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and used directly in subsequent procedures.

##### **Cells**

The SGBAF-1 cell line was established by transfection of bovine adrenal cortex zona fasciculata cells with pSV3neo as previously described for other cell types (Whitley et al, 1987). SGBAF-1 cells and COS-1 cells were maintained in Dulbecco's modified eagle medium (DMEM) containing a 10% foetal calf serum (FCS). Maintenance of *Spodoptera frugiperda* (Sf9) cells was carried out as described by Summers and Smith, 1987.

##### **Protein Purification and Amino Acid Sequence**

###### **Determination**

The purification of the p85 $\alpha$  and p110 proteins by chromatography on a peptide affinity column corresponding to amino acids 742-758 of the kinase insert region of the human PDGF- $\beta$  receptor has been described above. The method used for the final purification of p110 for amino acid sequence analysis was in accordance with the Protocol given hereinbefore. This procedure was carried out on three separate PI3-kinase preparations. A fourth preparation was eluted from the matrix as before and

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boiled for 5 min. After cooling, the sample was diluted with 25 mM Tris-HCl, pH 8.8 and digested directly with lysylendopeptidase for 72 h at 30°C. Peptides were separated as above. Peptide sequences were determined using a modified Applied Biosystems 477A automated pulse-liquid sequencer.

#### mRNA Isolation and cDNA Cloning

Total RNA was isolated from the SGBAF-1 by the method of Chirgwin et al. (1979) and poly(A)<sup>+</sup> mRNA selected by chromatography on oligo-dT cellulose (Maniatis et al., 1982). An oligo-dT primed cDNA library of 5 x 10<sup>6</sup> primary recombinants was constructed in lambda Uni-Zap (Stratagene) from 5 µg of this mRNA using the Stratagene Uni-Zap cDNA cloning system. The construction of the total bovine brain cDNA library in lambda Uni-Zap has been described previously (Otsu et al, 1991).

#### Library Screening and Hybridizations

The unamplified SGBAF-1 cDNA library (10<sup>6</sup> recombinants) was plated on E. coli K12 PLK-F' (Stratagene) at a density of 10<sup>5</sup> plaques per 15 cm dish and lifts taken in duplicate onto nitrocellulose membranes (Millipore). For screening, filters were prehybridized for at least 1 h at 42°C in 6 x SSPE, 0.5% SDS, 10 x Denhardt's solution, 100 µg ml<sup>-1</sup> denatured sonicated herring sperm DNA (Sigma). Hybridization was carried out in the same solution containing 10 ng ml<sup>-1</sup> radiolabelled oligonucleotide. Oligonucleotides used were: Peptide N (MDWIFHT) 5'-AA(G/A)ATGGA(T/C)TGGAT(C/T/A)TT(T/C)CA(T/C)AC-3'); Peptide J ( D D G Q L F H I D F G H F ) 5'-GATGATGGCCA(G/A)CTGTT(T/C)CA(T/C)AT(T/A)GA(T/C)TTGGCCA(T/C)TT. Oligonucleotides were labelled with <sup>32</sup>P at the 5' end in a 20 µl reaction containing 100 ng oligonucleotide, 1 x kinase buffer (Promega), 0.1 mM spermidine, 5 mM dithiothreitol, 100 µCi [ $\gamma$ -<sup>32</sup>P]ATP (5000 Ci mmol<sup>-1</sup>, Amersham) and 2 µl (20 U) T4 polynucleotide kinase (Amersham). Filters

were washed in 6 x SSC, 0.1% SDS at room temperature and then subjected to autoradiography using Kodak XAR film. Hybridizing clones were plaque-purified and rescued as plasmids according to the manufacturers instructions.

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#### Characterization of cDNA Clones

Sequencing was carried out by the chain termination method using the Sequenase system (United States Biochemicals).

Clones for sequencing were obtained by directed cloning of restriction fragments into M13 mp18 and mp19 vectors (Yanisch-Perron et al., 1985) and by making a series of exonuclease III mediated deletions (Henikoff, 1984; Pharmacia Exonuclease III deletion kit). DNA sequences were analysed on a MicroVAX computer using the Wisconsin (UWCGC: Devereux et al., 1984) sequence analysis package.

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#### RACE PCR

RACE PCR was carried out essentially as published previously (Frohman et al., 1988; Harvey and Garlison, 1991). Briefly, first strand cDNA primed with random hexamers (Amersham) was synthesized from 1 µg of SGBAF-1 cell mRNA using the Stratagene first strand cDNA synthesis kit. First strand cDNA was isolated by isopropanol precipitation and tailed with oligo-dA using terminal deoxynucleotidyl transferase (BRL).

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PCR was performed using oligo 2224

(5'-AATTCACACACTGGCATGCCGAT) and adaptor-dT

(5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT) as primers

using a Perkin Elmer/Cetus Tap polymerase PCR kit (conditions: 94°C 1 min, 35°C 1 min, 72°C 2 min, 30 cycles). Products were

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fractionated on a 1.5% low melting point agarose gel and

visualized by staining with ethidium bromide. The gel was

sliced into 6 bands (size range 150-2000 bp) and DNA isolated

from each gel slice. A further round of PCR was performed on

this DNA using oligonucleotide 2280

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(5'-TTAAGCTTAGGCATTCTAAAGTCACTATCATCCC) and adaptor

(5'-GACTCGAGTCGACATCGA) as primers (conditions: 94°C 1 min,

56°C 1 min, 72°C 2 min, 35 cycles). Products were fractionated

on an agarose gel and visualised by staining with ethidium

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bromide. A band 250 bp shorter than the size of the DNA in the gel slice used for the PCR was expected. An intensely staining band of 350 bp obtained from the ~600 bp gel slice was excised, digested with HindIII and Sall and ligated into Bluescript KS- digested with HindIII and XhoI to give plasmid pBS/race. Two independent inserts were completely sequenced.

#### Southern Transfer Hybridizations

High molecular weight DNAs were isolated from cell lines by standard techniques (Maniatis et al, 1982). DNAs were digested with restriction endonucleases, fractionated through 0.5% agarose gels and transferred to nitrocellulose (BA85, Schleicher and Schuell) as described in Maniatis et al (1982). Prehybridization was carried out in 1 M NaCl, 10 x Denhardt's solution, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.1% SDS and 100  $\mu\text{g ml}^{-1}$  denatured sonicated herring sperm DNA at 65°C. Hybridization was carried out overnight in the same solution containing 20 ng  $\text{ml}^{-1}$  radiolabelled probe fragment (0.88 kb XbaI-PstI fragment: Probe a, Figure 9, lower panel) of specific activity  $>10^8$  dpm  $\mu\text{g}^{-1}$ ). Probe fragments were isolated from agarose gels by electroelution (Maniatis et al, 1982) and labelled by nick translation (Rigby et al, 1977) using [ $\alpha$ - $^{32}\text{P}$ ] dATP ( $>3000$  Ci mmol $^{-1}$ , Amersham). Membranes were washed extensively in 0.1 x SSC, 0.1% SDS at 68°C or at 50°C in 0.5 x SSC, 0.1% SDS to detect related sequences, and subjected to autoradiography with Kodak XAR film.

#### Northern Transfer Hybridizations

Poly(A) $^+$  RNA from total bovine brain or the SGBAF-1 cell line was modified with DMSO and glyoxal and fractionated on a 0.9% agarose gel run in 10 mM phosphate buffer (pH 7.5) (Maniatis et al, 1982). Nucleic acid was transferred to nylon membranes (Hybond-N, Amersham) and filters baked dry. Prehybridization was carried out at 60°C in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.2% SDS, 200  $\mu\text{g ml}^{-1}$  denatured sonicated herring sperm DNA and 200  $\mu\text{g ml}^{-1}$  yeast RNA. Hybridization was carried out in the same solution containing  $1 \times 10^7$  cpm  $\text{ml}^{-1}$  antisense RNA probe. Probe was prepared by in vitro

transcription of a 2 kb fragment (nucleotides 598-2608; Probe b, Figure 9, lower panel) subcloned in pSPT19 (Boehringer), using SP6 RNA polymerase (Amersham) and [ $\alpha^{32}$ -P] UTP (Amersham) according to the manufacturers conditions. Membranes were washed in 0.1 x SSC, 0.1% SDS at 60°C. Filters were treated with 1  $\mu$ g ml<sup>-1</sup> RNAase A (Sigma) in 2 x SSC for 15 min at room temperature and the filter rinsed at 50°C in 0.1 x SSC. Filters were then subjected to autoradiography against Kodak XAR film at -70°C.

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#### PCR Determination of p85 $\alpha$ and p110 mRNA

For p85 $\alpha$  125 ng of poly (A)<sup>+</sup> RNA was reverse transcribed with 2.5 units rTth DNA polymerase (Perkin-Elmer-Cetus) at 70°C for 10 min in a 10  $\mu$ l reaction containing 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM MnCl<sub>2</sub>, 0.5 mM dNTP mixture and 1.2  $\mu$ M antisense primer (5'-CAGGCCTGGCTTCCTGT). For DNA polymerization the reaction volume was adjusted to 50  $\mu$ l by adding a single mix giving the following final concentrations: 5% (v/v) glycerol, 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.75 mM EGTA, 0.05% (v/v) Tween 20, 2 mM MgCl<sub>2</sub>, 0.24  $\mu$ M sense primer (5'-AACCAGGCTCAACTGTT). PCR was then performed under the following reaction conditions: 92°C 1 min, 58°C 1 min, 72°C 1 min for 25 cycles on a Perkin Elmer-Cetus DNA thermal cycler.

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Conditions for p110 were similar except concentration of the antisense primer (5'-TGCTGTAAATTCTAATGCTG) was increased to 4.8  $\mu$ M during the reverse transcription step. DNA polymerisation conditions were the same except the final MgCl<sub>2</sub> concentration was increased to 2.5 mM and both primers (sense primer = 5'-GTATTCATGAAACAAATGA) were present at a final concentration of 0.96  $\mu$ M. Taq DNA polymerase (Promega) was also added at 0.03 U  $\mu$ l<sup>-1</sup>. PCR was performed as follows: 92°C 30 sec, 54°C 5 sec, 72°C 30 sec for 35 cycles. 20  $\mu$ l of each reaction was run on a 3% agarose gel (Maniatis et al, 1982) and visualised by staining with ethidium bromide.

#### Antibodies and Immunoprecipitations

For the preparation of the anti C-terminal p110 antiserum,

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peptide CKMDWIFHTIKQHALN was synthesized by FMOC chemistry and purified by HPLC. It was then coupled to KLH using glutaraldehyde, and injected into the lymph nodes of rabbits using methods described in Kypta, R M et al., (1990), Cell 62, 481-492. Positive antisera as determined by enzyme-linked immunoassay were affinity purified on specific peptide-Actigel affinity columns. Anti-p85 $\alpha$  (Otsu et al, 1991) and anti CSF-1 receptor (Ashmun et al., 1989) antisera are previously documented. Immunoprecipitations were carried out as described in Otsu et al., 1991.

### PI3-kinase Assay

The assay for PI3-kinase activity was carried out as described by Whitman et al. (1985).

### Expression of p110 in Sf9 Cells

To clone the p110 coding region into the baculovirus transfer vector p36C (Page, 1989) a Sau 3A1 site (GGATCA) present 10 nucleotides upstream from the initiation codon (see Figure 9) was changed to a BamH1 (GGATCC site by PCR mediated mutagenesis. Briefly, a sense oligonucleotide substituting C for A at position 6 of the Sau3A1 site was used in a PCR reaction with an antisense primer comprising nucleotides (102-124) of the p110 sequence (see Figure 9) using Vent polymerase (New England Biolabs). Template DNA was random-primed first strand cDNA prepared from SGBAF-1 cell mRNA as described above; PCR conditions: 94°C 1 min, 50°C 1 min, 72°C 2 min, 35 cycles. The PCR product was digested with BamH1-EcoN1 and a 118 bp fragment isolated from a low melting point agarose gel. This BamH1-EcoN1 fragment was cloned into p110/2.2 digested with BamH1 (present in vector sequences) and EcoN1 (nucleotide = 108) giving plasmid p110/(BamH1). The BamH1-EcoN1 fragment of p110/(BamH1) was sequenced and found to agree with that previously determined. A 3.4 kb BamH1-Kpn1 (Kpn1 site present in the vector) fragment was isolated from p110/(BamH1) and ligated into p36C baculovirus transfer vector (Page, 1989) previously digested with the same enzymes. Recombinant viruses were obtained as described in Summers and Smith

(1987). Sf9 cells were infected at a multiplicity of infection of 10 with recombinant viruses in IPL-41 media supplemented with 10% FCS. Cells were harvested and lysed 2 days post-infection in EB lysis buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 1% NP40, 1 mM EDTA, 500  $\mu$ M sodium orthovanadate, 2 mM PMSF, 100 Kallikrein inhibitor units of Aprotinin  $\text{ml}^{-1}$ ) (Kazlauskas and Cooper, 1989) and lysates were analysed by immunoappreciation.

#### 10 **Association of p110 and p85 $\alpha$ with CSF-1 Receptor**

This assay was performed essentially as described by Kazlauskas and Cooper (1990). Sf9 cells were infected as already described and lysed 48 h post-infection in EB lysis buffer. CSF-1 receptor was immunoprecipitated from Sf9 cells

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and collected on Protein A-Sepharose\* beads. The immunocomplex was then subjected to extensive washing (3 times with EB lysis buffer, twice with kinase buffer; 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.02% Triton X-100, 12 mM  $\text{MgCl}_2$ , 2 mM  $\text{MnCl}_2$ , 10% glycerol, 500  $\mu$ M sodium orthovanadate) and the receptor phosphorylated for 15 min at 20°C with ATP. The precipitates were then washed again to remove free ATP and incubated for 2 h at 4°C with cell lysates prepared from Sf9 cells infected with viruses expressing (i) p85 $\alpha$ , (ii) p110 or (iii) co-infected with viruses expressing p85 $\alpha$  and p110. The immune complexes were washed and assayed for associated PI3-kinase activity.

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#### **Expression of p85 $\alpha$ and p110 in COS-1 Cells**

For transient expression of p85 $\alpha$  in COS-1 cells the coding region for p85 $\alpha$  was cloned into the adenovirus late promoter based expression vector pMT2 (Kaufman et al, 1989) as previously described (Otsu et al, 1991). For expression of the p110 cDNA plasmid pSG5-p110 was constructed as follows.

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The 3.4 kb BamH1-HindIII fragment from cDNA p2.1 was ligated into pSG5 (Stratagene) cut with BamH1 and BglII, the HindIII and BglII overhangs of p2.1 and pSG5 respectively, being filled in with Klenow polymerase. This gave construct pSG5.2.

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Plasmid pBS/race (above) was digested with EcoR1 and Hind111, the 350 bp band gel purified by electroelution (Maniatis et al, 1982) and further digested with Sau3A1 and Bsml. This mixture was then added to the gel purified Bsml-BstM1 fragment from p2.1 and ligated in a three fragment ligation to pSG5.2 digested with BamH1 and BstX1. 5 µg of each DNA was transfected into 10 cm dishes of 80% confluent COS-1 cells using Lipofectin (BRL) under conditions suggested by the manufacturers. Lysates were analysed by immunoprecipitation with anti-p85α polyclonal antiserum or with anti-p110 C-terminal peptide antiserum. Immunocomplexes collected on Protein A-Sepharose beads were analysed either on 10% SDS-PAGE gels followed by autoradiography or subjected to in vitro PI3-kinase assays as described.

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#### D. Results of Cloning

##### **cdNA Cloning and Deduced Amino Acid Sequence of p110**

Initially, an oligo(dT) primed bovine brain cdNA library (Otsu et al, 1991) was screened with oligonucleotide probes made against peptides J and N (see Figure 9). No hybridizing clones were detected. Therefore, a new cdNA library of  $5 \times 10^6$  primary recombinants was constructed from mRNA isolated from a pSV3neo transfected bovine adrenal cortex zona fasciculate cell line (SGBAF-1), which was known to contain PI3-kinase activity (Otsu et al, 1991). Screening of  $1 \times 10^6$  primary recombinants from this library with the same oligonucleotides led to the detection of 66 clones positive with both probes. Twenty overlapping clones were characterized and found to possess inserts from 1-4 kb. The clone with the longest insert representing coding sequence (clone p110/2.1) was completely sequenced. This revealed a potential open reading frame (ORF) of 1053 amino acids with a predicted molecular weight of 123 kD. The ORF contained all the sequenced peptides, but was not preceded by in-frame stop codons. Since the predicted size of the p110 protein from SDS gels is 110 kD, it was possible that the protein could initiate from an internal methionine within this ORF.

Expression studies carried out in COS-1 cells using methionines 16, 30, 123 and 130 as potential start codons (initiation at Met 123 would give rise to a protein of 110 kD) did not lead to the syntheses of a protein corresponding to p110 or any augmentation of PI3-kinase activity in these cells. This suggested p110/2.1 is missing 5' coding sequence and that either p110 protein runs anomalously on SDS-PAGE gels or that it is synthesized as part of a larger precursor molecule. Characterization of the remaining 46 positive clones initially isolated, showed that all had inserts shorter than that in clone p110/2.1. To further extend the p110/2.1 cDNA in the 5' direction a RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) (Frohman et al, 1988; Harvey and Garlison, 1991) was used. Two independent products which extended the known nucleotide sequence were characterized (see Figure 9, lower panel). The nucleotide and deduced amino acid sequences for the coding region of the composite cDNA are presented in Figure 9. The putative initiation codon is preceded by an in-frame stop codon and occurs in a Kozak consensus sequence (Kozak, 1987) for the initiation of translation (data not shown). The deduced amino acid sequence encodes a protein of 1068 amino acids with a calculated relative molecular mass of 124,247.

### 25 **Analysis of the p110 Nucleotide and Deduced Amino Acid Sequences**

The coding region of the cDNA for p110 is extremely A+T rich (G+C content = 39.3%) which is reflected in the failure to use codons TCG (Serine) and GTC (Valine). When the p110 amino acid sequence was compared with sequences in the Swissprot and NBRF protein databases, significant homology was found to only one protein, Vps34p (Figure 10). This is a rare 100 kD protein from *Saccharomyces cerevisiae* involved in the sorting of proteins into the yeast vacuole and in the vacuole morphogenesis during budding (Herman and Emr, 1990). A search of the p110 sequence for amino acids conserved in the active sites of kinases, reveals G<sub>842</sub>, K<sub>863</sub>, D<sub>916</sub>, N<sub>921</sub>, and the DFG triplet at residues 933-935 (these residues are marked in



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Figure 2B) which might be homologous to G<sub>52</sub>, K<sub>72</sub>, D<sub>166</sub>, N<sub>171</sub> and the DFG triplet at residues 184-186 in cAMP-dependent protein kinase (Knighton et al, 1991a,b). Equivalent residues are present in Vps34p and are also marked in Figure X. The  
5 glycine rich P-loop (Saraste et al., 1990), found in many kinases (Hanks et al., 1988), does not appear to be present in either p110 or Vps34p.

#### Genomic Southern Blot Analysis of p110 Genes

10 Given the occurrence of at least two forms of p85 (Otsu et al, 1991), Southern blot analysis was used to analyse the number of p110 related genes which occur in genomic DNA isolated from bovine, human and rat sources. The analysis clearly provides  
15 evidence for a second, closely related, gene in rat and human genomic DNA (e.g. compare Figure 11A lanes 4 and 9 with Figure 11B lanes 4 and 9). For bovine DNA there appear to be no hybridization signals removed by washing at higher stringency (compare Figure 11A lanes 1, 2 and 3 with Figure 11B lanes 1,  
20 2 and 3). However, it is possible that a related gene exists in bovine DNA, but, either it does not cross-hybridize under the conditions used, or it is too similar in sequence to be detected by differential washing.

#### Expression of p110 Cells and Tissues

25 A northern blot analysis carried out on mRNA isolated from the SGBAF-1 cell line and total bovine brain is shown in Figure 12A. Both mRNA samples contain major p110 specific transcripts of 4.8 kb and 9 kb, although there is substantially more p110 message present in mRNA isolated from SGBAF-1 cells (Figure  
30 12A, lane 2) than that isolated from total bovine brain (Figure 12A, lane 1). A PCR based study was performed to examine the distribution and conservation of p110 mRNA in cell lines and tissue from several species. Amplification of a p110 specific fragment is seen for three human mRNAs (218 bp;  
35 Figure 12B lanes 1, 2 and 3) and two bovine mRNAs (212 bp; Figure 12B, lanes 5 and 6). Similar sized fragments are amplified from cell lines of simian and porcine origin (Figure 12B, lanes 4 and 7, respectively), indicating the existence of

a p110 homologue in these species. An additional band of 300 bp is amplified from bovine brain mRNA (Figure 12B, lane 5) and its identity is currently being investigated. Since PI3-kinase activity may reside in a p85 $\alpha$ /p110 complex (Carpenter et al, 1990; Otsu et al., 1991; Shibasaki et al., 1991), some of these cell lines were examined to see whether messages for p85 $\alpha$  and p110 are co-expressed. Amplification of a p85 $\alpha$  specific 190 bp fragment is seen for the three human omission (Figure 12C, lanes 1, 2 and 3) cell lines and one simian (Figure 12C, lane 4) cell line analysed. Thus, at least in these four cell lines, messages for p85 $\alpha$  and p110 are co-expressed.

#### p110 cDNA Encodes a Protein of Apparent Molecular Weight 110 kD which Possesses PI3-kinase Activity

To demonstrate that the p110 cDNA encodes the 110 kD subunit of PI3-kinase, it was expressed in the baculovirus expression system (Summers and Smith, 1987). Immunoprecipitation with an anti-p110 antiserum from *Spodoptera frugiperda* (Sf9) cells infected with the p36C-p110 virus revealed a novel protein of apparent molecular weight 110 kD (Figure 13A, lane 4) which co-migrated with the p110 PI3-kinase subunit purified from bovine brain. No such protein was seen in anti-p110 immunoprecipitates prepared from cells infected with a control wild-type virus (Figure 13A, lane 2). This baculovirus expressed p110 was used to examine whether p110, alone, possesses catalytic activity or whether a p85 $\alpha$ /p110 complex is required. When assayed, p110-containing immunoprecipitates were found to possess significant levels of PI3-kinase activity (Figure 13B, lane 4), the identity of the lipid product being confirmed as PI(3)P by HPLC analysis. No activity was detected in anti-p110 immunoprecipitates prepared from control infected cells (Figure 13B, lane 2). These results clearly demonstrate that the p110 subunit of PI3-kinase is sufficient for catalytic activity.

