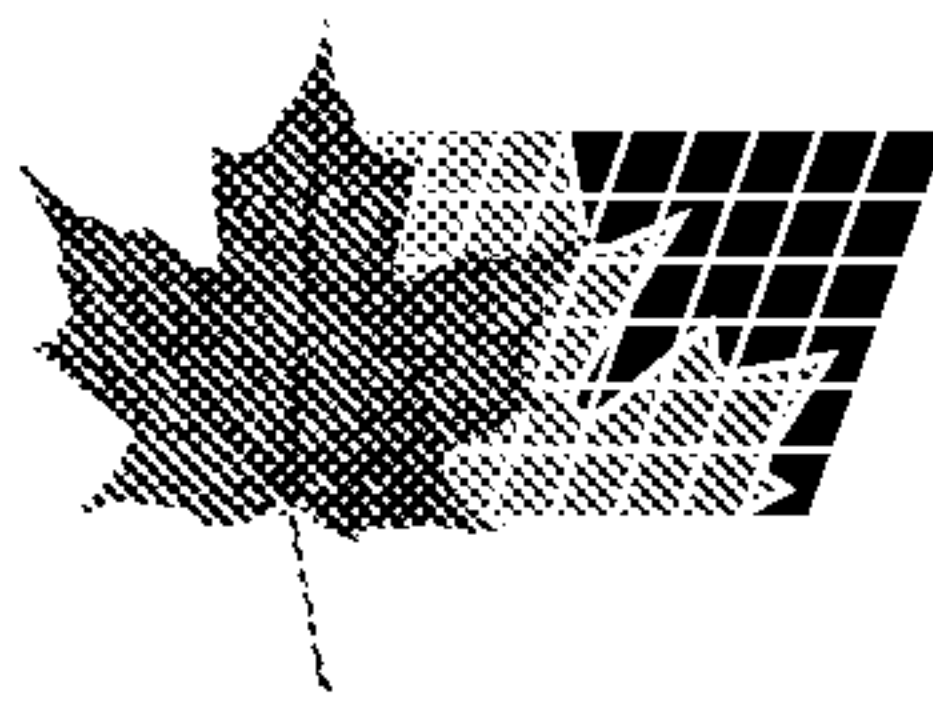




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(54) **PROCEDE SERVANT A EFFECTUER LE CONTROLE
SELECTIF DE LA PRESENCE D'UNE PROTEINE DE
MEMBRANE ET DE LA SECRETION DE PROTEINES DANS
DES CELLULES EUCARYOTES**

(54) **A METHOD FOR SELECTIVELY CONTROLLING MEMBRANE
PROTEIN DISPLAY AND PROTEIN SECRETION IN
EUKARYOTIC CELLS**

(57) Procédé et compositions correspondantes modulant la présence sur une membrane de surface cellulaire de protéines de membrane entières sélectionnées ou la sécrétion de protéines sécrétrices sélectionnées, ce qui consiste à augmenter ou à diminuer le transport intracellulaire de la protéine depuis le réticulum endoplasmique vers et à travers l'appareil de cis-Golgi. Des procédés associés permettent d'identifier si le transport intracellulaire d'une protéine spécifique a pour médiateur le système de trafic de protéines d'adaptation de spectrine et ankyrine (SAATS). Des procédés correspondants servent à déterminer si un composé candidat inhibe ou augmente le transport intracellulaire d'une protéine sélectionnée depuis le réticulum endoplasmique vers l'appareil de cis-Golgi par SAATS. Des agents et des procédés peuvent être mis en application dans une variété de domaines, y compris en tant qu'immunorégulateurs, inhibiteurs de transport d'ions, modulateurs vasculaires et agents chimiothérapeutiques contre le cancer.

(57) A method and related compositions are disclosed that modulate the presence on a cell surface membrane of selected integral membrane proteins, or modulate the secretion of selected secretory proteins, by increasing or decreasing the intracellular transport of the protein from the endoplasmic reticulum to and through the cis-Golgi apparatus. Associated methods are disclosed to identify whether the intracellular transport of a specific protein is mediated by the spectrin-ankyrin-adaptor protein trafficking system (SAATS). Related methods to determine whether a candidate compound inhibits or enhances the intracellular transport of a selected protein from the endoplasmic reticulum to the cis-Golgi apparatus by SAATS also are disclosed. Disclosed agents and methods are applicable for a variety of uses, including as immunoregulators, ion transport inhibitors, vascular modulators and cancer chemotherapeutics.

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(54) Title: A METHOD FOR SELECTIVELY CONTROLLING MEMBRANE PROTEIN DISPLAY AND PROTEIN SECRETION IN EUKARYOTIC CELLS (57) Abstract A method and related compositions are disclosed that modulate the presence on a cell surface membrane of selected integral membrane proteins, or modulate the secretion of selected secretory proteins, by increasing or decreasing the intracellular transport of the protein from the endoplasmic reticulum to and through the cis-Golgi apparatus. Associated methods are disclosed to identify whether the intracellular transport of a specific protein is mediated by the spectrin-ankyrin-adaptor protein trafficking system (SAATS). Related methods to determine whether a candidate compound inhibits or enhances the intracellular transport of a selected protein from the endoplasmic reticulum to the cis-Golgi apparatus by SAATS also are disclosed. Disclosed agents and methods are applicable for a variety of uses, including as immunoregulators, ion transport inhibitors, vascular modulators and cancer chemotherapeutics.		

**A METHOD FOR SELECTIVELY CONTROLLING
MEMBRANE PROTEIN DISPLAY AND
PROTEIN SECRETION IN EUKARYOTIC CELLS**

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Related Applications

This application is related to provisional application Serial No. 60/047,352, filed May 21, 1997, and provisional application Serial No. 60/053,218, filed July 18, 1997, both of which are herein incorporated by reference in their entirety. This application also claims priority to U.S. Provisional Application 60/060,559, which is herein incorporated by reference in its entirety. All other articles, publications, patents and documents referred to throughout this application are hereby incorporated by reference in their entirety.

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Technical Field

The present invention relates to methods for selectively modulating the sequestration of integral membrane or secretory proteins into transport vesicles and the trafficking of such vesicles between the endoplasmic reticulum, Golgi bodies, plasma membrane and other membrane compartments. The present invention, based on the discovery of a new biological phenomena, provides methods and compositions for use in identifying relevant integral membrane and secretory proteins, as well as agents that inhibit the sequestration of such proteins in transport vesicles. Related methods and compositions can be used to modulate the secretion and cell membrane display of various disease-related proteins.

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Background of the Invention

In all eukaryotic cells, a central and common process in integral membrane protein and secretory protein delivery is vesicular transport from their site of synthesis,
10 the endoplasmic reticulum, to and through the Golgi apparatus, and ultimately to specific plasma membrane or internal membrane domains. See, *e.g.*, Darnell *et al.*, **Molecular Cell Biology** (1990, Scientific American Books) at Chapter 17, Plasma-Membrane, Secretory, and Lysosome Proteins: Biosynthesis and Sorting. General mechanisms by which this process is achieved are now understood in reasonable detail, and involve
15 complex pathways of coated vesicle budding, transport, and fusion (reviewed in Kreis and Pepperkok, 1994; Schmid and Damke, 1995; Rothman and Wieland, 1996; Schekman and Orci, 1996; Bannykh and Balch, 1997). Collectively, these processes link the synthesis and folding of membrane proteins in the endoplasmic reticulum to a process of sequential vesicular transport through the various Golgi compartments and ultimately
20 their targeted delivery to specific plasma membrane or organelle domains. The molecular mechanisms that mediate these processes are less well understood. Typically, budding vesicles are encased in a closely adherent protein shell that favors their extrusion from a planar membrane compartment. Three general types of coats are now recognized: Clathrin/AP, COPI, and COPII. These coats are similar in many ways. They all form
25 easily discernible electron dense layers about their respective vesicles; they are of relatively simple and uniform composition; and they assemble onto vesicles under the control of ARF like GTP-binding proteins.

Homologues of the spectrin-ankyrin membrane cytoskeleton of erythrocytes have

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recently emerged as candidates for a fourth class of vesicle or Golgi coat protein. Immunologic, gel electrophoretic, and functional data strongly imply the presence of a novel isoforms of spectrin (termed $\beta I\Sigma^*$ and βIII) associated with Golgi membranes (Beck *et al.*,1994; for review see Devarajan and Morrow,1996; Devarajan *et al.*,1996; Morrow *et al.*,1997). Such isoforms of spectrin have been identified as components of the dynactin complex required for microtubule based vesicular transport (Holleran *et al.*,1996). Unique homologues of ankyrin, an adapter protein that links Na,K-ATPase and other transport proteins to the spectrin skeleton at the plasma membrane (for reviews see Bennett,1992; Devarajan and Morrow,1996; Morrow, *et al.*,1997), also exist in association with the Golgi. The Golgi associated ankyrins include a novel small ankyrin (Ank_{G119}) that has been cloned and characterized (Devarajan, *et al.*,1996), and larger isoforms that so far are only identified immunologically (Beck *et al.*,1997). Additional ankyrins also associate with other internal membrane compartments such as lysosomes (Hooock *et al.*,1997). While spectrin differs from the coatomer proteins in that it does not form geometrically precise coats and is more difficult to visualize by electron microscopy, as with other coat proteins spectrin's association with Golgi membranes is stimulated by ARF in a PtdInsP₂-dependent manner (Godi *et al.*,1997). Evidence disclosed below indicates a direct and specific role for spectrin-ankyrin and other adapter proteins in mediating the transport of integral and secretory proteins. This spectrin-ankyrin-adapter protein trafficking/tethering system is collectively termed SAATS.

Summary of the Invention

The present invention is based on our discovery of a covert intracellular processing and trafficking system that mediates the sequestration of integral membrane and secretory proteins into transport vesicles for transport from the endoplasmic reticulum to the cis-Golgi apparatus, from the cis-Golgi to the medial-Golgi apparatus, from the medial-Golgi to the trans-Golgi apparatus and from the trans-Golgi apparatus to

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the cell membrane or other cellular compartments. We identify specific adapter proteins and functionally active sites within components of the SAATS system that in isolation demonstrate pharmacologic activity, with the ability to control specifically the display or trafficking of selected membrane proteins. General methods are presented that will allow specific transport inhibitors for a selected membrane or secreted protein to be identified, and several examples of the application of these methods are presented.

Brief Description of the Drawings

Figure 1: Diagram of the spectrin/ankyrin/adapter protein trafficking/tethering system (SAATS) and one way protein transport may be blocked. (A) general schematic by which dynactin moves a vesicle from the endoplasmic reticulum (ER) to the Golgi. The transported tubular vesicular structure/vesicle emanating from the exit portals of the endoplasmic reticulum Golgi intermediate compartment (ERGIC) are linked to dynactin by SAATS, which is able to simultaneously bind the vesicle and dyNactin through an interaction with centractin, which is part of the dynactin complex. Centractin is an actin related protein, or ARP that binds to spectrin. (B) In wild-type cells, various proteins bind to vesicular associated spectrin directly or indirectly via adapter proteins such as ankyrin or by binding to another membrane carrier protein that is subsequently bound by SAATS (such as for secreted proteins). L, lumen of the ER. (C) Selected protein transport is achieved by blockage of its attachment to SAATS, such as in a cell transfected with a cDNA encoding a spectrin βI_{N-5} construct. In such cells, certain integral membrane or secretory proteins are not sequestered into the vesicle for transport from the endoplasmic reticulum to the Golgi.

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Figure 2: A series of appropriate and representative $\beta I\Sigma^*$ spectrin constructs useful for blocking membrane protein display and for determining suitable SAATS targets for pharmacologic agent identification. Some of the important functional domains are also depicted. Ankyrin binding occurs in repeat unit 15. The amino-terminal domain (green) bestows actin and presumably ARP binding on spectrin complexes. Membrane association domains (MAD1 and MAD2) are also shown. A third membrane association domain (MAD3) also resides somewhere between repeat units 3 and 9. Similar constructs prepared from other spectrins involved in protein trafficking (*e.g.*, βIII spectrin) can also be prepared.

Figure 3: Illustration of the use of cultured Madin Darby Canine Kidney (MDCK) cells to monitor the surface expression of Na,K-ATPase by indirect immunofluorescence and immunoprecipitation. In this example, the expression of amino terminal βI spectrin constructs in MDCK cells disrupts the native Golgi spectrin-ankyrin skeleton and Na,K-ATPase assembly. (A) The intracellular distribution of wild type Golgi spectrin was monitored using Mab VIIIC7, which reacts with full length Golgi spectrin but not the shorter FLAG tagged βI_{N-5} construct (Harris *et al.*, 1985; Devarajan, *et al.*, 1996; Godi, *et al.*, 1997). The distribution of $\beta I\Sigma^*$ spectrin in cells transfected with βI_{11-C} was monitored by Mab VD4, which does not react with the βI_{11-C} peptide. AnkG119 was monitored by the antibody "Jasmin" (Devarajan, *et al.*, 1996); α -Na,K-ATPase by a Mab from Upstate Biotechnology. Note the dispersal of the endogenous Golgi spectrin, Ank_{G119}, and α -Na,K-ATPase by βI_{N-5} (but not by βI_{11-C}). All shortened spectrin constructs containing region I and MAD1 displayed a similar effect, whereas constructs lacking these regions of βI spectrin did not significantly alter the native distributions of $\beta I\Sigma^*$ spectrin, AnkG119, or Na,K-ATPase. Bar = 10 μ m. (B) The interaction of Na,K-ATPase with the 14-15 repeat unit of spectrin occurs *via* an ankyrin G119 adapter protein intermediate, as detected by co-immunoprecipitation. In wt cells,

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β I Σ^* spectrin, AnkG119, centractin, and Na,K-ATPase are co-precipitated from detergent lysates by Mab VIIIC7 (lane IP). Soluble α II β II spectrin (as detected here by immunoblotting for α II spectrin), the form found predominately at the plasma membrane, is also present in these detergent lysates (lane lys) but is not part of the precipitable Golgi spectrin complex. Control experiments with preimmune or irrelevant antibody did not precipitate any of these components (data not shown). Similarly, immunoprecipitates with Mab VIIIC7 of the β I_{N-5} transfected cells also demonstrated co-precipitation of Ank_{G119}, and Na,K-ATPase with β I Σ^* spectrin, indicating that despite the dispersal of these elements induced by the β I_{N-5} construct, these elements remained associated in the detergent lysates (data not shown). Western blots after SDS-PAGE, visualized by ECL.

Figure 4: Illustration of alternative assays demonstrating the impairment of Na,K-ATPase trafficking from the ER induced by SAATS inhibition. Dispersal of Golgi spectrin by β I_{N-5} blocks Na,K-ATPase transport to the medial Golgi and its incorporation into a detergent stable skeletal complex. (A) The intracellular distribution of the endogenous β I Σ^* spectrin (determined by Mab VIIIC7, which does not react with the expressed β I_{N-5} peptide), and Na,K-ATPase in MDCK cells transfected with β I_{N-5} spectrin. (B) Golgi dependent glycosylation of β -Na,K-ATPase is blocked by the β I_{N-5} spectrin peptide. Control MDCK cells (wt) or cells transfected with the β I_{N-5} construct (β I_{N-5}) were analyzed by Western blot using a β -Na,K-ATPase specific antibody (Mab B1-13). The apparent MW of the various products are depicted. Note the broad band above 50 kDa (gly) representing mature glycosylated β -Na,K-ATPase, and the intense band at 44 kDa in the β I_{N-5} cells (core), representing the ER dependent core β -Na,K-ATPase glycosylation product. It thus appears that the β I_{N-5} spectrin peptide inhibits mature glycosylation of β -Na,K-ATPase, a process characteristic of medial Golgi processing, but does not interfere with the formation of the core glycosylation product, which occurs in the ER. In this experiment, approximately half of the β I_{N-5} cells are expressing the β I_{N-5} construct, as judged by immunofluorescence (data not shown), which

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appears to account for most of the mature glycosylated product observed in the βI_{N-5} cell lines. The band ≈ 106 kDa in the wild type cells (*) is inconstant in its appearance, and may represent a cross-linked adduct of β -Na,K-ATPase with α -Na,K-ATPase or other protein, as is commonly observed after reduced SDS-PAGE in MDCK cells (Morrow *et al.*, 1989). (C) Fraction of Na,K-ATPase present in the soluble and detergent insoluble pools of MDCK cells transfected with different βI spectrin constructs. Fx1 (soluble fraction) is that material solubilized by 0.5% Triton X-100 in 100 mM NaCl; Fx2 (cytoskeletal fraction) is the material solubilized by 0.5% Triton X-100 and 250 mM NH_4SO_4 (Devarajan *et al.*, 1994). Shown are Western blots for α -Na,K-ATPase after SDS-PAGE. (D) The results of three separate determinations of α -Na,K-ATPase extractability of wt and two transfected MDCK cell lines were quantified in-duplicate by densitometry, and all analyses averaged. Error bars represent ± 1 SD. Note the substantial loss of fully assembled and detergent insoluble Na,K-ATPase in the βI_{N-5} expressing cells.

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Figure 5: Illustration of selective blockage of Na,K-ATPase and VSV-G transport, but not E-cadherin, by βI_{N-5} . Comparison of the effect of βI_{N-5} spectrin on Golgi and the assembly of different proteins in MDCK cells. Wild-type (B,D,F,H) and βI_{N-5} transfected cells (A,C,E,G) are shown. The localization of VSV- G protein (E,F) was measured after transient infection. E-cadherin (C,D) was monitored with a Mab from Transduction labs, and also by the extent to which the precursor peptide was proteolyzed (inset, Western blot) from 135 kDa (*) to 120 kDa (EC) (a process that occurs in the trans-Golgi) (Shore and Nelson, 1991). There was no significant difference in the extent of E-cadherin processing or its level of assembly at the plasma membrane in the βI_{N-5} line vs. wt cells. Despite the disruption of Na,K-ATPase and VSV-G transport (and the wt Golgi spectrin skeleton, Fig. 3), the Golgi appears to remain largely intact as measured by the distribution of β -COP (A,B) and by the presence of normal appearing juxtannuclear Golgi structures in uranyl acetate and lead stained electron microscopy (G,H) (arrows).

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Bar = 10 μ in (A-F), 0.5 μ in (G,H). Original magnification (G,H) = 63,000x.

Figure 6: Illustrates the rescue of Na,K-ATPase trafficking by inclusion of a specific SAATS binding domain that in this case, binds to ankyrin. MDCK cells were transfected with either βI_{N-5} as above, or a with $\beta I_{N-5,15}$. This latter construct incorporates the ankyrin binding domain of spectrin into the βI_{N-5} peptide. Note the complete restoration of Na,K-ATPase transport, as measured in this case by its surface display and secondarily by a reduction in cell size (which is a consequence of cell swelling due to a deficiency of plasma membrane Na,K-ATPase in the βI_{N-5} transfected cells.

Figure 7: Illustrates how to make SAATS block VSV-G transport (but not Na,K-ATPase). MDCK cells infected with vesicular stomatitis virus were used to monitor the trafficking of VSV-G protein. The transport of this protein is normally to the basolateral membrane, similar to that for Na,K-ATPase (as shown in the wt cells and in Figure 5). This transport is blocked by SAATS inhibition with the βI_{N-5} construct, as is Na,K-ATPase (cf. Figure 6). However, unlike Na,K-ATPase, VSV-G trafficking is not restored by the inclusion of the ankyrin binding domain of spectrin (repeats 14-15) in the construct. Thus, it is possible to selectively block VSV-G transport without affecting Na,K-ATPase transport.

Figure 8: Illustrates the identification of a small peptide sequence responsible for association of Na,K-ATPase with ankyrin. (A) Schematic representation of the five cytoplasmic domains of α -Na,K-ATPase and their relationship to the ankyrin binding peptide sequences identified here. Codon positions defining each peptide are shown. Previous studies have established broad reactivity of cytoplasmic domains II and III with ankyrin, with domain II contributing most of the binding activity (Devarajan, *et al.*, 1994; Jordan *et al.*, 1995). (B) Each depicted peptide (II-IIC) was prepared as a fusion

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construct with SjGST, and examined for its ability to bind at various concentrations either purified ANK1 (from human red cells) or kidney ankyrin (ANK3) derived from whole MDCK cell lysates. Results from a single experiment are shown. The top panel shows Coomassie blue stained SDS-PAGE analysis of each peptide, as well as the entire MDCK cells extract applied to the affinity column to detect ANK3 binding. To detect ANK1 binding, purified erythrocyte ankyrin was used. Peptide IIA, sequence -**SYEQEAKSSKIMESFKNMVPQQALV**-, represents the minimal active sequence detected. We term this the minimal ankyrin binding domain (MAB).

Figure 9: Illustrates that the specific deletion of a SAATS attachment sequence in an integral membrane protein selectively blocks its transport alone. In this case, MDCK cells were transiently transfected with either wild-type Na,K-ATPase to which a FLAG epitope tag had been added to its NH₂-terminus, or by a similarly FLAG-tagged mutant Na,K-ATPase in which codons 142 to 166 had been deleted. These residues correspond to the minimal ankyrin binding domain (MAB) identified in Figure 8. In both transfections, the cells were also co-transfected with wt β -Na,K-ATPase, so as to assure sufficient β -Na,K-ATPase to pair with the α -Na,K-ATPase. **(top)** Flag tagged wt-Na,K-ATPase is delivered normally to the plasma membrane. **(bottom)** Flag tagged Na,K-ATPase lacking MAB does not assemble at the plasma membrane, and eventually is targeted for degradation in lysosomes. In these cells, the mutant Na,K-ATPase is the only protein whose transport is disrupted.

Figure 10: Illustrates selective blockage of Na,K-ATPase transport in normal cells by the expression of a small peptide inhibitor. Wild-type MDCK cells were transiently transfected with green fluorescent protein linked to the 25 residue MAB peptide. This is the same sequence whose deletion caused Na,K-ATPase to be retained in the ER and ultimately lysosomes in Figure 9. After approximately 1-2 days in culture, cells were fixed and stained for GFP (using anti-GFP antibodies, left panel) or for

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Na,K-ATPase (right panel, red). Note that the expression of GFP alone in MDCK cells (wt) did not affect Na,K-ATPase distribution, or cell size (which is dependent on Na,K-ATPase function. However, when the GFP carried the 25 residue MAB peptide, the binding of the endogenous Na,K-ATPase to SAATS was inhibited, resulting in its selective accumulation in the ER and reduced levels on the plasma membrane (two examples are shown, labeled "MAB"). Cells containing GFP-MAB are also markedly swollen, as expected. No other protein distributions were affected in these cells.

Figure 11: Illustrating the three-dimensional structure of MAB, which is well suited for the rational design of small molecule agents with similar pharmacologic action as MAB. This structure was determined using carrier mediated crystallization based on the active GST-MAB peptide shown in Figure 8 (Zhang *et al.*, 1997a). Three-axis views of the structure of the minimal ankyrin binding domain of α -Na,K-ATPase. The basic structural motif is that of a seven residue "loop" on a "stalk" composed of antiparallel β -strands. (A,C,E) Surface accessibility depictions showing the amphipathic surfaces formed by the "loop". (B,D,F) Ribbon diagrams demonstrating the back-bone contour. It is envisioned that the seven residue loop interacts with the β -hairpin tips of one or more ankyrin repeat units. The arrow in (C) marks the depth of the structural plane shown in (E and F). It is anticipated that small molecule congeners mimicking the 7 residue loop structure will prove to be effective and selective inhibitors of Na,K-ATPase trafficking.

Figure 12: Illustrating another integral membrane protein of significant medical interest that is linked to SAATS by a different ankyrin interaction. (A) Both CFTR (cystic fibrosis transmembrane conductance regulator) and its close homologue TNR (Transmembrane-Nucleotide binding -Regulatory domain) protein are present in the soluble (Fx1) and cytoskeletal pools (Fx2) of confluent MDCK cells. When these cells are surface labeled with biotin from either the apical surface (lane A) or

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the basolateral surface (lane B), it is also apparent that both CFTR and TNF are highly polarized, and unlike Na,K-ATPase, are expressed predominately in the apical domain of these polarized epithelial cells. (B) Despite the fact that CFTR and TNF are apical proteins, they nevertheless binds components of the SAATS system, which is responsible for their trafficking. Either whole cell lysates (L) of cells extracted with RIPA buffer, or Fx 1 and Fx 2, were immunoprecipitated with irrelevant antibody control (C) or with anti-CFTR antibody (IP). The washed immunoprecipitates were then analyzed by SDS-PAGE and examined by western blotting for either ankyrin. Note that both ankyrins (AnkG119 and Ank R) co-immunoprecipitate with CFTR, in a fashion exactly analogous to the way Na,K-ATPase binds SAATS. However, CFTR and Na,K-ATPase bind at distinct sites on SAATS, enabling their selective modulation.

Figure 13: Illustrating both down and up-regulation of integrin display in cultured endothelial cells by SAATS. SV40 transformed murine endothelial cells were transfected with βI_{N-5} or $\beta I_{N-5,15}$, and the surface display of three different integrins was measured by flow cytometry. Note the blockage of alpha V, beta 3, and beta 1 integrins by the βI_{N-5} peptide. Conversely, the $\beta I_{N-5,15}$ peptide markedly enhances the surface display of alpha V and beta 1 integrin (but not beta 3 integrin). This enhanced display (as opposed to blockage) is presumably achieved by enhancing the efficiency of SAATS directed cargo loading of vesicular transport by choosing peptides or agents that enhance the binding of a given membrane protein to SAATS.

Figure 14: Illustrating additional examples of SAATS regulation of integrin display in cultured endothelial cells. Experiment was carried out as in Figure 13. Note that alpha-V integrin is also modulated by SAATS. Also note that in this example, $\beta I_{N-2,15}$ was used to rescue beta-1 expression. βI_{N-2} is an even more broadly blocking inhibitor of SAATS trafficking (see Figure 1), but in this instance, supranormal levels of beta-1 can be achieved by inclusion of repeats 14,15 into the βI_{N-2} peptide.

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Figure 15: Illustration of PECAM (CD31) surface modulation by SAATS inhibitory peptides employing two different epitope tags, FLAG and GFP. Cells were transfected as above, and surface display of PECAM, a cell-cell adhesion molecule of the IgG superfamily, was monitored by flow cytometry. Note that for either construct, inclusion of the βI_{N-5} sequences indicated strong blockage of transport, indicating their involvement with SAATS. In both cases, surface display was rescued or accentuated by the $\beta I_{N-5,15}$ peptide. This effect was a bit more pronounced with the N-terminal FLAG tagged construct vs. the COOH-terminal GFP tagged construct, presumably due to interference of the GFP with the COOH-terminal 15th repeat unit needed to restore SAATS binding activity to PECAM.

Figure 16: Illustrates the modulation of CD45 and TNFR-1 in T-lymphocytes by SAATS inhibitors. In this experiment, Jurkat T-lymphocytes were transfected with the constructs indicated, including βI_{14-15} alone. Note the marked down regulation of CD45, a documented ankyrin binding protein, by constructs lacking repeat 15, as well as by the βI_{14-15} peptide itself (which lacks the constitutive Golgi targeting signal, see Fig. 2). These experiments also illustrate that TNFR-1 display is upregulated (rather than blocked) by βI_{N-2} or βI_{N-5} , suggesting that like E-cadherin, its attachment to SAATS is mediated either directly or indirectly by sequences contained within βI_{N-2} .

Figure 17: Illustrates the modulation of Fas and Fas-L in T-lymphocytes by SAATS. Experiments were as before. Note the changes in both Fas and Fas-L due to βI_{N-5} and $\beta I_{N-5,15}$ peptides.

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Figure 18: Illustrates the use of antibody or injected small peptides to modulate Na,K-ATPase in wild-type MDCK cells. Clusters of wild-type MDCK cells were micro injected with either (A) GST alone; or (B). with the βI_{N-4} peptide generated as a recombinant fusion peptide with GST, and then stained for Na,K-ATPase by indirect immunofluorescence. While micro injected GST is without effect, note the loss of Na,K-ATPase staining intensity in the injected cells on the left side of (B). The consequences of impaired Na,K-ATPase delivery to the membrane are also apparent in the larger size of cells with insufficient Na,K-ATPase, similar to the changes seen when βI_{N-5} is transfected. These results indicate that selective blockage of Na,K-ATPase can be achieved by exogenous small peptides. Similar experiments were also carried out using various monoclonal antibodies. In these experiments, the cells were subconfluent, and all cells in an isolated cluster were injected. (C) Control injections of buffer alone are without effect on the distribution of Na,K-ATPase. (D) Microinjection of Mab IID2, which reacts exclusively with αI spectrin (not considered to be a component of SAATS), is also without effect on Na,K-ATPase. (E) Microinjection of Mab VIIIC7, which reacts with βI spectrin (Harris et al., 1986), leads to severe disruption of Na,K-ATPase delivery, with extensive accumulation within the cells and cell swelling. In this micrograph, the loss of membrane Na,K-ATPase is so severe, that the membrane borders cannot be discerned. This experiment illustrates the effectiveness of SAATS specific monoclonal antibodies in blocking SAATS activity.

Figure 19: Presents the full-length cDNA sequence of a novel isoform of βIII spectrin that may be an additional component of SAATS. (A) A partial cDNA clone identified as an expressed sequence (EST) was identified as a spectrin family member, extended to complete the sequence by 5'RACE PCR amplification, and sequenced. (B) The resulting full-length sequence is predicted to encode a novel isoform of spectrin, termed βIII (Morrow, 1997), that is similar to both βI and βII , but shows significant differences in selected regions. (C) Sequence similarity between the various

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beta-spectrins. Also shown is a phylogenetic dendritogram showing the approximate relationship of β III spectrin to β I and β II spectrins.

5 **Figure 20: Illustrative examples of SAATS inhibitory peptide constructs, in two different vectors: pcDNA3 (FLAG tagged) and enhanced green fluorescent protein (eGFP).**

10 **Figure 21: Figure 4B represents a model of how Na,K-ATPases may interact with one or more ankyrin repeat units.**

Figure 22: Modulation of surface display of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by SAATS. MDCK cells were transfected with either the β I_{N-5} or β I_{N-5,15} constructs and then surface labeled with biotin. After labeling, cells were solubilized, and the total CFTR in each culture
15 immunoprecipitated with anti-CFTR antibodies. The precipitates were then analyzed by SDS-PAGE, transferred to nitrocellulose, and surface CFTR detected by avidin labeling. Total CFTR was also determined in these cellular extracts by western blotting (data not shown). In all cell lines, there was minimal change in the total CFTR in the cells. However, there was marked diminution of surface display of CFTR induced by the
20 SAATS blocker β I_{N-5}; whereas the β I_{N-5,15} construct markedly enhanced the surface display of CFTR. Collectively these results document the involvement of SAATS in CFTR trafficking, and suggest a possible therapeutic approach to the control of disorders such as cystic fibrosis, or the control of related proteins such as the multi-drug resistance gene product (MDR).

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Figure 23: Schematic representation of the Spectrin Heterodimer.

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Modes of Carrying Out the Invention

5 The following discussion presents a general description of the SAATS system and discusses ways in which this system can be modulated for general classes of integral membrane and secretory proteins as well as for specific proteins in order to affect cell membrane display and secretion.

10 The generality in terms of which classes of membrane proteins utilize this pathway remains to be fully determined. However, given the guidance presented herein, a skilled artisan will know how to determine which proteins participate in the SAATS and analogous intracellular transport systems, how to identify the vesicular skeletal proteins and associated adapter proteins that mediate protein transport from the ER to and through the Golgi apparatus, how to identify small molecule and other agents that mediate such transport, and how to utilize such agents for therapeutic purposes.

15

Table IA: Summary of mammalian spectrins

gene	Isoforms	chromosome	GenBank	Comment
α I	S1	1q21	M61877	No splice forms identified. Found in red cells & brain
α II	S1 to S8 (see ref. (Cianci <i>et al.</i> ,1997))	9q34.1	U83867 (S1) J05243 (S2)	Found in most tissues; three regions of alternative splicing, some tissue specific. S6-S8 believed to exist but not proven (Cianci, et al.,1997).
β I	S1 & S2 Possibly S3	14q23-q24	J05500 (S1) M37884 (S2)	Predominant in red cells, muscle. S2 isoform adds MAD2/PH domain & PIP ₂ binding. Putative β I Σ 3 may be Golgi form, but this is unproven.
β II	S1 & S2	2p21	M96803 (S1)	Found in most tissues. β IIS2 partially characterized(Lombardo <i>et al.</i> ,1994b). Other splice forms may exist, but not well documented.
β III	S1	??	AB002300	Partially identified by EST match. Full sequence shown in Figure 19

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Table IB: Interactions involving spectrin

	Membrane Interactions with spectrin band 3 (anion exchangers)	linker / cofactor ankyrin	tissue RBC, kidney, brain	selected references (Bennett and Branton,1977; Bennett and Stenbuck,1979b; Bennett and Stenbuck,1979a; Morgans and Kopito,1993) (Nelson and Veshnock,1987; Koob <i>et al.</i> ,1988; Morrow, <i>et al.</i> ,1989; Nelson and Hammerton,1989) (Smith <i>et al.</i> ,1991) (Srinivasan <i>et al.</i> ,1988; Srinivasan <i>et al.</i> ,1992) (Luna and Hitt,1992) (Joseph and Samanta,1993) (Lokeshwar and Bourguignon,1992a; Lokeshwar <i>et al.</i> ,1994) (Bourguignon <i>et al.</i> ,1992) (Davis and Bennett,1993; Davis <i>et al.</i> ,1993) (Smith <i>et al.</i> ,1993) (Hemming <i>et al.</i> ,1995) (Bourguignon <i>et al.</i> ,1986) (Lombardo <i>et al.</i> ,1994a) (Sinard <i>et al.</i> ,1994) (Pitcher <i>et al.</i> ,1992; Touhara <i>et al.</i> ,1994) (Bourguignon <i>et al.</i> ,1985; Lokeshwar and Bourguignon,1992b) (Molday <i>et al.</i> ,1990) (Cianci <i>et al.</i> ,1995) (Rotin <i>et al.</i> ,1994) (Pollerberg <i>et al.</i> ,1987) (Mombers <i>et al.</i> ,1977; Mombers <i>et al.</i> ,1979; Pradhan <i>et al.</i> ,1991; Diakowski and Sikorski,1994) (Andrews and Fox,1992; López <i>et al.</i> ,1992) (Rimm <i>et al.</i> ,1995)
5	Na/K-ATPase	ankyrin	kidney	
	amiloride-sensitive Na ⁺ channel voltage-gated Na ⁺ channel	ankyrin ankyrin	kidney brain	
10	ABGP 205 inositol triphosphate receptor CD44 (gp85)	ankyrin ankyrin ankyrin	brain brain lymphoma	
	CD44-like 116kD protein CAMS related to L1/neurofascin	ankyrin ankyrin	endothelium brain	
15	H/K-ATPase glycophorin C Thy-1 cadherin stomatin bg subunits of trimeric G-proteins	ankyrin protein 4.1 protein 4.1 α (E)-catenin adducin direct	gastric cells erythrocytes lymphocytes kidney RBC and others	
	CD45 (gp180) (tyrosine phosphatase)	direct	lymphocytes	
20	cGMP-gated cation channel dynamin (GTPase) epithelial Na ⁺ Channel N-CAM 180 Phospholipid	direct direct direct direct direct	retina (rods) MEL cells,brain MDCK cells brain RBC <i>et al</i>	
25	GP Ib-IX -- to actin cadherin -- to actin	ABP-280 α (E)-catenin	platelets MDCK cells	

Cytoskeletal Interactions with spectrin

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	actin	adducin	RBC	(Gardner and Bennett,1987;
		protein 4.1	RBC	Mische <i>et al.</i> ,1987)
				(Cohen and Foley,1980;
				Cohen and Korsgren,1980;
				Cohen and Foley,1982; Wolfe
	intermediate filaments	direct	brain, RBC (avian)	<i>et al.</i> ,1982)
				(Mangeat and Burridge,1984;
				Langley and Cohen,1986;
				Frappier <i>et al.</i> ,1987;
				Herrmann and Wiche,1987;
	tubulin	direct	brain	Frappier <i>et al.</i> ,1992)
				(Fach <i>et al.</i> ,1985; Riederer
				and Goodman,1990)
5	plectin	direct	glioma cells	(Herrmann and Wiche,1987)
	synapsin	direct	brain	(Sikorski <i>et al.</i> ,1991)
	A60	direct	brain (axons)	(Hayes <i>et al.</i> ,1995)
10	Other Interactions			
	synapsin to actin	direct	brain	(Bähler and Greengard,1987;
				Petrucci and Morrow,1987)
	tropomyosin to actin	direct	RBC, muscle	(Fowler and Bennett,1984;
				Fowler <i>et al.</i> ,1993)
	dematin (protein 4.9) to actin	actin	RBC	(Siegel and Branton,1985;
				Husain-Chishti <i>et al.</i> ,1988)
	protein 4.1 and glycophorin to p55	protein 4.1	RBC	(Marfatia <i>et al.</i> ,1994;
				Hemming, <i>et al.</i> ,1995;
				Marfatia <i>et al.</i> ,1995)
	ankyrin to vimentin	ankyrin	RBC (avian)	(Georgatos and
				Marchesi,1985; Georgatos <i>et</i>
				<i>al.</i> ,1985)
15	spectrin to IP3	direct	brain, etc	(Hyvonen <i>et al.</i> ,1995; Wang
				and Shaw,1995)

The practice of the present invention thus generally involves the modulation of
 specific interactions between SAATS and the recognized cargo or carrying proteins of the
 ER and Golgi. Also the elucidation of particular binding sites (which are often complex
 and involve homologues of spectrin + adapter proteins) is described in order to identify
 targets for blocking specific interactions. In some instances, the SAATS system can
 discriminate between H,K-ATPase, Na,K-ATPase, CFTR and other transport molecules,
 or can be modulated at the level of binding domains common to various classes of
 proteins.

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For example, identifying the binding domain for CFTR, the gene product responsible for cystic fibrosis, in SAATS by techniques disclosed herein, would allow early analysis of possible transport enhancing agents that might ameliorate the clinical severity of this disorder by enhancing the delivery of mutant (but functional) CFTR to the plasma
5 membrane. In Figures 12 and 22 the binding of CFTR to ankyrin components of SAATS is illustrated as well as its enhanced delivery to the membrane by the $\beta I_{N-5,15}$ construct.

An important discovery that remains under investigation is the actual gene structure of the spectrin(s) that participate in SAATS. Without intending to be bound by the identification of any particular spectrin species, we believe that βI spectrin or
10 homologues of βI spectrin such as βIII spectrin are participants in the SAATS process because they share extensive immunologic and functional similarity to the spectrin of SAATS, and because peptides based on βI spectrin (Winkelmann *et al.*, 1990) are functionally active as discussed below. A discussion of a particular Golgi-associated spectrum called $\beta I\Sigma^*$, so designated because it is still a somewhat uncharacterized
15 putative splice form of βI spectrin, is found in our earlier publication, *i.e.*, Devarajan *et al.*, *JCB* 133:819-830 (1996) and in Beck *et al.* (1994).

The participation of various adapter proteins, other than ankyrin, such as protein 4.1 homologues, adducin homologues, catenin homologues, and others are contemplated. The skilled artisan can explore the role of such adapter proteins by techniques disclosed
20 herein in order to determine their participation in the modulation of the interaction of SAATS with proteins trafficking through the ER and Golgi. The action of similar pathways acting on the delivery of proteins from the medial-Golgi and trans-Golgi to the plasma membrane or other internal membrane-bound compartments is also likely, but remains to be more fully explored with techniques disclosed herein.

25 The methods outlined here offer a novel approach to controlling the activity of specific membrane proteins, be they receptors, adhesion receptors, ion channels, or transporters. While most conventional therapeutic approaches target the activity of a given protein, the approach outlined herein targets the very appearance of a protein at

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the cell surface or in a secretory vesicle. This strategy is somewhat similar in concept to approaches that attempt to specifically suppress the synthesis of a given protein, such as by anti-sense RNA or by specific transcriptional regulators. However, the methods outlined herein are fundamentally different in that they: 1) do not attempt to suppress the
5 synthesis of a given protein, only its delivery to the correct cellular or tissue compartment; 2) do not require intracellular expression of RNA, obviating many hurdles inherent in such a task; and 3) lend themselves to high-throughput *in vitro* screening assays, and should be amenable to regulation by small molecule effectors. As such, the methods outlined here offer a novel therapeutic approach that is complementary to and
10 synergistic with other leading drug development strategies.

As used herein, the following terms have the meanings ascribed:

a. As used herein, *Transport vesicle* means any small membrane bounded structure containing protein and involved in the movement of such protein from one membrane
15 compartment to another.

b. As used herein, *Cell membrane* means a lipid and protein containing bilayer structure bounding any cellular compartment including the surface of the cell, the so called plasma membrane.

c. As used herein, *Adapter protein* means any protein that provides for a direct or
20 indirect linkage to the spectrin backbone of SAATS or to any other multifunctional proteins that directly or indirectly determine the specificity of cargo or cargo protein carrier capture by SAATS.

d. As used herein, an agent is said to be *randomly selected* when the agent is chosen randomly without considering the specific sequences of the vesicular skeletal
25 protein and associated adapter protein. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism or plant extract.

e. As used herein, an agent is said to be *rationally selected* or *rationally designed*

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when the agent is chosen on a nonrandom basis that takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the relevant vesicular skeletal protein or associated adapter protein. For example, a
5 rationally selected peptide agent can be a peptide whose amino acid sequence is identical to a fragment or relevant domain of a spectrin protein.

f. As used herein, "cell" absent any further designation, means any eukaryotic cell population, including primary and transformed immortalized cell lines, that express spectrin, or an equivalent protein. Madin Darby canine kidney (MDCK) cells can be
10 employed in the claimed methods. Transfection of some cell lines with broadly active SAATS inhibitors, such as the βI_{N-5} or βI_{N-2} constructs, may lead to poor cell survival and the activation of apoptosis or lethal swelling. This problem may be often circumvented by selecting less broadly active SAATS agents (eg. larger constructs, or constructs in which only small portions of spectrin, ankyrin, or one of the adapter proteins has been deleted or
15 modified). This strategy of scanning mutagenesis will minimize the global disruption of cellular transport that accompanies the most severe disruptors of SAATS, and improve cell viability so that determinations can be made on less hardy cell lines. This technique also has the advantage of more precisely pinpointing the locus of SAATS activity for a given protein. Transfection levels may also be modified or controlled to manage poor
20 cell survival.

If the induction of apoptosis becomes a problem with some broadly active SAATS constructs, improvement in the survivability of the cell based assays may be accomplished by the inclusion of available apoptosis inhibitors in the cell medium, such as caspase inhibitors or Fas blocking antibodies (see U.S. Patents 5,632,994, 5,656,725,
25 5,650,491 and 5,635, 187). Cells may also be engineered to express increased levels of *bcl-2* (see U.S. Patent 5,650,491).

g. As used herein, "stringent conditions" refers to conditions those commonly defined and available, such as those defined by Sambrook et al (*Molecular Cloning: A*

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Laboratory Approach, Cold Spring Harbor Press, NY, 1989) or Ausubel *et al.* (*Current Protocols in Molecular Biology*, Greene Publishing Co., NY, 1995).

Hybridization is a function of sequence identity (homology), G+C content of the sequence, buffer salt content, sequence length and duplex melt temperature (T_m) among other variables. See, Maniatis *et al.* (*Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1982). With similar sequence lengths, the buffer salt concentration and temperature provide useful variables for assessing sequence identity (homology) by hybridization techniques. For example, where there is at least 90 percent homology, hybridization is commonly carried out at 68° C. in a buffer salt such as 6XSSC diluted from 20XSSC. See Maniatis *et al.* The buffer salt utilized for final Southern blot washes can be used at a low concentration, e.g., 0.1XSSC and at a relatively high temperature, e.g. 68° C., and two sequences will form a hybrid duplex (hybridize). Use of the above hybridization and washing conditions together are defined as conditions of high stringency or highly stringent conditions. Moderately high stringency conditions can be utilized for hybridization where two sequences share at least about 80 percent homology. Here, hybridization is carried out using 6XSSC at a temperature of about 50-55° C. A final wash salt concentration of about 1-3XSSC and at a temperature of about 60-68° C are used. These hybridization and washing conditions define moderately high stringency conditions.

In particular, specific hybridization refers to conditions in which a high degree of complementarity exists between a nucleic acid comprising the sequence of at least one of the Figures and another nucleic acid. With selective hybridization, complementarity will generally be at least about 75%, 80%, 85%, preferably about 90-100%, or most preferably about 95-100%.

25

I. General Description

The present invention relates to a method for selectively controlling the transport of integral membrane and secretory proteins by controlling their sequestration into transport

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vesicles that mediate protein trafficking between the ER, Golgi, plasma membrane, and other membrane compartments. The present patent application expands on the discovery that specific isoforms of spectrin and various adapter proteins, such as ankyrin, associate with vesicle and Golgi membranes by multiple interactions (Devarajan *et al.*, 1994; 5 Devarajan and Morrow, 1995; Devarajan and Morrow, 1996; Devarajan *et al.*, 1996a; Devarajan *et al.*, 1996b; Devarajan *et al.*, 1997; Godi *et al.*, 1997). These interactions are necessary for the transport of broad classes (and possibly all) membrane and secretory proteins from the ER through the intermediate compartment to the *medial*-Golgi (Devarajan, *et al.*, 1996a; Devarajan, *et al.*, 1997; Godi, *et al.*, 1997). Moreover, by 10 modifying a specific interaction site within spectrin or in one of its associated adapter proteins, the transport and ultimately the rate of delivery of a specific protein to its final membrane target can be significantly altered or even blocked.

We call this newly discovered system mediating transport the spectrin-ankyrin-adapter protein trafficking system (SAATS). Since all membrane and 15 secretory proteins transit this pathway, and must be selectively and sequentially collected into different transport vesicles during their journey from the ER to the plasma membrane (or to other membrane compartments), this system has wide potential applicability and generality. We contemplate that the great diversity of proteins originating in the ER is accommodated by SAATS by a combinatorial process involving many weak but specific 20 interactions created by the polyvalent binding capacity of spectrin and ankyrin and other adapter proteins, as well as by the pairing of potentially non-binding proteins in oligomeric complexes with binding proteins. The control of secretory protein trafficking, *via* recognized cargo loading proteins resident in the ER, we envision also to be mediated by this pathway, in which the cargo loading membrane proteins bind to the 25 spectrin/adapter protein system.

Thus, by selectively controlling the binding of a specific membrane protein, or the binding of a specific set of cargo proteins to SAATS, the delivery of that specific protein into transport vesicles will be modified (blocked or enhanced), as will the

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delivery of that protein (but not other proteins) to the plasma membrane (or other targeted membrane compartment). This SAATS system and its functions are illustrated in Fig. 1.

We also believe that similar but distinct SAATS systems operate to mediate protein transport between the ER and Golgi, as well from the Golgi to the plasma membrane or other target (*i.e.*, post-Golgi). Therefore, the methods described here apply as well to the process of targeted membrane delivery post Golgi. An example of such a process would be spectrin linked microtubule mediated axonal transport of specific neuronal receptors destined for the pre or post-synaptic membranes.

In general, several methods are available for controlling the binding of selective proteins (or lipids) to SAATS using methods and technologies known in the biomedical field but heretofore applied for other purposes.

a) Using recombinant cDNA methodology, for example, the expression of β I spectrin related peptides and homologues from other spectrin related genes that preserve the MAD1 and actin binding motifs (Lombardo *et al.*, 1994) of β I spectrin, but delete its ankyrin binding domain (which binds Na,K-ATPase) (Kennedy *et al.*, 1991), selectively blocks the delivery of Na,K-ATPase to the plasma membrane (Devarajan, *et al.*, 1996a; Devarajan, *et al.*, 1997). Because other proteins participate in the SAATS transport processed, this opens the possibility of gene therapeutic approaches to modulating SAATS function in order to introduce genes that encode modified spectrin molecules that either inhibit or enhance the sequestration of selected membrane and secretory proteins into transport vesicles and, ultimately, their display on or secretion through the plasma membrane.

b) Certain polyclonal and monoclonal antibodies to spectrin block the transport of Na,K-ATPase (Figure 18) or VSV-G protein (Harris *et al.*, 1986; Devarajan, *et al.*, 1996a; Godi, *et al.*, 1997). While of limited therapeutic interest since such agents generally act extracellularly, we envision such agents as important tools for use in *in vitro* transport assays and in immunomicroscopy.

c) Inhibitors of ADP-ribosylation factor (ARF) block the binding of the

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PH domain of β I spectrin *via* a PtdInsP₂-dependent process, implying that SAATS may also contribute to the organization and trafficking of specific phospholipids. Agents modulating PtdInsP₂ phospholipid display, or ARF activity directly, may also thus form the basis for a class of therapeutics. An examples of such an agent would be a permeant
5 non-hydrolyzable homologue of GTP, such as a permeant GTP- γ -S.

d) We have prepared recombinant peptides representing specific functional domains of β I spectrin (Kennedy, *et al.*,1991; Lombardo, *et al.*,1994; Weed *et al.*,1996) that also block transport (see figures, also Godi, *et al.*,1997) of selected integral membrane and secretory proteins. These demonstrate the potential activity of small
10 peptide or peptide mimetic drugs.

e) Many of the interactions involving SAATS, based on our understanding of the spectrin skeleton that underlies the plasma membrane (for review, see Morrow *et al.*,1997), are likely to be subject to post-translational regulation by pathways involving protein kinase C, Ca⁺⁺, calmodulin, casein kinase, etc. Agents that therefore modulate
15 these regulatory pathways, while less specific, may also form the basis of potential SAATS oriented therapeutics.

f) We envision that small molecule modulators of SAATS specific interactions will form the basis for a broad and effective category of therapeutics. Such small molecule agents would either block or enhance the delivery of specific proteins to
20 the plasma membrane or other sites. We have developed several *in vitro* assays that detect specific interactions between SAATS and other proteins, based on *in vitro* binding, surface plasmon resonance, and genetic screening (two-hybrid systems). Many of these *in vitro* assays are amenable to high-throughput screening, facilitating the identification of lead compounds. We have also developed sophisticated algorithms that allow us to
25 model the three-dimensional structure of certain spectrin repeat units, together with the variant sequences that mediate specific binding interactions (Cianci and Morrow, 1997; Gallagher *et al.*,1997; Stabach *et al.*,1997). We envision that the availability of these sophisticated 3-D protein models will facilitate rational drug design initiatives.

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The specific examples presented below are illustrative only and are not intended to limit the scope of the invention.

II. Specific Embodiments

5 The following discussion describes specific embodiments of our invention and provides procedures for: (A) identifying integral membrane and secretory proteins whose vesicular transport to or through the Golgi is mediated by the SAATS or analogous systems; (B) identifying which vesicular skeletal protein, *i.e.*, spectrin, or associated adapter proteins, *i.e.*, ankyrin, are specifically involved in mediating
10 sequestration of particular integral membrane and secretory proteins into a transport vesicle; (C) identifying peptides and small molecules that modulate sequestration and/or vesicle transport of such proteins; and (D) utilizing agents that modulate the sequestration and/or vesicular transport of such proteins for therapeutic and research purposes.

15 **EXAMPLES**

***Example 1:* Identification of integral membrane and secretory proteins whose vesicular transport is mediated by SAATS**

20 The identification of broad inhibitors of SAATS may generally be approached by comparing the ability of a series of overlapping and complimentary spectrin peptides to disrupt the transport in cultured cell systems of any particular integral membrane or secretory protein. This information allows identification of a minimal region within spectrin that is responsible for the cryptic sorting and docking of the targeted protein to the SAATS system. Other potential blocking agents also follow from knowledge of
25 adapter proteins that bind to this region of spectrin, as discussed below. The following steps are preferred.

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Step A1: Select a model cultured cell line that expresses the membrane or secretory protein that is being evaluated, and for which an assay exists that will allow the detection of surface or secreted target proteins. Examples would be immunofluorescent detection of surface protein; flow cytometry; surface labeling assays, or transport assays using pulsed ^{35}S methionine labeling. Assays that monitor the transport of a specific protein may also be performed by the use of GFP - labeled proteins and time lapse vital fluorescent microscopy. Also envisioned is the detection of a specific transport or electrical activity associated with the surface display or secretion of the targeted protein. As a specific example, cultured Madin Darby Canine Kidney cells (MDCK) may be used to monitor the surface expression of Na,K-ATPase by indirect immunofluorescence (See Figs 3 through 10, 18).

Step A2: Prepare using standard transfection methods of the model cell line a series of individual clonal lines or lines transiently transfected or lines infected using retroviral vectors or other virus based infection strategies, each expressing one of a complimentary and overlapping series of $\beta\text{I}\Sigma^*$ spectrin cDNA constructs of varying lengths under the control of a strong eukaryotic expression vector. A series of suitable $\beta\text{I}\Sigma^*$ spectrin constructs is listed in Figure 2. For example, stable MDCK cell lines expressing the $\beta\text{I}_{\text{N-5}}$ spectrin peptide (See Figs. 3 and others), which competitively displaces or substitutes for the endogenous SAATS system, as one appropriate line.

The transformation of appropriate cell hosts with an rDNA (recombinant DNA) molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*, Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment

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methods are typically employed. See, for example, Graham *et al.*, *Virol* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

5 Successfully transformed cells, *i.e.*, cells that contain an rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method. If tags
10 such as green fluorescent protein are employed in the construction of the recombinant DNA, the transfected cells may also be detected *in vivo* by the fluorescence of such molecules by cell sorting.

Step A3: Evaluate each clonal line or cells expressing the desired
15 construct (in assays in which transient expression is used, *e.g.* Figure 9) for disruption of the surface display or secretion of the selected protein. Expression of full length $\beta I\Sigma^*$ spectrin (or other full length spectrins) will not disrupt SAATS. Expression of at least one of the truncated constructs shown in Figure 2 or analogous constructs for other spectrins and other vesicular skeletal proteins will act in a dominant negative way to
20 disrupt SAATS. A comparison of the inhibitory ability of different constructs will reveal the boundaries of an inhibitory peptide or cDNA construct encoding such inhibitory peptide. For example, inhibition of surface display of Na,K-ATPase in MDCK cells by expression of the $\beta I_{N,5}$ spectrin construct (Fig. 3).

25 Step A4: Knowledge of the minimal inhibitory peptide can be further refined by repeating steps A2 and A3, above, using $\beta I\Sigma^*$ spectrin constructs or similar constructs prepared from other spectrins in which specific regions deleted in the inhibitory construct are returned to the inhibitory construct, thereby identifying the

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regions required to rescue surface display or secretion of the selected (targeted) protein. By this approach, minimal sequences required for docking the targeted protein to SAATS can be identified.

For example, the surface display of Na,K-ATPase in MDCK cells, which
5 is inhibited by βI_{N-5} , is rescued by attaching βI spectrin repeats 14 and 15 to the βI_{N-5} construct (See Fig. 6 and others). These results indicate that βI spectrins, 14-15 repeat region is the site of docking (either directly or indirectly) of Na,K-ATPase to SAATS.

Step A5: A further refinement of the SAATS docking site for the targeted protein
10 may be obtained by determining whether it attaches directly to spectrin at the site identified in Steps A1-A4, or whether an intermediary adapter protein is utilized. In most instances, this can be readily determined by *in vitro* binding or co-immunoprecipitation assays (eg Figure 3), in which the interaction of the targeted protein with spectrin and/or adapter proteins (such as ankyrin, protein 4.1, adducin, α -catenin, see TABLE IB) is
15 evaluated. Such methods are well known in the art as exemplified by Harlow *et al.*, 1988.

For example, the interaction of Na,K-ATPase with the 14-15 repeat unit of spectrin is *via* an ankyrin G119 adapter protein intermediate, as detected by co-immunoprecipitation (Figure 3), and by direct binding assays indicating that ankyrin
20 binds to this region of spectrin (Kennedy *et al.*, 1991) and that Na,K-ATPase binds to ankyrin (Morrow *et al.*, 1989) and at a specific locus within Na,K-ATPase (Devarajan, *et al.*, 1994).

Preparation of Spectrin and associated cDNA constructs

25 Recombinant peptides are prepared as fusion proteins with glutathione-S-transferase, using the prokaryotic expression vector pGEX (Smith and Johnson, 1988; Kennedy *et al.*, 1991). Spectrin clones used for these are identical to sequences published for βII spectrin (Hu *et al.*, 1992; Chang *et al.*, 1993; Chang and

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Forget, unpublished), β I spectrin (Winkelmann *et al.*, 1988; Winkelmann *et al.*, 1990b; Winardi *et al.*, 1993), and β III spectrin as shown in figure 19. In general, the desired segment of cDNA is excised by EcoRI or other suitable restriction enzymes from pGEM 7z(-), purified from agarose gels by electroelution and subcloned into the appropriate

5 pGEX vector, usually pGEX-2T. In the examples shown (Figure 2 and (Kennedy, *et al.*, 1991; Kennedy *et al.*, 1994; Lombardo, *et al.*, 1994b)) the polymerase chain reaction was typically used in conjunction with Pfu polymerase and selected primers to amplify regions of the spectrin clones to facilitate plasmid construction and in some cases linker-insertion mutagenesis (Sambrook *et al.*, 1989; Kennedy, *et al.*, 1991). Occasionally

10 Taq polymerase was used to facilitate cloning of PCR products into the pCRII vector of the TA cloning kit (Invitrogen). The validity of all PCR generated constructs is confirmed by DNA sequencing. In general, it is useful but not required if PCR primers are engineered to have BamHI sites 5' and EcoRI sites 3' to ease directional subcloning into pGEX-2T. Two to five additional bases were usually added at the ends of each

15 primer to enhance their susceptibility to restriction endonucleases. The amino acid boundaries for the amplification of the spectrin structural repeats are selected based upon the phasing of *Drosophila* α -spectrin (Winograd *et al.*, 1991). Peptides representing the unique alternatively spliced COOH-terminal region of the β I spectrin found in brain and muscle are prepared from plasmid pMSP6AC (Winkelmann, 1990) by digestion with

20 Xma I. The resulting 767 bp fragment containing the alternatively spliced unique C-terminus of spectrin β ISII is gel purified and subcloned into pGEX-2T at the Sma I site. Other typical details of primer selection and plasmid construction are summarized as in Kennedy, *et al.*, 1991; Lombardo, *et al.*, 1994b. The various β I spectrin peptides are expressed as above from existing human β I spectrin constructs (Winkelmann *et*

25 *al.*, 1990a; Winkelmann, *et al.*, 1990b; Weed *et al.*, 1996). Constructs for eukaryotic cell expression are typically prepared in the pcDNA3- vector (Invitrogen) or other comparable vector incorporating a suitable eukaryotic promoter. With the pcDNA3 vector, stable MDCK cell lines can be established by selection with G418 after

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lipofectamine transfection (Weed, *et al.*,1996). Transient experiments can also be carried out in this system or in several other systems well suited for transient assays, such as COS cells. For work with VSV-G, newly confluent cells are infected for 60 min. with VSV-G in Transwell® filters (100 ml, 108 titer virus Pimplikar *et al.*,1994). Infected cells
5 are incubated for 3 hours and fixed prior to analysis.