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### **p110 Expressed in Insect Cells Forms a Stable Complex with p85 $\alpha$**

Since PI3-kinase purified from bovine brain is a complex of p85 $\alpha$  and p110, the ability of p85 $\alpha$  and p110 expressed in insect cells to reconstitute an active p85 $\alpha$ /p110 complex was examined. Baculoviruses expressing either p85 $\alpha$  (pACC4-p85 $\alpha$ ; Otsu et al, 1991) or p110 (p36C-p110) were infected separately, or together, into Sf9 cells and expressed proteins analysed as described in experimental procedures.

Immunoprecipitates of p85 $\alpha$  alone (Figure 13A, lane 3) were inactive in a PI3-kinase assay (Figure 13B, lane 3) as previously demonstrated (Otsu et al, 1991). In double infection experiments, both p85 $\alpha$  and p110 were detected in either anti-p85 $\alpha$  (Figure 13A, lane 5) or anti-p110 (Figure 13A, lane 6) immunoprecipitates. As neither subunit-specific antiserum recognises the other subunit (see Figure 15A, lane 3; Figure 15C, lane 2), the simplest interpretation of this data is that, when expressed in Sf9 cells, p110 and p85 $\alpha$  (Figure 13B, lane 5) or the anti-p110 antisera (Figure 13B, lane 6) were both active. Neither antiserum immunoprecipitated endogenous PI3-kinase activity from Sf9 cells infected with wild-type virus (Figure 13B, lanes 1 and 2).

### **PI3-kinase Activity Expressed in Sf9 Cells Can Associate with the Activated CSF-1 Receptor**

PI3-kinase activity has been shown to associate with many activated PTK receptors, but particularly well studied have been those receptor PTKs possessing a kinase insert region, e.g., PDGF- $\beta$  receptor (Coughlin, S R et al., (1989), Science 243, 1191-1193 and the CSF-1 receptor (Varticovski et al, 1989; Shurtleff et al, 1990). An in vitro association assay (Kazlauskas and Cooper, 1990) was used to study the association of PI3-kinase activity expressed in insect cells with the activated CSF-1 receptor. Figure 14 shows that baculovirus expressed PI3-kinase activity can associate with the CSF-1 receptor, but only from an Sf9 cell lysate containing both p85 $\alpha$  and p110 (Figure 14, lane 2), and only

when the receptor has been phosphorylated prior to incubation with Sf9 cell lysate (compare Figure 14, lanes 2 (+ ATP) and 3 (- ATP)). No PI3-kinase activity associates with CSF-1 receptors incubated with Sf9 cells lysates containing p85 $\alpha$  alone (Figure 14, lane 4) or p110 alone (Figure 14, lane 5). No activity is found associated with the CSF-1 receptor immunoprecipitated from Sf9 cells (Figure 14, lane 1). Thus, PI3-kinase subunits expressed in insect cells can be used to reconstitute an active p85 $\alpha$ /p110 complex that binds to a phosphorylated PTK receptor.

#### Expression of PI3-kinase in COS-1 Cells

The results shown above were all obtained from expression studies carried out in insect cells. In order to study p110 and its interaction with p85 $\alpha$  in a mammalian cell system, transient co-expression studies in COS-1 cells were performed. The p110 cDNA was cloned into the SV40 based expression vector, pSG5 (giving plasmid pSF5-p110) and transfected into COS-1 cells, either alone or together with a p85 $\alpha$  expression construct, pMT2-p85 $\alpha$  (Otsu et al., 1991). To enable proteins to be more easily visualised transfected COS-1 cells were metabolically labelled with <sup>35</sup>S-methionine for 3-4 h prior to lysis. Radiolabelling at this time results in preferential labelling of proteins synthesized from transfected constructs. Cell lysates were immunoprecipitated with either anti-p85 $\alpha$  (Figure 15, panels A and B) or anti-p110 antisera (Figure 15, panels C and D). Immunoprecipitated proteins were either visualised by autoradiography following fractionation on SDS-PAGE gels (Figure 15, panels A and C) or subjected to an in vitro PI3-kinase assay (Figure 15, panels B and D).

Transfection of pMT2-p85 $\alpha$  resulted in a significant elevation of p85 $\alpha$  over the background level due to endogenous simian p85 $\alpha$  - compare Figure 15A, lanes 2 and 4 with Figure 15A, lane 1. In p85 $\alpha$ /p110 co-transfectants, the anti-p85 $\alpha$  antiserum co-immunoprecipitates p85 $\alpha$  and p110 (Figure 15A, lane 4), demonstrating the existence of a p85 $\alpha$ /p110 complex. When assays for PI3-kinase activity were performed on the anti-

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p85 $\alpha$  immunoprecipitates, enhanced activity (10 fold over the background due to endogenous simian PI3-kinase) was only detected with immunoprecipitates which contained both p85 $\alpha$  and p110 (compare Figure 15B, lane 4 with Figure 15B lanes 1, 2 and 3). These results demonstrate that in COS-1 cells, as in Sf9 cells, the p110 cDNA directs the synthesis of a protein of molecular weight 110 kD, which associates with p85 $\alpha$  to give a p85 $\alpha$ /p110 complex that possesses PI3-kinase activity.

10 However, when proteins were immunoprecipitated from the same lysates with the anti-p110 antiserum and PI3-kinase assays performed, the results were surprising. As expected, the anti-p110 antiserum immunoprecipitated p110 from cells transfected with pSG5-p110 (Figure 15C, lane 3). However, in addition, it would only immunoprecipitate free p110 from lysates prepared from cells co-transfected with p85 $\alpha$  and p110 (Figure 15C, lane 4) even though p85 $\alpha$ /p110 complex was present in these lysates (Figure 15A, lane 4). When assayed for PI3-kinase activity, no activity above that present in control immunoprecipitates (Figure 15D, lanes 1 and 2), was present in p110 containing immunoprecipitates prepared from either p110-transfected (Figure 15D, lane 3) or, p85 $\alpha$  and p110 co-transfected, cells (Figure 15D, lane 4). Thus, the anti-p110 antiserum is capable of immunoprecipitating p110 from cell lysates of both infected Sf9 cells (Figure 13A, lane 4) and transfected COS-1 cells (Figure 15C, lane 3), but only the immunoprecipitates prepared from Sf9 cell lysates possess elevated levels of PI3-kinase activity (compare Figure 13B, lane 4 and Figure 15D, lane 3). Also, the anti-p110 antiserum immunoprecipitates the p85 $\alpha$ /p110 complex when expressed in Sf9 cells, but not when expressed in COS-1 cells.

As indicated above, analysis of the cloned p110 cDNA shows it to encode a protein of 1068 amino acids with a calculated molecular weight of 124 kD. The reason for the difference in size between the calculated (124 kD) and observed molecular weight value 110 kD is unclear, but it is known that many proteins migrate anomalously on SDS-PAGE gels. Expression of

the protein encoded by this ORF in Sf9 cells, COS-1 cells, reticulocyte lysate and E. coli all result in the production of a protein of apparent molecular weight 110 kD.

5 The deduced amino acid sequence of p110 contains all the peptide sequences determined by protein sequence analysis. Since the peptides were obtained from a lysylendopeptidase digestion, it is expected that they should all be preceded by an arginine residue. This is true in every case, except for  
10 peptide A which is preceded by an arginine residue (Arg 162). Nucleotide sequence data obtained from another cDNA clone covering this region confirms the presence of an arginine residue in this position. Thus, it seems likely that cleavage at this site by lysylendopeptidase results from a sequence  
15 polymorphism.

When a database search was performed on the p110 sequence no significant homology was detected with any proteins known to be involved in inositol lipid metabolism. However, as noted,  
20 p110 did show significant homology throughout its C-terminal half to the *Saccharomyces cerevisiae* protein Vps34p. The possibility that Vps34p is a yeast PI-kinase is currently being investigated. If p110 and Vps34p are homologous  
25 proteins then it is interesting to speculate that p110 might also be involved with protein targeting and/or vesicular transport. PI3-kinase activity has previously been implicated in vesicle mediated responses in higher eukaryotes. Hence, PI3-kinase activity is seen to increase following stimulation of platelets with thrombin (Kucera and Rittenhouse, 1990) and  
30 neutrophils with f-Met-Leu-Phe (Traynor-Kaplan et al, 1988). In both cases, ligand stimulation promotes the fusion of vesicular structures necessary for the biological response. A role for PI3-kinase in intracellular vesicles following the activation of PTKs has also been suggested (Cantley et al,  
35 1991; Kelly et al, 1992).

Southern blotting data suggests there may be two genes for PI3-kinase in rats and humans. Evidence for the existence of

a second gene in rat DNA is also provided by the results of Carpenter et al., (1990), who identified two forms of p110 in their purified PI3-kinase preparation. In situ hybridization confirms the presence of two closely related sequences in human DNA, although one could be a pseudogene. Two forms of p85 (p85 $\alpha$  and p85 $\beta$ ) have been characterized (Otsu et al, 1991), although only p85 $\alpha$  is found associated with p110 in PI3-kinase from bovine brain. It is possible to speculate that p85 $\beta$  associates with a second form of p110.

Although, at present, the function of the 3-phosphorylated phosphoinositides produced by PI3-kinase is unclear, the availability of expression systems which allow their generation will aid in the determination of their function.

#### Example 2

Using the bovine cDNA probe constituted by the XbaI-PstI fragment of the sequence of Figure 9 (probe a, bottom panel) and genomic DNAs from several species, Southern blot analyses prove positive against the bovine probe in the following species:- bovine (calf thymus), human (HeLa cells), rat (liver), simian (COS cells), porcine (ZNR cells), chicken (from Promega), and Xenopus (liver).

The human cDNA was isolated from a cDNA library, made from mRNA isolated from the human cell line KG1a using standard techniques. The probe was a partial cDNA from the second half of the bovine p110 cDNA. The probe was labelled with <sup>32</sup>P and hybridised overnight to the library filters at 65°C in 1M NaPi, 7% SDS buffer. The filters were washed in 2xSSC at 50°C, and exposed to X-ray film at -70°C. The nucleotide sequence is shown in Figure 16 together with the corresponding amino acid sequence. The human p110 sequence has 95% homology to the bovine p110 sequence at the DNA level and is 98% identical at the protein level (Figures 17 and 18). The protein sequence is shown in Figure 19. Primers (357) AAG GAT CAG AAC AAT GCC T and (416) AGG CTT TCT TTA GCC ATC A were

used to amplify, using RT-PCR (94°C 30 sec 50°C 30 sec, 72°C 60 secs; for 35 cycles) the partial sequence of a highly related p110 gene (p110-11). P110-11 has 96% nucleotide homology to p110 (sequence not provided).

5

Two novel cDNAs related to p110 have been cloned. Degenerate primers were designed to conserved sequences between human p110 and the related yeast gene VPS34 (Sense (GDDLQRD) 5' GGN GAT/C GAT/C T/C TA/G CGN CAA/G GA-3' antisense (FHIDFGHF) 5'A/GAA A/GTG ICC A/GAA A/GTC A/G/TAT A/GTG A/GAA-3). These were used in RT-PCR reactions using mRNA from the human cell lines MOLT4 and U937 (94°C 30 sec, 50°C 30 sec, 72°C 30 sec for 35 cycles). Two novel cDNA's, P1TR-c and P1TR-f, related to p110, were isolated. The P1TR-c nucleotide sequence is shown in Figure 20. This gene is highly related to the yeast gene VPS34, the VPS34 protein is involved in the protein sorting from the golgi to the vacuole and has an intrinsic PI3-kinase activity. The P1TR-f nucleotide sequence is shown in Figure 21 and is more similar to p110 than P1TR-c and is likely also to possess PI3-kinase activity. The alignment of human p110, the human PI3-kinase related genes P1TR-c and P1TR-f and the yeast PI3-kinase VPS34 are shown in Figure 22. The amino acids conserved in 3 or more of the proteins are shown in the upper case.

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The interaction of the p85 and p110 subunits of PI3-kinase are thought to be required for the activity of the kinase in mammalian cells. Thus inhibiting the interaction between the subunits could provide a means of inhibiting the activity of this signal transduction pathway. In order to design reagents to p110 which will block the interaction, it is useful to define the region of p110 which binds to the p85 subunits. To do this a series of mutants were constructed which express various domains of the p110 protein (Figure 23B). These fragments were expressed as GST fusion proteins in bacteria. The proteins were then bound to a glutathione sepharose column (Pharmacia) according to the manufacturer's instructions (Panayotou G et al (1992) EmboJ 11:4261-4272). The ability of

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these protein fragments to bind the p85 subunits was assessed by the ability of the column specifically to retain p85 subunits purified from baculovirus (Otsu et al(1991) Cell 65:91-104). As shown in Figure 23A, only p110-N ( $\alpha$ 1-128) was capable of binding the p85 $\alpha$  and  $\beta$  subunits. To further characterise the binding domain, deletion mutants and PCR fragments were made from the p110-N fragment as shown in Figure 24. The results in Figure 25 demonstrate that a domain containing amino acids 19-110 of human p110 is sufficient to associate with the p85 subunits. Removal of a further 20 amino acids from either the amino or carboxy termini led to loss of binding activity. Now that this domain has been identified it allows the design of specific peptides, antibodies or small molecules which can inhibit the interaction between the subunits.

The invention includes a human PI3-kinase p110 subunit sequence comprising amino acids 19 to 110 of human p110, or an amino terminal truncated or carboxy terminal truncated derivative thereof having less than 20 amino acids deleted from the amino terminal or carboxy terminal end, respectively, but which is capable of binding to a PI3-kinase p85 subunit; and also included is a method of inhibiting p85 and p110 mammalian PI3-kinase subunit interaction, which comprises utilizing a molecule which blocks the binding domain located between amino acids 19 and 110 of human p110.

The invention further provides the use of a sequence or derivative as defined above in screening for a therapeutic or prophylactic agent which operates by inhibiting interaction between p85 and p110 mammalian PI3-kinase subunits.

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**CLAIMS:**

1. An isolated DNA sequence comprising the sequence set out in Figure 16, or a portion thereof, wherein the polypeptide being the expression product of the isolated DNA sequence and the polypeptide being the expression product of the portion of the isolated DNA sequence has:
  - (i) PI3-kinase activity when expression is in an insect cell; and
  - (ii) PI3-kinase activity after complexing with a p85 PI3-kinase subunit when expression is in a COS-1 cell.
2. A portion of the isolated DNA of claim 1, which portion comprises a sequence encoding amino acid residues 272-1068 of Figure 16.
3. Isolated DNA having a base sequence which hybridizes to the complement of the DNA sequence of claim 1 or claim 2 under conditions comprising 1 M NaCl/10x Denhardt's solution/50 mM Tris-HCl (pH 7.4)/10 mM EDTA/0.1% SDS/ 100  $\mu$ g/ml denatured herring sperm DNA at 65°C for 16 h followed by washing at 2 x SSC/0.1% SDS at 42°C; then 0.5 x SSC/0.1% SDS at 50°C; then 0.1 x SSC/0.1% SDS at 65°C; then 0.1 x SSC/0.1% SDS at 68°C; wherein the polypeptide being the expression product of the isolated DNA sequence has:
  - (i) PI3-kinase activity when expression is in an insect cell; and
  - (ii) PI3-kinase activity after complexing with a p85 PI3-kinase subunit when expression is in a COS-1 cell.
4. A DNA construct comprising DNA having a base sequence as defined in any one of claims 1 to 3 under the control of a control sequence and in proper reading frame in an expression vector.
5. A DNA construct according to claim 4 wherein the control sequence includes a regulatable promoter.

6. Host cells which have been genetically altered by the incorporation therein of a construct as defined in claim 4 or 5 so as to permit the expression of the encoded polypeptide.
7. Host cells according to claim 6 which are insect cells.
8. A method for the preparation of a polypeptide comprising cultivating the host cells of claims 6 or 7, wherein the polypeptide has:
  - (i) PI3-kinase activity when preparation is in an insect cell; and
  - (ii) PI3-kinase activity after complexing with a p85 PI3-kinase subunit when preparation is in a COS-1 cell.
9. An isolated polypeptide comprising the amino acid sequence set out in Figure 16, or a portion thereof wherein the polypeptide and said portion thereof independently have:
  - (i) PI3-kinase activity when said polypeptide or portion thereof is produced in an insect cell; and
  - (ii) PI3-kinase activity after complexing with a p85 PI3-kinase subunit when said polypeptide or portion thereof is produced in a COS-1 cell.
10. A portion of the isolated polypeptide according to claim 9 which comprises amino acid residues 272-1068.
11. An isolated polypeptide obtained by expression of a DNA sequence according to claim 3.
12. An antibody which specifically binds to a polypeptide as defined in any one of claims 9 to 11.
13. The use of the polypeptide of any one of claims 9 to 11 to provide PI3-kinase activity.

14. The use of a polypeptide as defined in any one of claims 9 to 11 in a screening method to identify molecules which bind specifically to said polypeptide.
15. The use of a polypeptide as defined in any one of claims 9 to 11, or of an active complex containing said polypeptide, or of an antibody as defined in claim 12, in the manufacture of a medicament; said medicament
  - (a) encouraging or discouraging cell proliferation mediated through a cell surface receptor interactive with said PI3-kinase activity; or
  - (b) effecting the level of stimulation of platelets or neutrophils; or
  - (c) regulating blood glucose levels; or
  - (d) blocking the binding domain located between amino acids 19 and 110 of human p110.
16. The use of a polypeptide as defined in any one of claims 9 to 11, or of an active complex containing said polypeptide, in the in vitro enzymatic production of 3-phosphorylated phosphoinositides.
17. A pharmaceutical or veterinary formulation comprising an antibody as defined in claim 12, formulated for pharmaceutical or veterinary use, together with an acceptable diluent, carrier or excipient and/or in unit dosage form.
18. A human PI3-kinase sequence comprising amino acids 19-110 of the amino acid sequence set out in Figure 16, or an amino terminal truncated or carboxy terminal truncated derivative of said sequence comprising amino acids 19-110 of the amino acid sequence set out in Figure 16, having less than 20 amino acids deleted from the amino terminal or carboxy terminal end, respectively, but which is capable of binding to a PI3-kinase p85 subunit.

19. The use of a sequence or derivative as defined in claim 18 in screening for a therapeutic or prophylactic agent which operates by inhibiting interaction between p85 and p110 mammalian PI3-kinase subunits.