Protein expression and purification

Recombinant proteins are expressed in *E. Coli* strains HB101, DH5a or the protease deficient strain CAG-456 and purified as before (Smith and Johnson,1988;
10 Kennedy, *et al.*,1991). When required, peptides were further purified by gel filtration on a 1 x 100 cm column of Sephacryl S-200 HR in the presence of 1 M NaBr, 10 mM Na₂HPO₄, 0.5 mM EGTA, 0.5 mM EDTA, 15 mM sodium pyrophosphate, 1 mM NaN₃, 0.5 mM DTT, pH 8.0. Purified proteins were dialyzed into 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaN₃, 1 mM DTT, 0.2 mM PMSF, 25% glycerol, pH 7.5 and stored
15 at -80 °C. Bovine brain spectrin is prepared from fresh brain membranes by low ionic strength extraction of demyelinated brain membranes followed by gel filtration on Sephacryl-S-500 HR (Harris, *et al.*,1985; Bennett,1986) .

Antibodies and immunodetection procedures.

20 Constructs epitope labeled by the 8 aa FLAG marker (IBI Scientific) were detected with Mab M5 (IBI Scientific); β I spectrin antibodies included: Mab VIIIC7, Mab VD4, Mab IVC9, and Mab IVF8 (Harris, *et al.*,1986); Pab C19 against 19 COOH-terminal residues of β I Σ 1 spectrin (gift, V. Marchesi); and Pab MUS1 against region III of β I Σ 2 spectrin (Malchiodi-Albedi *et al.*,1993). Pab 10D detected β II spectrin
25 (Devarajan, *et al.*,1996). Other antibodies were: Mab β -COP, I. Melman; Pab E-cadherin, Transduction labs; Mab α -Na,K-ATPase, UBI; Mab β -Na,K-ATPase, M. Caplan; Mab VSV-G, J. Rose; and Pab centractin, E. Holzbaur. Cells were grown on glass cover slips or culture dishes, washed x3 with PBS, fixed 15 min. in acetone, blocked 30 min. in goat

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serum, and incubated with 1° antibody in 2% BSA in PBS + 10% goat serum at RT for 60 min. All spectrin Mab's were used at 1:100; E-cadherin at 1:5000; b-COP, 10D, C19 at 1:200. Detection used 2° antibodies conjugated to CY3 or CY2 (Vector).

5 *Immunoprecipitations.*

Confluent MDCK cells were PBS washed, lysed 20 min. at 4°C by gentle rocking in IP buffer (10 mM Tris-HCl, pH 7; 150 mM NaCl; 5mM EDTA; 1mM EGTA; 2 mg/ml BSA; 0.5% deoxycholate; 1% NP-40; 0.5 mM pefablock; 1mM PMSF; and 1mM leupeptin. The lysate (2 ml) was decanted and centrifuged 1 min. at 10,000xg, 10 precleared by 60 min. incubation with 25 ml of non-immune rabbit serum with 200 ml of a 50% Protein A Sepharose (Pharmacia). The remaining supernatant was treated 4 hours at 4° with 200 ml of 1° antibody, pelleted by Protein A Sepharose, and analyzed.

15 **Example 2: Identification of Na,K-ATPase as an integral membrane protein participating in SAATS**

In wild type MDCK cells, Golgi spectrin and Ank_{G119} are localized with the Golgi, while α -Na,K-ATPase is predominately distributed at the plasma membrane where it is tethered *via* the plasma membrane form of ankyrin to α II β II spectrin (Morrow, *et al.*, 1989; Nelson and Hammerton, 1989) (Figure 3).

20 To determine the intracellular distribution of Golgi spectrin, Ank_{G119}, and α -Na,K-ATPase, cells were grown on glass cover slips or culture dishes, washed x3 with PBS, fixed 15 min. in acetone, blocked 30 min. in goat serum, and incubated with 1° antibody in 2% BSA in PBS + 10% goat serum at RT for 60 min. The intracellular distribution of wt Golgi spectrin was monitored using Mab VIIIC7, which reacts with full 25 length Golgi spectrin but not the shorter FLAG tagged β I_{N-5} construct (Harris *et al.*, 1985; Devarajan, *et al.*, 1996; Godi, *et al.*, 1997). The distribution of β I Σ * spectrin in cells transfected with β I_{11-C} was monitored by Mab VD4, which does not react with the β I_{11-C} peptide. AnkG119 was monitored by the antibody "Jasmin" (Devarajan, *et al.*, 1996);

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α -Na,K-ATPase by a Mab from Upstate Biotechnology. Detection was by indirect immunofluorescence.

Electron microscopy was performed by fixing cell monolayers *in situ* for 1 hr (Karnovsky's fixative 18). Fixed monolayers were washed in 0.1 M Na-cacodylate, pH 7.4, and postfixed in 1% OsO₄ in 0.1M s-collidine. After dehydration in graded ethanol and washing with propylene oxide, samples were embedded in Epox-812 (Ernest Fullman, Inc. N.Y.). Ultrathin sections stained with aqueous uranyl acetate and lead citrate were viewed on a Zeiss EM-910 at 80 kV.

In Figure 3A, the results of the indirect-immunofluorescence labeling are presented. Note the dispersal of the endogenous Golgi spectrin, Ank_{G119}, and α -Na,K-ATPase by β I_{N-5} (but not by β I_{11-C}). All shortened spectrin constructs containing region I and MAD1 displayed a similar effect, whereas constructs lacking these regions of β I spectrin did not significantly alter the native distributions of β I Σ^* spectrin, AnkG119, or Na,K-ATPase. Bar = 10 μ .

The distribution of the endogenous Golgi spectrin-ankyrin skeleton, as well as membrane assembly of Na,K-ATPase, was disrupted by β I spectrin constructs that contained the region I/MAD1 Golgi targeting signal. (Fig. 3A). In the presence of such peptides, endogenous Golgi β I Σ^* spectrin and Ank_{G119} were dispersed into coarse punctate cytosolic patches. (Figs. 3A & 4A). Even more dramatic is the effect of these peptides on the distribution of Na,K-ATPase, which becomes widely dispersed throughout the cytoplasm in a pattern resembling the endoplasmic reticulum, with very little protein identifiable at the cell surface (Figs 3A, 4A). The β I_{N-5} expressing cells are also swollen compared to control cells (mean diameter 29 $\pm$ 6.7 μ m vs. 17 $\pm$ 3.7 μ m), and grow more slowly presumably due to insufficient plasma membrane Na,K-ATPase (or other membrane proteins, see below). This effect on Na,K-ATPase distribution is not evident with constructs lacking region I/MAD1 (*e.g.* β I_{11-C}, Fig. 3A), nor is it apparent in cells expressing full length β I Σ 1 spectrin (that contains the ankyrin-Na,K-ATPase binding domain but lacks MAD2 and PtdInsP₂-dependent Golgi binding).

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To determine the means of interaction between Na,K-ATPase and spectrin, co-immunoprecipitation studies are performed. Briefly, confluent MDCK cells were PBS washed and lysed 20 min. at 4°C by gentle rocking in IP buffer (10 mM Tris-HCl, pH 7; 150 mM NaCl; 5mM EDTA; 1mM EGTA; 2 mg/ml BSA; 0.5% deoxycholate; 1% NP-40; 0.5 mM pefablock; 1mM PMSF; and 1mM leupeptin. The lysate (2 ml) was decanted and centrifuged 1 min. at 10,000xg, precleared by 60 min. incubation with 25 ml of non-immune rabbit serum with 200 ml of a 50% Protein A Sepharose (Pharmacia). The remaining supernatant is treated 4 hours at 4° with 200 ml of 1° antibody, pelleted by Protein A Sepharose, analyzed by Western blots after SDS-PAGE and visualized by ECL. The co-immunoprecipitation studies demonstrate that the interaction of Na,K-ATPase with the 14-15 repeat unit of spectrin occurs *via* an ankyrin G119 adapter protein intermediate (see Figure 3B). In wt cells, $\beta I\Sigma^*$ spectrin, AnkG119, centractin, and Na,K-ATPase are co-precipitated from detergent lysates by Mab VIIIC7 (lane IP). Soluble $\alpha II\beta II$ spectrin (as detected here by immunoblotting for αII spectrin), the form found predominately at the plasma membrane, is also present in these detergent lysates (lane lys) but is not part of the precipitable Golgi spectrin complex. Control experiments with preimmune or irrelevant antibody did not precipitate any of these components. Similarly, immunoprecipitates with Mab VIIIC7 of the βI_{N-5} transfected cells also demonstrated co-precipitation of Ank_{G119}, and Na,K-ATPase with $\beta I\Sigma^*$ spectrin, indicating that despite the dispersal of these elements induced by the βI_{N-5} construct, these elements remained associated in the detergent lysate.

As exemplified by Figure 3B, immunoprecipitation with Mab VIIIC7 reveals that a fraction of α -Na,K-ATPase exists in a complex with $\beta I\Sigma^*$ spectrin, Ank_{G119}, and centractin (Fig. 3B). Centractin (ARP-1) is an actin related protein that is part of the dynactin complex. This pool of α -Na,K-ATPase associated with $\beta I\Sigma^*$ spectrin, Ank_{G119}, and centractin appears to be distinct from the pool of plasma membrane α -Na,K-ATPase, since αII spectrin was not present in these complexes (Fig. 3B), and presumably represents Na,K-ATPase not yet assembled at the plasma membrane.

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Na,K-ATPase is blocked at the ER to medial-Golgi transition by βI_{N-5} spectrin

Normally, after synthesis and core glycosylation in the ER, β -Na,K-ATPase undergoes further glycosylation in the medial Golgi and then is vectorally incorporated as an α , β -Na,K-ATPase heterodimer into a detergent insoluble α II β II spectrin and ankyrin cortical skeletal lattice (Nelson and Veshnock, 1986; Morrow, *et al.*, 1989; Nelson and Hammerton, 1989; Mays *et al.*, 1995). The distribution of Na,K-ATPase in the βI_{N-5} expressing cells suggested that the exit of Na,K-ATPase from the ER might be blocked (Fig. 4A).

Western blotting confirmed that the spectrin βI_{N-5} polypeptide blocked Golgi mediated glycosylation of β -Na,K-ATPase (Fig. 4B). Control MDCK cells (wt) or cells transfected with the βI_{N-5} construct (βI_{N-5}) were analyzed by Western blot using a β -Na,K-ATPase specific antibody (Mab B1-13). The apparent MW of the various products are depicted. Note the broad band above 50 kDa (gly) representing mature glycosylated β -Na,K-ATPase, and the intense band at 44 kDa in the βI_{N-5} cells (core), representing the ER dependent core β -Na,K-ATPase glycosylation product. It thus appears that the βI_{N-5} spectrin peptide inhibits mature glycosylation of β -Na,K-ATPase, a process characteristic of medial Golgi processing, but does not interfere with the formation of the core glycosylation product, which occurs in the ER. In this experiment, approximately half of the βI_{N-5} cells are expressing the βI_{N-5} construct, as judged by immunofluorescence, which appears to account for most of the mature glycosylated product observed in the βI_{N-5} cell lines. The band $\approx$ 106 kDa in the wild type cells (*) is inconstant in its appearance, and may represents a cross-linked adduct of β -Na,K-ATPase with α -Na,K-ATPase or other protein, as is commonly observed after reduced SDS-PAGE in MDCK cells (Morrow *et al.*, 1989).

While these experiments did not seek to quantitatively measure the degree of transport inhibition, the results of several separate determinations suggest that the efficiency of β -Na,K-ATPase transport inhibition by the βI_{N-5} peptide is quite high, since the level of processed β -Na,K-ATPase that can be detected correlates inversely with the

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fraction of cells in these cultures that actually express the recombinant peptide. Also blocked in these cells was the transition of α -Na,K-ATPase to detergent insolubility (Fig. 4C), indicating a failure of Na,K-ATPase to join a stable plasma membrane associated spectrin lattice. Shown are Western blots for α -Na,K-ATPase after SDS-PAGE. Fx1 (soluble fraction) is that material solubilized by 0.5% Triton X-100 in 100 mM NaCl; Fx2 (cytoskeletal fraction) is the material solubilized by 0.5% Triton X-100 and 250 mM NH_4SO_4 (Devarajan *et al.*, 1994). The presence of the βI_{11-15} polypeptide, with its competent ankyrin binding domain but lacking region I/MAD1 sequences (*cf.* Fig 2C), only marginally impacted the assembly of Na,K-ATPase into a detergent insoluble skeleton. Collectively, these data indicate that truncated βI spectrin peptides containing the Golgi targeting signal, but lacking downstream sequences including the ankyrin/Na,K-ATPase binding domain, block the ER to medial Golgi transport of both subunits of Na,K-ATPase.

15 *Na,K-ATPase interaction with SAATS occurs via an adapter protein*

As set forth above, co-immunoprecipitation of wild-type cells with antibodies to βI spectrin precipitate complexes containing spectrin, ANKG119, centractin, and Na,K-ATPase (Figure 3). Independent studies using *in vitro* assays have determined that α -Na,K-ATPase binds directly to ankyrin (Morrow, *et al.*, 1989; Devarajan, *et al.*, 1994), and that a small 25 residue region of Na,K-ATPase, codons 142 to 166, accounts for most of this binding activity (this region is termed MAB, for minimal ankyrin binding domain) (Figure 8) (Zhang, *et al.*, 1997a). Other studies have also established that ankyrin binds to the 15th repeat unit of spectrin (Kennedy, *et al.*, 1991).

The requirement for ankyrin binding to SAATS in Na,K-ATPase transport can then be demonstrated by i) showing that the ankyrin binding domain of spectrin is required for transport, as evidenced by the rescue of Na,K-ATPase delivery to the plasma membrane in cells carrying $\beta\text{I}_{\text{N-5,15}}$ spectrin construct (Figure 2) in which the ankyrin binding domain has been returned to the otherwise $\beta\text{I}_{\text{N-5}}$ peptide which blocks SAATS

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mediated transport of Na,K-ATPase (Figure 6); and, ii) by showing that the removal of the specific ankyrin binding sequence from Na,K-ATPase renders the protein incapable of being transported from the ER (Figure 9). Further proof of the importance of this linkage to an adapter protein may also be obtained by inhibiting its transport selectively
5 by the expression (or micro-injection) of specific inhibitory peptides that block the interaction of (in this case) α -Na,K-ATPase with ankyrin (Figures 10, 18).

In Figure 6, MDCK cells were transfected with either βI_{N-5} as above, or a with $\beta I_{N-5,15}$. This latter construct incorporates the ankyrin binding domain of spectrin into the βI_{N-5} peptide. Note the complete restoration of Na,K-ATPase transport, as measured in
10 this case by its surface display and secondarily by a reduction in cell size (which is a consequence of cell swelling due to a deficiency of plasma membrane Na,K-ATPase in the βI_{N-5} transfected cell.

In Figure 9, MDCK cells were transiently transfected with either wild-type Na,K-ATPase to which a FLAG epitope tag had been added to its NH2-terminus, or by a
15 similarly FLAG-tagged mutant Na,K-ATPase in which codons 142 to 166 had been deleted. These residues correspond to the minimal ankyrin binding domain (MAB) identified in Figure 8. In both transfections, the cells were also co-transfected with wt β -Na,K-ATPase, so as to assure sufficient β -Na,K-ATPase to pair with the α -Na,K-ATPase. (top) Flag tagged wt-Na,K-ATPase is delivered normally to the plasma
20 membrane. (bottom) Flag tagged Na,K-ATPase lacking MAB does not assemble at the plasma membrane, and eventually is targeted for degradation in lysosomes. In these cells, this is the only protein whose transport apparently is disrupted.

25

Example 3: Identification of VSV-G as an integral membrane protein participating in SAATS

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Infection with vesicular stomatitis virus demonstrated that VSV-G transport to the plasma membrane, which is packaged exclusively by the COPII coat (see review Schekman and Orci,1996), like Na,K-ATPase, was blocked by the βI_{N-5} spectrin construct (Fig. 5E,F). The pattern of VSV-G staining observed suggests a block prior to the

5 intermediate compartment. Wild-type (B,D,F,H) and βI_{N-5} transfected cells (A,C,E,G) are shown. The localization of VSV- G protein (E,F) was measured after transient infection. E-cadherin (C,D) was monitored with a Mab from Transduction labs, and also by the extent to which the precursor peptide was proteolyzed (inset, Western blot) from 135 kDa (*) to 120 kDa (EC) (a process that occurs in the trans-Golgi/Shore and

10 Nelson,1991). There was no significant difference in the extent of E-cadherin processing or its level of assembly at the plasma membrane in the βI_{N-5} line vs. wt cells. Despite the disruption of Na,K-ATPase and VSV-G transport (and the wt Golgi spectrin skeleton, Fig. 3), the Golgi appears to remain largely intact as measured by the distribution of β -COP (A,B) and by the presence of normal appearing juxtannuclear Golgi structures in

15 uranyl acetate and lead stained electron microscopy (G,H) (arrows). Bar = 10 μ in (A-F), 0.5 μ in (G,H). Original magnification (G,H) = 63,000x.

Unlike α -Na,K-ATPase, VSV-G protein is transported by SAATS without using ankyrin as its adapter protein, as illustrated by its failure to be rescued by return of the spectrin ankyrin binding domain (repeat 15) to the inhibitory βI_{N-5} construct (Figure 7).

20 MDCK cells infected with vesicular stomatitis virus were used to monitor the trafficking of VSV-G protein. Newly confluent cells are infected for 60 min. with VSV-G in Transwell® filters (100 ml, 108 titer virus Pimplikar *et al.*,1994). Infected cells are incubated for 3 hours and fixed prior to analysis. Mab VSV-G (J. Rose) was used as the primary antibody in 2% BSA in PBS + 10% goat serum at room temperature for 60

25 minutes after blocking with goat antiserum for 30 minutes. The transport of this protein is normally to the basolateral membrane, similar to that for Na,K-ATPase (as shown in the wt cells and in Figure 5). This transport is blocked by SAATS inhibition with the βI_{N-5} construct, as is Na,K-ATPase (cf. Figure 6). However, unlike Na,K-ATPase,

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VSV-G trafficking is not restored by the inclusion of the ankyrin binding domain of spectrin (repeats 14-15) in the construct. Thus, it is possible to selectively block VSV-G transport without affecting Na,K-ATPase transport.

5 **Example 4: Determination that E-cadherin sequestration is not blocked by βI_{N-5} spectrin**

E-cadherin, another type I basolaterally targeted membrane protein was assayed for its interactions with SAATS. Wild-type (Figure 5D) and βI_{N-5} transfected cells (Figure 5C) were assayed for the distribution of E-cadherin. E-cadherin (C,D) was
10 monitored with a Mab from Transduction labs as the primary antibody in 2% BSA in PBS + 10% goat serum at room temperature for 60 minutes after blocking with goat antiserum for 30 minutes., and also by the extent to which the precursor peptide was proteolyzed (inset, Western blot) from 135 kDa (*) to 120 kDa (EC) (a process that occurs in the trans-Golgi Shore and Nelson,1991). There was no significant difference in
15 the extent of E-cadherin processing or its level of assembly at the plasma membrane in the βI_{N-5} line vs. wt cells. Despite the disruption of Na,K-ATPase and VSV-G transport (and the wt Golgi spectrin skeleton, Fig. 3), the Golgi appears to remain largely intact as measured by the distribution of β -COP (A,B) and by the presence of normal appearing juxtannuclear Golgi structures in uranyl acetate and lead stained electron microscopy
20 (G,H) (arrows). Bar = 10 μ in (A-F), 0.5 μ in (G,H). Original magnification (G,H) = 63,000x., was fully expressed at the plasma membrane (Fig. 5C,D).

Western blot analysis of E-cadherin processing, which involves a protease cleavage in the *trans*-Golgi compartment that reduces the precursor protein from 135 to 120 kDa (Shore and Nelson,1991), could detect no increase in uncleaved E-cadherin
25 pools (Fig. 5D inset). Thus, unlike Na,K-ATPase and VSV-G, the transport of E-cadherin was not blocked at the ER to Golgi transition, indicating that its attachment to SAATS occurs within the βI_{N-5} peptide, or else is assembled by a separate pathway.

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Example 5: Identification of $\alpha 5$, αV , $\beta 3$, and βI Integrins as integral membrane proteins participating in SAATS in endothelial cells.

A rapid way to evaluate the participation of a variety of integral membrane proteins in SAATS trafficking is to use flow cytometry to monitor their surface display in cells transfected with the various constructs shown in Figure 2, or related constructs. Examples of this approach are shown in Figures 13 and 14. Note, that in Figure 14, another somewhat more severe blocker of SAATS function, the βI_{N-2} peptide, was used with comparable results

In Figure 13, SV40 transformed murine endothelial cells were transfected with βI_{N-5} or $\beta I_{N-5,15}$, and the surface display of three different integrins was measured by flow cytometry. Note the blockage of alpha V, beta 3, and beta 1 integrins by the βI_{N-5} peptide. Conversely, the $\beta I_{N-5,15}$ peptide markedly enhances the surface display of alpha V and beta 1 integrin (but not beta 3 integrin). This enhanced display (as opposed to blockage) is presumably achieved by enhancing the efficiency of SAATS directed cargo loading of vesicular transport by choosing peptides or agents that enhance the binding of a given membrane protein to SAATS.

In Figure 14, experiments were carried out as in Figure 13. SV40 transformed murine endothelial cells were transfected with βI_{N-5} , $\beta I_{N-5,15}$, or $\beta I_{N-2,15}$ and the surface display of three different integrins was measured by flow cytometry. Note that alpha-V integrin is also modulated by SAATS. Also note that in this example, $\beta I_{N-2,15}$ was used to rescue beta-1 expression. βI_{N-2} is an even more broadly blocking inhibitor of SAATS trafficking (see Figure 1), but in this instance, supranormal levels of beta-1 can be achieved by inclusion of repeats 14,15 into the βI_{N-2} peptide.

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Example 6: Identification of other proteins participating in SAATS transport in lymphocytes.

Using flow cytometry in conjunction with the methods outlined here, other

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proteins can be identified as modulated by SAATS, and those modulated by the adapter protein ankyrin are also apparent, since only those modulated by ankyrin will be rescued by the inclusion of repeats 14,15 in the inhibitory spectrin construct. In Figure 15, PECAM (CD31) is shown to be ankyrin/SAATS transported in lymphocytes. Cells were
5 transfected as above, and surface display of PECAM, a cell-cell adhesion molecule of the IgG superfamily, was monitored by flow cytometry. Note that for either construct, inclusion of the βI_{N-5} sequences resulted in strong blockage of transport, indicating their involvement with SAATS. In both cases, surface display was rescued or accentuated by the $\beta I_{N-5,15}$ peptide. This effect was a bit more pronounced with the N-terminal FLAG
10 tagged construct vs. the COOH-terminal GFP tagged construct, presumably due to interference of the GFP with the COOH-terminal 15th repeat unit needed to restore SAATS binding activity to PECAM.

In Figure 16, CD45 is shown to be SAATS regulated with ankyrin its putative adapter, while TNFR-1 is modulated by SAATS, but does not involve ankyrin as its
15 adapter molecule. In this experiment, Jurkat T-lymphocytes were transfected with the constructs indicated, including βI_{14-15} alone. Note the marked down regulation of CD45, a documented ankyrin binding protein, by constructs lacking repeat 15, as well as by the βI_{14-15} peptide itself (which lacks the constitutive Golgi targeting signal, see Fig. 2). These experiments also illustrate that TNFR-1 display is upregulated (rather than
20 blocked) by βI_{N-2} or βI_{N-5} , suggesting that like E-cadherin, its attachment to SAATS is mediated either directly or indirectly by sequences contained within βI_{N-2} .

In Figure 17, Fas and Fas-L are shown to be SAATS dependent, but only Fas appears to utilize ankyrin. Jurkat T-lymphocytes were transfected with the constructs indicated. Experiments were as before. Note the changes in both Fas and Fas-L due to
25 βI_{N-5} and $\beta I_{N-5,15}$ peptides.

Example 7: Selective inhibition of transport of a single protein.

By utilizing a specific inhibitor of the docking of a single integral membrane

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protein to SAATS, rather than a more general blocker of SAATS function, great specificity may be achieved. For example, the ankyrin binding domain sequence of α -Na,K-ATPase is highly conserved across all species of Na,K-ATPase, but is not found in any other protein (Zhang *et al.*, 1997b). Expression of this peptide sequence alone in
5 cells (Figure 10) selectively blocks the transport of Na,K-ATPase, without blocking the transport of any other known protein. Thus, by the identification of selective inhibitors of protein docking to SAATS, great specificity can be achieved.

In Figure 10, wild-type MDCK cells were transiently transfected with green fluorescent protein linked to the 25 residue MAB peptide. This is the same sequence
10 whose deletion caused Na,K-ATPase to be retained in the ER and ultimately lysosomes in Figure 9. After approximately 1-2 days in culture, cells were fixed and stained for GFP (using anti-GFP antibodies, left panel) or for Na,K-ATPase (right panel, red). Note that the expression of GFP alone in MDCK cells (wt) did not affect Na,K-ATPase distribution, or cell size (which is dependent on Na,K-ATPase function. However, when
15 the GFP carried the 25 residue MAB peptide, the binding of the endogenous Na,K-ATPase to SAATS was inhibited, resulting in its selective accumulation in the ER and reduced levels on the plasma membrane (two examples are shown, labeled "MAB"). Cells containing GFP-MAB are also markedly swollen, as expected. No other protein distributions were affected in these cells.

20 In Figure 18, clusters of wild-type MDCK cells were micro injected with either (A) GST alone; or (B).with the βI_{N-4} peptide generated as a recombinant fusion peptide with GST, and then stained for Na,K-ATPase by indirect immunofluorescence. While micro injected GST is without effect, note the loss of Na,K-ATPase staining intensity in the injected cells on the left side of (B). The consequences of impaired Na,K-ATPase
25 delivery to the membrane are also apparent in the larger size of cells with insufficient Na,K-ATPase, similar to the changes seen when βI_{N-5} is transfected. These results indicate that selective blockage of Na,K-ATPase can be achieved by exogenous small peptides. Similar experiments were also carried out using various monoclonal antibodies.

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In these experiments, the cells were subconfluent, and all cells in an isolated cluster were injected. (C) Control injections of buffer alone are without effect on the distribution of Na,K-ATPase. (D) Microinjection of Mab IID2, which reacts exclusively with α spectrin (not considered to be a component of SAATS), is also without effect on Na,K-ATPase. (E) Microinjection of Mab VIIIC7, which reacts with β I spectrin (Harris et al., 1986), leads to severe disruption of Na,K-ATPase delivery, with extensive accumulation within the cells and cell swelling. In this micrograph, the loss of membrane Na,K-ATPase is so severe, that the membrane borders cannot be discerned. This experiment illustrates the effectiveness of SAATS specific monoclonal antibodies in blocking SAATS activity.

Example 8: The cystic fibrosis transmembrane conductance regulator participates in SAATS transport.

Figure 12 illustrates another integral membrane protein of significant medical interest that is linked to SAATS by different ankyrin interaction. (A) Both CFTR (cystic fibrosis transmembrane conductance regulator) and its close homologue TNR (Transmembrane-Nucleotide binding-Regulatory domain) protein are present in the soluble (Fx1) and cytoskeletal pools (Fx2) of confluent MDCK cells. When these cells are surface labeled with biotin from either the apical surface (lane A) or the basolateral surface (lane B), it is also apparent that both CFTR and TNR are highly polarized, and unlike Na,K-ATPase, are expressed predominately in the apical domain of these polarized epithelial cells. (B) Despite the fact that CFTR and TNR are apical proteins, they nevertheless binds components of the SAATS system, which is responsible for their trafficking. Either whole cell lysates (L) of cells extracted with RIPA buffer, or Fx 1 and Fx 2, were immunoprecipitated with irrelevant antibody control (C) or with anti-CFTR antibody (IP). The washed immunoprecipitates were then analyzed by SDS-PAGE and examined by western blotting for either ANK_{G119} or ANK_R. Note that both ankyrins co-immunoprecipitate with CFTR, in a fashion exactly analogous to the way

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Na,K-ATPase binds SAATS. However, CFTR and Na,K-ATPase bind at distinct sites on SAATS, enabling their selective modulation.

Figure 22 illustrates the modulation of surface display of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by SAATS. MDCK cells were
5 transfected with either the βI_{N-5} or $\beta I_{N-5,15}$ constructs and then surface labeled with biotin. After labeling, cells were solubilized, and the total CFTR in each culture immunoprecipitated with anti-CFTR antibodies. The precipitates were then analyzed by SDS-PAGE, transferred to nitrocellulose, and surface CFTR detected by avidin labeling. Total CFTR was also determined in these cellular extracts by western blotting (data not
10 shown). In all cell lines, there was minimal change in the total CFTR in the cells. However, there was marked diminution of surface display of CFTR induced by the SAATS blocker βI_{N-5} ; whereas the $\beta I_{N-5,15}$ construct markedly enhanced the surface display of CFTR. Collectively these results document the involvement of SAATS in CFTR trafficking, and suggest a possible therapeutic approach to the control of disorders
15 such as cystic fibrosis, or the control of related proteins such as the multi-drug resistance gene product (MDR).

Example 9: Selective enhancement of integral membrane protein display by SAATS

20 The rate of exit of integral membrane and secretory proteins from the ER and Golgi appears to be at least partially kinetically regulated by their rate and efficiency of docking to SAATS. Therefore, upregulation of the surface display or secretion of a given protein, as well as its blocking, can be achieved by modulation of SAATS. This is illustrated in Figures 13 to 16, where it can be noted that for those molecules utilizing
25 ankyrin as an adapter complex, supranormal levels are achieved by augmenting their transport with the $\beta I_{N-5,15}$ peptide, which increased ankyrin dependent transport at the expense of ankyrin independent transport (e.g., the fall in TNFR-1 with $\beta I_{N-5,15}$ expression, Figure 16).