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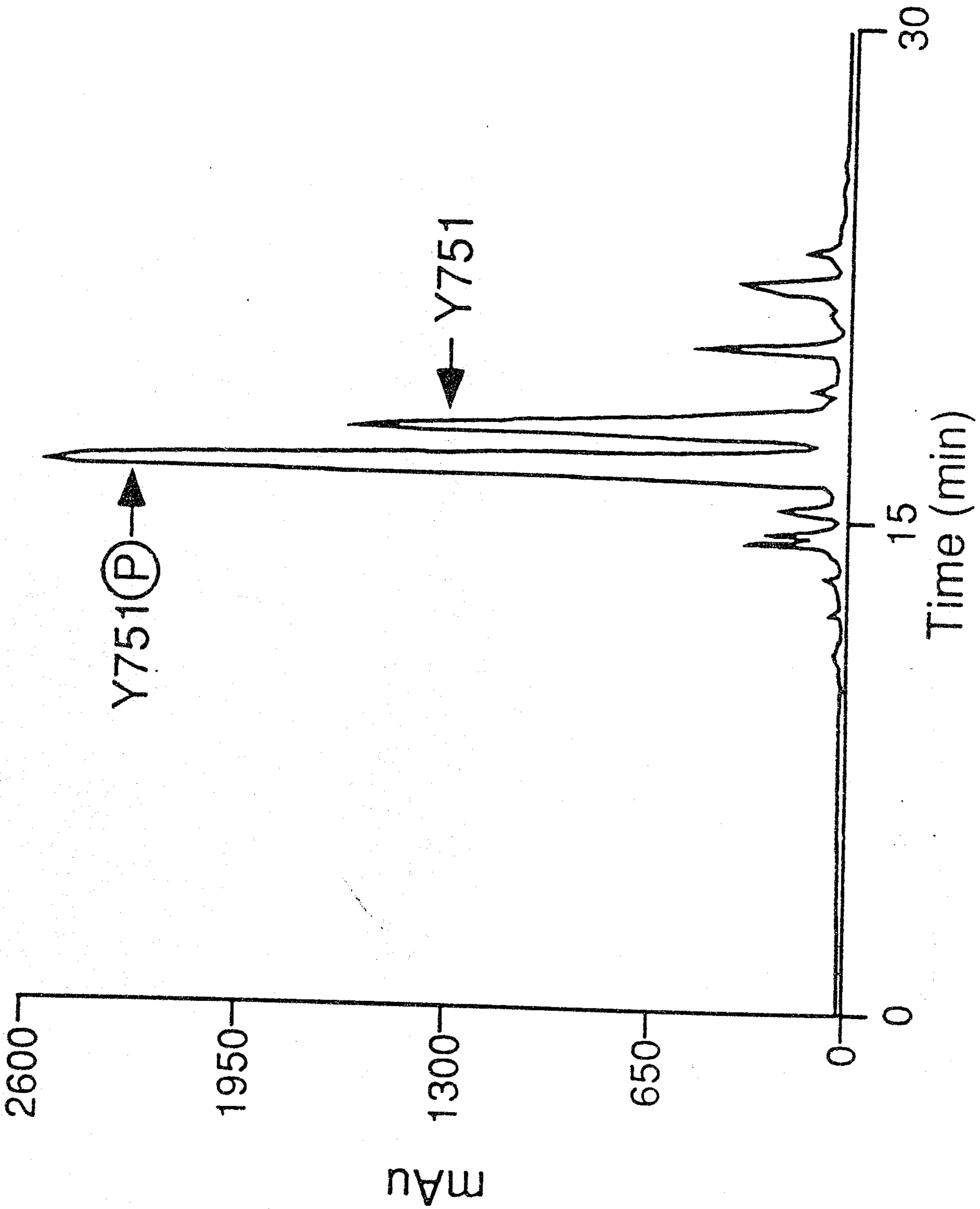


Figure 1

A

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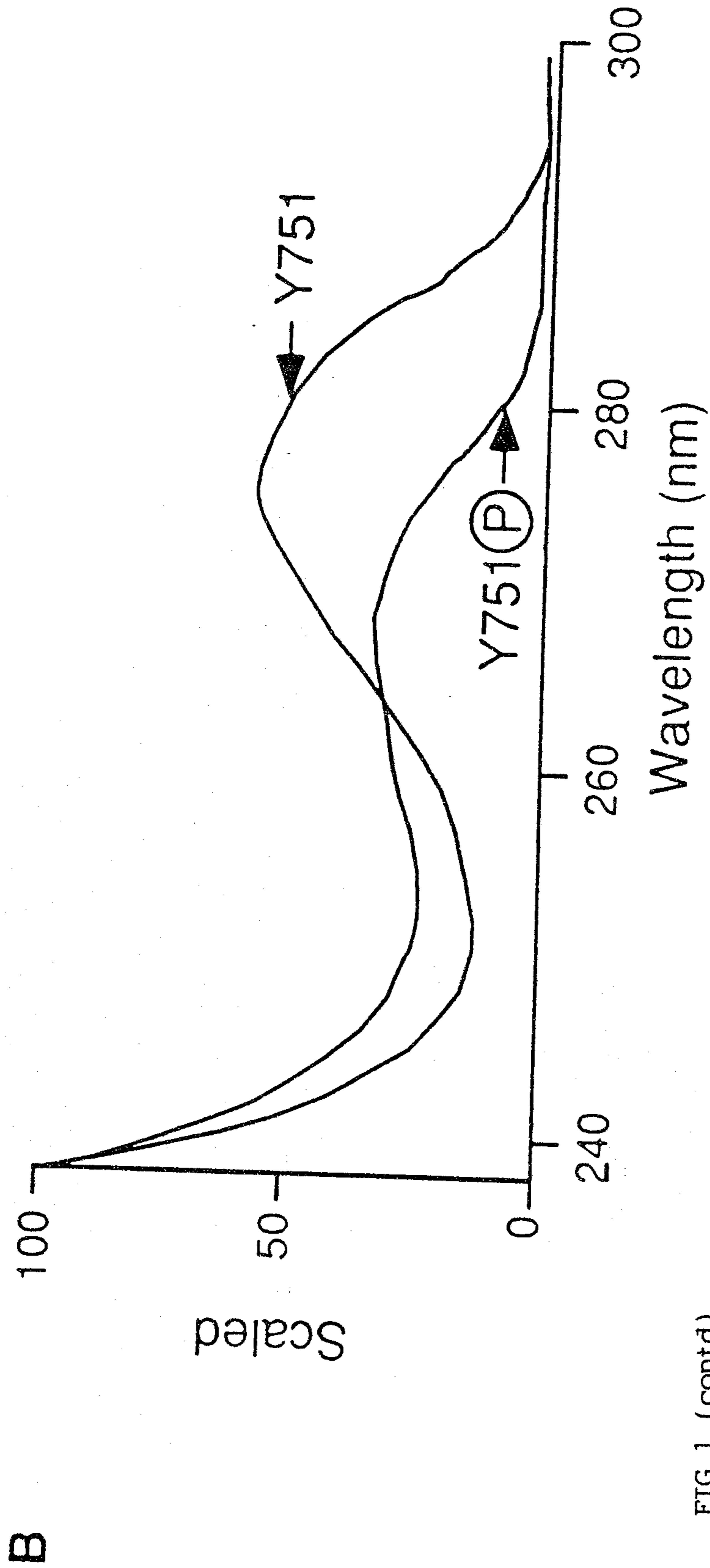
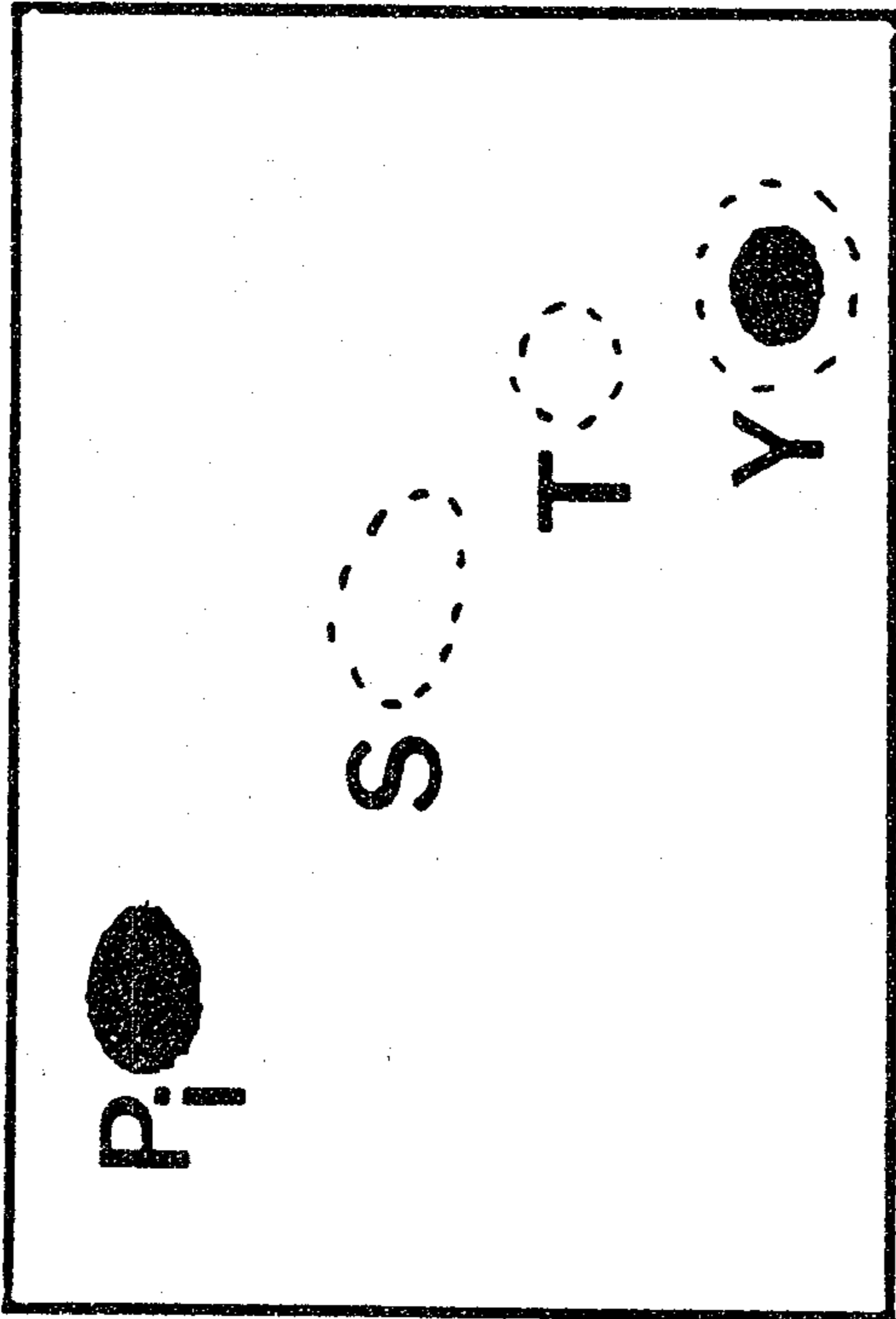
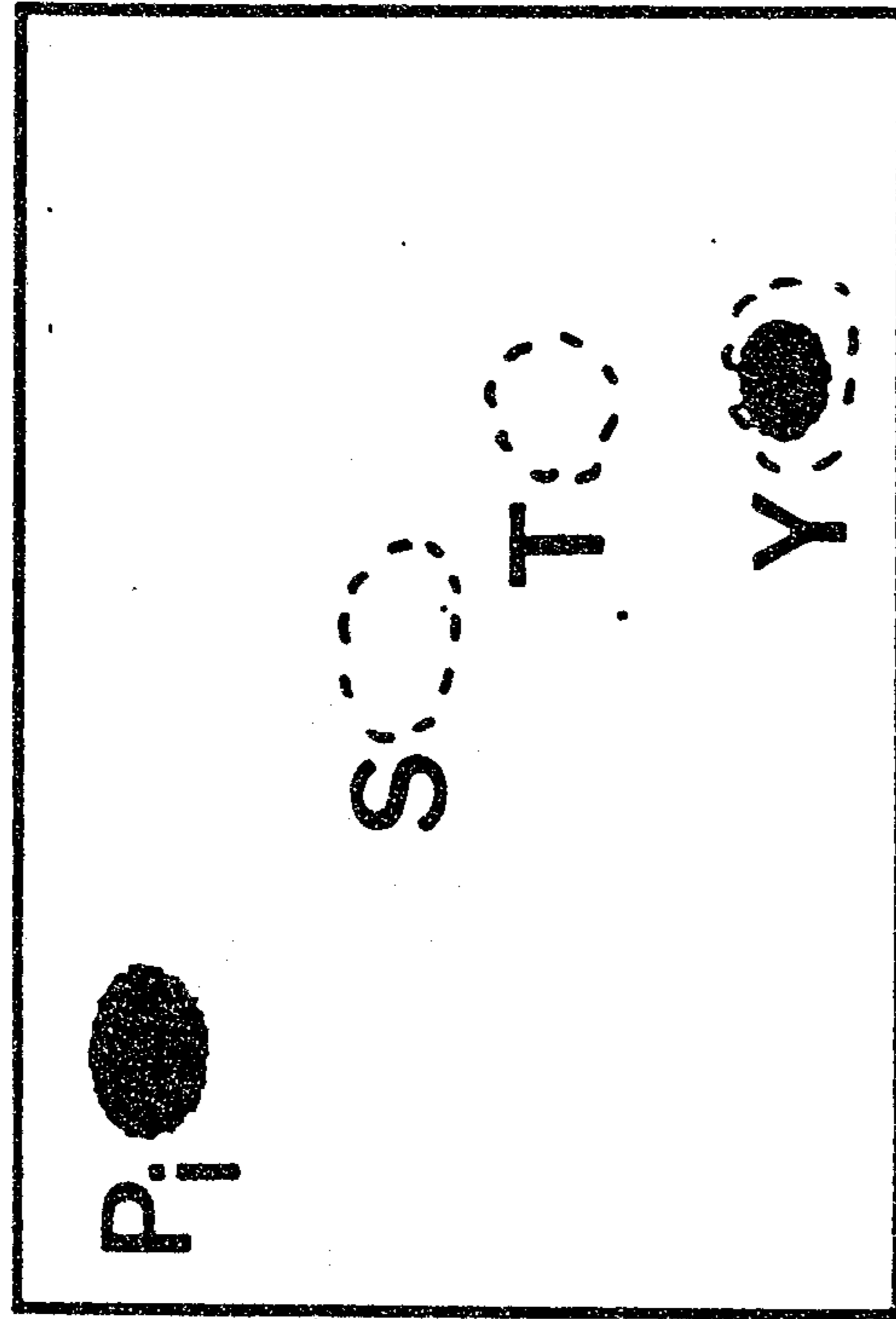


FIG 1 (contd)

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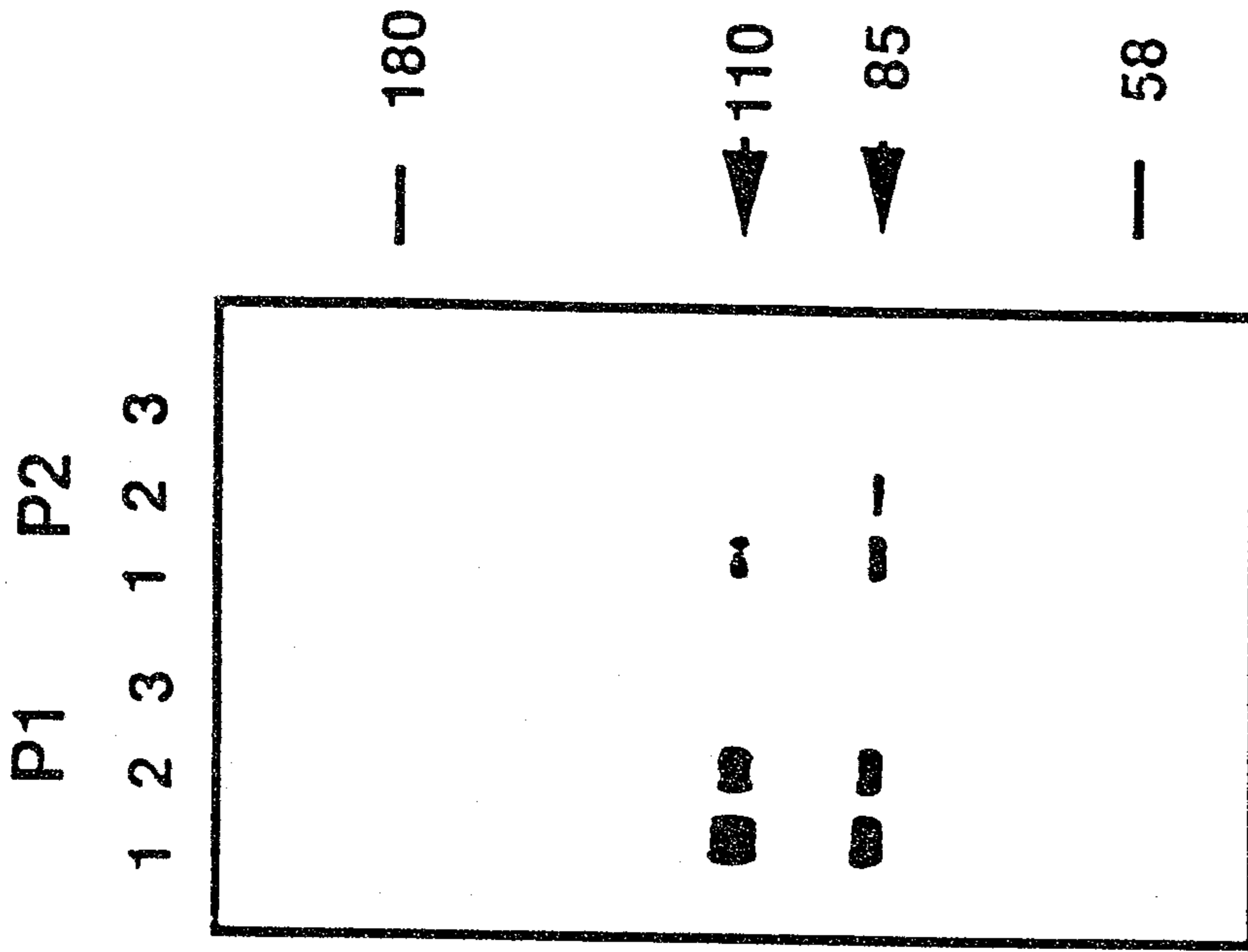
C

FIG 1 (contd)

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B



A

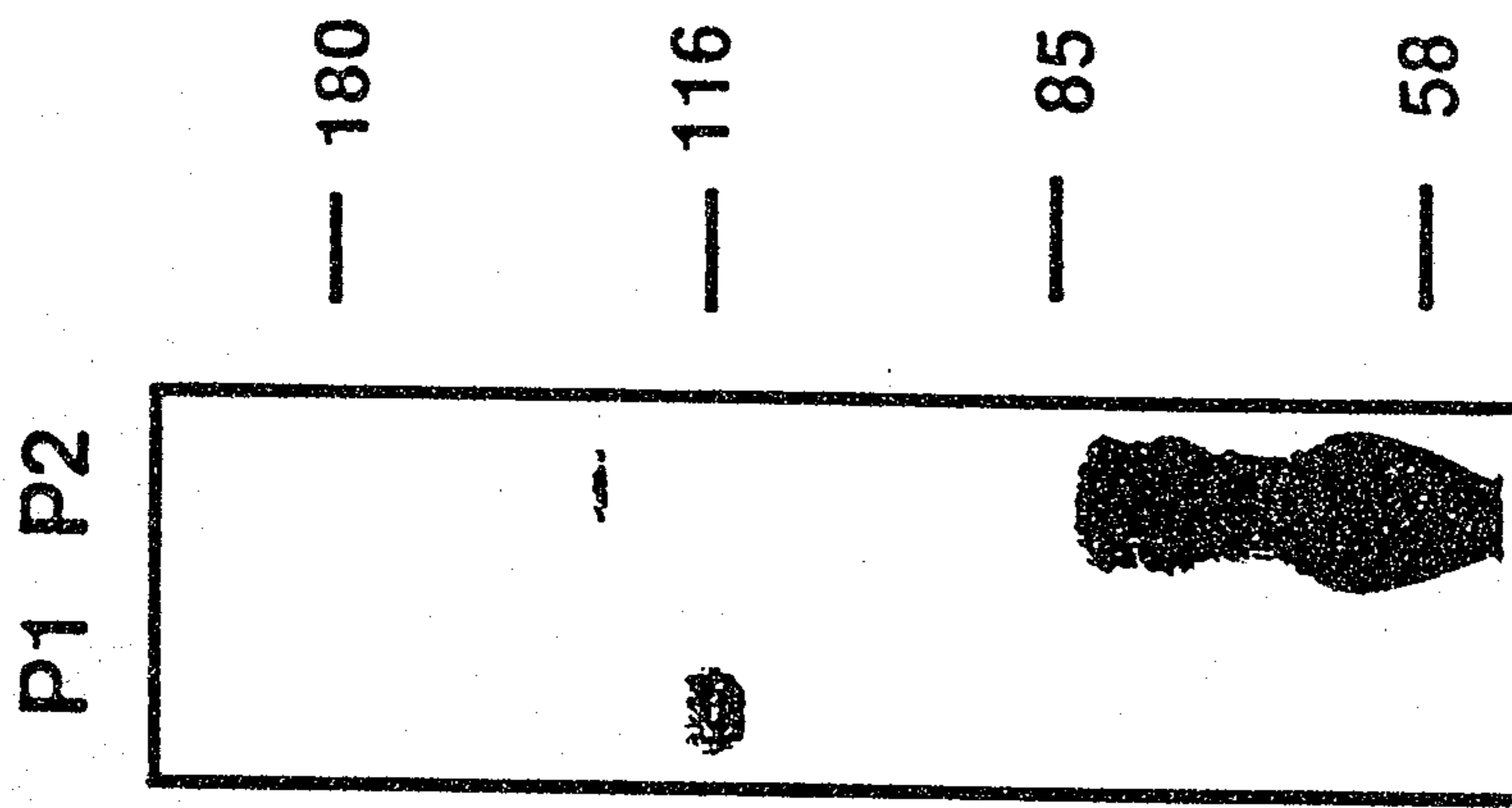
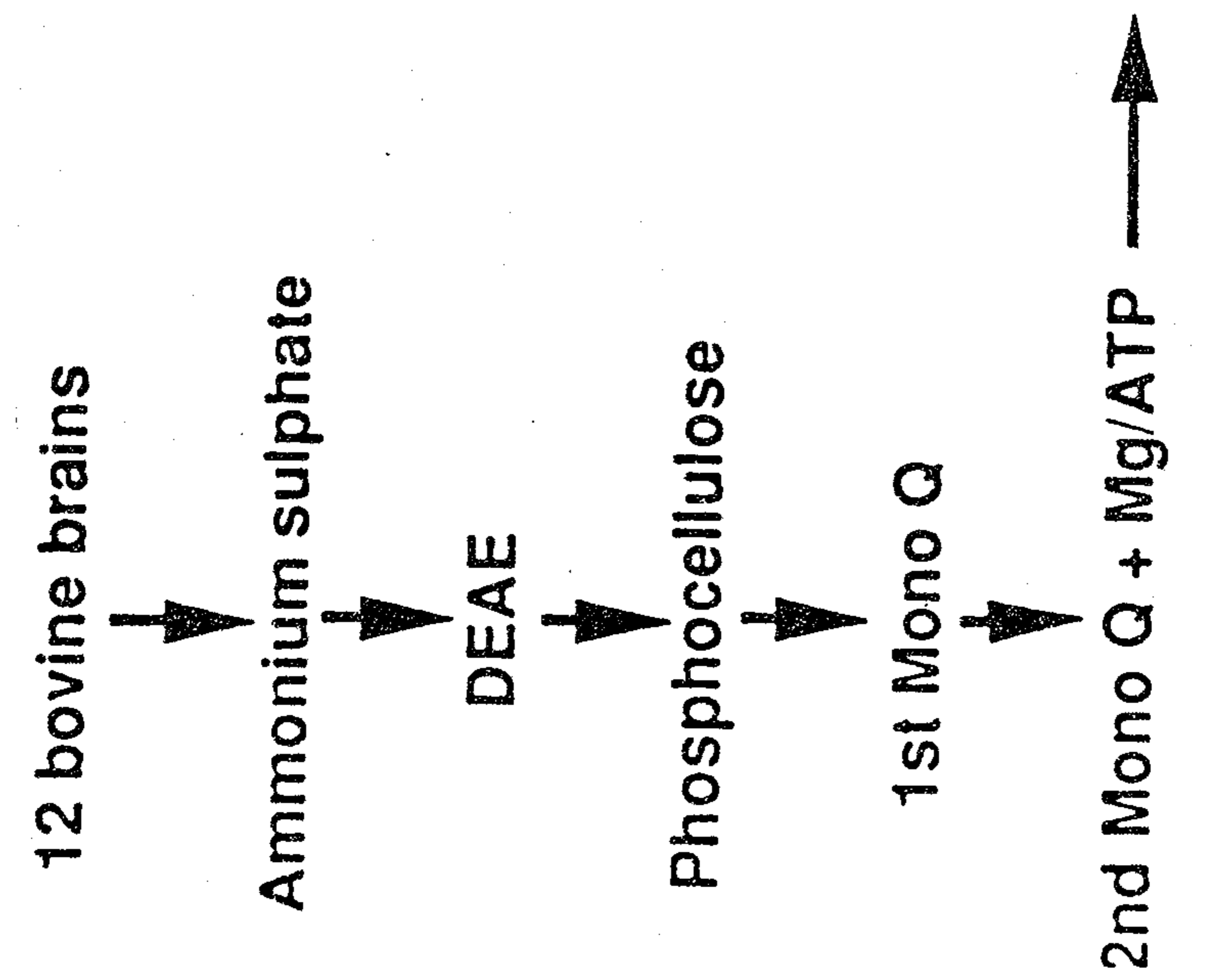
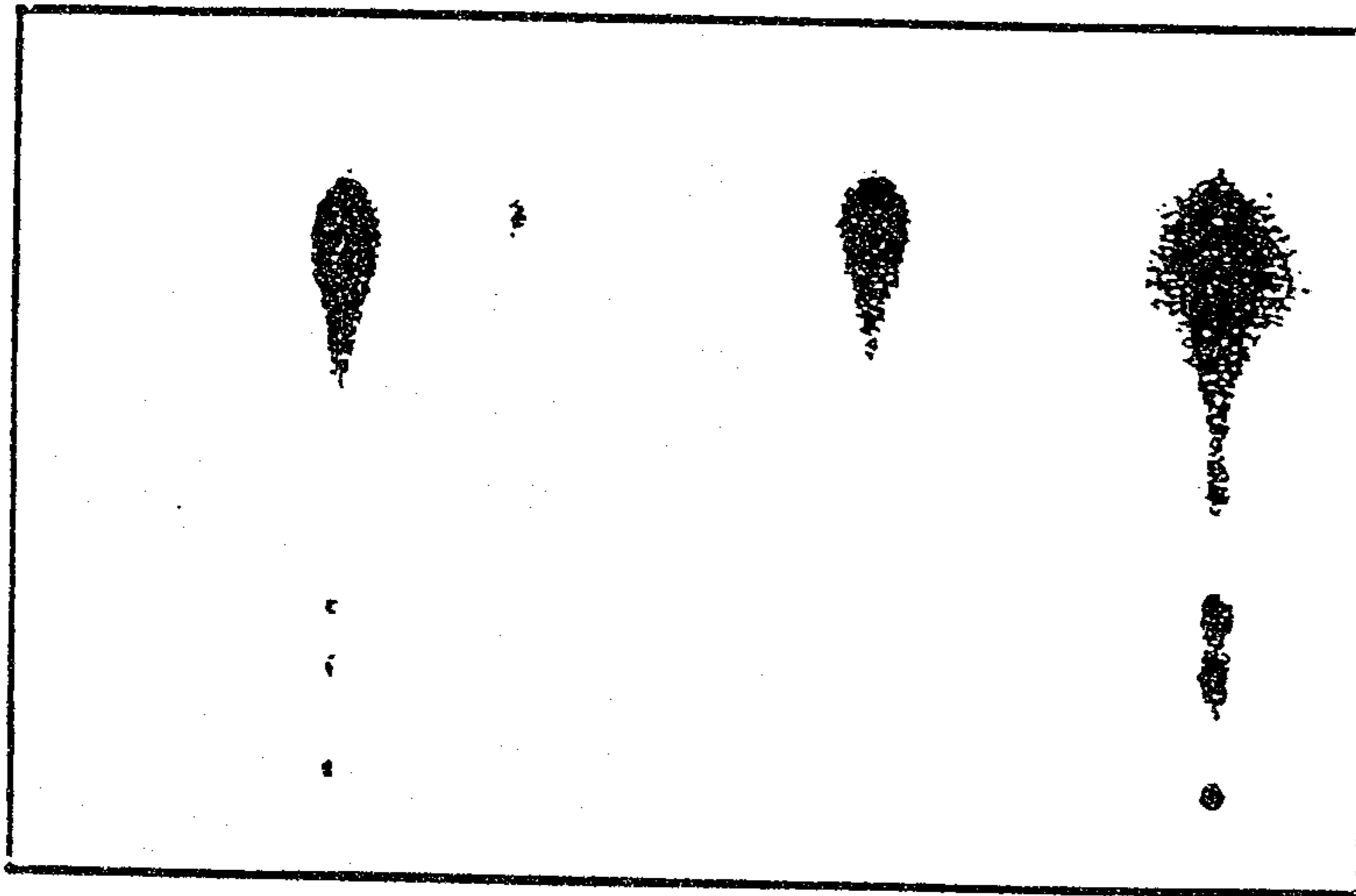