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Example 10: Determination of which vesicular skeletal proteins and associate adapter proteins are specifically involved in mediating sequestration of a given cell membrane or secretory proteins into transport vesicles.

5 The general method described herein is based on the discovery that the delivery of a given membrane protein or class of membrane proteins to the plasma membrane of eukaryotic cells may be inhibited or augmented by altering the binding of such proteins to the spectrin/ankyrin/adapter protein trafficking system (SAATS). The secretion of proteins that bind to so-called cargo receptors in the endoplasmic reticulum may also be
10 controlled by controlling the binding of such cargo receptor proteins to SAATS.

 As disclosed here, SAATS is a cryptic protein sorting apparatus operating to concentrate specific proteins in the endoplasmic reticulum (ER), and to facilitate their inclusion into vesicles undergoing transport or diffusion between the ER and Golgi compartments. A similar pathway is also active between the Golgi and the plasma
15 membrane of many cells, but the most general use of this method of control is achieved by blocking SAATS at the ER to *cis* Golgi transition. The method is implemented by 1) identifying to which component of the SAATS system a given integral membrane protein binds, discussed as follows; and 2) identifying a peptide, small molecule analog, antibody, or antisense oligonucleotide that will disrupt *in vivo* the binding interaction
20 between the target protein and SAATS. This latter aspect of the present invention is discussed in the next section of this specification.

 Previous efforts have only identified binding sites for a small number of what we now know to be SAATS ligands (See Table 1B). We believe this is due to the fact that many interactions will occur in solution with only low affinity, affinities that will become
25 significant in the restricted environment of SAATS-membrane interaction. Specifically, once entropic contributions of to the binding energy are largely accommodated by way of a high affinity SAATS-membrane binding (such as *via* MAD1 or MAD2) (Lombardo *et al.*, 1994), then other interactions between membrane proteins and SAATS will be

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facilitated. Assays to detect such interactions must therefore be sensitive to detecting low affinity but specific interactions.

Standard techniques that measure protein-protein interactions at equilibrium are thus well suited to detect the potentially weak interactions ($K_a = 10^{-4}$ to 10^{-5}) that may typically characterize binary interactions between membrane proteins and many ligand sites in SAATS. Typical of such assays would be equilibrium dialysis; fluorescent based assays in which the change in intrinsic fluorescence of a protein is monitored as a function of binding; assays based on osmolarity or light scattering; and others commonly known to practitioners of the art. In addition, surface plasmon resonance, such as implemented in Pharmacia's BiaCore instrument, is also well suited to detecting specific macromolecular interactions withing SAATS, often even in unpurified solutions prepared from cell or bacterial lysates. Other ways to enhance detection sensitivity is by employing radioactive labeled compounds, or other sensitive detection techniques such as enhanced chemiluminescence in conjunction with standard microtiter or blot overlay techniques. Genetic selection assays are also useful, such as two-hybrid assays in yeast, or phage epitope display library screening.

Example 11: Identification of peptides and small molecules that modulate sequestration and/or vesicle transport of integral membrane and secretory proteins

In general, several approaches can be used to identify an agent able to block (or augment) the interaction between a target protein and SAATS. In general, this method may be approached by either: A) identifying broad inhibitors of SAATS function that disrupt a class or multiple classes of membrane or secretory proteins, such as peptide agents or expressed recombinant genes that encode specific functional domains in SAATS, and that block the docking of classes of membrane proteins by competitive inhibition with the endogenous SAATS system; or B) following a drug design strategy that can be either rational, with inhibitory agents determined based on detailed

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knowledge of binding sites mediating the attachment of the target protein to SAATS, or random, based on *in vitro* surrogate assays of SAATS function. This latter approach is based on identifying compounds that block interactions with SAATS as measured by *in vitro* binding assays.

5 Examples of approach A) were presented in Section A of this detailed description, above. Examples utilizing approach B are outlined below, together with a general protocol for identifying such agents.

B) Strategies based on rational drug design. To block or modulate the display of membrane protein X using a rational drug design approach, detailed knowledge of its
10 SAATS binding site, up to and including its three dimensional structure, will be required. Completion of Steps A4 and A5 above provide the starting point for a systematic approach.

Step B1: Determine whether protein X when mature is monomeric or
15 homopolymeric, or whether it exists in its mature state as a heterocomplex with another membrane protein. This step may not be necessary if protein X can be shown to bind SAATS directly, but many membrane and all secretory proteins will only attach to SAATS *via* their interaction with another membrane protein. If this is the case, either
20 agents must be identified that will prevent the formation of the heterocomplex, or the complex will be controlled as a unit (*i.e.*, the display of all components of the functional heterocomplex) will be controlled together by controlling their SAATS assembly. Standard protein biochemical and biophysical techniques can be used to make these determinations, including co-immunoprecipitation from non-ionic detergent extracts of cultured cells expressing protein X.

25 For example, the demonstration of direct binding of α -Na,K-ATPase (as opposed to β -Na,K-ATPase) to ankyrin (Morrow, *et al.*, 1989, Devarajan, 1994) can be demonstrated by a variety of *in vitro* and *in vivo* techniques, including co-sedimentation, gel overlay binding assays, and surface plasmon resonance. In these assays (*e.g.* see

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Morrow *et al.* 1989), direct binding of ankyrin to the α -Na,K-ATPase, but not β -Na,K-ATPase can be demonstrated. However, since β -Na,K-ATPase binds stoichiometrically to the alpha subunit, both are efficiently transported by SAATS, but this transport is dependent on the binding of α -Na,K-ATPase to SAATS (*e.g.* see Figure 4 demonstrating failure of β -Na,K-ATPase transport).

Step B2: Determination of the specific binding site within protein X that links it to SAATS. Many conventional methods may be used to accomplish this. A common one is the use of deletional mutagenesis using a series of recombinant peptides, often generated as fusion peptides linked to glutathione-S-transferase (GST). For example, a deletional analysis of GST-fusion peptides representing the cytoplasmic domains of Na,K-ATPase (Devarajan *et al.*, 1994a; Devarajan *et al.*, 1994b).

(Go to Step B3 or B3 alternate).

15

Step B3: Use of conventional structure determination methods to resolve the 3-D structure of the binding domain. If a complete structure determination of protein X is not possible, a useful method for determining the relevant structure of the binding site is carrier mediated crystallization, in which the binding domain of interest is crystallized as a fusion protein with GST (for example, see Lim *et al.*, 1994). Subsequent determination of the structure of this complex yields the structure of the key binding site. Other structural determination methods, such as multidimensional NMR, are of course applicable as well.

For example, the determination of the 3-D structure of the minimal ankyrin binding domain in α -Na,K-ATPase by crystallization of the active GST-fusion peptide would be useful, as we have recently demonstrated (Zhang, *et al.*, 1997b). (See Figs. 4B, 4C, and 11). To prove the efficacy of agents based on the structure of such as binding domain, it is also useful to demonstrate the specific inhibition of the SAATS

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transport of the native protein using the binding domain alone (as illustrated in Figures 10 & 18), as well as the ability of the binding domain to bind *in vitro* to its ligand receptor site with an affinity and stoichiometry comparable to that of the native protein.

5

Step B4: Use of the structure determined above to design candidate small molecules that will inhibit the binding interaction and assay of these compounds by their ability to block the *in vitro* interaction between the active site of protein X and its SAATS binding site. Alternatively, if the goal is to identify small molecules that
10 augment a given SAATS interaction, so as to identify a therapeutic that enhances the display or transport or secretion of a given protein (such as would be desirable in the treatment children with cystic fibrosis, Figure 12B), molecules that augment *in vitro* the interaction between protein X and SAATS would be sought.

15

Step B3 alternate: Alternatively, an empirical approach identifying lead compounds based on a surrogate measures of SAATS function is possible. Since all or most proteins exit the ER compartment via a direct or indirect binding to SAATS, *in vitro* assays based on specific binding sites for each membrane or secretory protein of interest provide an ideal basis for high-throughput *in vitro* screens of SAATS function. For
20 example, high-throughput screens in microtiter plates, seeking to identify all agents that block or diminish the binding of spectrin to AnkG119, or AnkG119 to protein X, would be one such suitable assay. Alternatively, one could prepare similar assays in which protein X or ankyrin or other SAATS component was immobilized onto an inert substrate (eg. nitrocellulose, immobilon, 96 well titer plate, microchip, etc), and various agents
25 compared with respect to their ability to block or enhance a the binding of a soluble protein to the immobilized protein. The details of the implementation of such assays is well known in the art. The key feature of these assays, as applied to SAATS, is that the direct binary interaction between an integral membrane protein, or even an oligomeric

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complex of such proteins even partially purified, can be used as a suitable target for drug discovery since i) such proteins can be immobilized even in impure form; ii) purified and well characterized components of SAATS can be monitored for binding to such immobilized or impure proteins; and iii) the presence of the binary interaction is itself a well proven and good surrogate for in vivo SAATS activity.

Example 12: Strategies based on regulation of spectrin binding to the Golgi complex

We envision that naturally the rate of transport of specific secretory and integral membrane proteins from the ER to the plasma membrane will be regulated by control of the binding to SAATS. Several levels of post-translational control of the spectrin cortical cytoskeleton have been identified, and we anticipate that several of these will also be active in regulating SAATS (Morrow, *et al.*, 1997). In addition, regulation of SAATS also appears to share at least one regulatory pathway that also regulates the assembly of other coatamer components in the secretory pathway, and that is modulation of SAATS affinity for the Golgi by ADP-ribosylation factor (ARF) (Godi *et al.*, 1997).

In one embodiment of the present invention, to evaluate the activity of a candidate compound to modulate interaction of SAATS with selected integral membrane or secretory proteins, such a compound is mixed with the selected protein and whatever vesicular skeletal protein or adapter protein is relevant. After mixing under conditions that allow association of these components, the mixture is analyzed to determine if the agent inhibited or enhanced binding of the selected protein to its SAATS binding partner.

Inhibitors and enhancers are thus confirmed as being able to modulate this interaction. A skilled artisan can readily employ numerous art-known techniques for determining whether a particular agent modulates the binding of such selected proteins to their SAATS binding partner. Agents can be further tested for the ability to modulate binding using a cell-free assay system or a cellular assay system. Example 14 provides one such method that can be used to assay for relevant activity.

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As used herein, an agent is said to inhibit SAATS binding activity when the agent reduces the binding of a selected integral membrane or secretory protein to its SAATS binding partner. The preferred inhibitor will selectively inhibit such binding specifically, not affecting the interaction of any other proteins to SAATS. Further, the preferred
5 inhibitor will reduce such binding by more than about 50%, more preferably by more than about 90%, most preferably eliminating substantially all binding of the selected protein to SAATS.

As used herein, an agent is said to enhance SAATS binding activity when the agent increases the binding of a selected integral membrane protein to SAATS. The
10 preferred binding enhancer will increase SAATS binding activity by more than about 50%, more preferably by more than about 90%, most preferably more than doubling the level of binding or transport of such proteins to SAATS.

The preferred inhibitors and enhancers will be selective for a specific species of integral membrane or secretory protein. The agents of the present invention can be, as
15 examples, peptides, small molecules, and vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of various spectrin homologues. Small peptide agents can serve as competitive
20 inhibitors of SAATS binding, sequestration and transport.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard
25 recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical epitopes of vesicular skeletal proteins such as spectrin and associated adapter

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proteins such as ankyrin. Antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of, *e.g.*, spectrin, which binds the selected integral membrane or secretory. Critical regions for the transport of Na,K-ATPase include the domains identified in Figure 3 or the
5 cytoplasmic 29 residues of VSV-G. Such agents can be used in competitive binding studies to identify second generation inhibitory agents.

Example 13: Use of agents that modulate the sequestration and/or vesicular transport of integral membrane and secretory proteins for therapeutic purposes
10

We contemplate that the methods described above will be useful in a wide variety of therapeutic contexts. For example, the following Table 2 illustrates several possibilities.

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Table 2: Therapeutics envisioned for the following disorders

General Area	Specific Conditions	Comment
Cancer	Growth factor and Growth factor receptor modulation. Multi-drug resistance transporter down-regulation. Hormone receptor down-regulation in breast, endocrine, and prostatic tumors. Adhesion molecule modulation; Integrin modulation.	Blockage of MDR to sensitize tumors favorable early therapeutic target.
Cardiovascular	Hypertension; Cholesterol and hyperlipidemia conditions, anti-arrhythmic agents (e.g. Ca ⁺⁺ channel antagonists), Thrombosis control; anti-platelet agents; endothelial surface modifying agents; restenosis control	Modification of membrane channels, thrombin receptors, endothelial adhesion receptors.
5 Degenerative Disorders	Osteoporosis,	BMP regulation, calcitonin/PTH balance regulation; calcium homeostasis.
Dermatologic/ cosmetic	Psoriasis, scleroderma, cutaneous hypersensitivity, poison ivy,	Topical application may offer early therapeutic advantage
Genetic Disease	Cystic fibrosis (eg D508 mutation reduces transport of CFTR to membrane.)	Enhanced binding to SAATS may ameliorate disease without correcting the mutation.
Hematological Disease	Transfusion incompatibilities	surface epitope display modulation;
Immune-modulation	Type I Diabetes; Arthritis; Asthma; Allergy; Scleroderma; lupus;	pro inflammatory cytokines; moderate immunoregulators
10 Infectious Disease	Viral infections of all types, including influenza and HIV. Enteric and other bacterial infections. Malaria, protozoan infections, clamidia.	Inhibition of surface display of viral proteins. Infections mediated by binding to tissue receptors.
Inflammation	Chronic ulcers; diabetes; wound healing; osteomyelitis; inflammatory cell/ endothelial cell adhesion; fibrosis control.	Modification of selectin display, endothelial adhesion receptors, cytokine secretion and cytokine receptors. Interleukins and receptors, Tumor necrosis factor and receptors. Collagen secretion.
M a l a b s o r p t i o n	Hemochromatosis; Celiac disease/non-tropical sprue;	transferrin receptor modulation,
Nutritional disorders	gluten enteropathy; ulcer disease	immunogens and immunogen receptors; mucosal IgA display.
Neurological Disorders	Depression; Manic-depressive illness; Schizophrenia; Parkinson's disease; Neural regeneration; APP protein display and secretion in Alzheimer's Disease.	Neuropeptide receptor modulation; Neurotransmitter uptake and secretion; axonal guidance molecule display and receptors. Axonal transport modulators also.
15 Transplantation	Epitope modulation; HL-A antigen display in grafts. Moderate immunosuppression. Bone marrow transplantation	antigen presentation control; epitope display, cross-reactivity suppression
Diabetes	autoimmune control. (Type I). Insulin receptor modulation; glucagon regulation.	Glucose transporter and Insulin receptor control.

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Reproductive biology	Sperm/ovum receptors, contraception, infertility,
	maternal/fetal immune disorders
Endocrine Disorders	Goiter, hyper/hypo thyroidism, growth hormone and receptors, pituitary disorders

**5 Traumatic and Ischemic
Injury**

to accelerate, *e.g.* recovery of mislocated
Na,K-ATPase following ischemia/heart,
kidney, brain, salvage of myocardium,
recovery of infarction, limitation of infarct.

10

The administration of agents identified by the foregoing techniques will be dependent on their intended purpose, *i.e.*, to enhance or inhibit the transport of a selected integral membrane protein or secretory protein, and on their chemical nature, *i.e.*, peptide or small molecule. For example, to treat cystic fibrosis, as noted above, by identifying the

15 SAATS binding domain for CFTR would allow the identification of agents that selectively increase the delivery of mutant (but functional) CFTR to the plasma membrane. Appropriate agents might optimally be delivered by inhalation therapy (see U.S. Patents 5,669,376 and 5,655,516).

For vascular regulators, an appropriate agent can be administered via parenteral,

20 subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

As described below, there are many methods that can readily be adapted to

25 administer such agents once identified by the methods herein described. The determination of optimal ranges of effective amounts of each component is within the ordinary level of skill in the therapeutic art and is based on the intended use. In addition to the particular agent of choice, the compositions of the present invention may contain other ingredients, such as suitable pharmaceutically acceptable carriers comprising

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excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble variants, for example, water-soluble salts. In
5 addition, suspensions of the active compounds, and as appropriate, oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose,
10 sorbitol, and/or dintran.

Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell. The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used
15 simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release variants thereof.

The agents of the present invention that modulate sequestration and trafficking of
20 selected integral membrane and secretory proteins can be provided alone, or in combination with another appropriate pharmacoactive. For example, an agent of the present invention that reduces the presence of various cell surface receptors involved in the attachment of viral envelopes might be administered in combination with other anti-viral agents. As used herein, two agents are said to be administered in combination
25 when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

Example 14: Identification of the spectrin or adaptor protein domain(s)

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responsible to binding and transport of an integral membrane or secretory protein.

As set forth in Figure 2, β I Σ spectrin contains a series of functional domains that have been shown to confer or may confer the ability to bind specific adaptor proteins and/or proteins to be transported. Similarly, ankyrin contains a number of repeats (13 to 5 24 repeat units characteristic of most ankyrins), with each ankyrin-repeat structure composed of two alpha helices and a β -hairpin loop. The complexity of both the individual repeats and the number of repeats in the structure of both structural proteins such as spectrins and adaptor proteins such as ankyrin may allow for the varied ability of both molecules to bind to multiple distinct integral membrane or secretory proteins.

10 As an example, in ankyrins, multiple repeat units create a structure in which interactions between the helices form a central core structure, while the tips of the exposed β -hairpin turns provide putative protein-protein interaction surfaces (See Figure 21). In one scenario, the seven residue loop within MAB of α -Na,K-ATPase interacts with a specific site in ankyrin created by the tips of one or more of these β -hairpin turns. 15 Since a multiplicity of potential binding pockets would be created by the 13 to 24 repeat units characteristic of most ankyrins, specific and unique binding sites presumably also exist for the other short peptide sequences responsible for ankyrin binding activity in other proteins.

The ability of adaptor proteins to bind to spectrins or integral membrane or 20 secretory proteins to bind directly to spectrins can be assayed to identify the spectrin domain, or combinations of domains responsible for specific binding of a given adaptor protein, adaptor protein and integral membrane or secretory protein or integral membrane or secretory protein directly. Likewise, the ability of an given integral membrane or secretory protein to bind to an adaptor protein such as ankyrin can be assayed to identify 25 the ankyrin domain or combination of domains responsible for specific integral membrane or secretory protein binding. Identification of the specific domains responsible for binding then allows for the development of peptide based molecules, or mimetopes with similar structures, to modulate or disrupt specific binding of the many

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protein-protein interactions involved in SAATS.

As an example, to determine the identity of ankyrin domains responsible for specific binding to a given integral membrane, deletional analysis of the ankyrin repeat region is used to produce a series of fragments of the protein. The fragments are then
5 tested for their ability to bind to the integral membrane protein of interest. Any means commonly available of detecting the ability of the ankyrin fragment to bind to the specific integral membrane protein can be used. For instance, cell free systems can be used wherein the binding is detected by the ability of the ankyrin fragment, detected by
10 radiolabelling, fluorescent tag, or specific antibody, to be co-immunoprecipitated by antibodies to the protein to which binding is suspected. Alternatively, the ability of the ankyrin fragment to co-sediment with permeabilized cells or membrane preparations enriched in the proteins of interest (*e.g.* the co-sedimentation of ankyrin with
Na,K-ATPase enriched membranes, Morrow et al., 1989) is another method of detecting binding. Other methods include binding to immobilized ligands; co-sedimentation,
15 co-elution on gel filtration chromatography; surface plasmon resonance, fluorescent energy transfer; osmolarity; light-scattering; resonance raman spectroscopy, NMR spectroscopy, yeast two-hybrid systems; expression cloning, and phage display. Other methods will also be known to the practitioner of the art. Since individual ankyrin repeat units, if that is the target of the assay, are often insoluble or unstable, it will often be of
20 value to examine smaller internal sequences, such as those from the beta-hairpin loops of the ankyrin repeat structure, for binding activity. . High through-put assays can also be developed to detect binding the a library of ankyrin fragments comprising all individual repeat units plus all possible combinations of the repeat units. Examples of such assays would include incorporating single, two-mer, and trimer combinations of the beta-hairpin
25 loop sequences of all known ankyrin repeat units into pseudo-libraries suitable for screening in the yeast two-hybrid or CHO two-hybrid KISS assays (Fearon et al., 1992) against the ligand of interest. Similar strategies could also be employed utilizing the same peptide sequences, generated either recombinantly or by synthetic methods,

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immobilized on nitrocellulose or immobilon or similar membranes, or on plastic multiwell plates, or with the sequences incorporated into bacteriophage so as to create a phage display library suitable for binding to the protein of interest. (Scott, 1992)

5 It should be understood that the foregoing discussion and examples present merely present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All articles, patents and patent applications that are identified above are incorporated by reference in their entirety.

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Claims:

1. A method to modulate the intracellular sequestration of a selected integral membrane or secretory protein into a transport vesicle for transport from the endoplasmic reticulum to the cis-Golgi apparatus, or from the cis-Golgi to the medial-Golgi apparatus,
5 or from the medial-Golgi to the trans-Golgi apparatus or from the trans-Golgi apparatus to the plasma cell membrane, comprising the steps of:
selecting a compound effective to enhance or inhibit the binding of the selected protein to the transport vesicle; and
contacting the cell with said compound, whereby the sequestration
10 of the protein is enhanced if binding is enhanced or inhibited if binding is inhibited.
2. The method of claim 1, wherein the binding and sequestration of the selected protein is inhibited and wherein said compound is selected from the group consisting of: i) a modified or truncated skeletal component of the transport vesicle, ii)
15 an associated adapter protein, or iii) a small molecule mimetic of either i) or ii).
3. The method of claim 1, wherein the sequestration of the selected protein is inhibited and wherein said compound is a modified or truncated adapter protein, or a small molecule mimetic thereof, which protein or mimetic mediates binding or
20 association of the selected protein to a skeletal component of the transport vesicle.
4. The method of claim 3, wherein the adapter protein is ankyrin and the skeletal component of the transport vesicle is spectrin.
- 25 5. The method of claim 4, wherein the ankyrin is AnkG119.

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6. The method of claim 2 or 3, wherein the skeletal component or associated adapter protein is selected from the group consisting of ADP-ribosylation factor (ARF), ankyrin, AnkG119 spectrin, spectrin β I, spectrin β II*, centractin and tubulin and small molecule mimetics of any of the foregoing.

7. The method of any of claims 1-5, wherein the transport of the selected protein from the endoplasmic reticulum to the cis-Golgi apparatus is inhibited.

8. A method to determine whether a candidate compound inhibits or enhances the transport of a selected integral membrane or secretory protein via a transport vesicle from the endoplasmic reticulum to the cis-Golgi apparatus, or from the cis-Golgi to the medial-Golgi apparatus, or from the medial-Golgi to the trans-Golgi apparatus or from the trans-Golgi apparatus to the cell membrane, comprising the steps of:

contacting a cell in which the selected protein is expressed with the candidate compound; and

determining whether intracellular vesicular transport of the selected protein is enhanced or inhibited.

9. The method of claim 8 wherein the determination that inhibition of intracellular transport has occurred is made by: i) detecting a substantial decrease in the presence of the selected protein in the cell membrane or in the extracellular medium; or ii) detecting the dispersed intracellular presence of the selected protein outside of its normal pattern of transport to and through the Golgi apparatus.

10. A method to determine whether the vesicular transport of a selected integral membrane protein or secretory protein from the endoplasmic reticulum to and through the Golgi apparatus is mediated by the binding of the selected protein to a

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component of the skeleton of the vesicle, comprising the steps of:

treating cells that express the selected protein with an agent that interferes with the sequestration of integral membrane proteins or secretory protein into transport vesicles; and

5 determining whether the selected protein is found substantially in its normal distribution pattern in said cells, whereby an abnormal distribution pattern indicates mediated transport of the vesicle.

10 11. The method of claim 10 in which said agent is one of a series of progressively more truncated portions of a protein that is a structural component of the vesicular skeleton or is an associated adapter protein, and said cells separately are transfected with cDNAs encoding said series of vesicular skeleton or associated adapter proteins.

15 12. The method of claim 11, wherein said vesicular skeleton or associated adapter proteins are selected from the group consisting of ADP-ribosylation factor (ARF), ankyrin, AnkG119 spectrin, spectrin β I, spectrin β I Σ^* , contractin and tubulin.

20 13. The method of claim 10, wherein the step of determining whether the distribution pattern of the selected protein is substantially normal includes an assessment of whether there is: i) a substantial decrease in the presence of the selected protein in its normal intracellular distribution pattern; ii) or a substantial decrease in the presence of the selected protein on the outer cell membrane or in the extracellular media of said cells.

25 14. The method of claim 12, wherein said vesicular skeleton protein is spectrin.

15. The method of claim 14, wherein said spectrin is β I Σ^* spectrin.

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16. The method of claim 12, wherein said adapter protein is ankyrin.

17. The method of claim 16, wherein said ankyrin is AnkG119.

5

18. A group of β I Σ * spectrin cDNA constructs of varying lengths that each encode one of a complimentary and overlapping series of truncated β I Σ * spectrin peptides.

10

19. A set of individual clonal cell lines each expressing one of the β I Σ * spectrin cDNA constructs of claim 18.

20. An isolated and purified nucleic acid fragment encoding a β III spectrin.

15

21. The nucleic acid of claims 20, wherein the β III spectrin comprises the amino acid sequence of Figure 19B.

22. The nucleic acid of claim 20 comprising the sequence of Figure 19A.

20

23. An isolated nucleic acid that specifically hybridizes to the nucleic acid of claim 22 under stringent conditions.

24. A vector comprising a nucleic acid of any one of claims 20 to 23.

25

25. A host cell comprising the vector of claim 24.

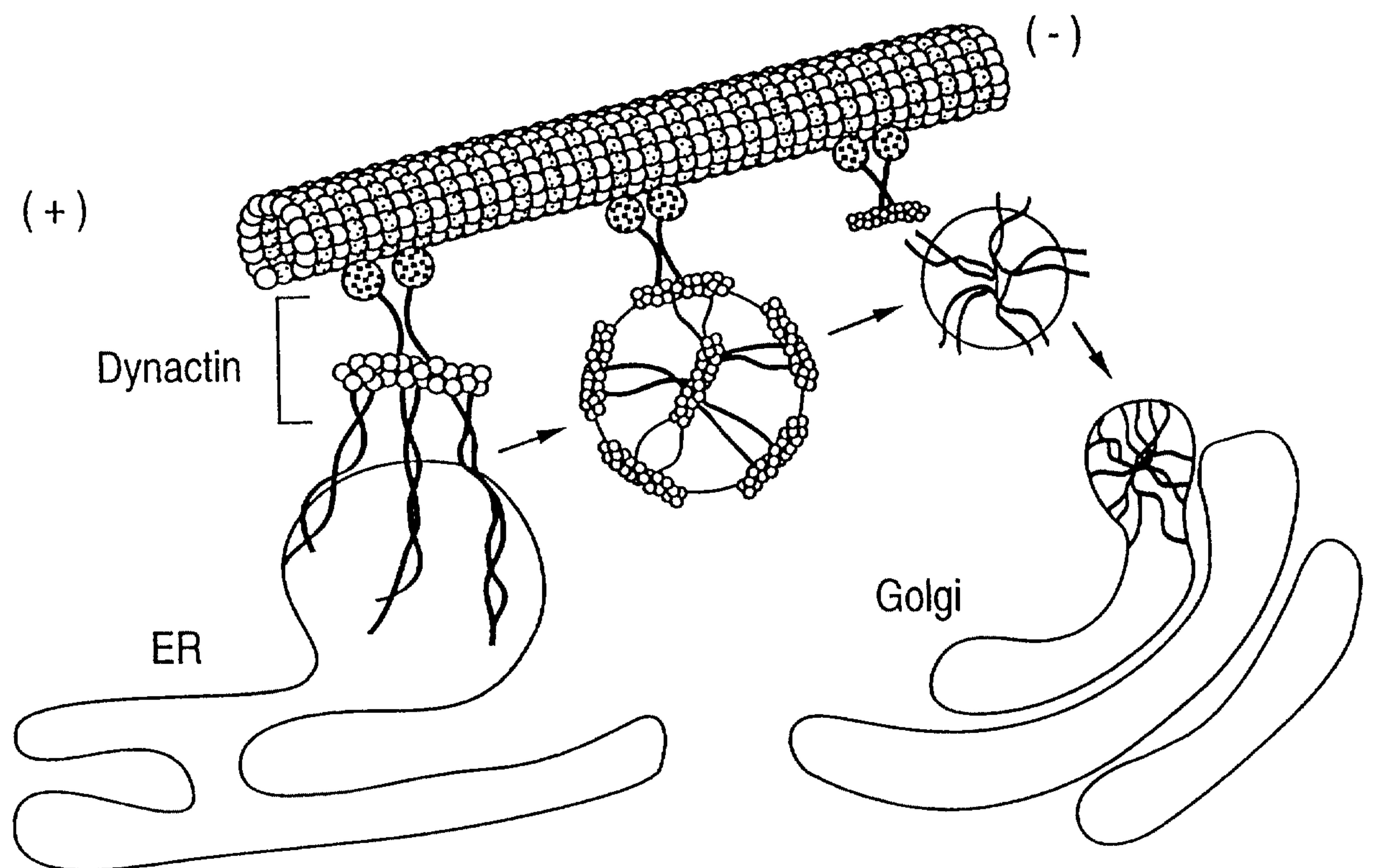
26. A method to ameliorate the effects of a disease caused by an relative excess

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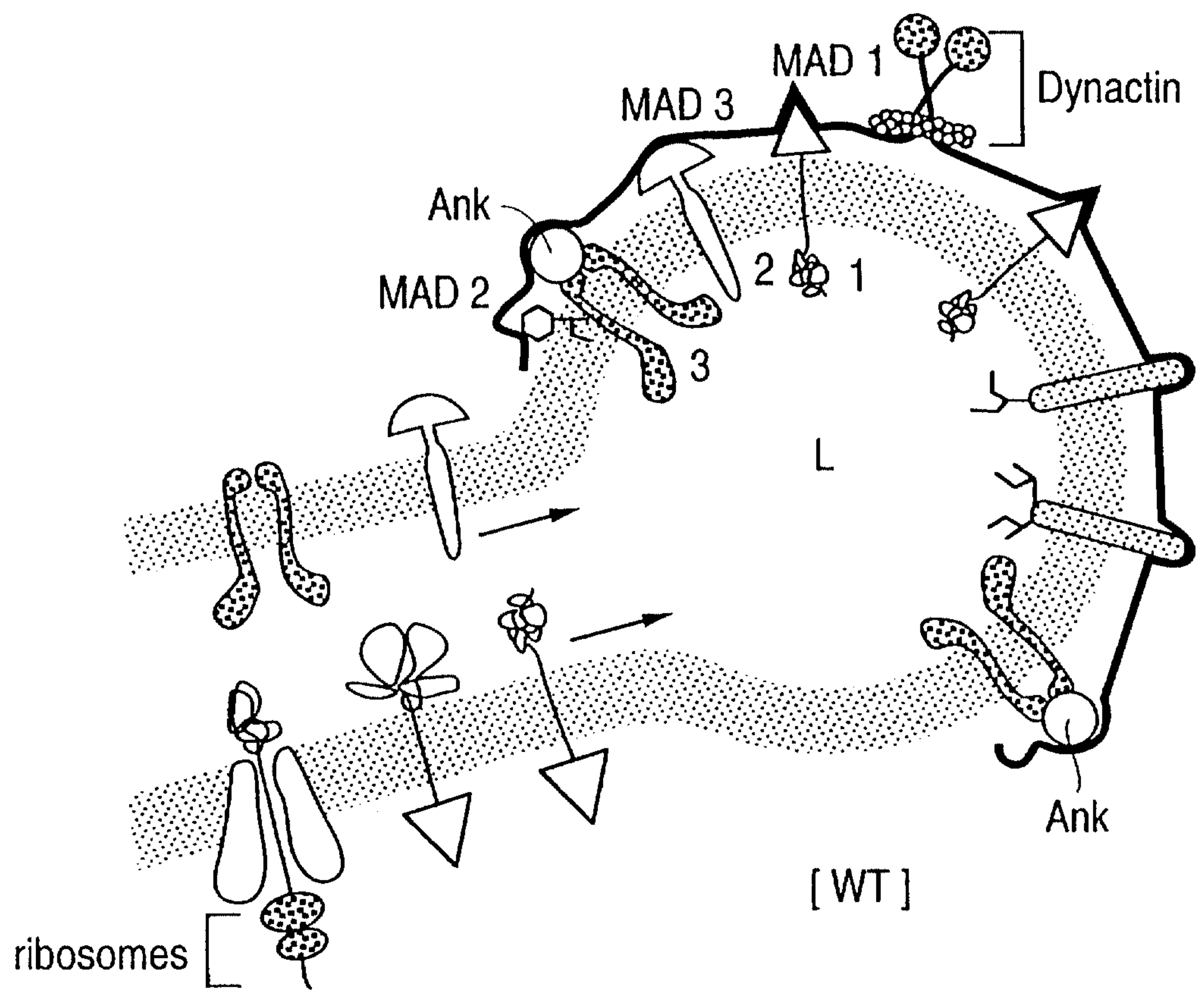
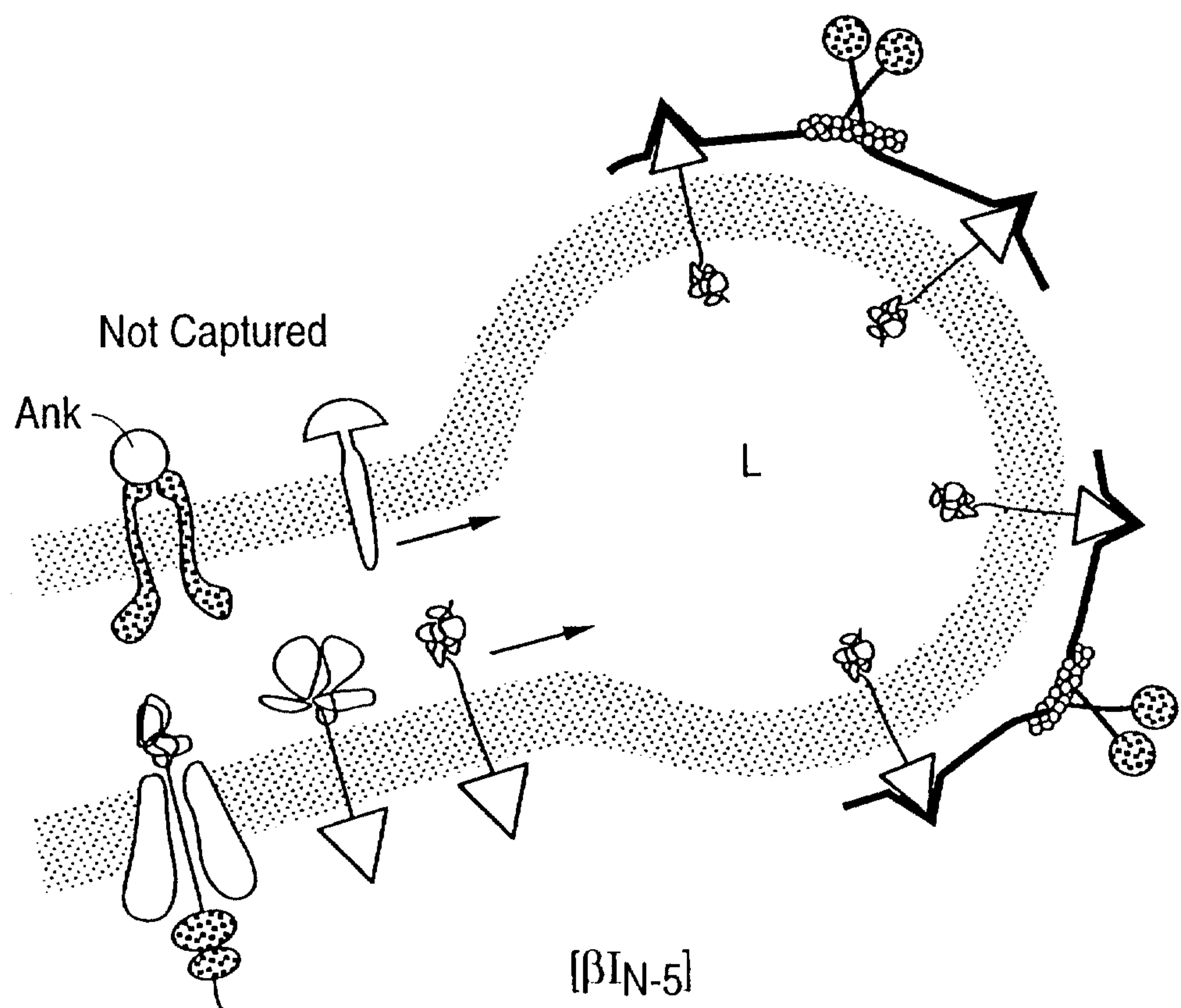
of a particular integral membrane protein, comprising the administration of a compound in a unit dose that is effective to inhibit the intracellular sequestration of a selected integral membrane or secretory protein into a transport vesicle for transport from the endoplasmic reticulum to the cis-Golgi apparatus, or from the cis-Golgi to the
5 medial-Golgi apparatus, or from the medial-Golgi to the trans-Golgi apparatus or from the trans-Golgi apparatus to the cell membrane.

27. A method to ameliorate the effects of a disease caused by an relative deficit
10 in the amount of a particular integral membrane protein, comprising the administration of a compound in a unit dose that is effective to enhance the intracellular sequestration of a selected integral membrane or secretory protein into a transport vesicle or the transport thereof from the endoplasmic reticulum to the cis-Golgi apparatus, or from the cis-Golgi to the medial-Golgi apparatus, or from the medial-Golgi to the trans-Golgi apparatus or
15 from the trans-Golgi apparatus to the cell membrane.

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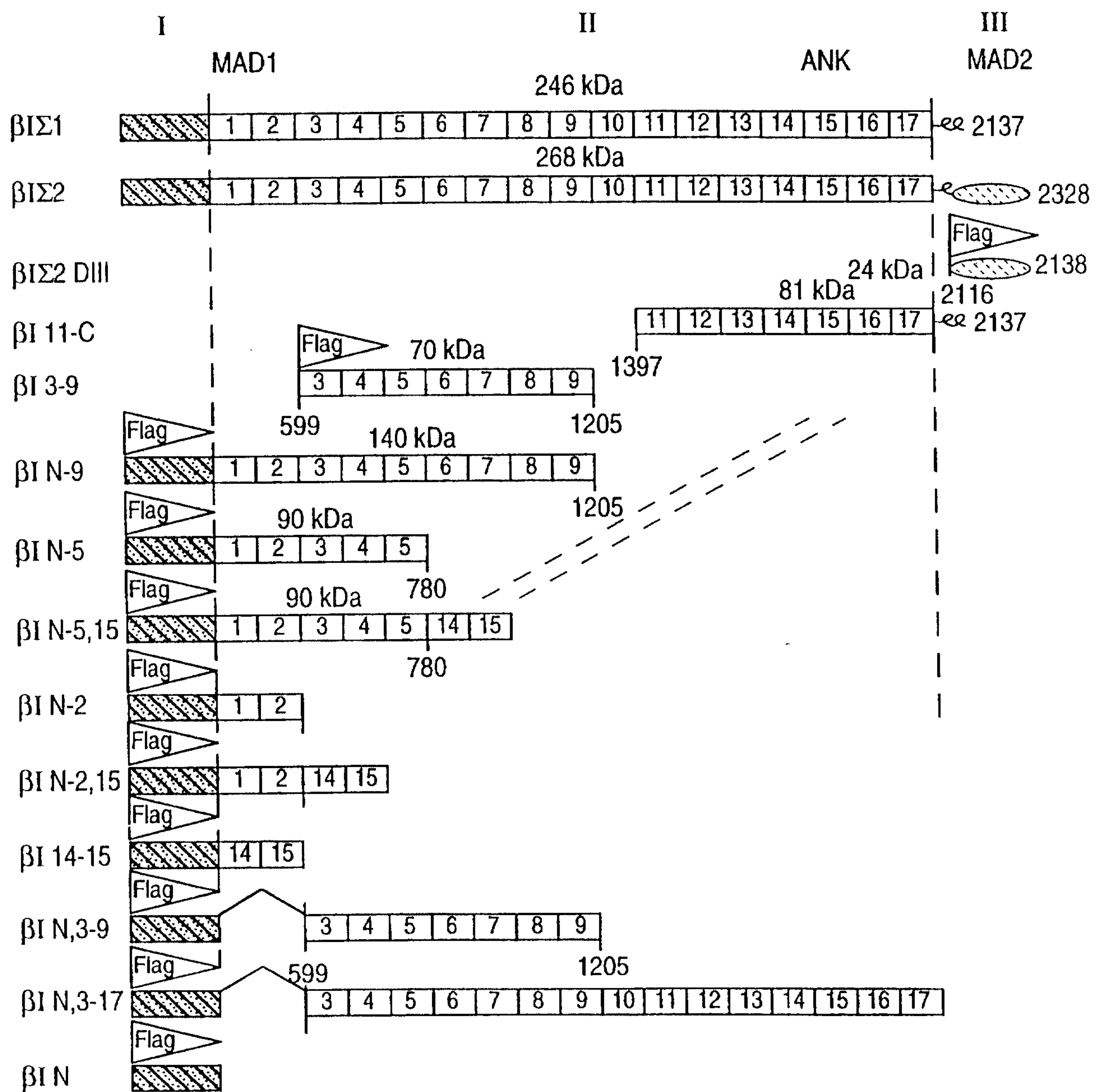
FIG. 1A

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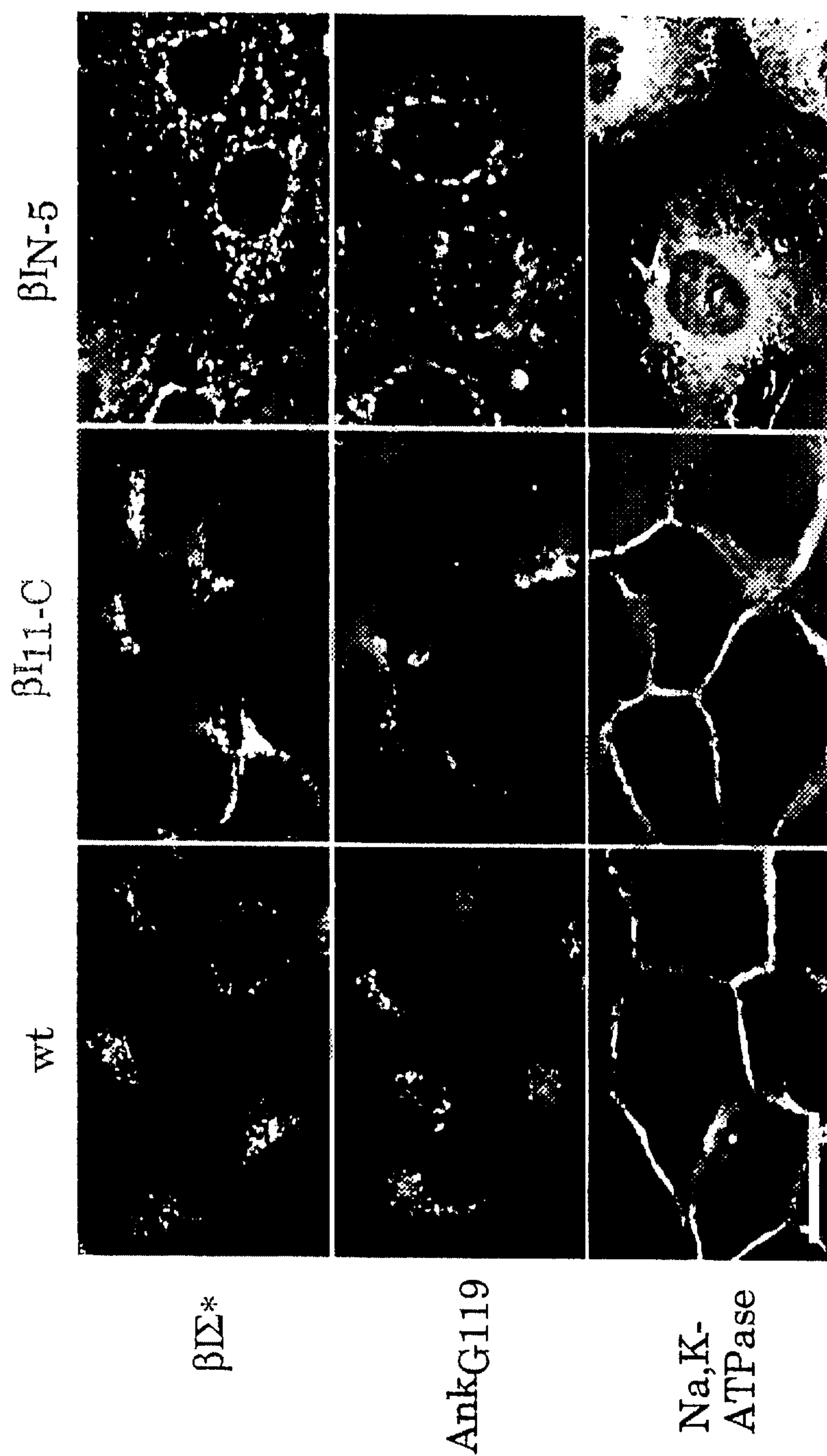
FIG. 1B**FIG. 1C**

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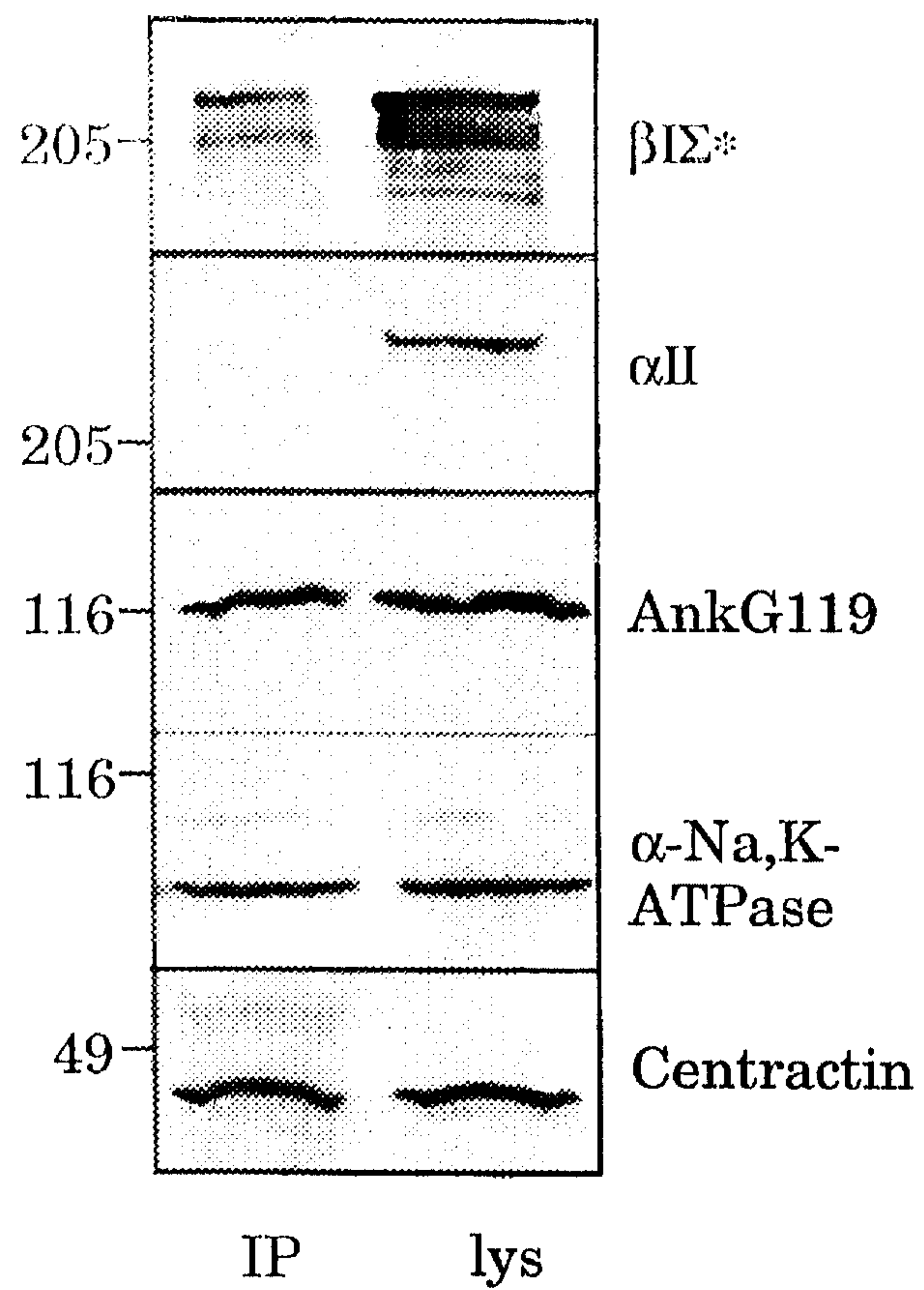
FIG. 2



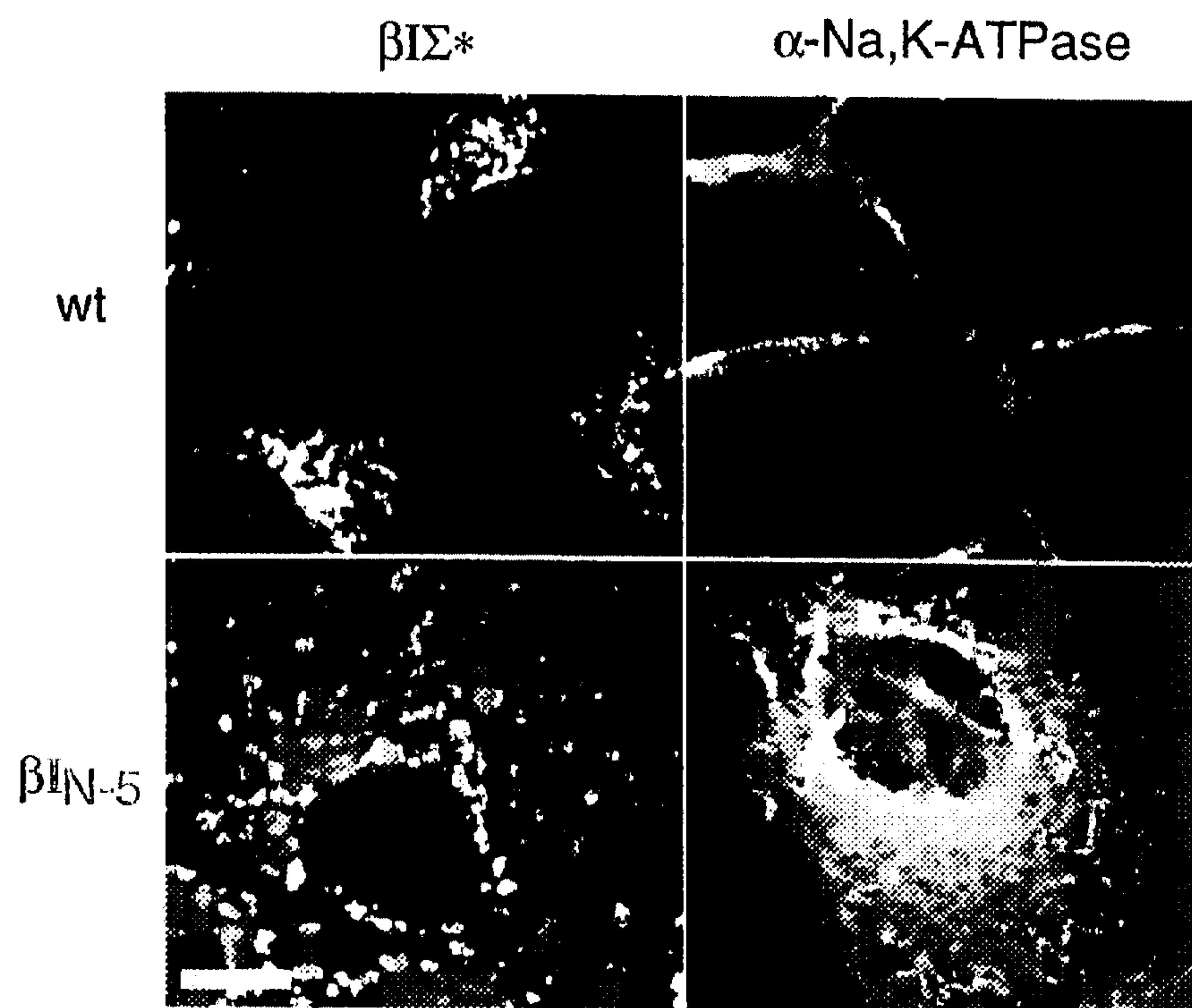
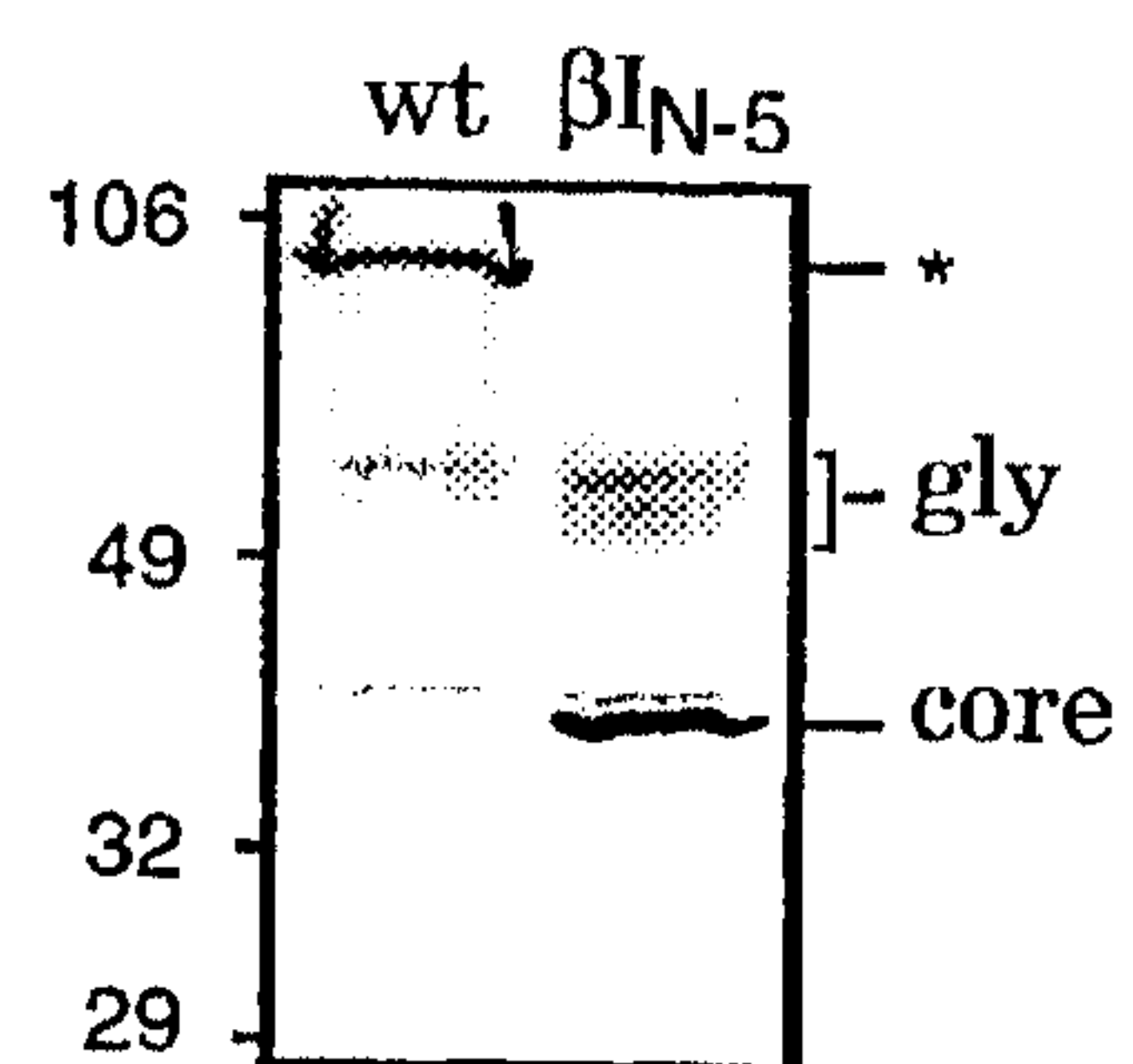
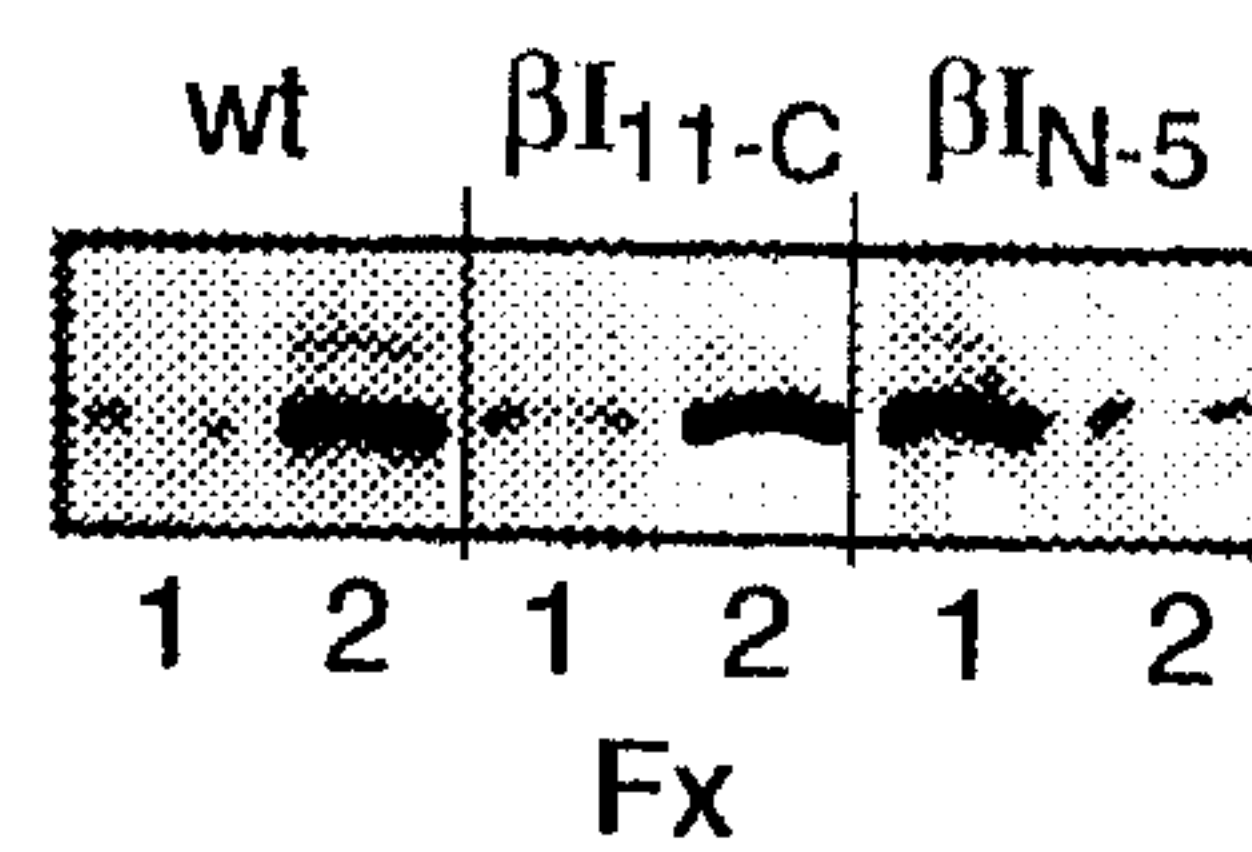
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FIG. 3A