Figure 2

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1 2 3 4 5 6



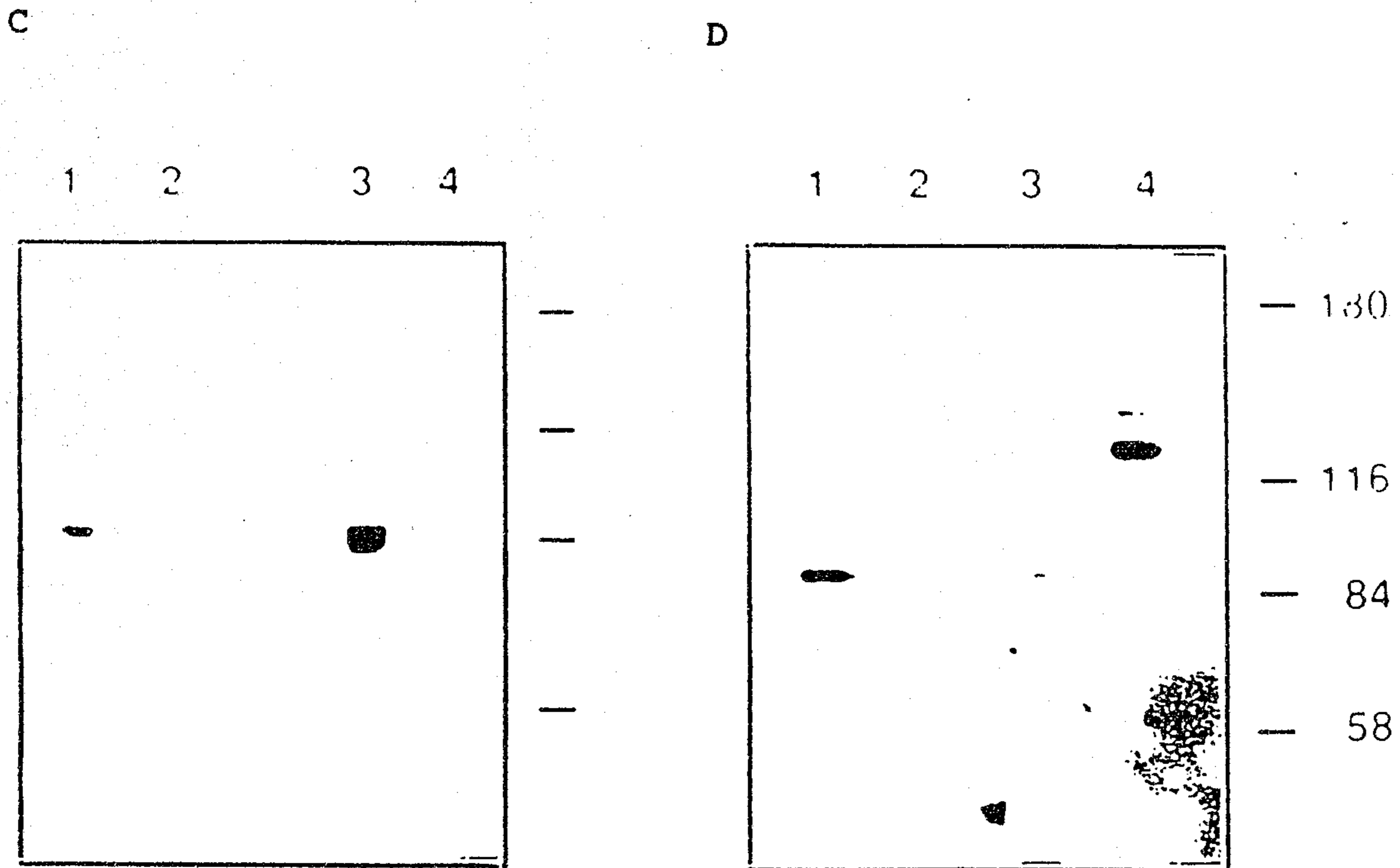
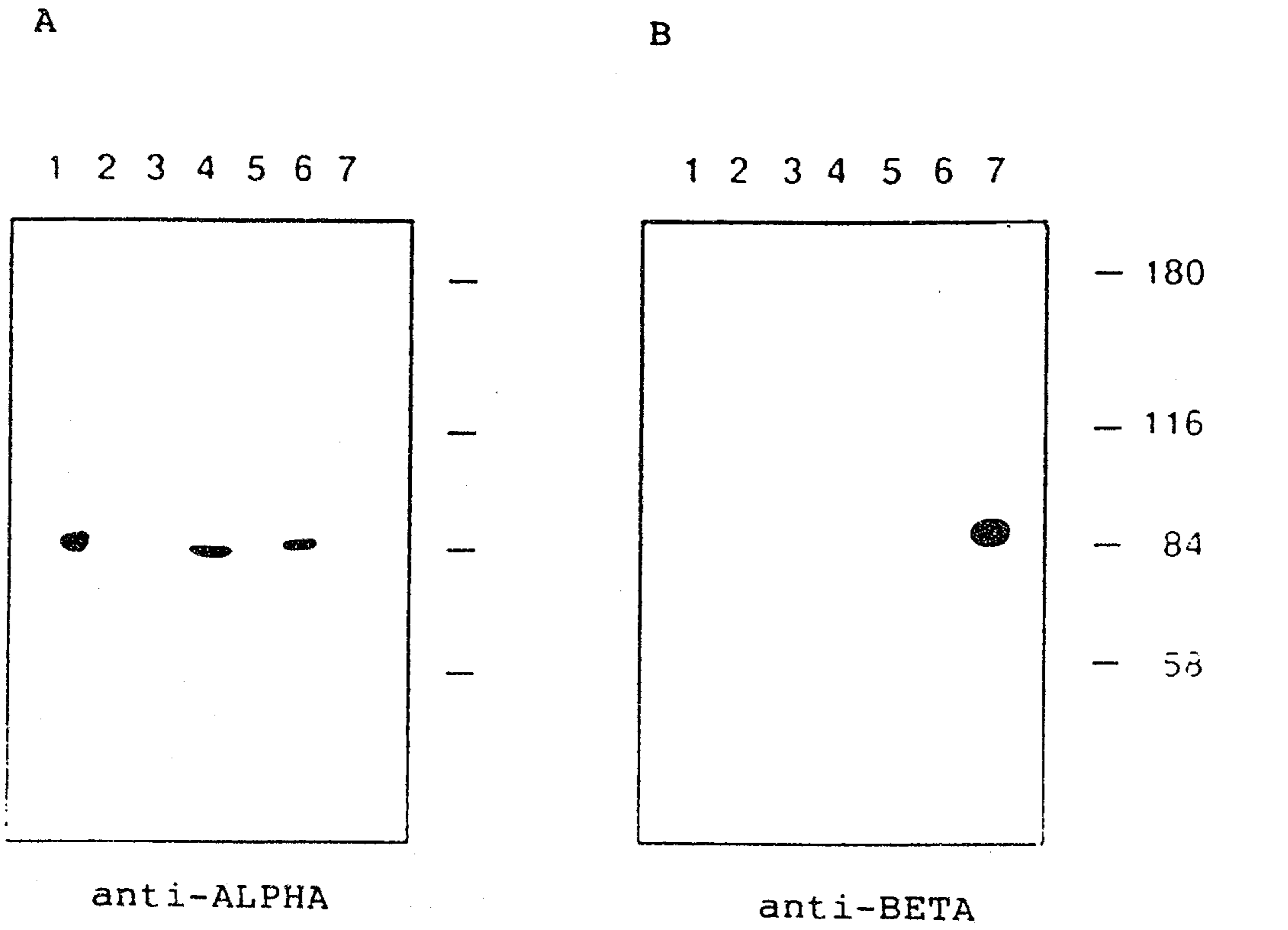
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Figure 3

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Figure 4



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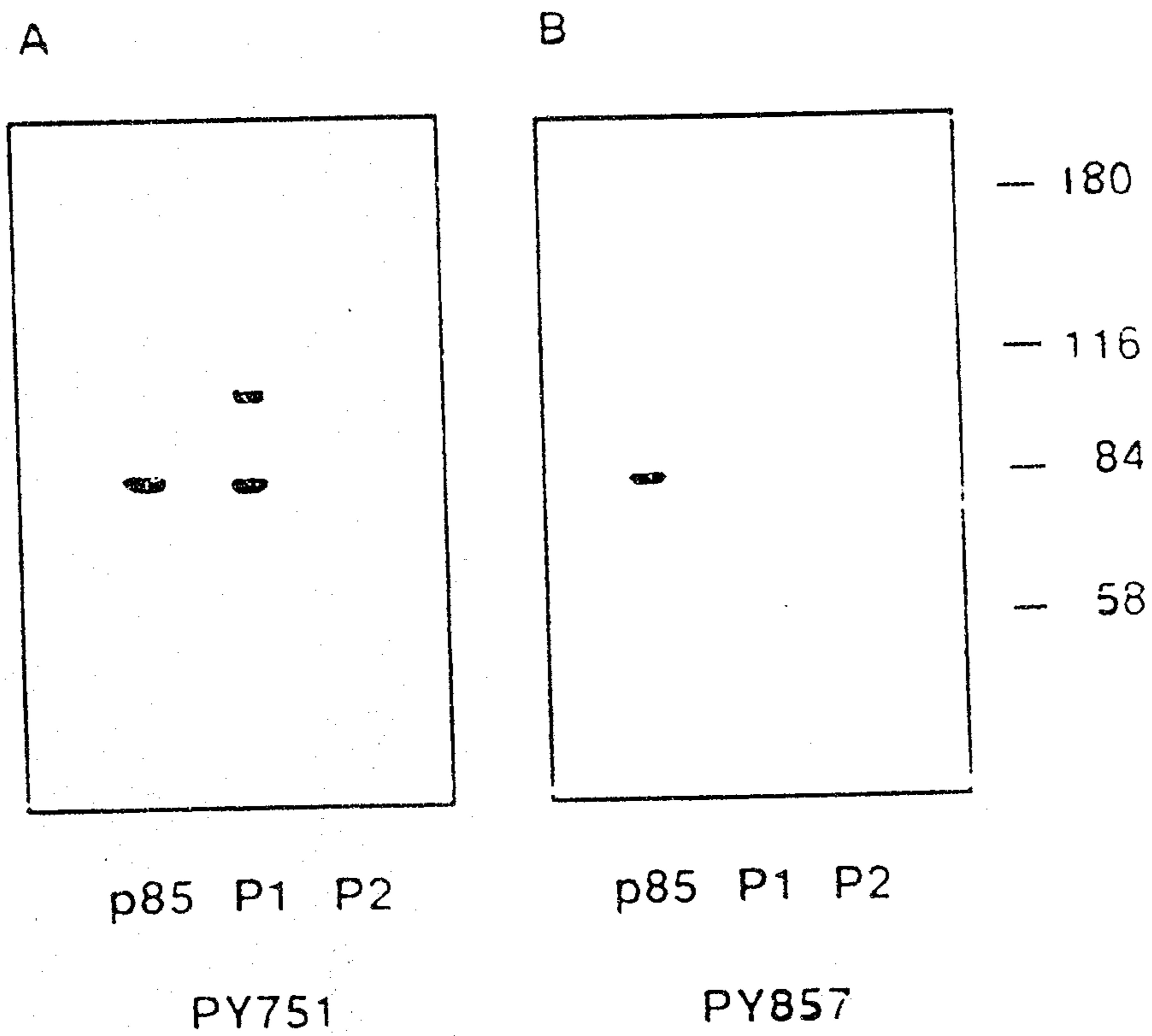