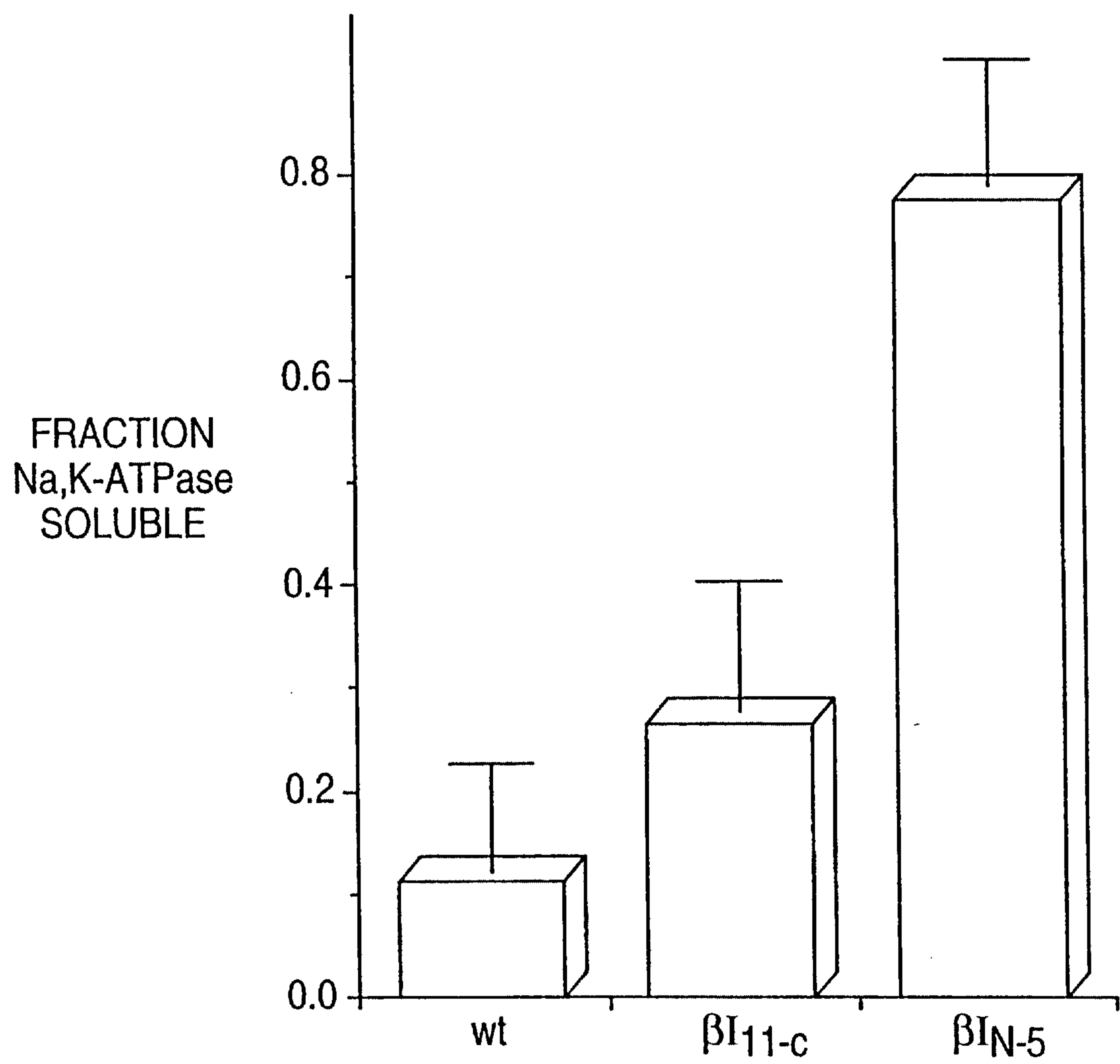
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FIG. 3B

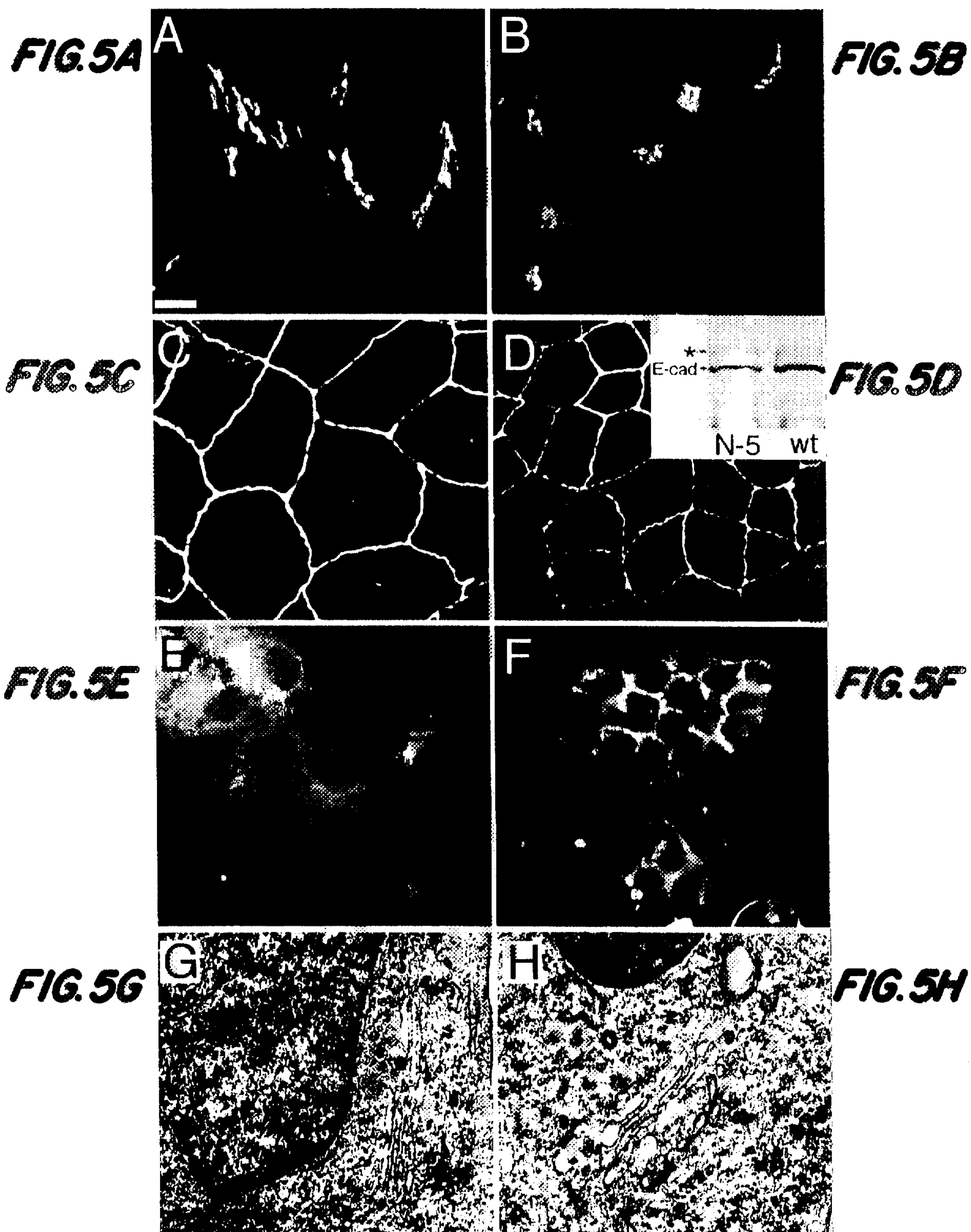
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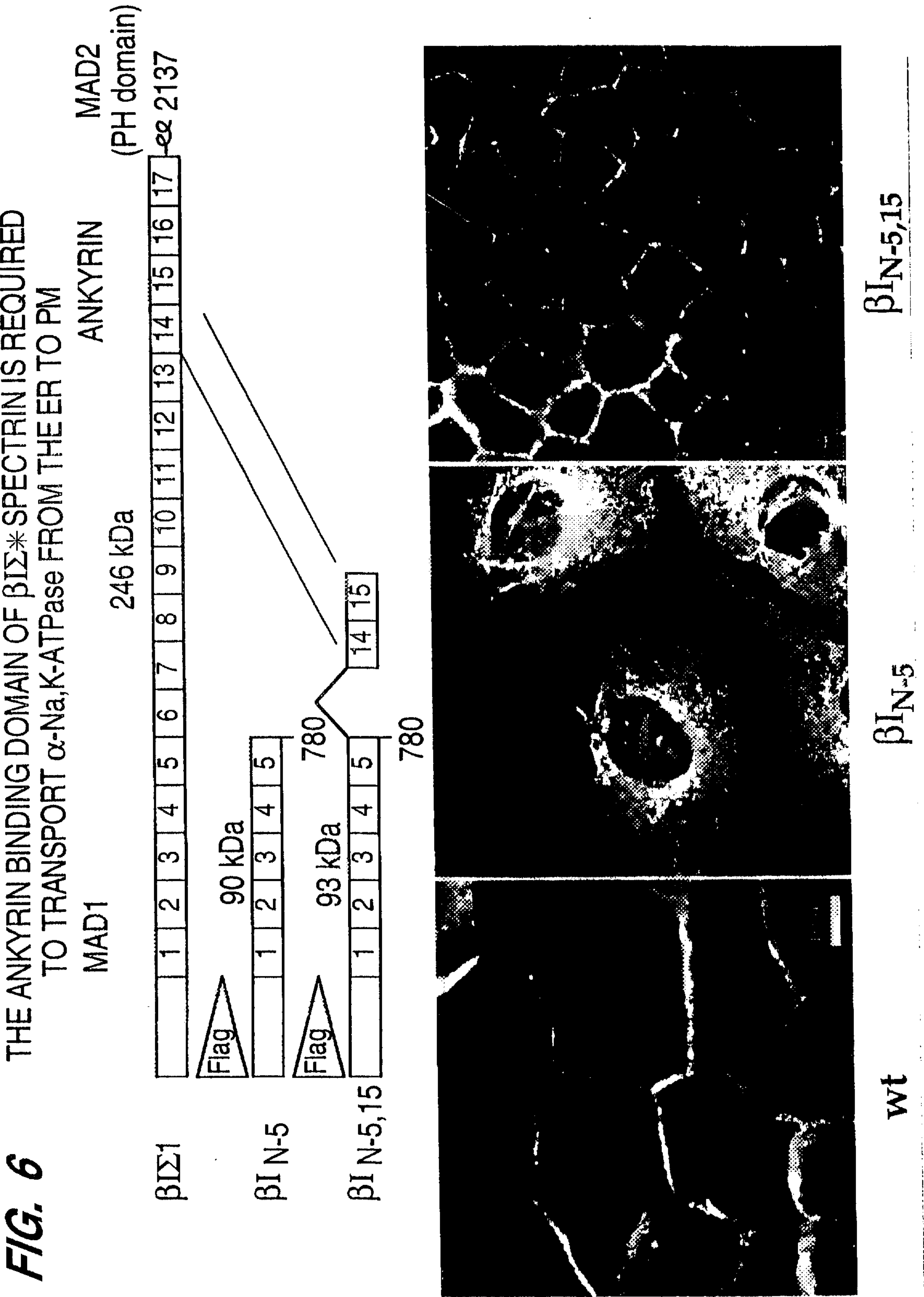
FIG. 4A**FIG. 4B****FIG. 4C**

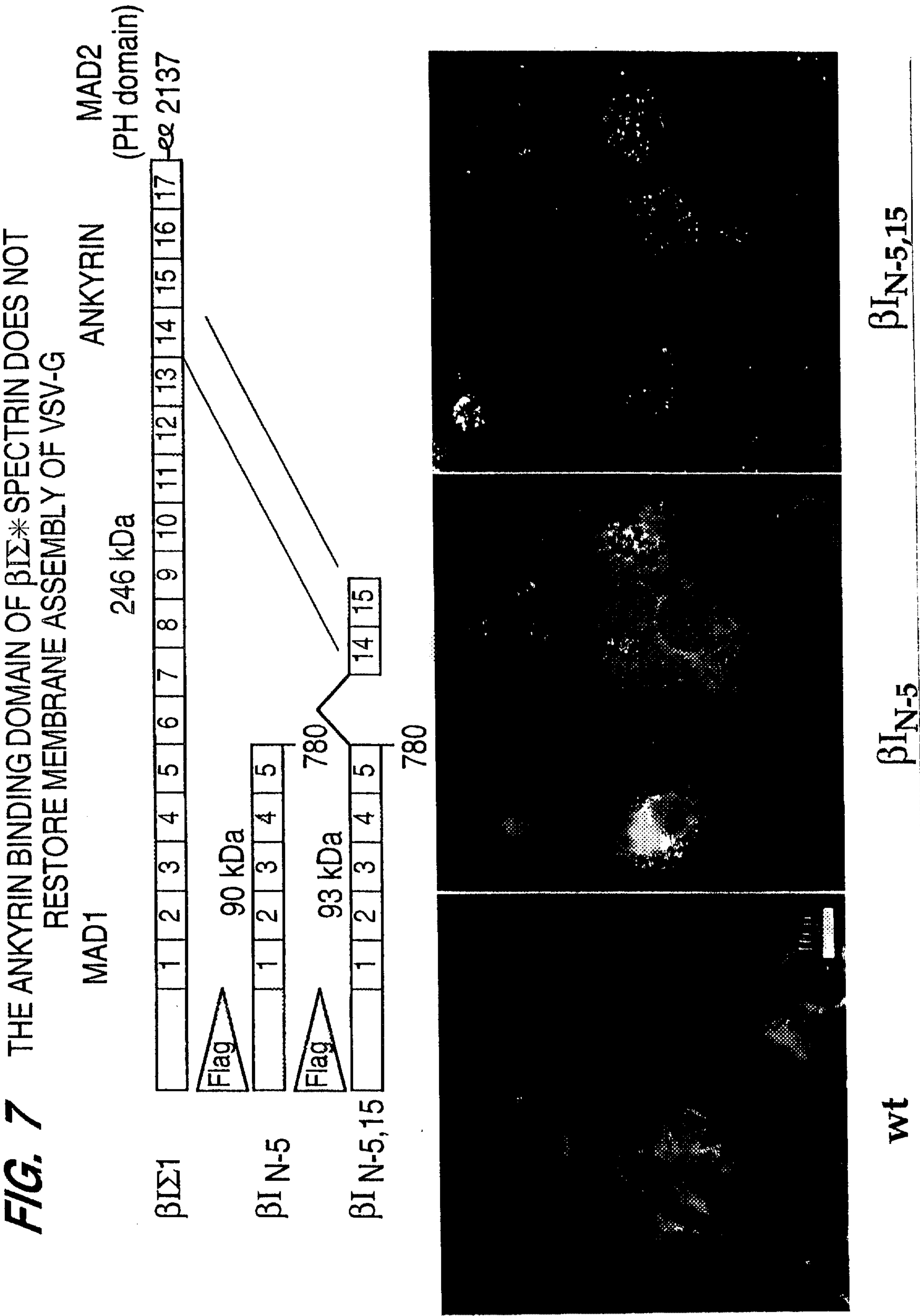
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FIG. 4D

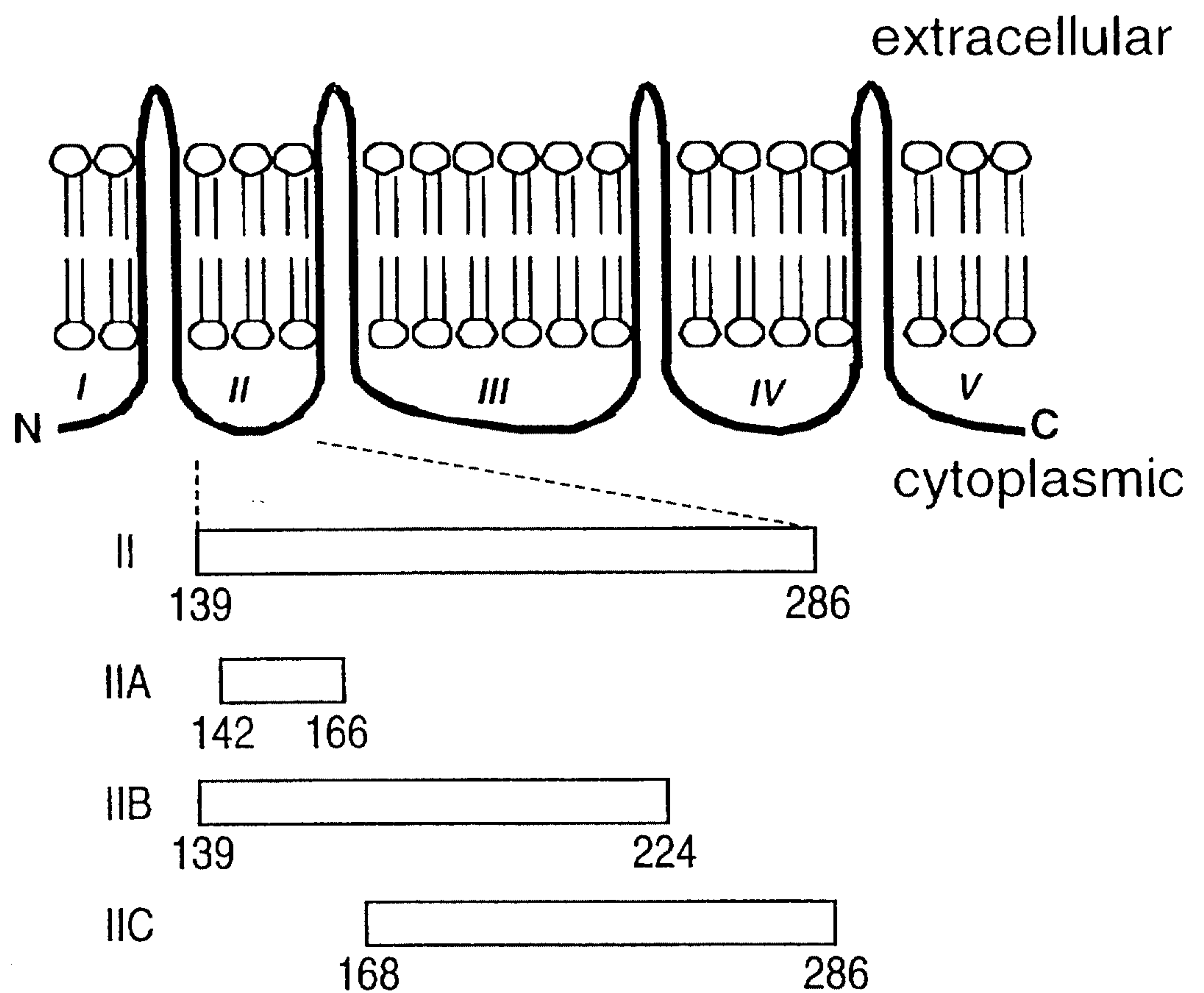
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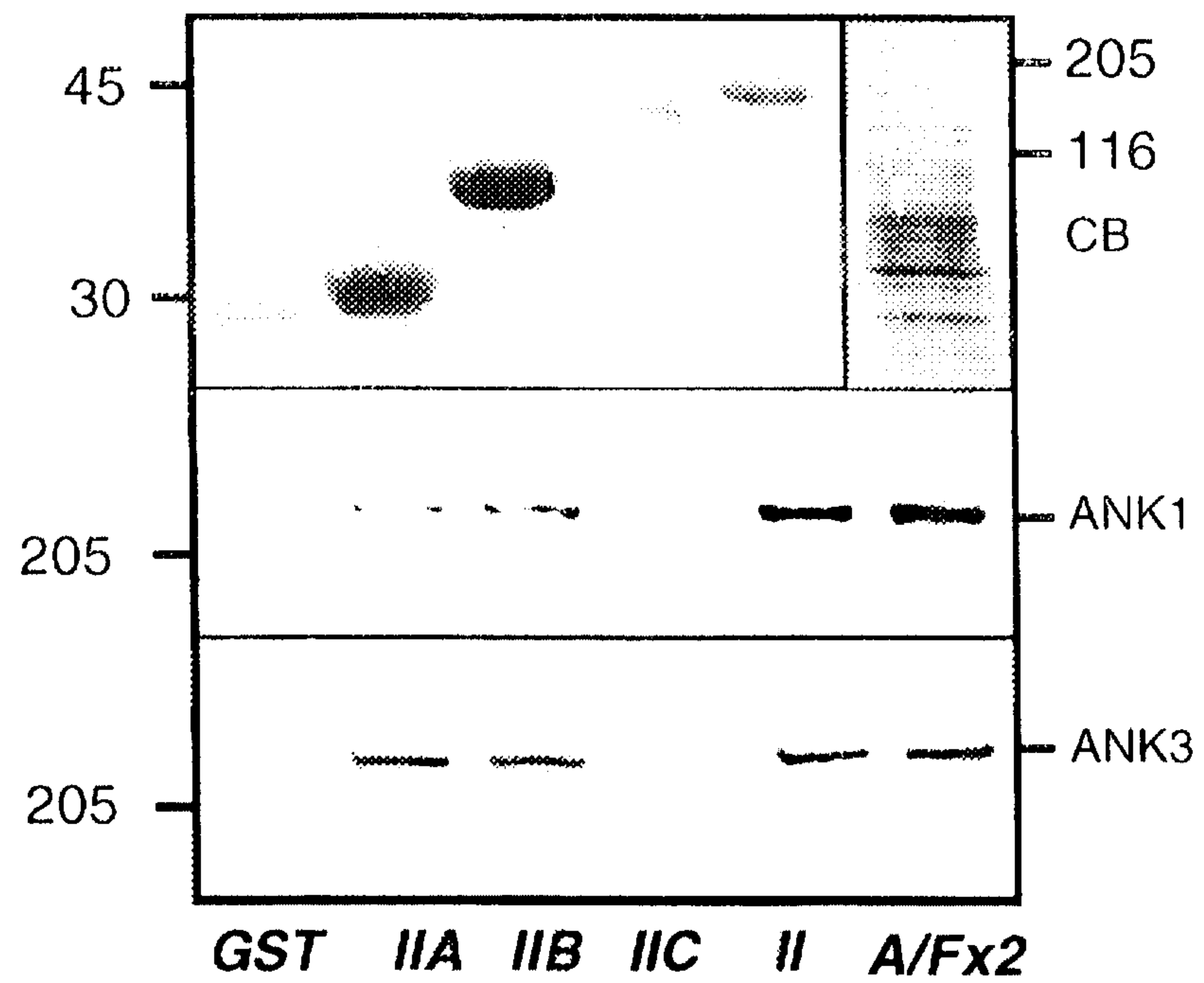




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FIG. 8A

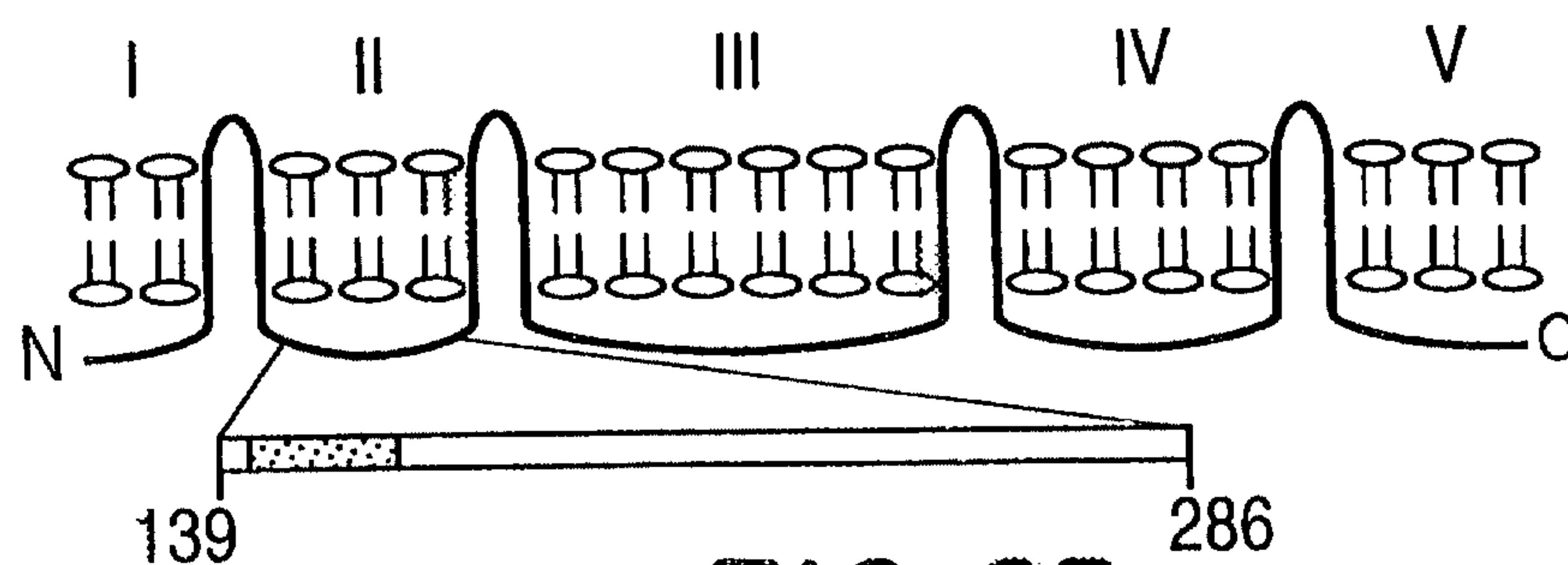
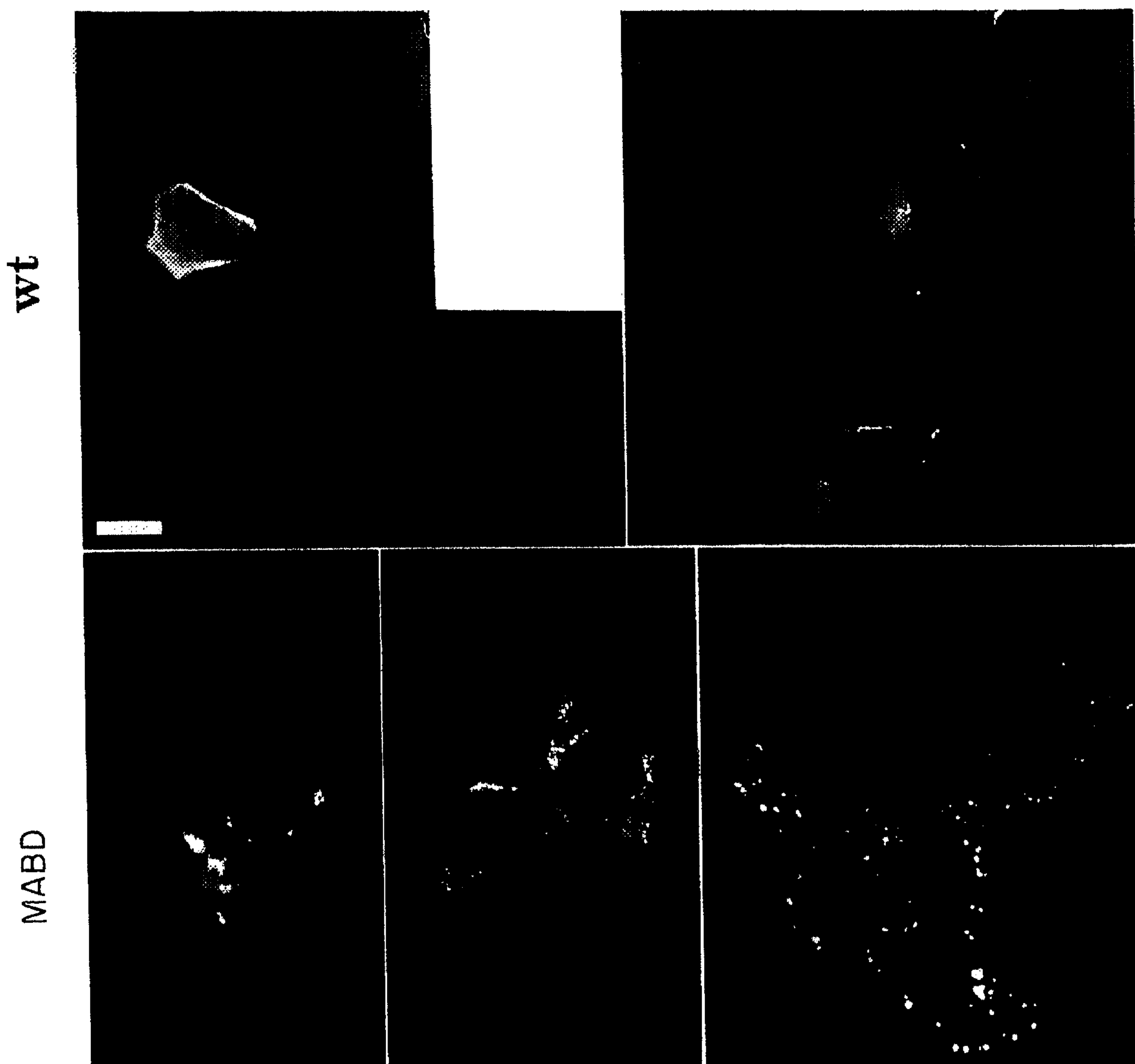
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FIG. 8B

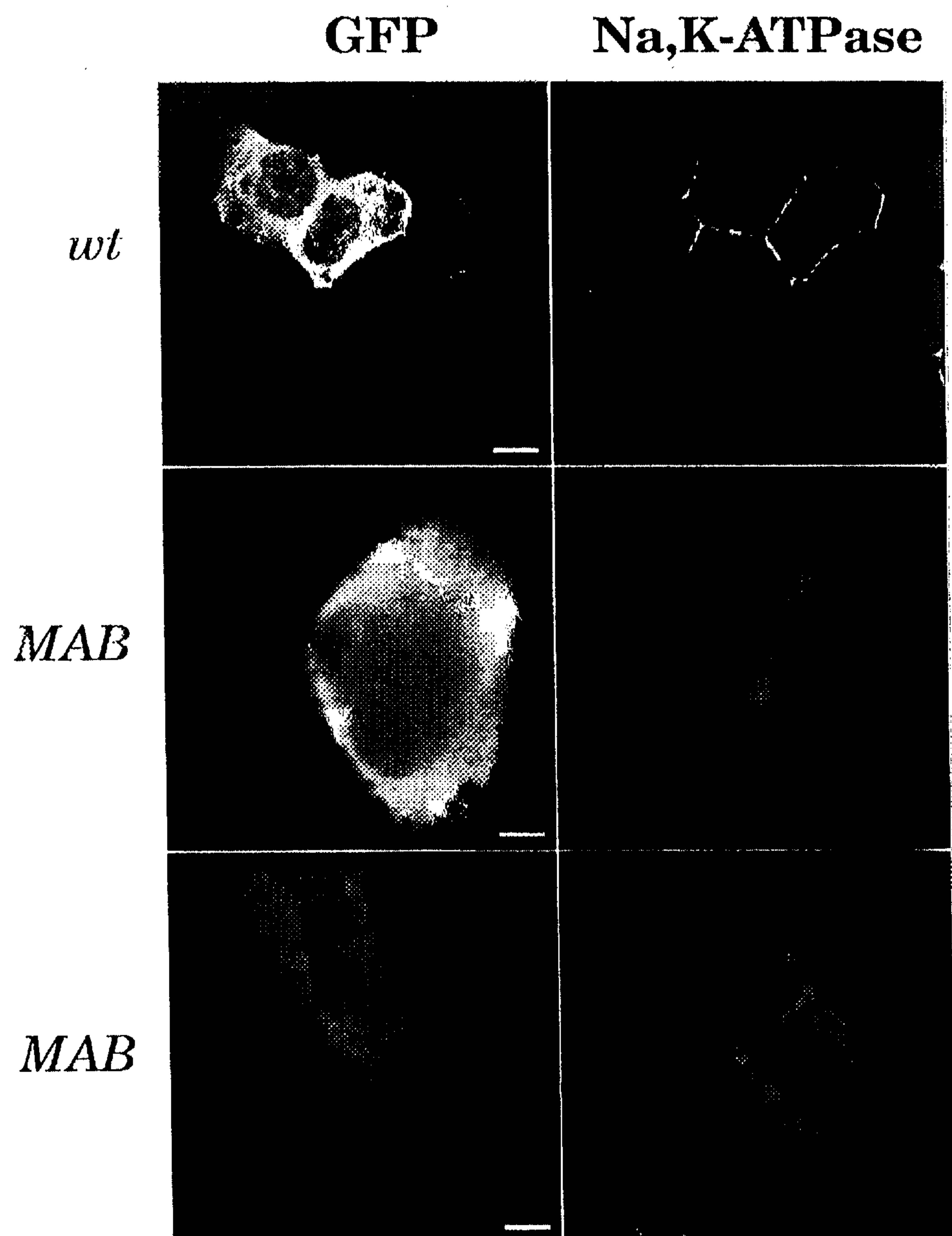
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FIG. 9A

THE ANKYRIN BINDING DOMAIN OF α -Na,K-ATPase
IS REQUIRED FOR ITS MEMBRANE ASSEMBLY

**FIG. 9B**

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FIG. 10A

SUBSTITUTE SHEET (RULE 26)

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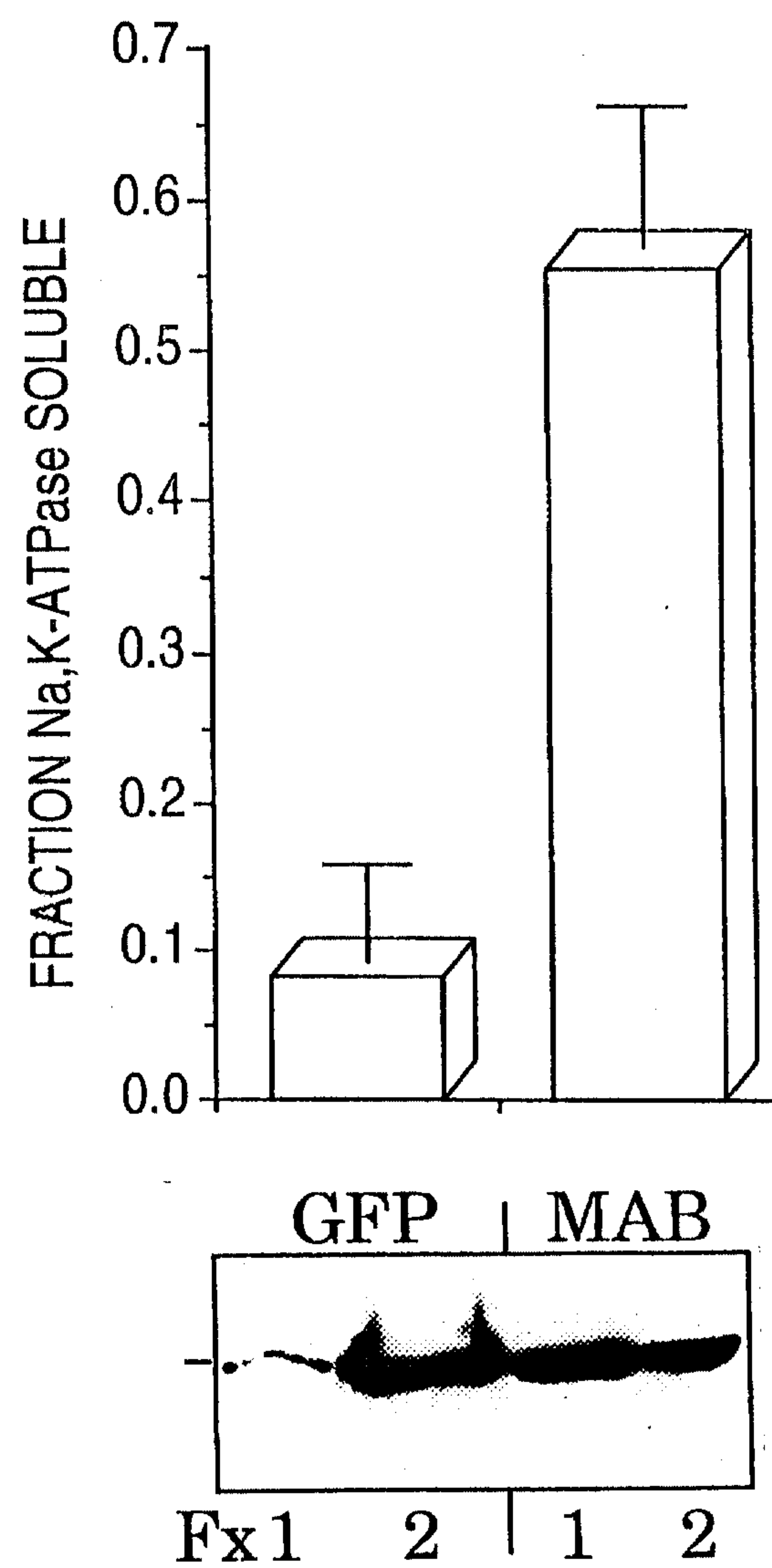
FIG. 10B

FIG. 11B

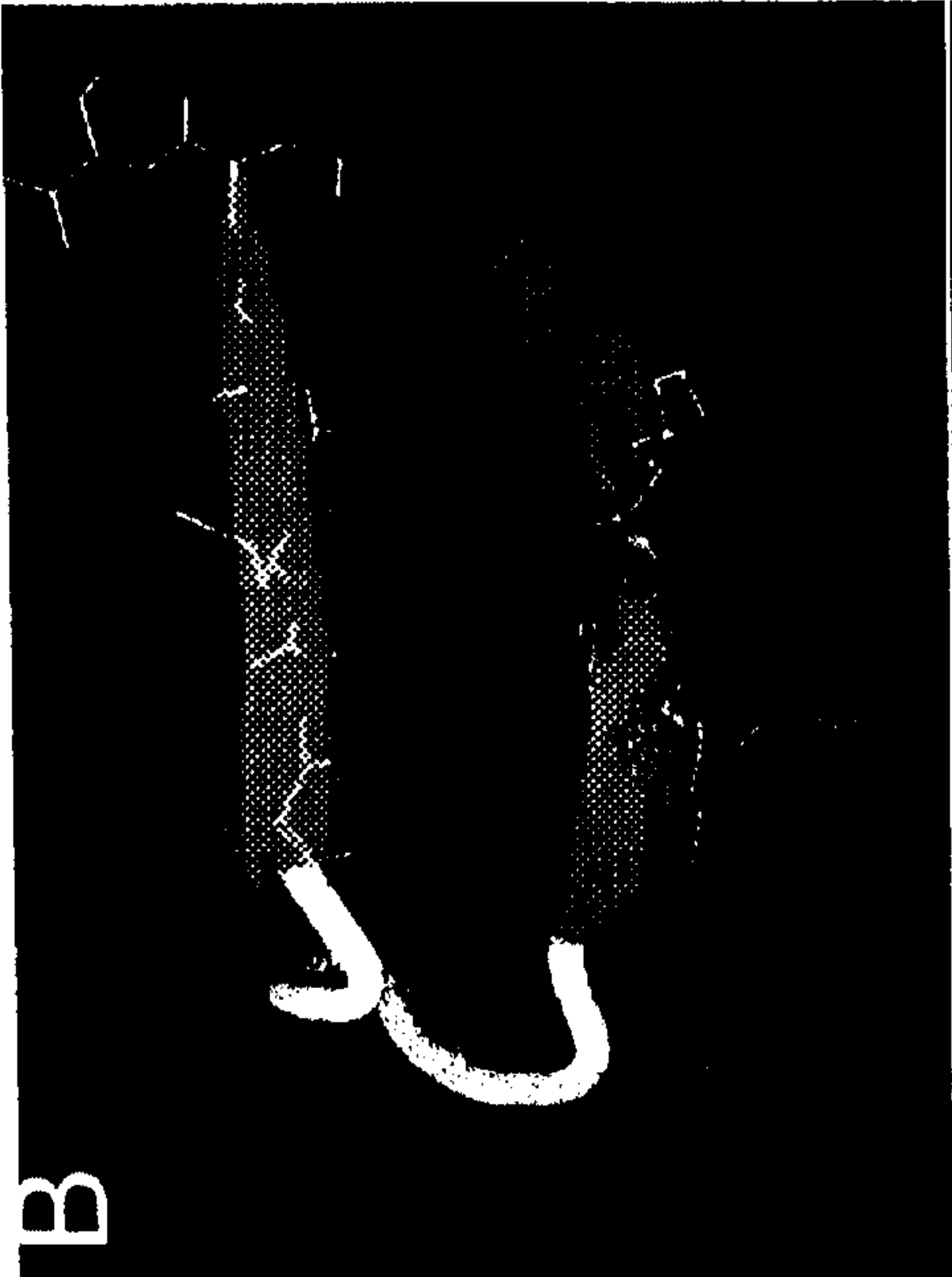


FIG. 11D

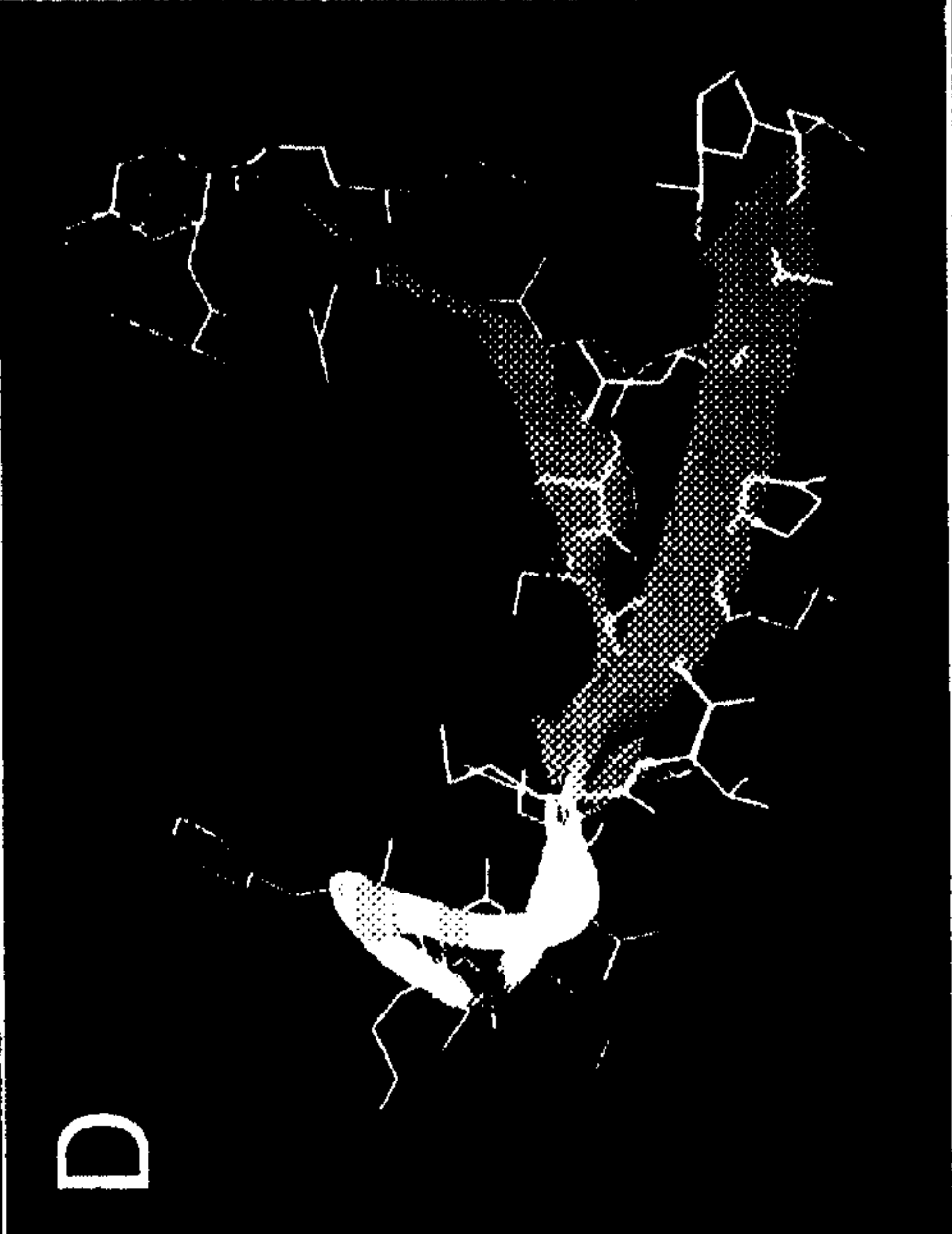


FIG. 11F

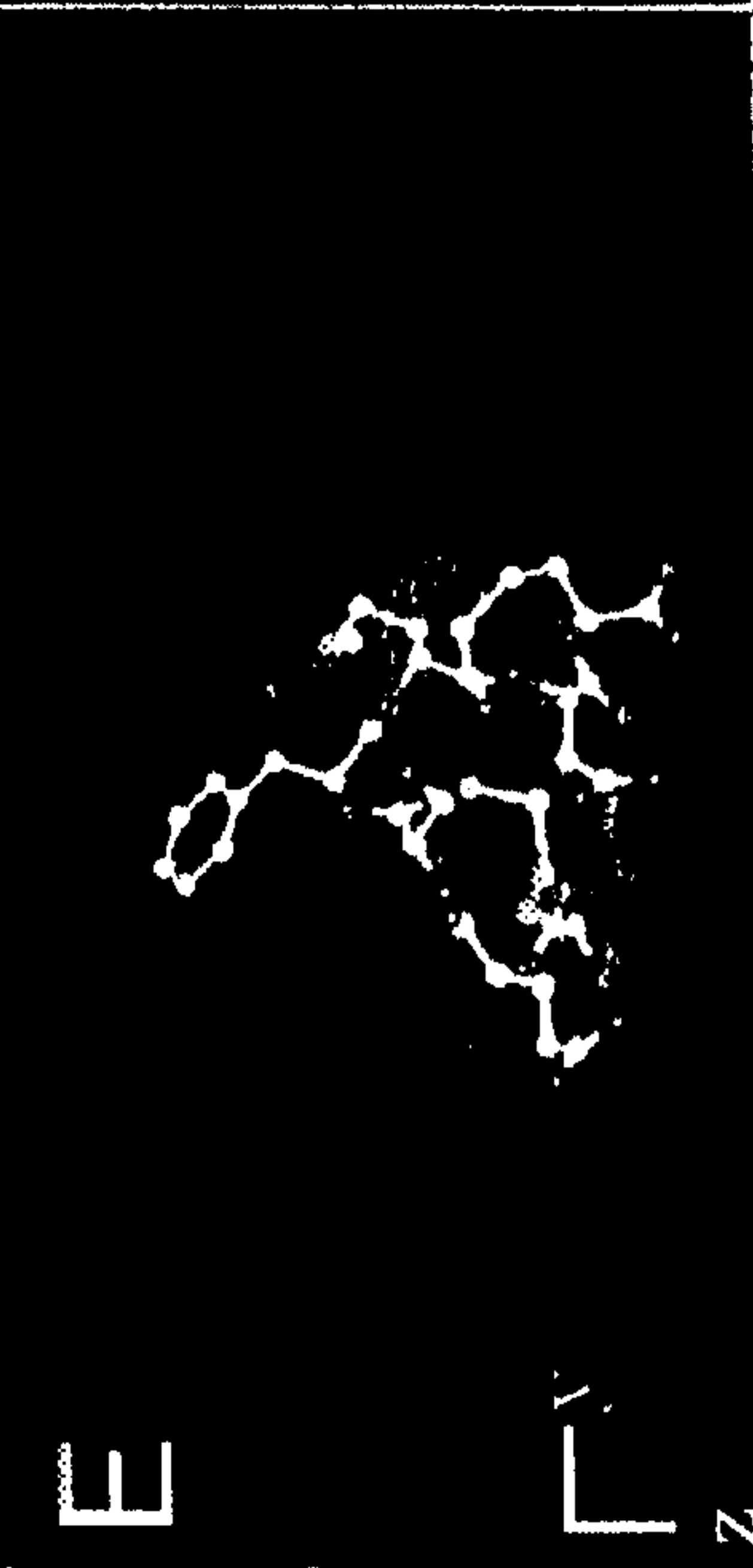
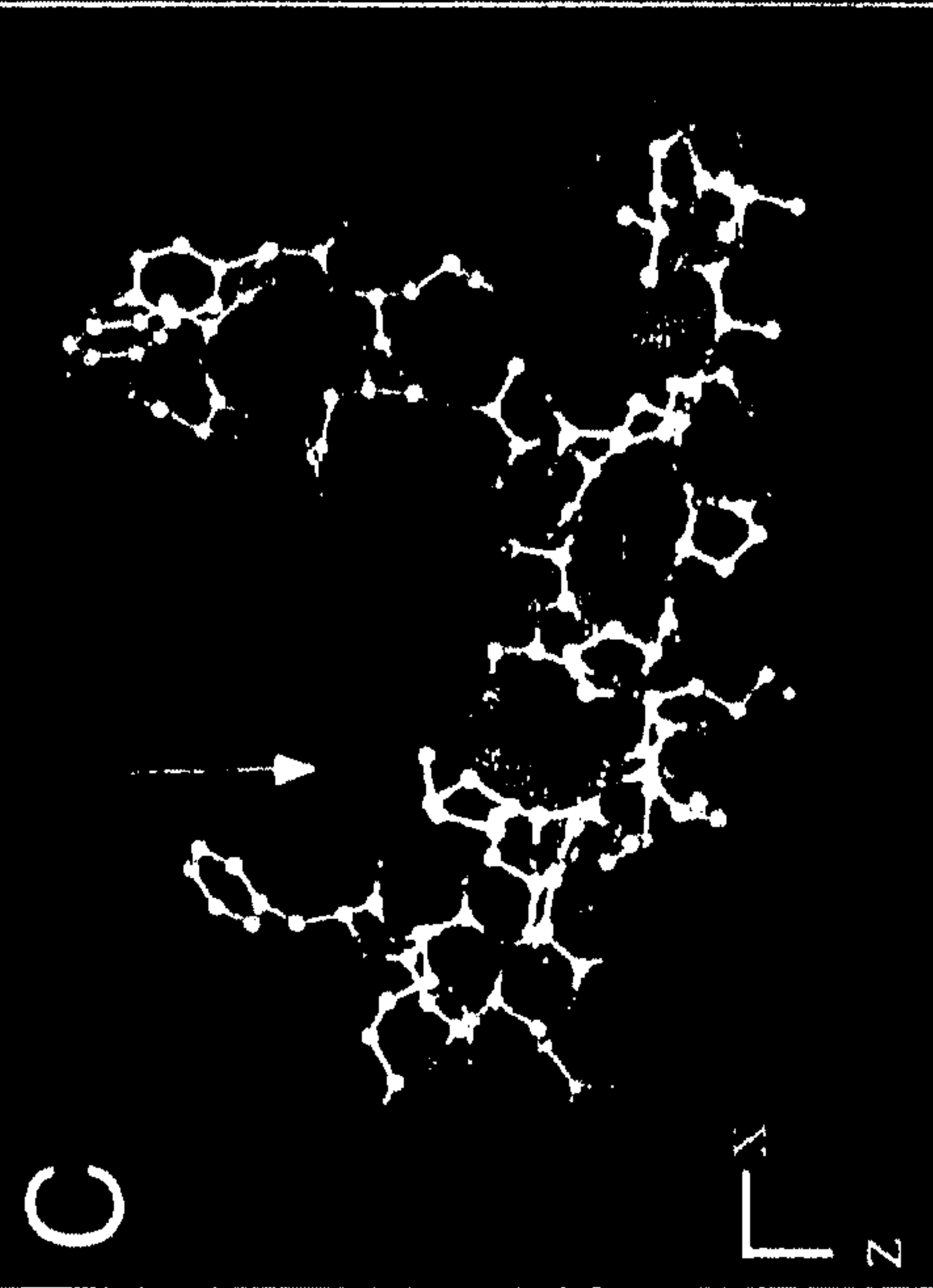
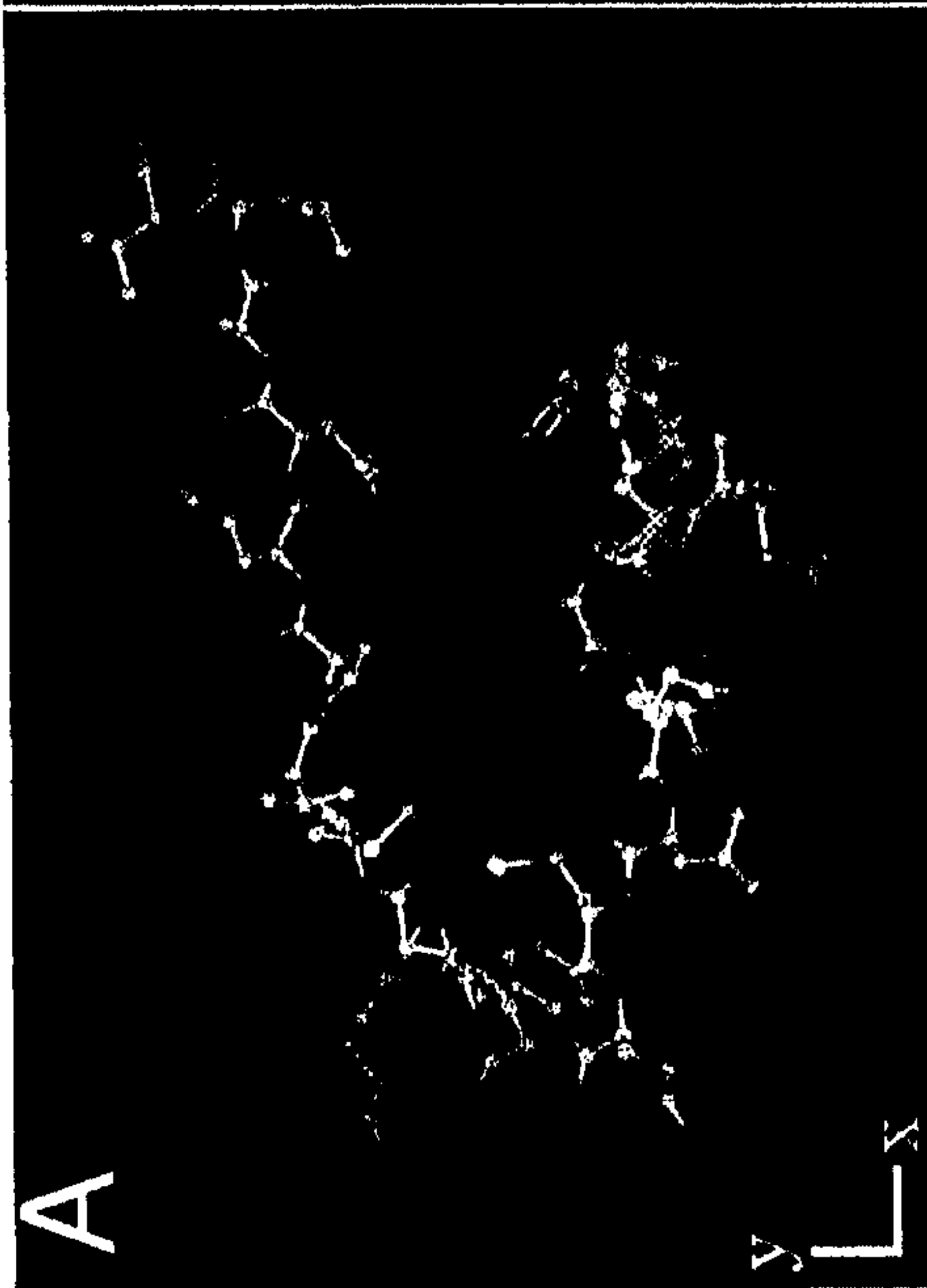