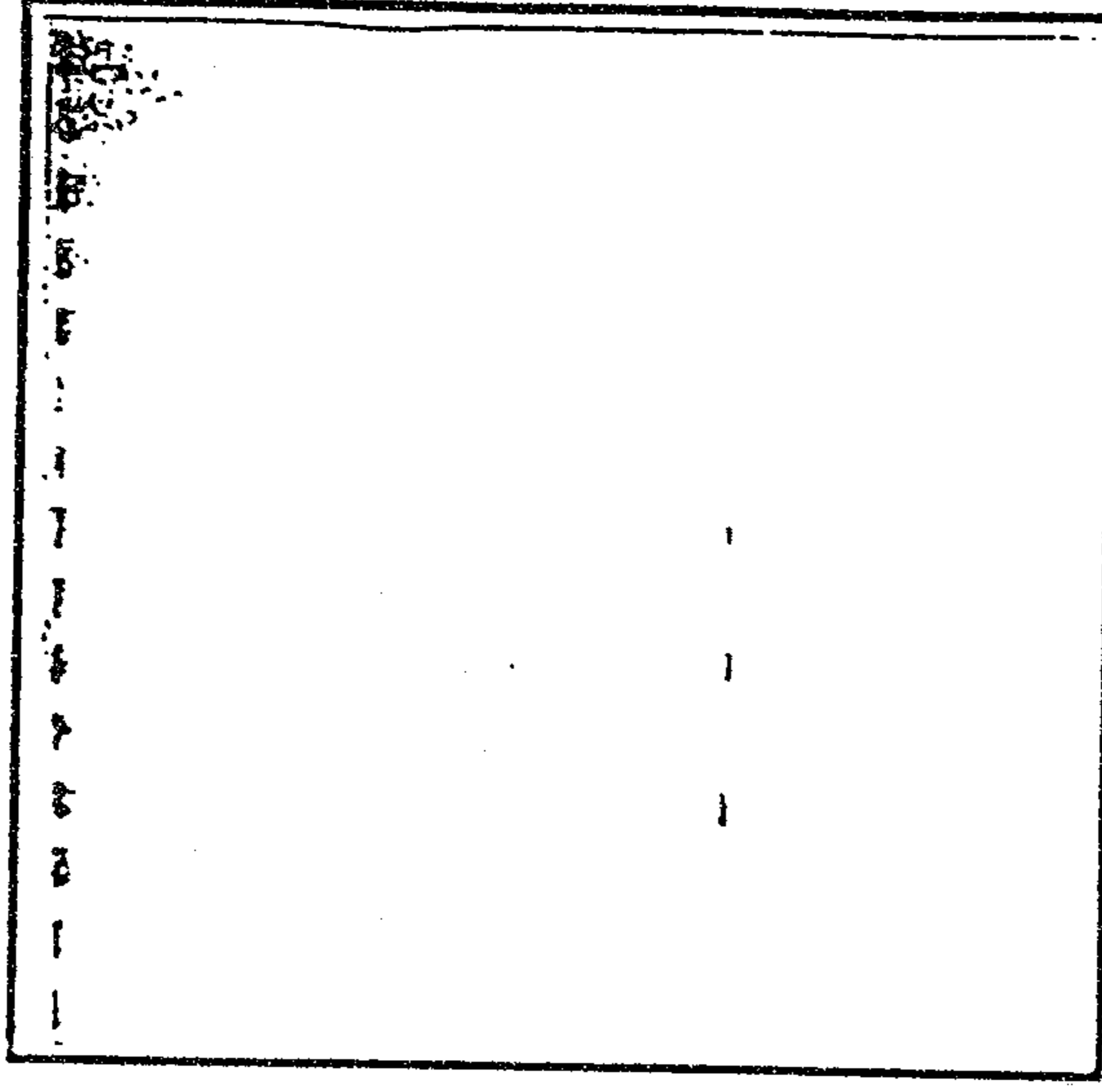
Figure 5

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—180  
—116  
—85  
—58

p85 alpha

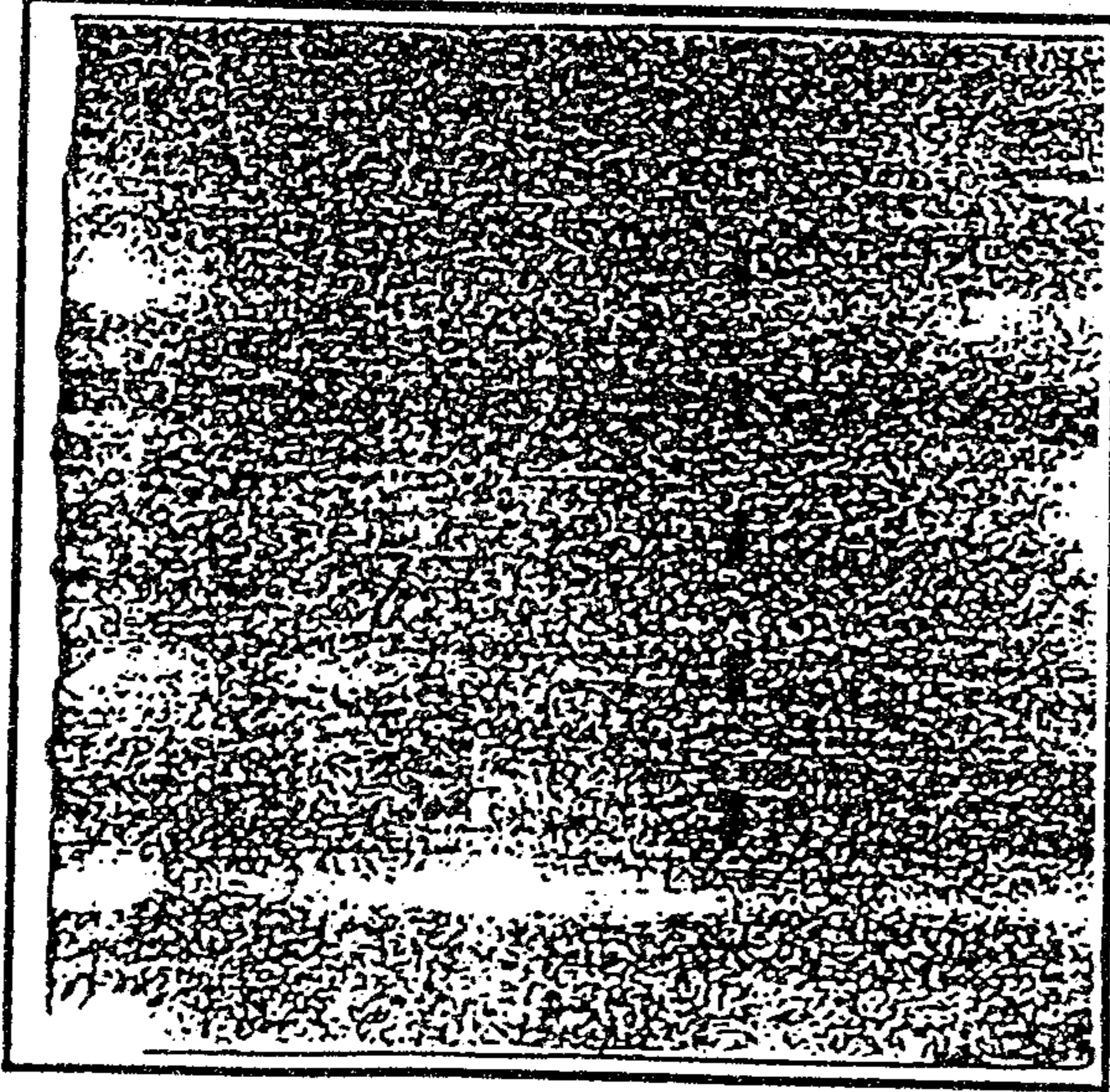


C O N  
Y 7 5 1  
Y 7 5 1 S  
Y 8 5 7  
pG A T  
Y 4 1 6  
Y 5 2 7

A

—180  
—116  
—85  
—58

p85 beta



C O N  
Y 7 5 1  
Y 7 5 1 S  
Y 8 5 7  
pG A T  
Y 4 1 6  
Y 5 2 7

B

Figure 6

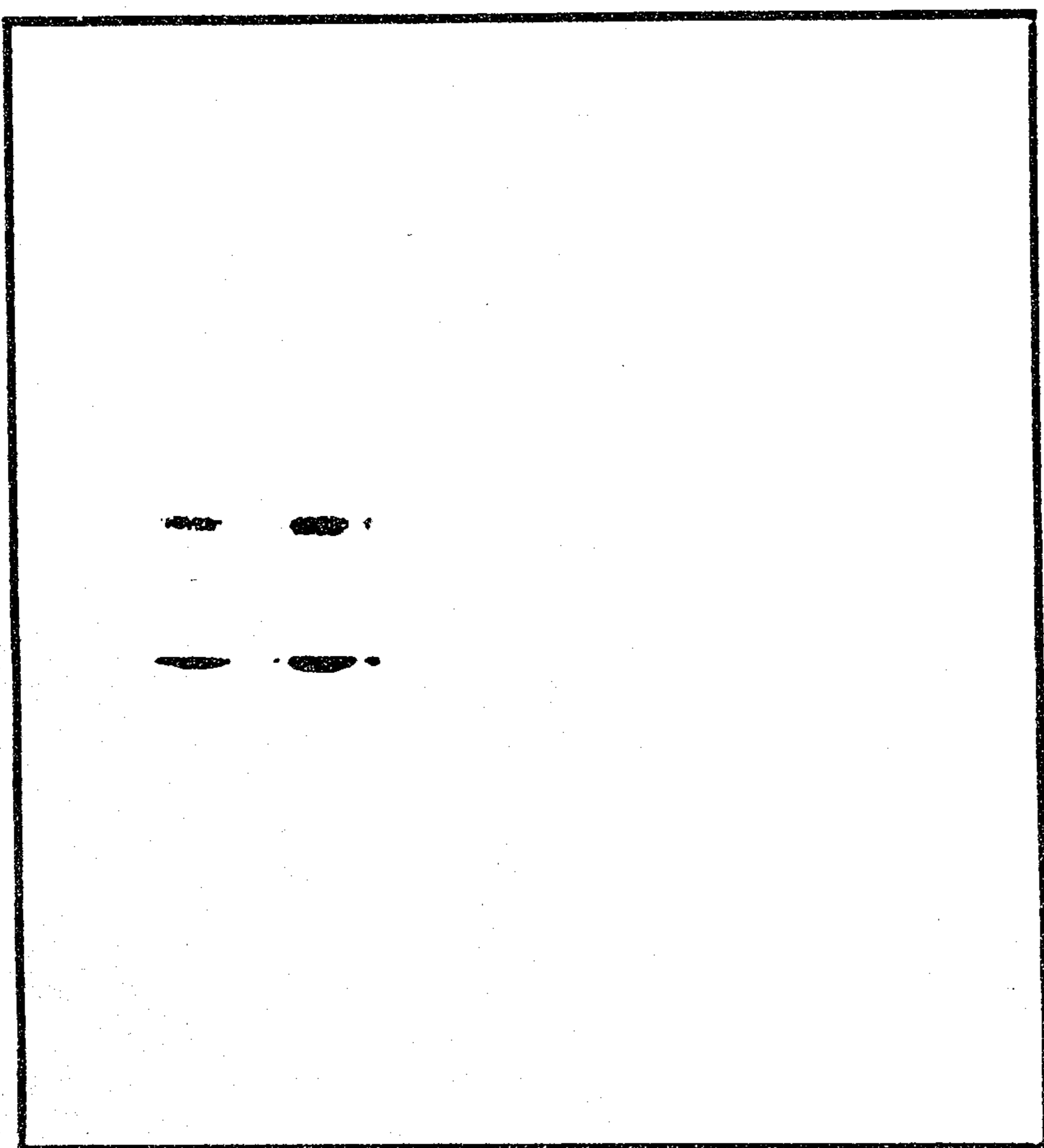


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Figure 7

A



— 180

— 116

— 85

— 58

C	Y	Y	Y	pG	Y	Y
O	7	7	8	A	4	5
N	5	5	5	T	1	2
	1	1	7		6	7
		S				

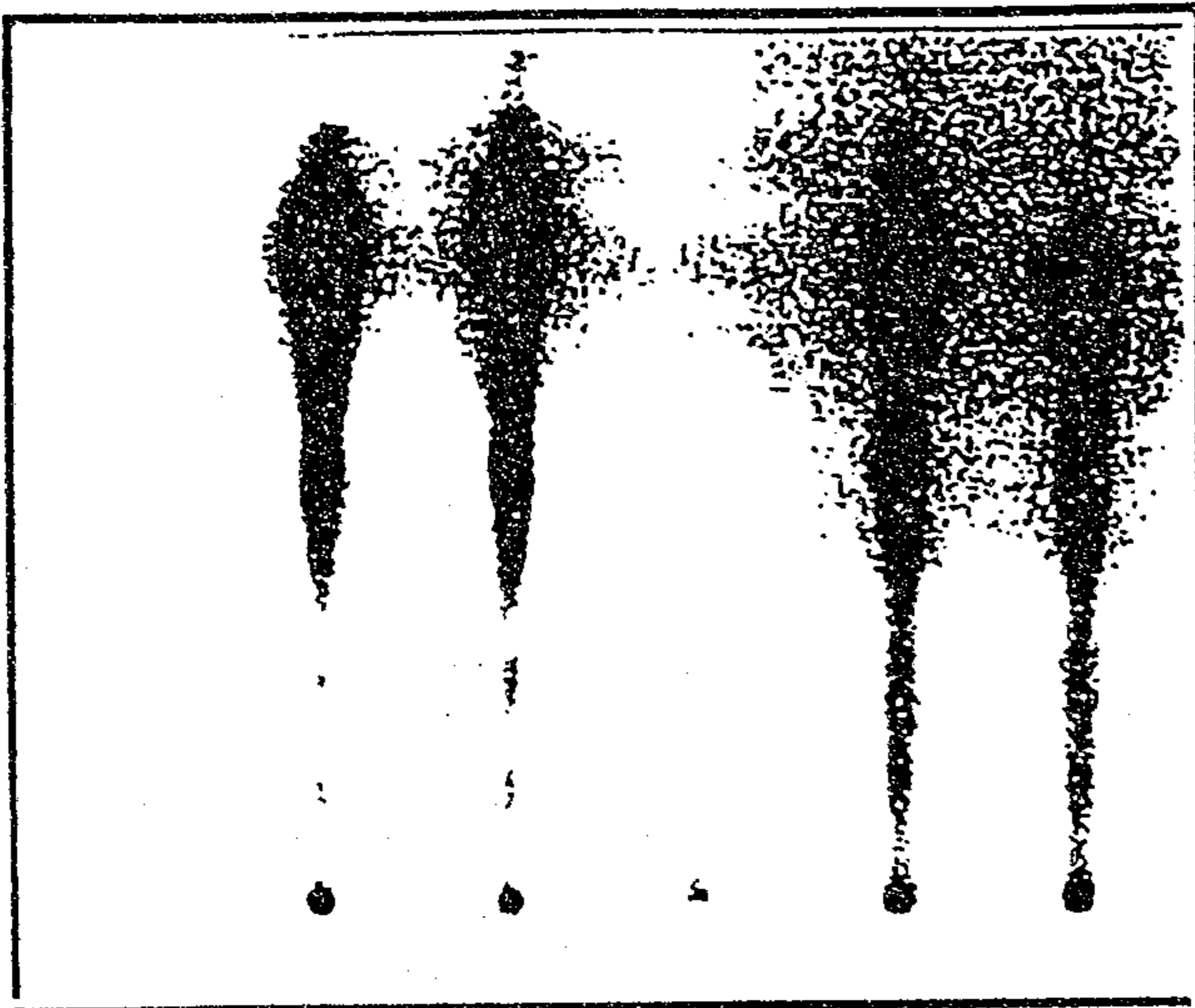


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Figure 8

1 2 3 4 5 6



< PIP

< Ori

751	D	M	S	K	D	E	S	V	D	Y	V	P	M	L	D	M	K
751 S				C	D	E	S	V	D	Y	V	P	M	L			
740				G	E	S	D	G	G	Y	M	D	M	S	K		
1313			E	F	C	P	D	P	L	Y	E	V	M	L	K		
Consensus					E	E	E	E	E	Y	M	P	M	X	X		
					D	D	D	D	D		V						

Figure 9

M P P R P S S G E L W G I H L M 16  
ATGCCCTCCAAGACCATCATCAGGTGAACCTGTGGGCATCCACTTGTATG 48  
  
P P R I L V E C L L P N G M I V 32  
CCCCAAGAATCCTAGTAGAATGTTTACTACCAAAATGGGATGATAGTG 96  
  
T L E C L R E A T L I T I K H E 48  
ACTTTAGAAATGCCCTCCGTGAGGCTACGTTAATAACGATAAAGCATGAA 144  
  
L F K E A R K Y P L H Q L L Q D 64  
CTATTTAAGAAGCAAGAAATAACCTCTCCATCAACTTCTTCAAGAT 192  
  
E S S Y I F V S V T Q E A E R E 80  
GAATCTTCTTACATTTTCGTAAGTGTACCCTCAAGAACGAAAGGGAA 240  
  
E F F D E T R R L C D L R L F Q 96  
GAATTTTGTGATGAAACAAGACGACTTTGTGACCTTCGGCTTTTTCAA 288  
  
P F L K V I E P V G N R E E K I 112  
CCCTTTTAAAGTAATTTGAACCAGTAGGCAACCGTGAAGAAAAGATC 336  
  
L N R E I G F A I G M P V C E F 128  
CTCAATCGAGAAATTTGGTTTGTGCTATCGGCATGCCAGTGTGTAATTC 384  
  
D M V K D P E V Q D F R R N I L 144  
GATATGGTTAAAGATCCAGAAGTACAGGACTTCCGAAGAAATATTCCTC 432

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N V C K E A V D L R D L N S P H  
AATGTTTGTAAGAAGCTGTGGATCTTAGGGATCTTAATTCACCTCAT

160  
480

A

S R A M Y V Y P P N V E S S P E  
AGTAGAGCAATGTATGTTTATCTCCAAATGTAGAATCTTCACCAGAA

176  
528

L P K H I Y N K L D K G Q I I V  
CTGCCAAAGCACATATAATAAATGGATAAAGGGCCAATAATAGTG

192  
576

V I W V I V S P N N D K Q K Y T  
GIGATTTGGTAATAGTTTCTCCAAATAATGACAAACAGAAAGTATACT

208  
624

L K I N H D C V P E Q V I A E A  
CTGAAATCAACCATGACTGTGTGCCAGAACAAAGTAATTTGCTGAAGCA

224  
672

I R K K T R S M L L S S E Q L K  
ATCAGGAAAAAACTCGAAGTATGTTGCTATCATCTGAACAACATAAA

240  
720

L C V L E Y Q G K Y I L K V C G  
CTCTGTGTTTAGAATATCAGGGCAAGTATATTTTAAAGTGTGTGGA

256  
768

C D E Y F L E K Y P L S Q Y K Y  
TGTGATGAATACTTCTAGAAAATAATCCTCTGAGTCAGTATAAGTAT

272  
816

I R S C I M L G R M P N L M L M  
ATAAGAAGCTGTATAATGCTTTGGAGGATGCCCAATTTGATGCTGATG

288  
864

FIG 9 (contd)

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304 A K E S L Y S Q L P M D C F T M  
912 GCTAAGAAAGCCCTCTATTCTCAACTGCCAATGGACTGTTTACAAATG

320 P S Y S R R I S T A T P Y M N G  
960 CCAATCATATCCAGACGCATCTCCACAGCTACGCCATATATAATGAATGGA

B

336 E T S T K S L W V I N S A L R I  
1008 GAAACATCTACAATAATCCCTTIGGGTTATAAATAGTGCACCTCAGAATA

352 K I L C A T Y V N V N I R D I D  
1056 AAAATTCTTIGIGCAACCTATGTGAATGTAATAATATTCGAGACATTTGAC

368 K I Y V R T G I Y H G G E P L C  
1104 AAGATTATGTTCGAACAGGTATCTACCATGGAGGAGAACCCCTTATGT

384 D N V N T Q R V P C S N P R W N  
1152 GATAATGTGAACACTCAAAGAGIACCTTGTTCCTTCCAAATCCAGGTGGAAT

400 E W L N Y D I Y I P D L P R A A  
1200 GAATGGCTGAATFACGATATAATACATTCCTGATCTTCCTCGTCTGCT

416 R L C L S I C S V K G R K G A K  
1248 CGACTTIGCCCTTCCATTGTCTGTAAAGGCCGAAAGGGTGTAA

432 E E H C P L A W G N I N L F D Y  
1296 GAGGAACACTGTCCATTGGCCCTGGGAAATATAAATCTTGTGATTAC

FIG 9 (contd)

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448  
1344

T D T L V S G K M A L N L W P V  
ACAGATACTCTAGTATCTGGAAAATGGCTTTTGAATCTTTGGCCAGTAA

464  
1392

P H G L E D L L N P I G V T G S  
CCTCATGGACTAGAAAGATTTGCTGAAACCCCTATTTGGTGTACTGGATCA

480  
1440

N P N K E T P C L E L E F D W F  
AATCCAAATAAAGAAACTCCATGTTTAGAGTTGGAGTTTGACTGGTTC

496  
1488

S S V V K F P D M S V I E E H A  
AGCAGTGGTAAAGTTTCCAGATATGTCAGTGTGATTTGAAGAGCATGCC

512  
1536

N W S V S R E A G F S Y S H A G  
AATTGGTCTGTATCCCGTGAAGCAGGATTTAGTTATTTCCCATGCAGGA

528  
1584

L S N R L A R D N E L R E N D K  
CTGAGTAACAGACTAGCTAGAGACAATGAATTAAGAGAAAATGATAAA

544  
1632

E Q L R A I C T R D P L S E I T  
GAACAGCTCCGAGCAATTTGTACACGAGATCCCTCTATCTGAAATCACT

560  
1680

E Q E K D F L W S H R H Y C V T  
GAGCAAGAGAAAGATTTTCTGTGGAGCCACAGACACTATTGTGTAACT

576  
1728

I P E I L P K L L L S V K W N S  
ATCCCCGAAATTTCTACCCAAATTTGCTTCTGTCTGTAAATGGAACTCT

FIG 9 (contd)

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FIG 9 (contd)

R D E V A Q M Y C L V K D W P P 592  
AGAGATGAAGTAGCTCAGATGTACTGCTTGGTAAAGATTTGGCCCTCCA 1776

I K P E Q A M E L L D C N Y P D 608  
ATCAAGCCCTGAACAGGCTATGGAGCTTCTGGACTGCAATTTACCCAGAT 1824

P M V R G F A V R C L E K Y L T 624  
CCTATGGTTCGAGGTTTTCCTGTTGGTCTTAGAAAATAATTTAACA 1872

*D*

D D K L S O Y L I O L V O V L K 640  
GATGACAAACTTTCAGTACCCTAATTCAGCTAGTACAGTACTAAAA 1920

Y E O Y L D N L L V R F L L K K 656  
TATGAACAGTATTTGGATAACCTGCTTGTGAGATTTTACTCAAAAA 1968

*E*

A L T N O R I G H F F F W H L K 672  
GCGTTAACTAATCAAGGATCGGTCACCTTTTCTTTGGCATTTAAAA 2016

*F*

S E M H N K T V S O R F G L L L 688  
TCTGAGATGCACAATAAACAGTTAGTACAGAGGTTTGGCCCTGCTTTTG 2064

E S Y C R A C G M Y L K H L N R 704  
GAGTCCCTATTGCCGTCATGTGGATGTATCTGAAGCACCTTTAATAGG 2112

*G*

Q V E A M E K L I N L T D I L K 720  
CAAGTTGAGGCTATGGAAAAGCTCATTTAACTTGACTGACATTTCTCAAA 2160



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FIG 9 (contd) Q E K K D E T Q K V Q M K F L V 736  
 CAAGAGAAGGATGAAACACAAAGGTACAGATGAAGTITTTAGTT 2208  
  
 E Q M R R P D F M D A L Q G F L 752  
 GAGCAAATGCGGACCAGATTTCATGGATGCTCTCCAGGGCTTTCIG 2256  
  
 S P L N P A H Q L G N L R L E E 768  
 TCTCCTTAACCCCTGCTCATCAGCTGGGAATCTCAGGCTTGAAGAG 2304  
  
 C R I M S S A K R P L W L N W E 784  
 TGTCGAATTATGTCITCTGCAAAAGGCCACTGTGGTTGAATTTGGGAG 2352  
  
 N P D I M S E L L F Q N N E I I 800  
 AACCCAGACATCATGTCAGAATTACTCTTTCAGAACAAATGAGATCATC 2400  
  
 F K N G D D L R Q D M L T L Q I 816  
 TTTAAAATGGGGATGATTTACGGCAAGATAATGCTAACCCCTTCAGATT 2448  
  
 I R I M E N I W Q N Q G L D L R 832  
 ATTCCGATTATGGAAAATATCTGGCAAATCAAGGTCITTGATCTTCGA 2496  
  
 M L P Y G C L S I G D C V G L I 848  
 ATGTTACCTTATGGATGTCITGCAATCGGTGACTGTGTGGACTTATC 2544  
  
 E V V R N S H T I M Q I Q C K G 864  
 GAGGTGGAGAAAATCTCACACTATAATGCAGATTTCAGTAAAGGA 2592

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H

G L K G A L O F N S H T L H O W 880  
GGCCTGAAAGGTGCACCTGCAGTTTAAACAGCCACACACTCCATCAGTGG 2640

L K D K N K G E I Y D A A I D L 896  
CTCAAAGACAAGAACAGGGGAATATATGATGCGGCCATCGATTGG 2688

I

F T R S C A G Y C V A T F I L G 912  
TTTACACGATCATGTGCTGGATAATTTGTTGCCACCTTCATTTTGGGA 2736

I G D R H N S N I M V K D D G O 928  
ATTGGAGATCGTCACAATAGTAATATCATGTTTAAAGATGATGGACAA 2784

J

L F H I D F G H F L D H K K K K 944  
CTGTTTCATATAGATTTTGGACACTTTTGGATCACAAGAGAAAAA 2832

K

F G Y K R E R V P F V L T Q D F 960  
TTTGGTTATAAACGAGAGCGCGTCCGTTTGTGTTTGACACAAGATTTC 2880

L I V I S K G A Q E C T K T R E 976  
TTAATAGTGATTAGTAAGGAGCCCAAGAATGCACAAGACAAGAGAA 2928

F E R F Q E M C Y K A Y L A I R 992  
TTTGAGAGGTTTCAGGAGATGTGTTACAAGGCTTATCTAGCTATTCGG 2976

FIG 9 (contd)

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L

Q H A N L F I N L F S M L G S 1008  
CAGCATGCCAATCTTCATATAAATCTTTCTCAATGATGCTTGGCTCT 3024

G M P E L Q S F D D I A Y I R K 1024  
GGAATGCCAGAACTGCAATCTTTGATGATATTTGCATACATTCGAAAG 3072

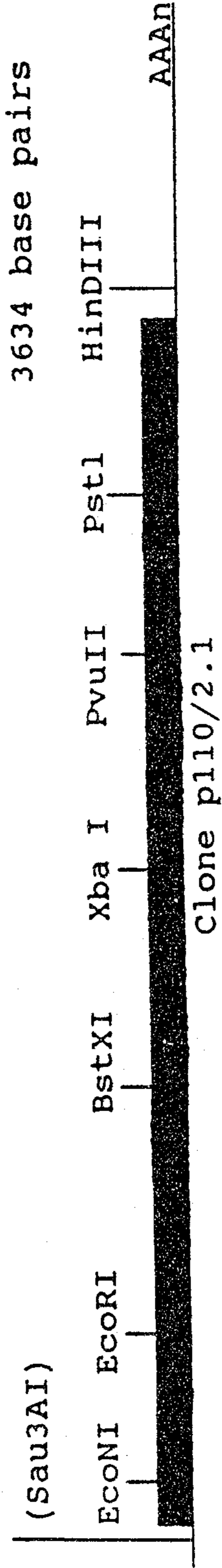
M

T L A L D K T E O E A L E Y F M 1040  
ACCTAGCTTTAGATAAAACTGAGCAAGAGGCTTTGGAGTATTTCATG 3120

K Q M N D A H H G G W T T K M D 1056  
AAACAAATGAATGATGCACACCATGGTGGCTGGACAAACAATAATGGAT 3168

N

W I F H T I K Q H A L N \* 1069  
TGGATCTTCCACACAATTAAGCAGCATGCTTTGAAC TGA 3207



\_\_\_\_ Race Product \_\_\_\_\_  
A \_\_\_\_\_  
B \_\_\_\_\_

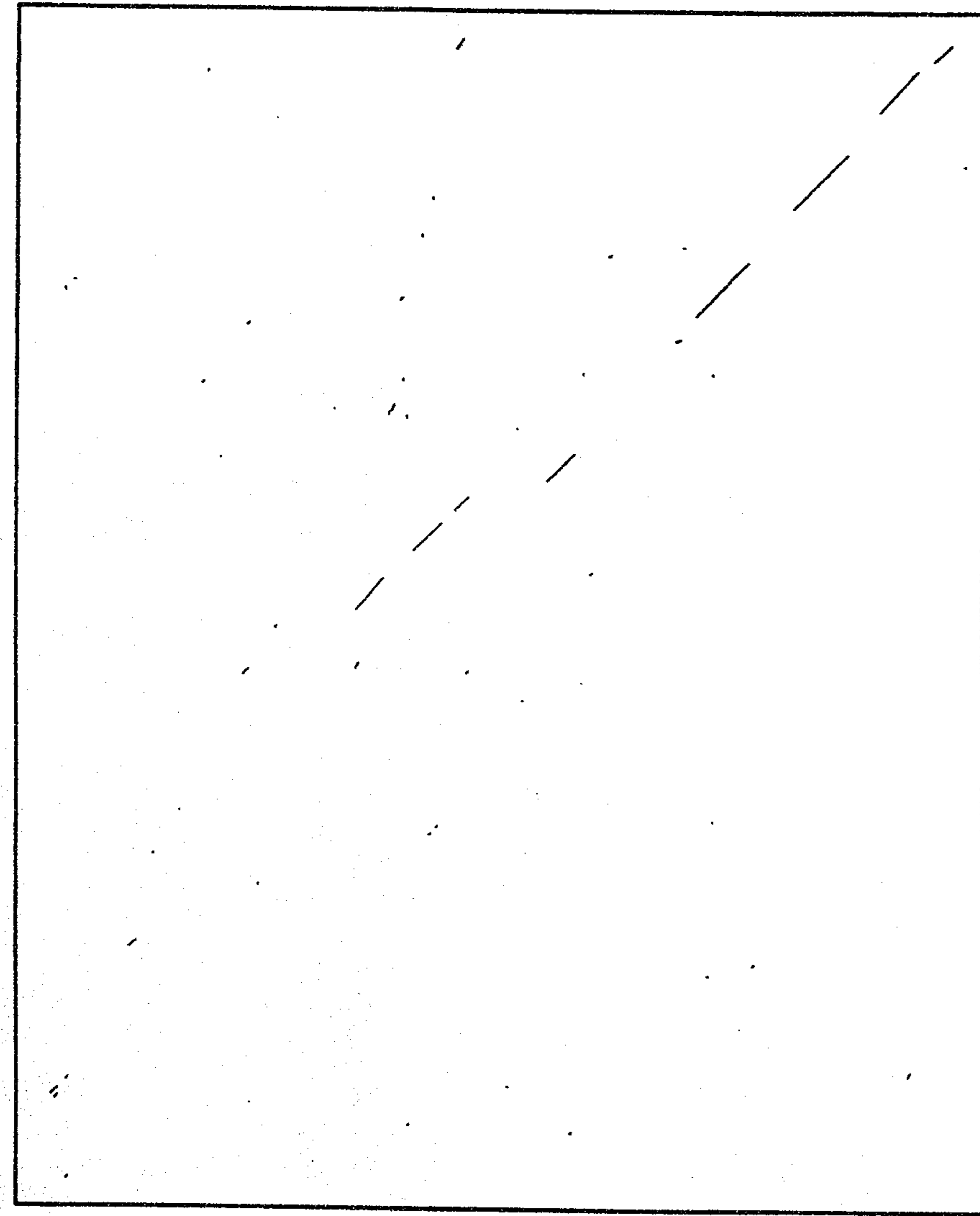
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1068

P110

1



1

VPS34

875

Figure 10 A











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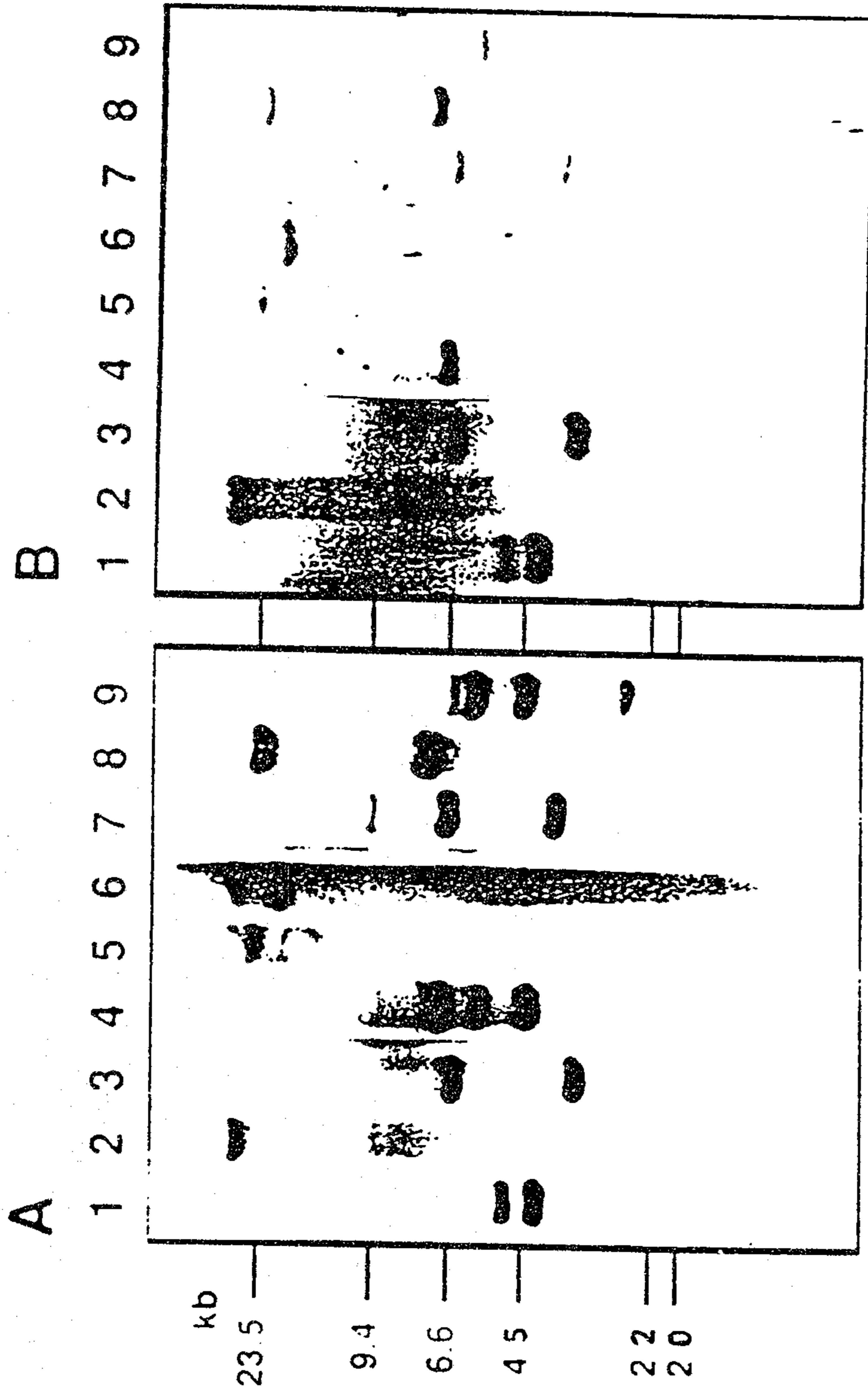


Figure 11

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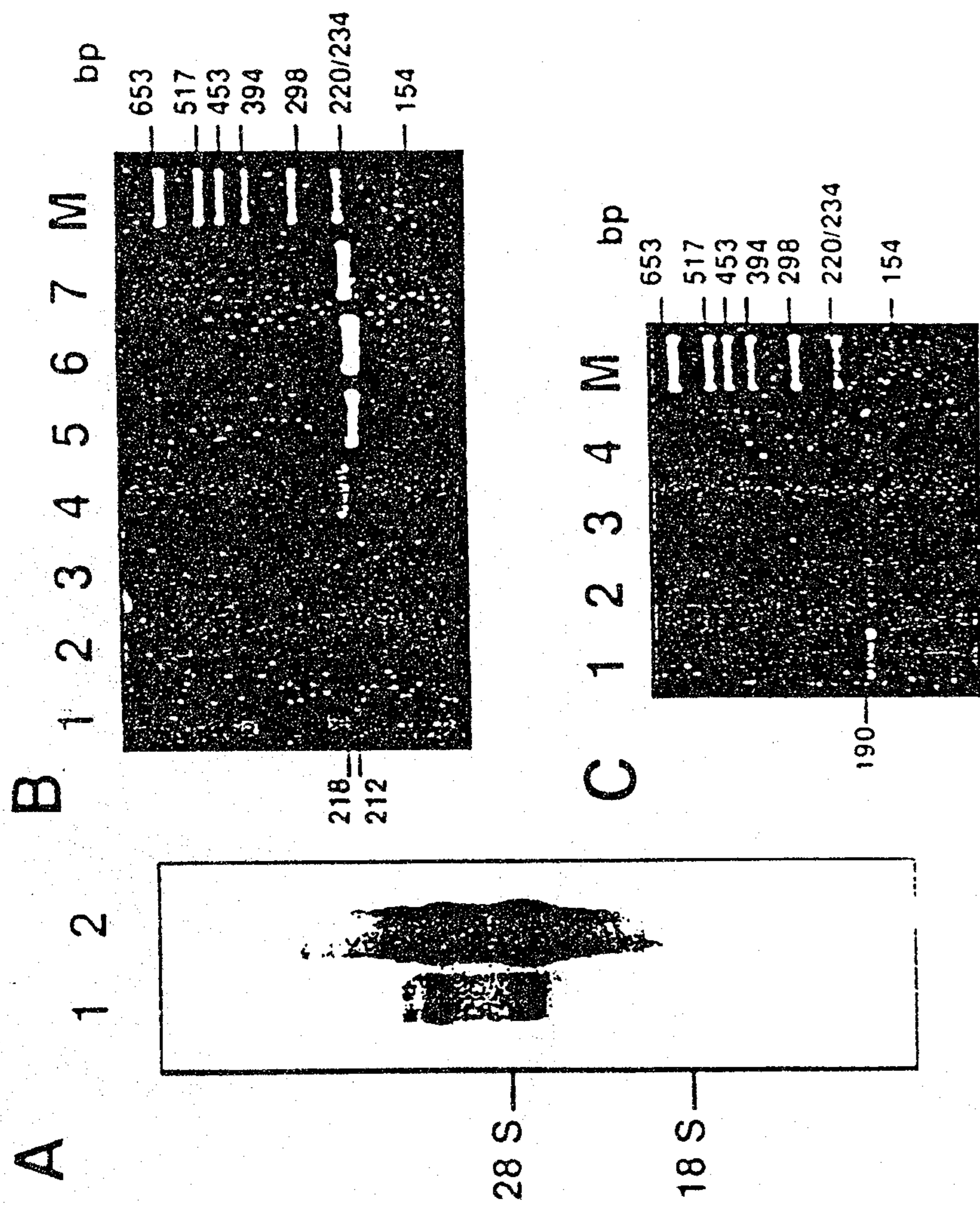


Figure 12

211205

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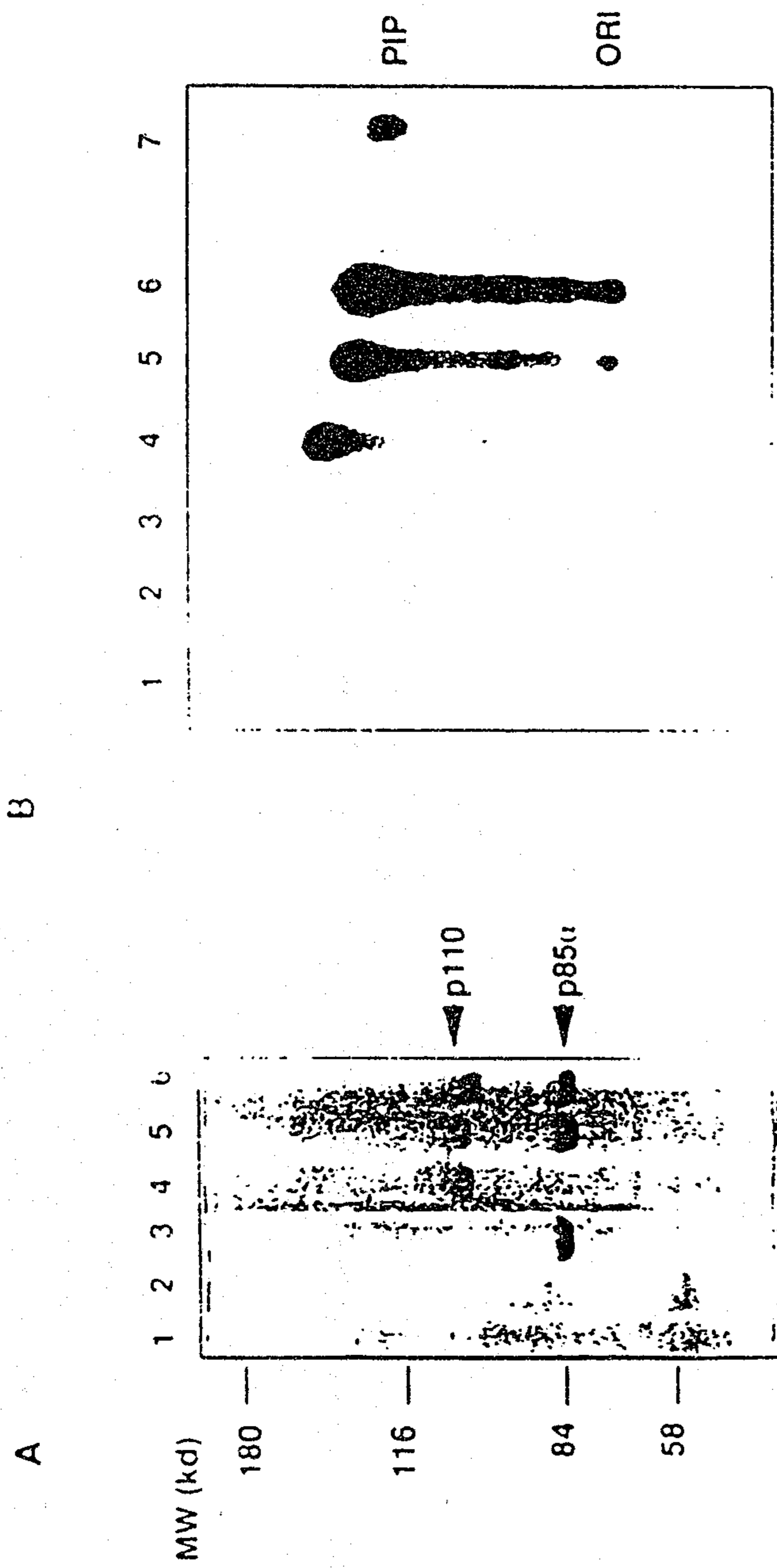


Figure 13

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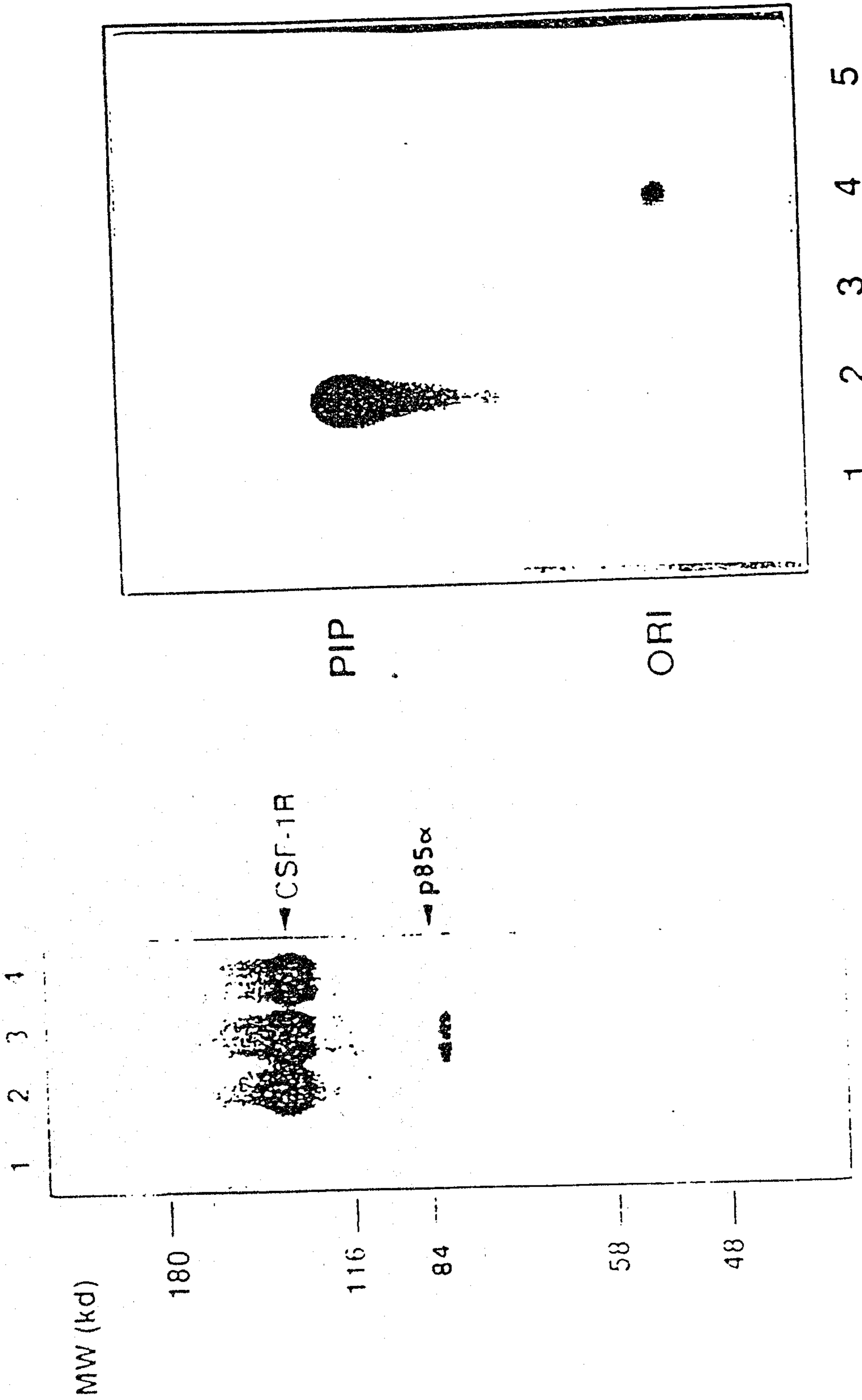
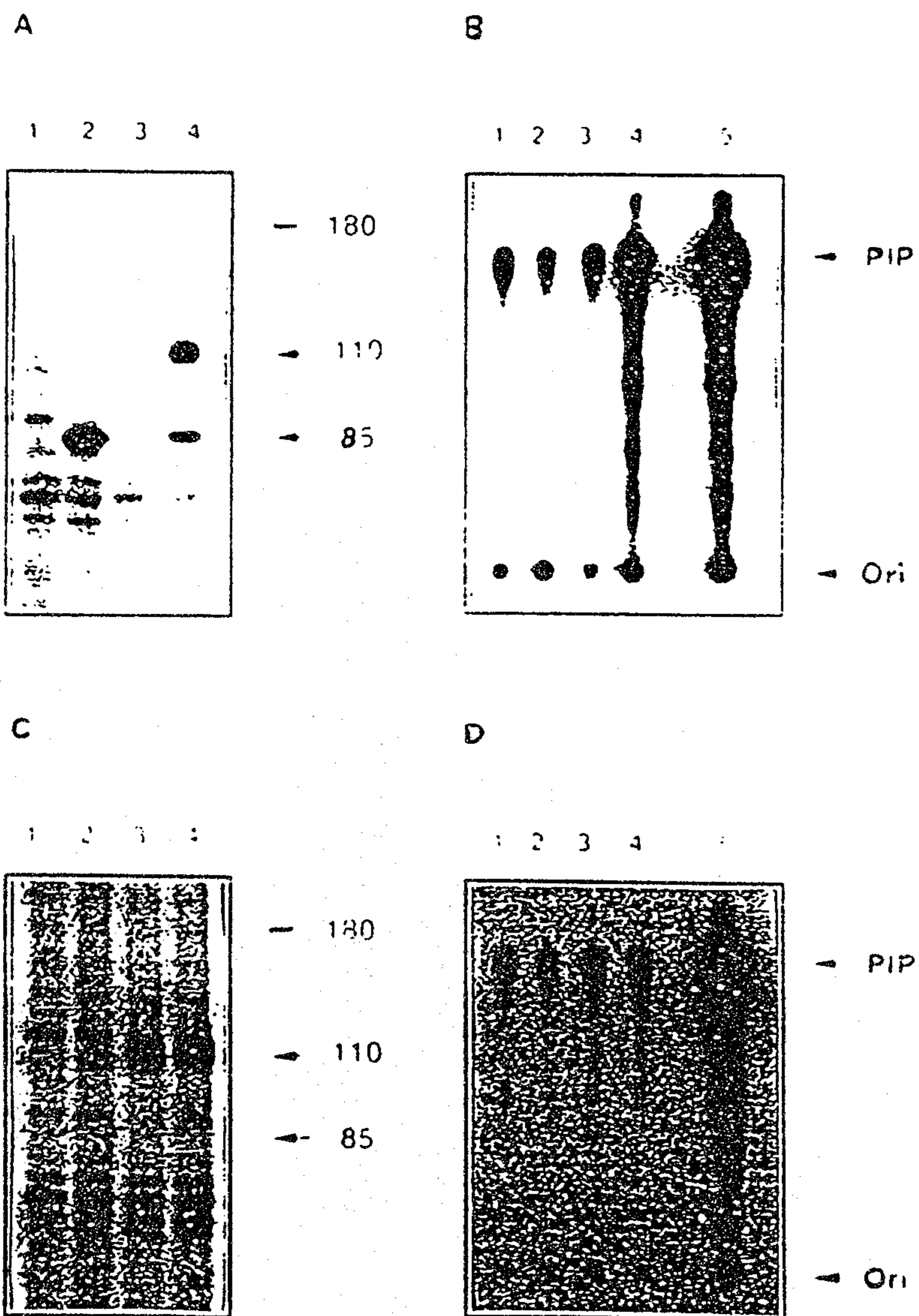


Figure 14

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Figure 15



The human p110 cDNA nucleotide and deduced amino acid sequence.

1 ATGCCCTCCAAGACCATCATCAGGTGAACCTGTGGGCATCCACTTGATG 48

-----+-----+-----+-----+-----+-----+-----  
TACGGAGGTTCTGGTAGTAGTCCACTTGACACCCCGTAGGTGAACCTAC  
M P P R P S S G E L W G I H L M

49 CCCCCAAGAAATCCTAGTGGAAATGTTTACTACCAAATGGAATGATAGTG 96

-----+-----+-----+-----+-----+-----+-----  
GGGGTCTTAGGATCACCTTACAATGATGGTTTACCTTACTATCAC  
P P R I L V E C L L P N G M I V

97 ACTTTAGAAATGCCCTCCGTGAGGCTACATTAAGTAACTATAAAGCATGAA 144

-----+-----+-----+-----+-----+-----+-----  
TGAAATCTTACGGAGGCACCTCCGATGTAATCATGATAATTTCGTACTT  
T L E C L R E A T L V T I K H E

145 CTATTTAAAGCAAGAAATAACCCCTCCTCCATCAACTTCTTCAAGAT 192

-----+-----+-----+-----+-----+-----+-----  
GATAAATTCTTCGTTCTTTATGGGAGAGGTAGTTGAAGAAGTTCTA  
L F K E A R K Y P L H Q L L Q D

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Figure 16

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193 GAATCTTCTTACATTTTCGTAAGTGTACCCAAGAAGCAGAAAGGGAA 240  
 -----+-----+-----+-----+-----+-----+-----+  
 CTTAGAAGATGTAAAGCATTCACAATGGGTTCTTCGTCTTCCCTT  
 E S Y I F V S V T Q E A E R E

241 GAATTTTGTGATGAAACAAGACGACTTGTGATCTTCGGCTTTTCAA 288  
 -----+-----+-----+-----+-----+-----+-----+  
 CTTAAAACACTACTTGTCTGCTGAAACACTAGAACCCGAAAAGTT  
 E F D E T R R L C D L R L F Q

289 CCATTTTAAAAGTAATTGAACCAGTAGGCCAACCGTGAAGAAAAGATC 336  
 -+-----+-----+-----+-----+-----+-----+-----+  
 GGTA AAAATTTTCACTTGGTCA TCCGTTGGCACTTCTTTCTAG  
 P F L K V I E P V G N R E E K I

337 CTC AATCGAGAAATTGGTTTGTGCTATCGGCATGCCAGTGCGGAATTT 384  
 ---+-----+-----+-----+-----+-----+-----+-----+  
 GAGTTAGCTTTAACC A AAACGATAGCCGTACGGTCACACGCTTAAA  
 L N R E I G F A I G M P V C E F

FIG 16 (contd)





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577 GTGATTTGGGTAATAGTTTCTCCAAATAATGACAAGCAGAAGTATACT  
 ---+-----+-----+-----+-----+-----+-----+-----+  
 CACTAAACCCATTATCAAAAGAGGTTTATTACTGTTCGTTTCATATGA  
 V I W V I V S P N N D K Q K Y T

624

625 CTGAAATCAACCATGACTGTGTGCCAGAACAAGTAATTGCTGAAGCA  
 ---+-----+-----+-----+-----+-----+-----+-----+  
 GACTTTAGTTGGTACTGACACACGGTCTTGTTCATTAACGACTTCGT  
 L K I N H D C V P E Q V I A E A

672

673 ATCAGGAAAAAATAAGTATGTGCTATCATCTGAACAATTAATA  
 ---+-----+-----+-----+-----+-----+-----+-----+  
 TAGTCCTTTTGGATCTTCAACAACGATAGTACTTGTAAATTT  
 I R K K T R S M L L S S E Q L K

720

721 CTCGTGTTTAGAATAATCAGGGCAAGTACATTTTAAAAAGTGTGTGGA  
 ---+-----+-----+-----+-----+-----+-----+-----+  
 GAGACAAAAATCTTATAGTCCCCTTCATGTAAAATTTTCACACACCT  
 L C V L E Y Q G K Y I L K V C G

768

FIG 16 (contd)

111995

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769 TGATGAACTTCTAGAAAAATATCCTCTGAGTCAGTATAAGTAT 816  
 -+-----+-----+-----+-----+-----+-----+-----+-----+  
 AACTACTTATGAAGGATCTTTTATAGGAGACTCAGTCATATTCATA  
 C D E Y F L E K Y P L S Q Y K Y

817 ATAAGAAGCTGATAATGCTTGGGAGGATGCCCAATTGAAGATGATG 864  
 ---+-----+-----+-----+-----+-----+-----+-----+-----+  
 TATTCTTCGACATATTACGAACCCCTCCTACGGTTAAACTTCTACTAC  
 I R S C I M L G R M P N L K M M

865 GCTAAAGAAAGCCTTTATTCTCAACTGCCAATGGACTGTTTACAATG 912  
 ---+-----+-----+-----+-----+-----+-----+-----+-----+  
 CGATTTCTTCGGAAATAAGAGTTGACGGTTACCTGACAAAATGTTAC  
 A K E S L Y S Q L P M D C F T M

913 CCATCTTATCCAGACGCATTTCCACAGCTACACCATAATGAATGGA 960  
 ---+-----+-----+-----+-----+-----+-----+-----+-----+  
 GGTAGAATAAGGCTCGGTAAGGTGTCGATGTGGTATATACTTACCT  
 P S Y S R R I S T A T P Y M N G

FIG 16 (contd)



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1153 GAATGGCTGAATATGATATATACATTCCTGATCCTCCTCGTGCTGCT  
 -----+-----+-----+-----+-----+-----+-----+  
 1200 CTTACCGACTTAATACTATAATGTAAGGACTAGAGGAGCAGCAGCGA  
 E W L N Y D I Y I P D L P R A A  
  
 1201 CGACTTTGCCCTTCCATTTGCTCTGTTAAAGGCCGAAAGGGTGCTAAA  
 -----+-----+-----+-----+-----+-----+-----+  
 1248 GCTGAAACGGAAAGGTAACGAGACAAATTCCTCGGCTTCCACCGATTT  
 R L C L S I C S V K G R K G A K  
  
 1249 GAGGAACACTGTCCATTTGGCATGGGAAATATAAACTTGTGTTGATTAC  
 -+-----+-----+-----+-----+-----+-----+-----+  
 1296 CTCCTTGTGACAGGTAACCGTACCCCTTATAATTGAAACAACCTAATG  
 E E H C P L A W G N I N L F D Y  
  
 1297 ACAGACACTCTAGTATCTGGAAAATGGCTTTGAATCTTTGGCCAGTA  
 ---+-----+-----+-----+-----+-----+-----+-----+  
 1344 TGTCTGTGAGATCATAGACCTTTTACCGAAACTTAGAAACCGGTCAT  
 T D T L V S G K M A L N L W P V

FIG 16 (contd)

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1345 CCTCATGGATTAGAAAGATTGCTGAACCCCTATTGGTGTACTGGATCA 1392  
-----+-----+-----+-----+-----+-----+-----+  
GGAGTACCTAATCTTCAAACGACTTGGGATAACCACCAATGACCCTAGT  
P H G L E D L L N P I G V T G S  
1393 AATCCAAATAAAGAAACTCCATGCTTAGAGTTGGAGTTTGACTGGTTC 1440  
-----+-----+-----+-----+-----+-----+-----+  
TTAGGTTATTCTTTGAGGTACGAACTCAACCTCAAACTGACCCAAG  
N P N K E T P C L E L E F D W F  
1441 AGCAGTGTGGTAAAGTCCCAGATAATGTCAGTGATTGAAGAGCATGCC 1488  
-----+-----+-----+-----+-----+-----+-----+  
TCGTCACACCATTTCAAGGGTCTATAACAGTCACTAACTTCTCGTACGG  
S S V V K F P D M S V I E E H A  
1489 AATTGGTCTGTATCCCGAGAAGCAGGATTTAGCTATTCACCACGCAGGA 1536  
-+-----+-----+-----+-----+-----+-----+-----+  
TTAACGACATAGGGCTTTCGTCCTAAATCGATAAGGGTGCCTCCT  
N W S V S R E A G F S Y S H A G

FIG 16 (contd)

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1537 CTGAGTAACAGACTAGCTAGAGACAATGAATTAAGGAAAATGACAAA  
 1584  
 GACTCATTTGCTGATCGATCTGTACTTAATTCCTTTTACTGTTT  
 L S N R L A R D N E L R E N D K

1585 GAACAGCTCAAAGCAATTTCTACACGAGATCCTCTCTGAAATCACT  
 1632  
 CTTGTCGAGTTTCGTTAAAGATGTGCTCTAGGAGAGACTTTAGTGA  
 E Q L K A I S T R D P L S E I T

1633 GAGCAGGAAAAGATTTCTATGGAGTCACAGACACTATTTGTGTAAC  
 1680  
 CTCGTCCTTTCTAAAGATACCTCAGTGTCTGTGATAACACATTTGA  
 E Q E K D F L W S H R H Y C V T

1681 ATCCCCGAAATTTCTACCCAAATTTGCTTCTGTCTGTTAAATGGAAATCT  
 1728  
 TAGGGCTTTAAGATGGGTTTAAACGAAGACAGACAAATTTACCTTAAGA  
 I P E I L P K L L L S V K W N S

FIG 16 (contd)







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2113 CAAGTCGAGGCAATGGAAAAGCTCATTAACCTAACTGACATTCCTCAA  
 -----+-----+-----+-----+-----+-----+-----+  
 2160 GTTCAGCTCCGTTACCTTTCCGAGTAATTGAATTGACTGTAAGAGTTT  
 Q V E A M E K L I N L T D I L K

2161 CAGGAGGAGGATGAAACACAAAAGGTACAGATGAAGTTTATTAGTT  
 -----+-----+-----+-----+-----+-----+-----+  
 2208 GTCCTCCTCCTACTTTGTGTTTCCATGTCTACTTCAAAAATCAA  
 Q E R K D E T Q K V Q M K F L V

2209 GAGCAAATGAGGCGACCAGATTTCAATGGATGCCCTACAGGGCTTGCTG  
 -+-----+-----+-----+-----+-----+-----+-----+  
 2256 CTCGTTACTCCGCTGGTCTAAAGTACCTACGGGATGTCCCAGAACGAC  
 E Q M R R P D F M D A L Q G L L

2257 TCTCCTAAACCCTGCTCATCAACTAGGAAACCCTCAGGCTTAAAGAG  
 -+-----+-----+-----+-----+-----+-----+-----+  
 2304 AGAGGAGATTTGGGACGAGTAGTTGATCCTTTGGAGTCCGAAATTCTC  
 S P L N P A H Q L G N L R L K E

FIG 16 (contd)

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2305 TGTCGAATTATGTCCTTCTGCCAAAAGGCCACTGTGGTTGAATTGGGAG  
 2352  
 ACAGCTTAATACAGAAGACGTTTCCGGTGACACCAACTTAACCCCTC  
 C R I M S S A K R P L W L N W E

2353 AACCCAGACATCATGTCAGAGTACTGTTTCAGAACAAATGAGATCATC  
 2400  
 TTGGGTCGTAGTACAGTCTCAATGACAAAGTCTTGTACTCTAGTAG  
 N P D I M S E L L F Q N N E I I

2401 TTAAAAATGGGATGATTTACGGCAAGATATGCTAACACTTCAAATT  
 2448  
 AAATTTTACCCCTACTAAATGCCGTTCTATACGATTGTGAAGTTAA  
 F K N G D D L R Q Q D M L T L Q I

2449 ATTCCGTATTATGGAAAATACTGGCAAATACTAAGGCTTGATCTTCGA  
 2496  
 TAAGCATAATACCTTTTATAGACCCGTTTAGTTCAGAACTAGAAGCT  
 I R I M E N I W Q N Q G L D L R

FIG 16 (contd)

2497 ATGTTACCTTATGGTTGTCTGTCAATCGGTGACTGTGTGGGACTTATT  
 2544 TACAATGGAATACCAACAGACAGTTAGCCACTGACACACCCCTGAATAA  
 M L P Y G C L S I G D C V G L I

2545 GAGGTGGTCCGAAATTCTCACACTATTATGCAAAATTCAGTGCAAAAGGC  
 2592 CTCCACCACGCTTAAAGAGTGTGATAAATACGTTTAAAGTCACGTTTCCG  
 E V V R N S H T I M Q I Q C K G

2593 GGCTTGAAAGGTGCACTGCAGTTC AACAGCCACACACTACATCAGTGG  
 2640 CCGAACTTCCACGTCGACGTC AAGTTGTCGGTGTGATGTAGTCACC  
 G L K G A L Q F N S H T L H Q W

2641 CTCAAAGACAAGAACAAAGGAGAAATATATGATGCAGCCATTGACCCTG  
 2688 GAGTTTCTGTTGTTCCCTCTTATA TACTACGTCGGTAACTGGAC  
 L K D K N K G E I Y D A A I D L

FIG 16 (contd)

2689 TTTACACGTTCAATGCTGGATACTGTAGCTACCTTCAATTTGGGA  
 -+-----+-----+-----+-----+-----+-----+-----+-----+  
 2736 AAATGTGCAAGTACACGACCTATGACACATCGATGGAAGTAAACCCT  
 F T R S C A G Y C V A T F I L G

2737 ATTGGAGATCGTCAACAATAGTAACATCATGGTGAAAGACGATGGACAA  
 ---+-----+-----+-----+-----+-----+-----+-----+-----+  
 2784 TAACCTAGCAGTGTATCATTTGTAGTACCACCTTCTGCTACCTGTT  
 I G D R H N S N I M V K D D G Q

2785 CTGTTTCAATAGATTTGGACACTTTTGGATCACAAAGAAA  
 ---+-----+-----+-----+-----+-----+-----+-----+-----+  
 2832 GACAAAGTATCTAAAACCTGTGAAAACCTAGTGTCTTCTTTT  
 L F H I D F G H F L D H K K K K

2833 TTTGGTTATAACGAGAACGTGTGCCATTTGTTTGACACAGGATTC  
 ---+-----+-----+-----+-----+-----+-----+-----+-----+  
 AAACCAATATTTGCTCTTGCCACACGGTAAACAAACTGTGTCCTAAAG  
 F G Y K R E R V P F V L T Q D F

FIG 16 (contd)

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2881 TTAATAGTGATTAGTAAAGGAGCCCAAGAAATGCACAAGACAAGAGAA 2928  
 -----+-----+-----+-----+-----+-----+-----  
 AATTACTACTAATCATTTCCCTCGGTTCTTACGTGTTCTGTTCTCTT  
 L I V I S K G A Q E C T K T R E

2929 TTGAGAGGTTTCAGGAGATGTGTTACAAGGCTTATCTAGCTATTCCGA 2976  
 -----+-----+-----+-----+-----+-----+-----  
 AAActCCAAAGTCCCTACACAATGTTCCGAAATAGATCGATAAGCT  
 F E R Q E M C Y K A Y L A I R

2977 CAGCATGCCAATCTTCTATAAATCTTTTCTCAATGATGCTTGGCTCT 3024  
 -----+-----+-----+-----+-----+-----+-----  
 GTCGTACGGTTAGAGAAGTATTTAGAAAAGAGTTACTACGAACCGAGA  
 Q H A N L F I N L F S M L G S

3025 GGAATGCCAGAACTACAATCTTTTGATGACATTGCCATACATTCCGAAAG 3072  
 -----+-----+-----+-----+-----+-----+-----  
 CCTTACGGTCTTGATGTTAGAAAACACTACTGTAACTGTGTAAGCTTTC  
 G M P E L Q S F D I A Y I R K

FIG 16 (contd)



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3265 CTCAGCAGGCAAGACCGGATTGCATAGGAAATGCACAAATCCATGAACA  
 ---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+  
 3312 GAGTCGTCGGTTCTGGCTAACGTAATCCTTAACGTTAGGTTACTTGT

3313 GCATTAGATTACAGCAAGAACAGAAATAAAATACTATAATAATTAAA  
 ---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+  
 3360 CGTAATCTAAATGTCGTTCTTGTCTTTATTTATGATATATATAAATTT

3361 TAATGTAAACGCAACAGGGTTTGATAGCACTTAAACTAGTTCATTC  
 ---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+  
 3408 ATTACATTTGCCGTTTGTCCCAAACTATCGTGAAATTTGATCAAGTAAAG

3409 AAAA  
 -+-- 3412  
 TTTT

FIG 16 (contd)

Alignment of human and bovine p110 cDNA nucleotide sequences.

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Nucleotide Similarity: 96%

```

hum110 1 ATGCCCTCCAAGACCATCATCAGGTGAACTGTGGGCATCCACTTGATGCC 50
      |||
bov110 1 ATGCCCTCCAAGACCATCATCAGGTGAACTGTGGGCATCCACTTGATGCC 50

51 CCCAAGAATCCTAGTGGAATGTTACTACCAATGGAATGATAGTACTT 100
      |||
51 CCCAAGAATCCTAGTAGAATGTTACTACCAATGGATGATAGTACTT 100

101 TAGAATGCCCTCCGTGAGGCTACATTAGTAACTATAAAGCACTAATT 150
      |||
101 TAGAATGCCCTCCGTGAGGCTACGTTAATAACGATAAAGCACTAATT 150

151 AAAGAAGCAAGAAATAACCCCTCCTCAACTTCTTCAAGATGAATCTC 200
      |||
151 AAAGAAGCAAGAAATAACCCCTCCTCAACTTCTTCAAGATGAATCTC 200

```

Figure 17



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201 TTACATTTTCGTAAGTGTACCCAGAAAGCAGAAAGGGAAGAAATTTTTC 250  
 |||  
 201 TTACATTTTCGTAAGTGTACCCAGAAAGCAGAAAGGGAAGAAATTTTTC 250

251 ATGAAACAAGACGACTTGTGATCTTCGGCTTTTCAACCATTTTAAA 300  
 |||  
 251 ATGAAACAAGACGACTTGTGACCTTCGGCTTTTCAACCCTTTAAA 300

301 GTAAATGAACCAGTAGGCAACCGTGAAGAAGAATCCTCAATCGAGAAAT 350  
 |||  
 301 GTAAATGAACCAGTAGGCAACCGTGAAGAAGAATCCTCAATCGAGAAAT 350

351 TGGTTTGCTATCGGCATGCCAGTGTGCGAATTTGATATGGTTAAAGATC 400  
 |||  
 351 TGGTTTGCTATCGGCATGCCAGTGTGGAATTCGATATGGTTAAAGATC 400

401 CTGAAGTACAGGACTTCCGAAGAAATATCTTAATGTTGTAAGAAGCT 450  
 |  
 401 CAGAAGTACAGGACTTCCGAAGAAATATCTCAATGTTGTAAGAAGCT 450

FIG 17 (contd)





951 TATGAATGGAGAAACATCTACAAAATCCCTTGGGTTATAAATAGAGCAC 1000  
 |||  
 951 TATGAATGGAGAAACATCTACAAAATCCCTTGGGTTATAAATAGAGCAC 1000

1001 TCAGAAATAAAAATCTTGTGCAACCCTACGTGAATCTAAATATTCGAGAC 1050  
 |||  
 1001 TCAGAAATAAAAATCTTGTGCAACCCTATGTGAATGTAAATATTCGAGAC 1050

1051 ATTGACAAGATTTATGTTCCGAACAGGTATCTACCATGGAGGAGAACCCCTT 1100  
 |||  
 1051 ATTGACAAGATTTATGTTCCGAACAGGTATCTACCATGGAGGAGAACCCCTT 1100

1101 ATGTGACAATGTGAACACTCAAAGAGTACCTTGTCCAAATCCAGGTGGA 1150  
 |||  
 1101 ATGTGATAATGTGAACACTCAAAGAGTACCTTGTCCAAATCCAGGTGGA 1150

1151 ATGAATGGCTGAATTATGATATATACATTCCTGATCTTCCTCGTGCTGCT 1200  
 |||  
 1151 ATGAATGGCTGAATTACGATATATACATTCCTGATCTTCCTCGTGCTGCT 1200

FIG 17 (contd)



















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3201 GAACTGAAAGATAAAGTGAAGAAATGAAAGCTCACTCTGGA  
 |||||  
 3201 GAACTGA.....

FIG 17 (contd)

The alignment between human and bovine p110 proteins.

```

10          20          30          40          50          60
h MPPRSSGELWGIHLMPPRIILVECLLPNGMIVTLECLREATLTIKHELFKYARKYPLHQ
  |||
b MPPRSSGELWGIHLMPPRIILVECLLPNGMIVTLECLREATLTIKHELFKYARKYPLHQ

10          20          30          40          50          60

70          80          90          100         110         120
h LLQDESSYIFVSVTQEAEREFFDETRRLCDLRLFQPFVKVIEPVGNGRREKILNREIGFA
  |||
b LLQDESSYIFVSVTQEAEREFFDETRRLCDLRLFQPFVKVIEPVGNGRREKILNREIGFA

130         140         150         160         170         180
h IGMPVCEFDLVKDPVQDFRRNILLNVCKEAVDLRDLNSPHSRAMYVYPPHVESSELPKH
  |||
b IGMPVCEFDLVKDPVQDFRRNILLNVCKEAVDLRDLNSPHSRAMYVYPPHVESSELPKH
130         140         150         160         170         180

```

Figure 18



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370 380 390 400 410 420  
 h YHGGELCDNVNTQRVPCSNPRWNEWLNLDIYIPDLPRAAARLCLSVKGRKGAKEEHC  
 |||  
 b YHGGELCDNVNTQRVPCSNPRWNEWLNLDIYIPDLPRAAARLCLSVKGRKGAKEEHC  
 370 380 390 400 410 420

430 440 450 460 470 480  
 h PLAWGNINLFDYTDTLVSGKMALNLWPVPHGLEDLLNPIGVGTGSNPNKETPCLELEFDWF  
 |||  
 b PLAWGNINLFDYTDTLVSGKMALNLWPVPHGLEDLLNPIGVGTGSNPNKETPCLELEFDWF  
 430 440 450 460 470 480

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490 500 510 520 530 540  
 h SSVVKFPDMSVIEEHANWSVSREAGFSYSHAGLSNRLARDNELRENDKEQLKAISTRDPL  
 |||  
 b SSVVKFPDMSVIEEHANWSVSREAGFSYSHAGLSNRLARDNELRENDKEQLRAICTRDPL  
 490 500 510 520 530 540

FIG 18 (contd)





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730 740 750 760 770 780  
 h QERKDETQKVQMKFLVEQMRRPDMDALQGLLSPLNPAHQGLNLRKKECRIMSSAKRPLW  
 ||:|||||  
 b QEKKDETQKVQMKFLVEQMRRPDMDALQGLLSPLNPAHQGLNLRLEECRIMSSAKRPLW  
 730 740 750 760 770 780

790 800 810 820 830 840  
 h LNWENPDIMSELLFQNNIEIFKNGDDL RQDMLTLQIIRIMENIWQNQGLDLRMLPYGCLS  
 |||||||  
 b LNWENPDIMSELLFQNNIEIFKNGDDL RQDMLTLQIIRIMENIWQNQGLDLRMLPYGCLS  
 790 800 810 820 830 840

850 860 870 880 890 900  
 h IGDCVGLIEVVRNSHTIMQIQCKGGLK GALQFNSHTLHQWLKDKNKGEIYDAAIDLFTRS  
 |||||||  
 b IGDCVGLIEVVRNSHTIMQIQCKGGLK GALQFNSHTLHQWLKDKNKGEIYDAAIDLFTRS  
 850 860 870 880 890 900

FIG 18 (contd)

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910 920 930 940 950 960  
 h CAGYCVATFILGIGDRHNSNIMVKDDGQLFHIDFGHFLDHKKKKFGYKRERVPFVLTQDF  
 |||  
 b CAGYCVATFILGIGDRHNSNIMVKDDGQLFHIDFGHFLDHKKKKFGYKRERVPFVLTQDF  
 910 920 930 940 950 960

970 980 990 1000 1010 1020  
 h LIVISKGAQECTKTREFFERFQEMCYKAYLAIRQHANLFINLFSMMLGSGMPELQSFDDIA  
 |||  
 b LIVISKGAQECTKTREFFERFQEMCYKAYLAIRQHANLFINLFSMMLGSGMPELQSFDDIA  
 970 980 990 1000 1010 1020

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1030 1040 1050 1060 1070 1080  
 h YIRKTLALDKTEQEALEYFMKQMNDAAHHGGWTTKMDWIFHTIKQHALNXXKITEKMKAHSG  
 |||  
 b YIRKTLALDKTEQEALEYFMKQMNDAAHHGGWTTKMDWIFHTIKQHALNX  
 1030 1040 1050 1060

FIG 18 (contd)

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Figure 19. The predicted amino acid sequence of human p110 CDNA.

1    MPPRPSSGEL WGIHLMPPRI LVECLLPNGM IVTLECLREA TLVTIKHELEF  
 51    KEARKYPLHQ LLQDESSYIF VSVTQEAERE EFFDETRRLC DLRLFQPFK  
 101    VIEPVGNREE KILNREIGFA IGMPVCEFDM VKDPEVQDFR RNILNVCKEA  
 151    VDLRDINSPH SRAMYVYPPH VESPELPHK IYNKLDRGQI IVVIWVIVSP  
 201    NNDKQKYTLK INHDCVPEQV IAEAIRKKTR SMLLSSEQLK LCVLEYQGKY  
 251    ILKVCGCDEY FLEKYPLSQY KYIRSCIMLG RMPNLKMMAK ESLYSQLPMD  
 301    CF'TMPSYSRR ISTATPYMNG ETSTKSLWVI NRALRIKILC ATYVNLNIRD  
 351    IDKIYVRTGI YHGGEPLCDN VNTQRVPCSN PRWNEWLNVD IYIPDLPRAA  
 401    RLCLSICSVK GRKGAKKEHC PLAWGNINLF DYTDTLVSGK MALNLWVPH  
 451    GLEDLLNPIG VTGSNPNKET PCLELEFDWF SSVVKFPDMS VIEEHANWSV

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501 SREAGFSYSH AGLSNRLARD NELRENDKEQ LKAISTRDPL SEITEQEKDF  
551 LWSHRHYCVT IPEILPKLLL SVKWNRSRDEV AQMYCLVKDW PPIKPEQAME  
601 LLDNCNYPDPM VRGEAVRCLF KYLTDDKLSQ YLIQLVQVLK YEQYLDNLLV  
651 RFLKKALTN QRIGHEFFWH LKSEMHNKTV SQRFGLLES YCRACGMYLK  
701 HLNROVEAME KLINLTDILK QERKDETQKV QMKFLVEQMR RPDEFMDALQG  
751 LLSPLNPAHQ LGNLRKKECR IMSSAKRPLW LNWENPDIMS ELLEQNNELI  
801 FKNGDDLROD MLTLQIRIM ENIWQNQGLD LRMLPYGCLS IGDCVGLIEV  
851 VRNSHTIMQI QCKGGLKGAL QFNSHTLHQW LKDKNKGEIY DAAIDLFTRS  
901 CAGYCVATFI LGIGDRHNSN IMVKDDGOLF HIDE GHFLDH KKKKFGYKRE  
951 RVPFVLTQDF LIVISKAQE CTKTREFEF QEMCYKAYLA IRQHANLEIN  
1001 LFSMMLGSGM PELQSFDDIA YIRKTLALDK TEQEALEYFM KOMNDAHGG  
1051 WTTKMDWIEH TIKQHALN\*

FIG 19 (contd)

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The human P1TR-c partial cDNA nucleotide and deduced amino acid sequence.

```

1  GGAGACGACTGGACAGGATCAACTTATCTTCAAATCATTTCACTC
   GlyAspLeuArgGlnAspGlnLeuIleLeuGlnIleIleSerLeu
49  ATGGACAAGCTGTACGGAAGAAATCTGGACTTGAAATTGACACCT
   MetAspLysLeuLeuArgLysGluAsnLeuAspLeuLysLeuThrPro
97  TATAAGGTGTAGCCACCAGTACAAACAATGGCTTCATGCAGtTTATC
   TyrLysValLeuAlaThrSerThrLysHisGlyPheMetGlnPheIle
145  CAGTCAGTtCCTGTGGCTGaaGTTCTTGATACAGAGGGAAGCATTCAG
   GlnSerValProValAlaGluValLeuAspThrGluGlySerIleGln
193  AACTTTTTAGAAAATATGCACCAGTGAGAAATGGCCAAATGGGATT
   AsnPhePheArgLysTyrAlaProSerGluAsnGlyProAsnGlyIle
241  AGTGCTGAGGTCATGGACACTtACGTTAAAGCTGTGCTGGATATTGC
   SerAlaGluValMetAspThrTyrValLysSerCysAlaGlyTyrCys
289  GTGATCACCTATACTTGGAGTTGGAGACAGGCACCTGGATAACCTT
   ValIleThrTyrIleLeuGlyValGlyAspArgHisLeuAspAsnLeu

```

Figure 20

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337 TTGCTAACCAAAACAGGCAACTCTTCCACATCGATTTCGGCCAC  
LeuLeuThrLysThrGlyLysLeuPheHisIleAspPheGlyHis

FIG 20 (contd)

SUBSTITUTE SHEET

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The human P1TR-f partial cDNA nucleotide and deduced amino acid sequence.

1 GGGGATGACTTACGGCAGGACATGCTAACGCTGCAGATGATTCGCATC  
 GlyAspLeuArgGlnAspMetLeuThrLeuGlnMetIleArgIle

49 ATGAGCAAGATCTGGTCCAGGAGGGCTGGACATGGCATGGTCATC  
 MetSerLysIleTrpValGlnGlyLeuAspMetArgMetValIle

97 TTCCGCTGCTTCCACCGGCCGAGAGGGATGGTGGAGATGATC  
 PheArgCysPheSerThrGlyArgGlyMetValGluMetIle

145 CCTAATGCTGAGACCCTGCGTAAGATCCAGGTGGAGCATGGGTGACC  
 ProAsnAlaGluThrLeuArgLysIleGlnValGluHisGlyValThr

193 GGCTCGTTCAAGGACCGGCCCTGGCAGACCGGCTGCAGAAACAAC  
 GlySerPheLysAspArgProLeuAlaAspArgLeuGlnLysHisAsn

241 CCTGGGAGGACGAGTATGAGAAGGCTGTGGaGAACTTATCTACTCC  
 ProGlyGluAspGluTyrgluLysAlaValGluAsnPheIleTyrsSer

289 TCGGCTGGCTGCTGGCCACGTCACGTCCTGGGCATCTGTGACCga  
 CysAlaGlyCysCysValAlaThrTyrvAlleuGlyIleCysAsparg

Figure 21



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337 CATAATGACAACATCATGCTGAAGACCACTGGTCCACATGTTCCACATC  
HisAsnAspAsnIleMetLeuLysThrThrGlyHisMetPheHisIle

385 GACTTCGGC  
AspPheGly

FIG 21 (contd)

ALIGNMENT OF HUMAN P110, HUMAN PI-3 KINASE RELATED GENES  
 PITR-C AND PITR-F AND YEAST PI-3 KINASE VPS34.

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In upper case are shown amino acids that are conserved in 3 or more of the proteins. Underlined are the residues involved in ATP binding.

	1	50
vps34	GDDL <u>R</u> QDqLvVQIIsImnellknEnvDLkLtPYkiLaTGpqeGaIEfIpN	
PITR-c	GDDL <u>R</u> QDqLiLQIIsImdkllrkENLDLkLtPYkvLaTstkhGFmqfIqs	
hump110	GDDL <u>R</u> QDmLtLQIIRiMeniwqnqGLDlRMlPYgCLsiGdcvGLIEvVrN	
PITR-f	GDDL <u>R</u> QDmLtLQmIRiMskiwvqEGLDmRmviFrcFSTGrgrGMVEMIpN	
Consensus	GDDL <u>R</u> QD-L-LQII-M-----E-LDL---PY--L-TG---G-IE-I-N	
	51	100
vps34	dtlasiLSkyhGILGy.....LklhypdeNatlgVqgwvLDnFVksCA	
PITR-c	vpvaeVldtegsIqnf.....FrkYapseNgpNgIsaevmDtYVksCA	
hump110	shtimqIQckGkGalqfnshTlhqWlkdkNkge.IydaaiDLFtrSCA	
PITR-f	aetlRkiqvehGvtGs..fkdrpLadrLqkhNpgedeyekavEnFIySCA	
Consensus	-----GI-G-----L-----N-----I-----D-FV-SCA	

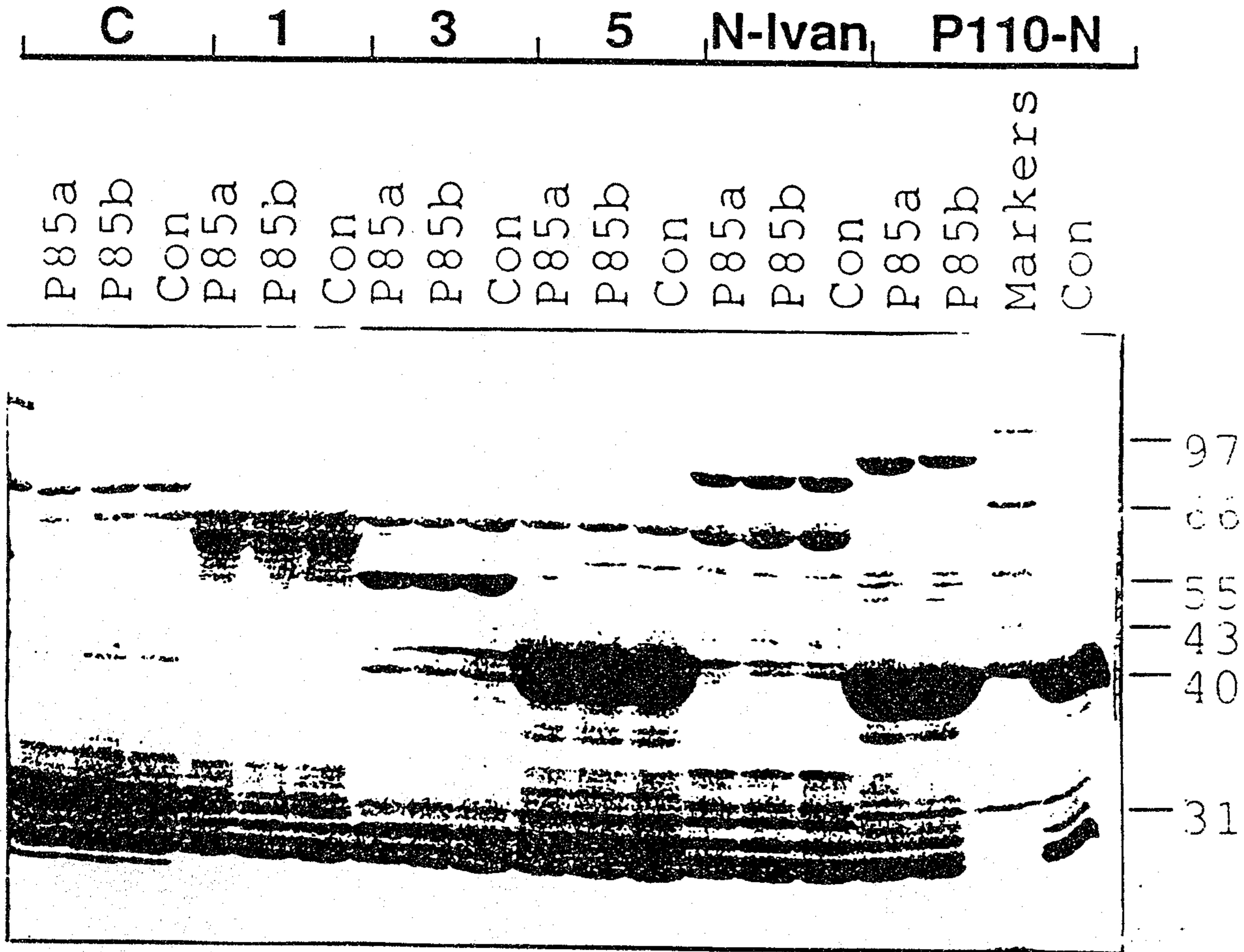
Figure 22

	101		133
vps34		GYCViTYILGVGDRH1DN1LlvtPdGhFFHaDFG	
P1TR-c		GYCViTYILGVGDRH1DN1LlktGkLFHIDFG	
hump110		GYCVaTFILGIGDRHnsNiMvkddGqLFHIDFG	
P1TR-f		GCCVaTYVLGICDRHnDNiMlktGhMFHIDFG	
Consensus		GYCV-TYILG-GDRH-DN-----G-LFHIDFG	

FIG 22 (contd)

Figure 23 A

# MAPPING THE P85 BINDING SITE ON P110



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Figure 23 B

# Locating the P85 binding site on P110



P85 association

PI 3-Kinase activity

P110N



N-IVAN



C



1



3



5

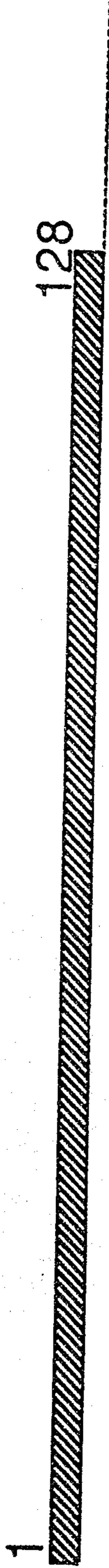


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Figure 24

# Mapping of the P85 Binding Site on P110



## P85 BINDING

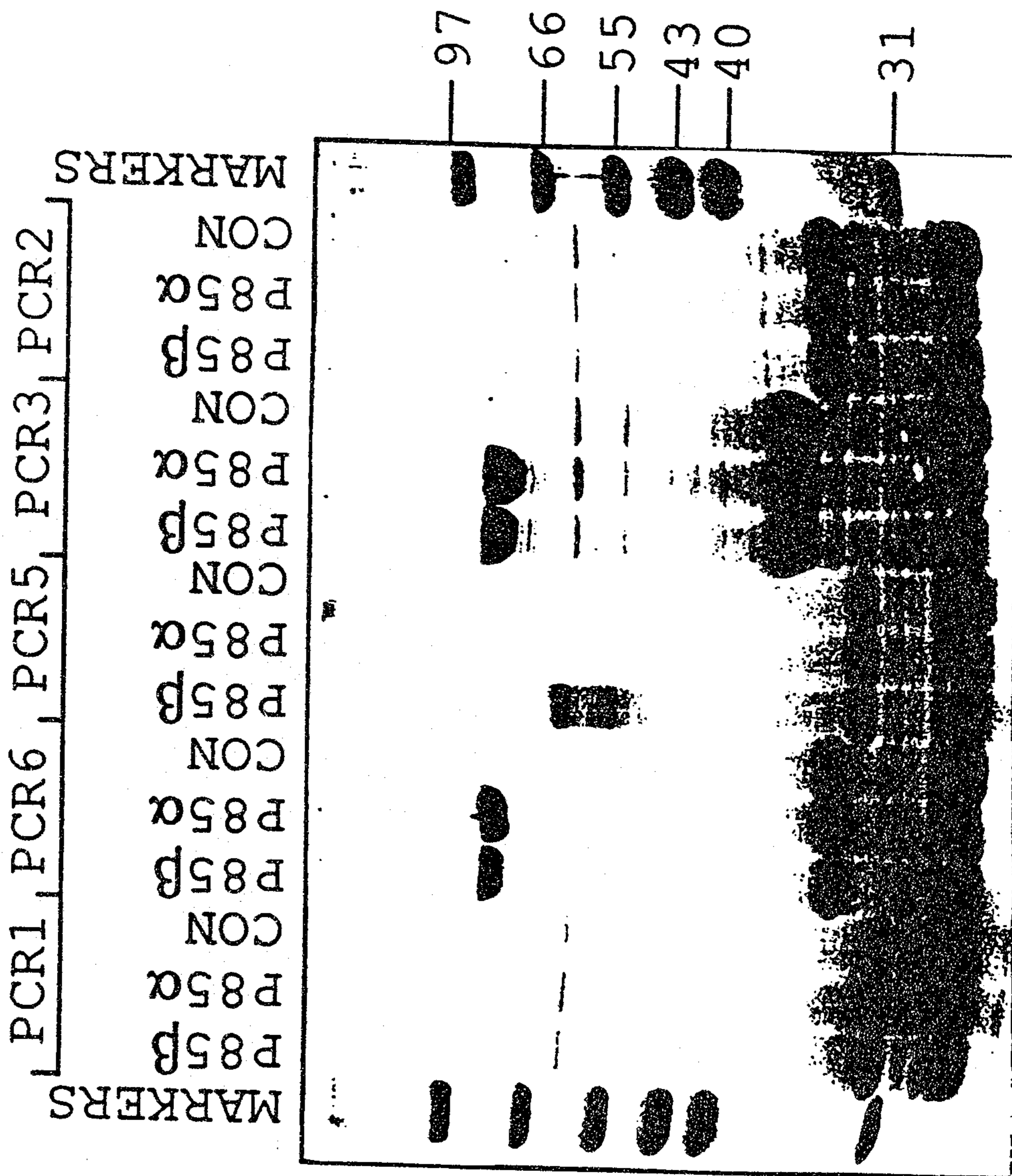
pcr1	1	49		-
pcr2	1	81		-
pcr3	1	108		+
pcr4	19	49		nd
pcr5	19	81		-
pcr6	19	108		+
N8	1	35		-
N13	37	128		-
110N	1	128		+

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Figure 25 A

# MAPPING THE P85 BINDING SITE ON P110



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Figure 25 B

# MAPPING THE P85 BINDING SITE ON P110 (2)