FIG. 11A

FIG. 11C

FIG. 11E

FIG. 12A

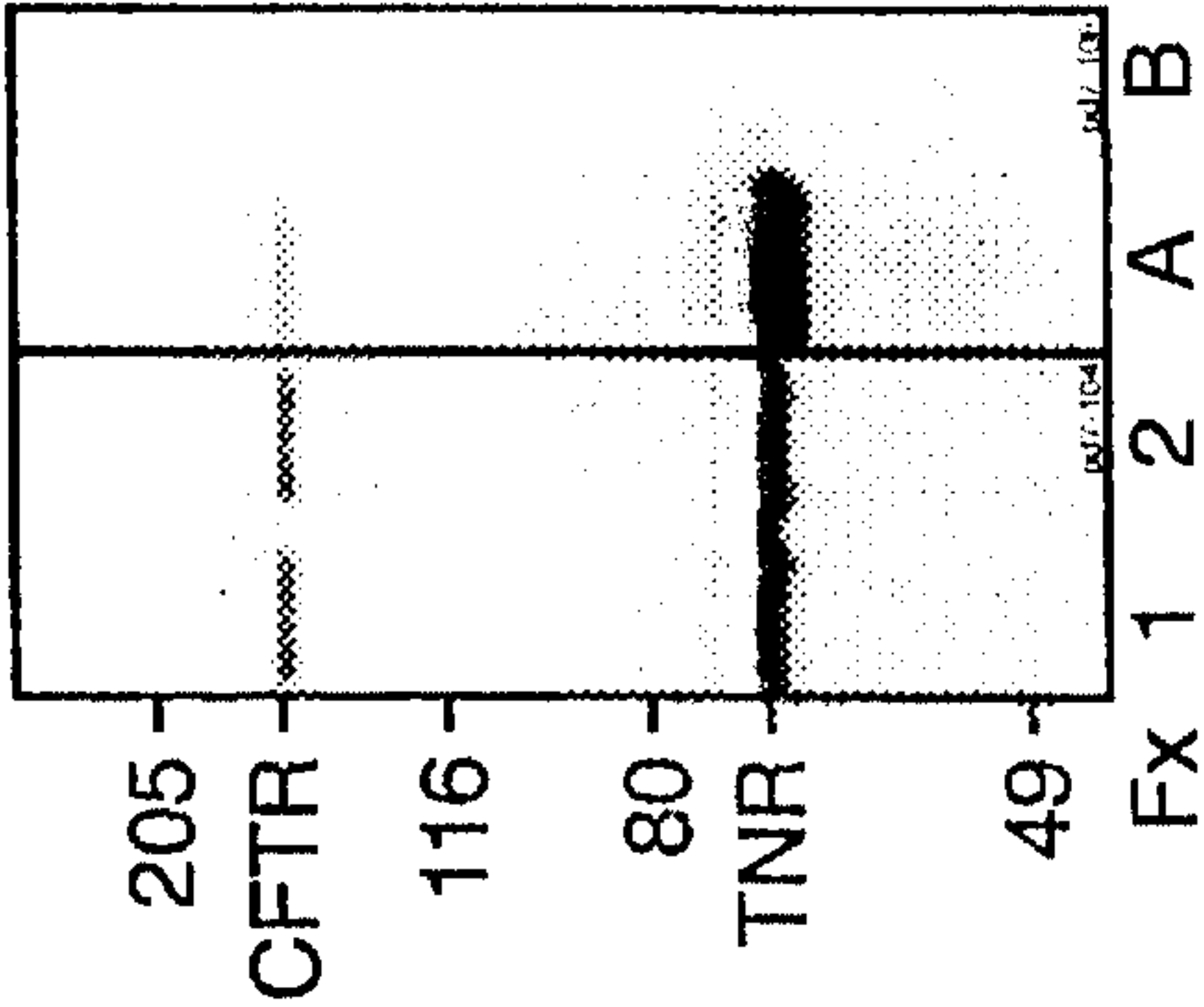
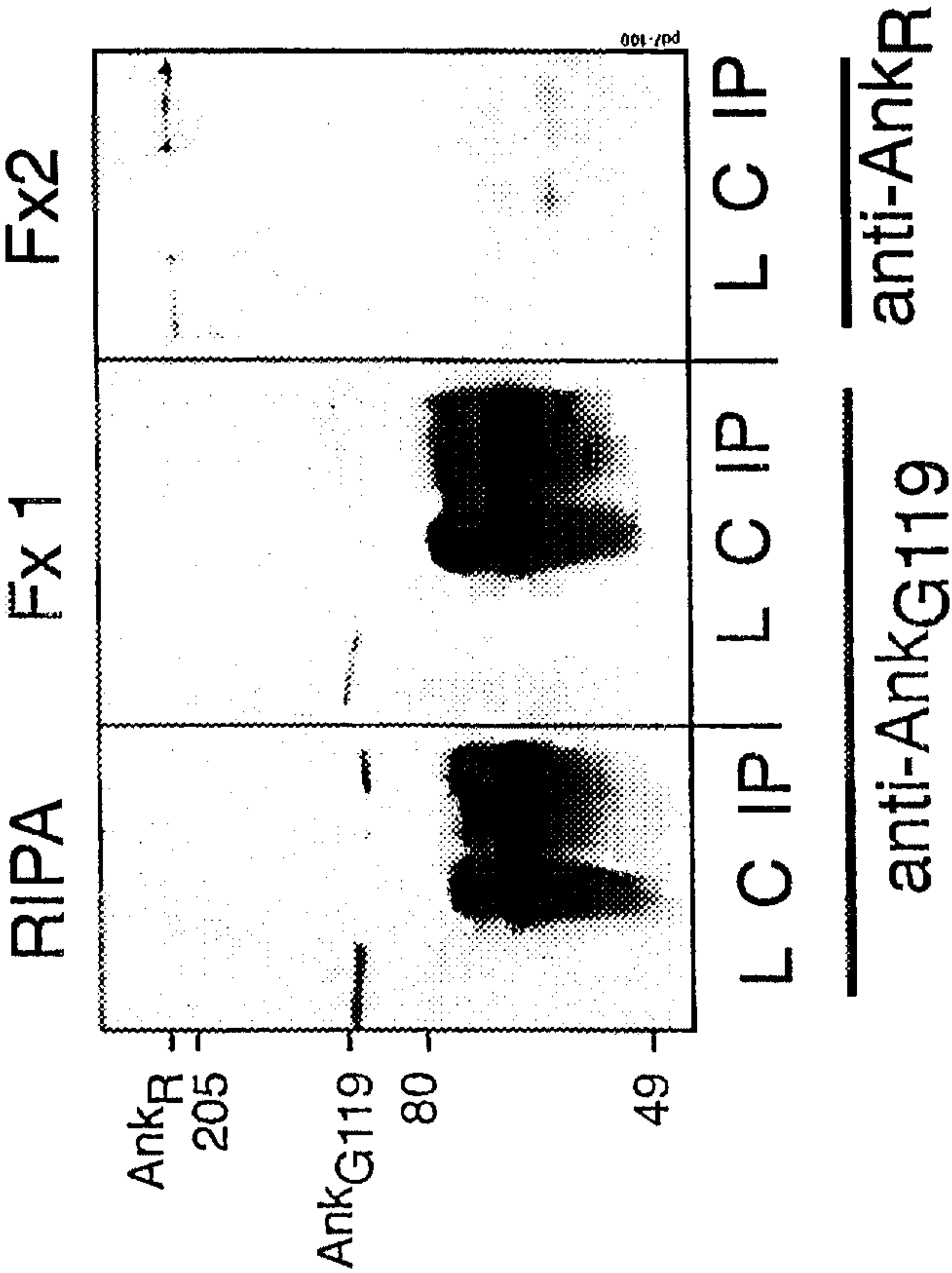
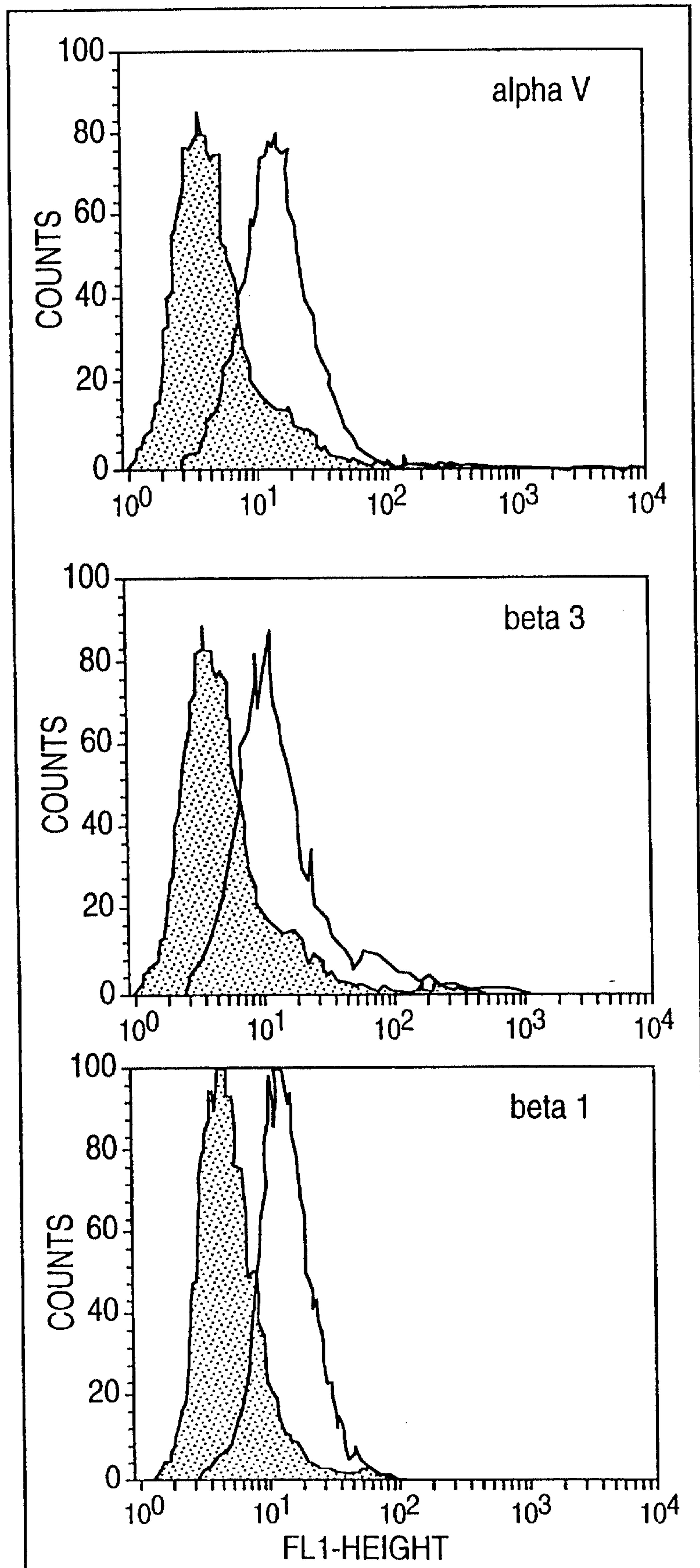


FIG. 12B



CFTR and TNR are apical, associated with the cytoskeleton, & bind both AnkG119 and AnkR

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FIG. 13A

SVEC cytometry

SUBSTITUTE SHEET (RULE 26)

FIG. 13B

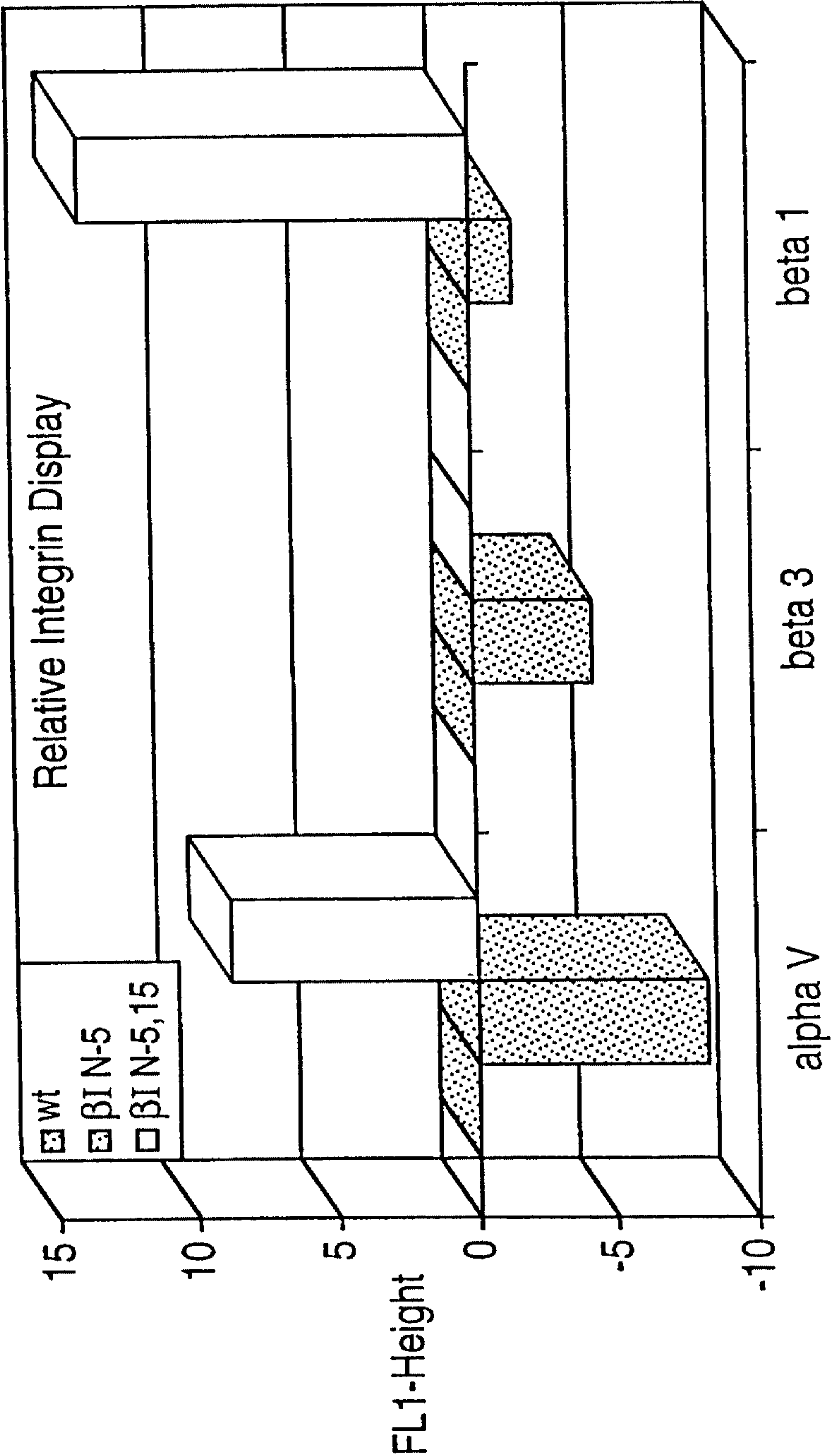
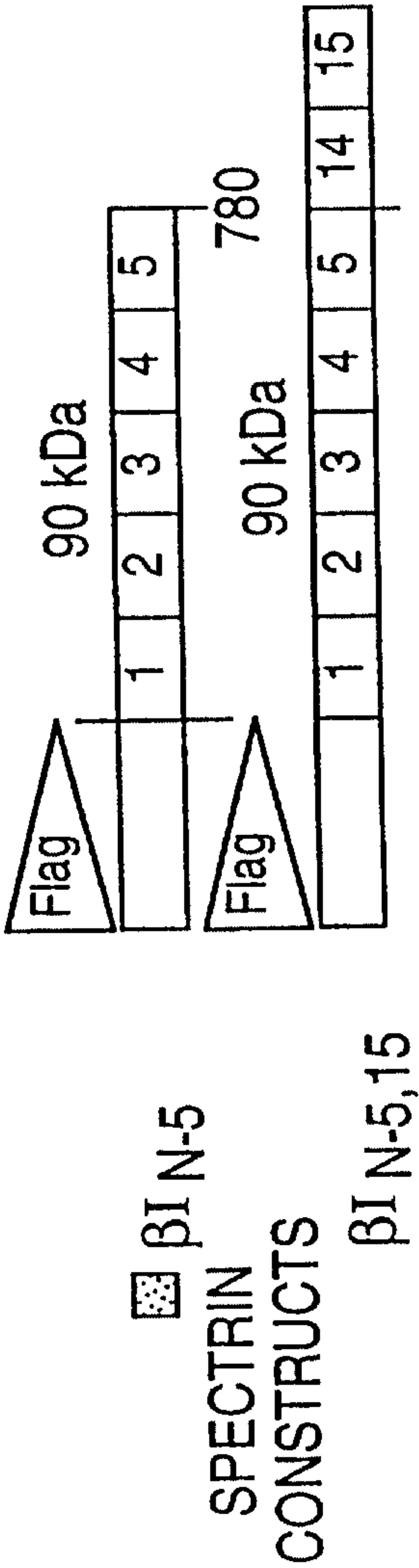


FIG. 14C

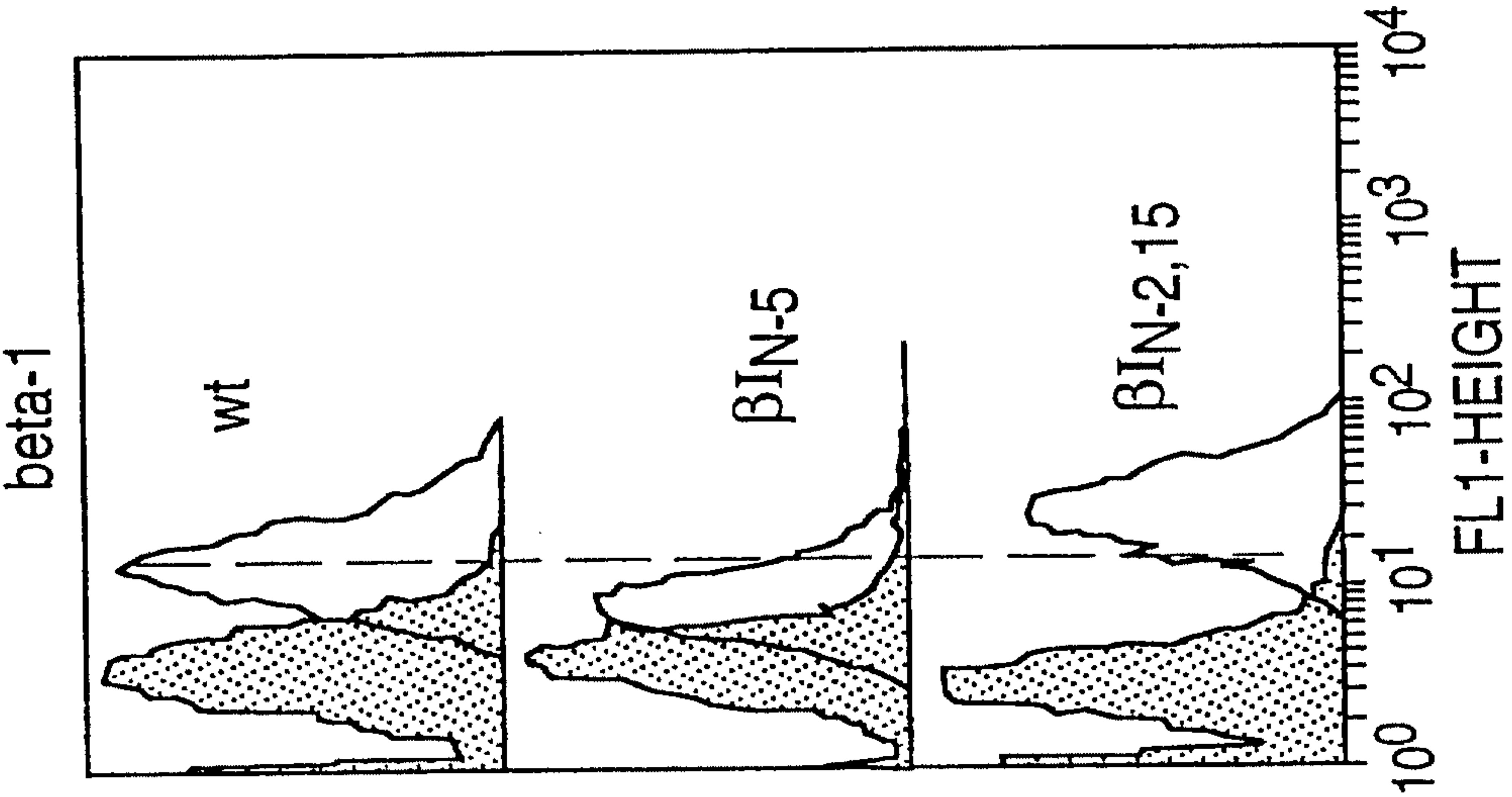


FIG. 14B

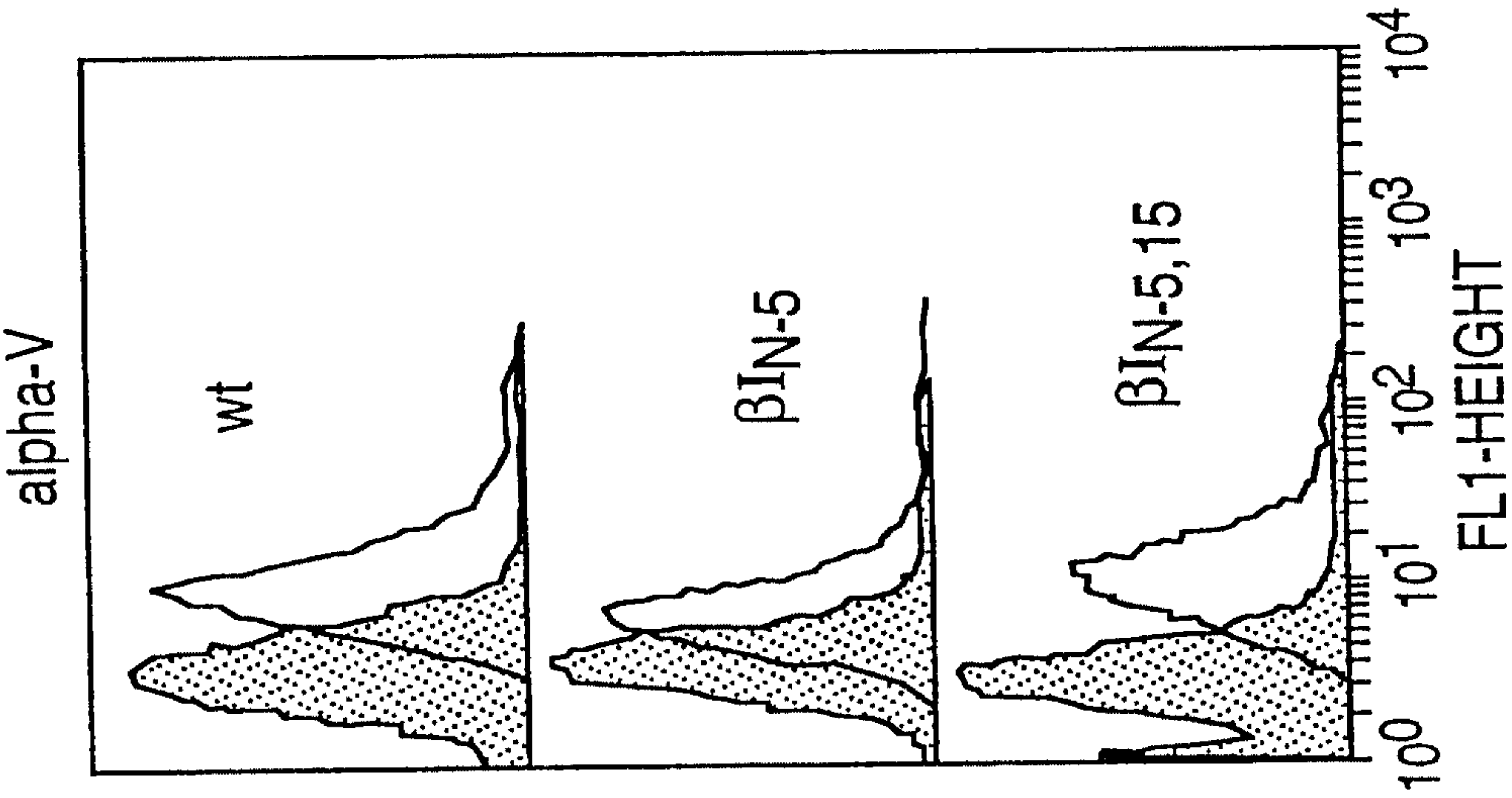
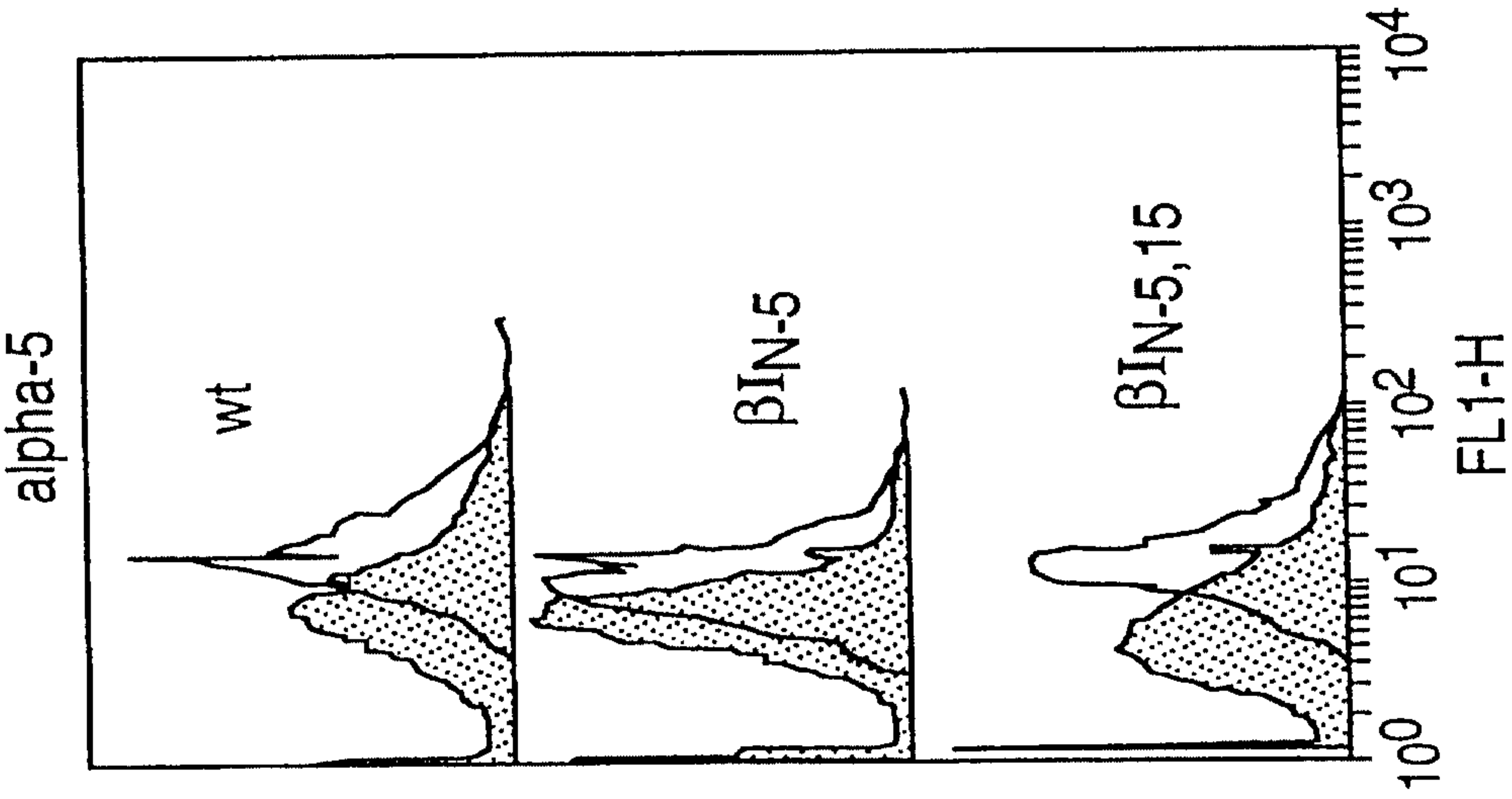
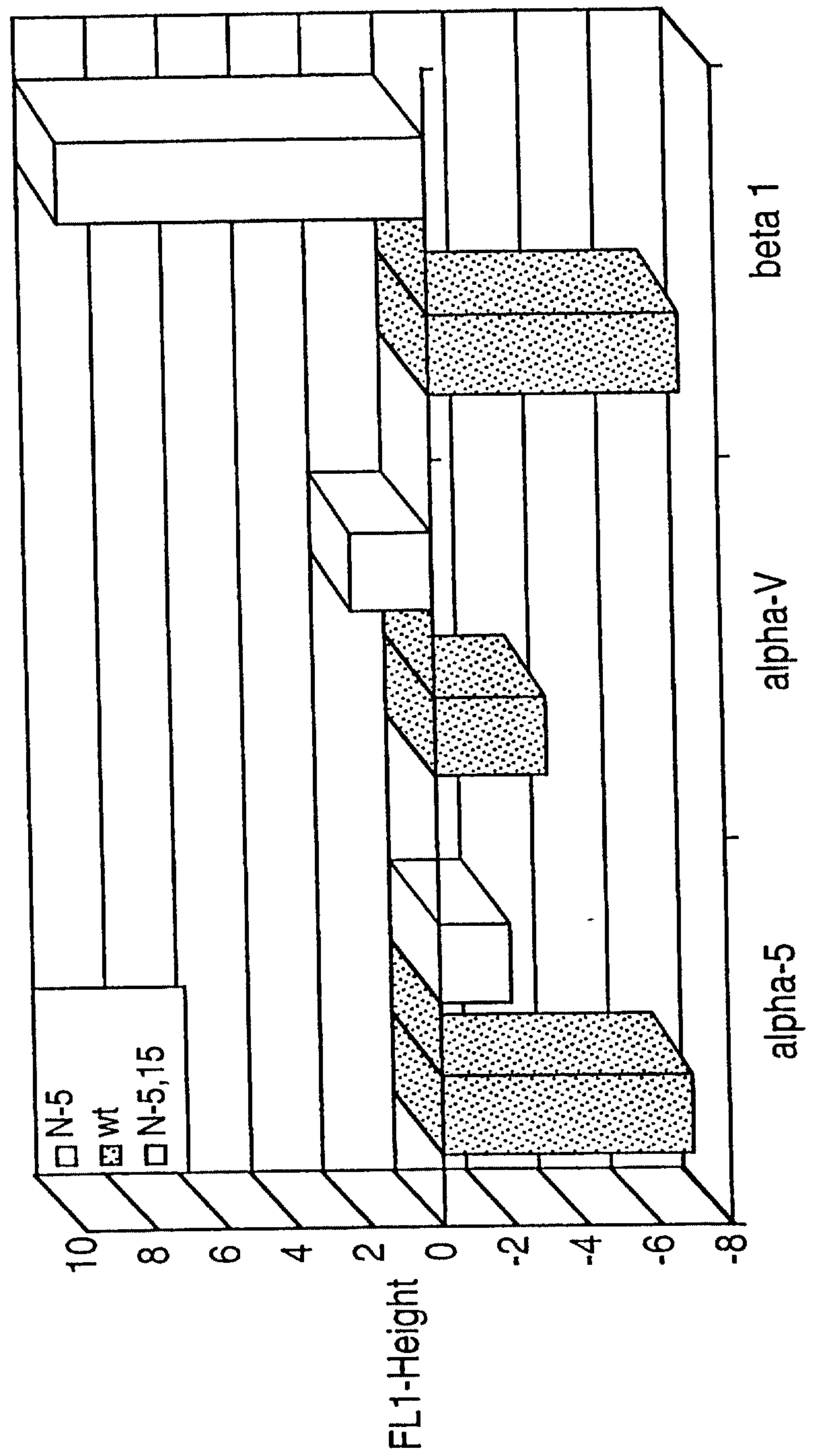


FIG. 14A

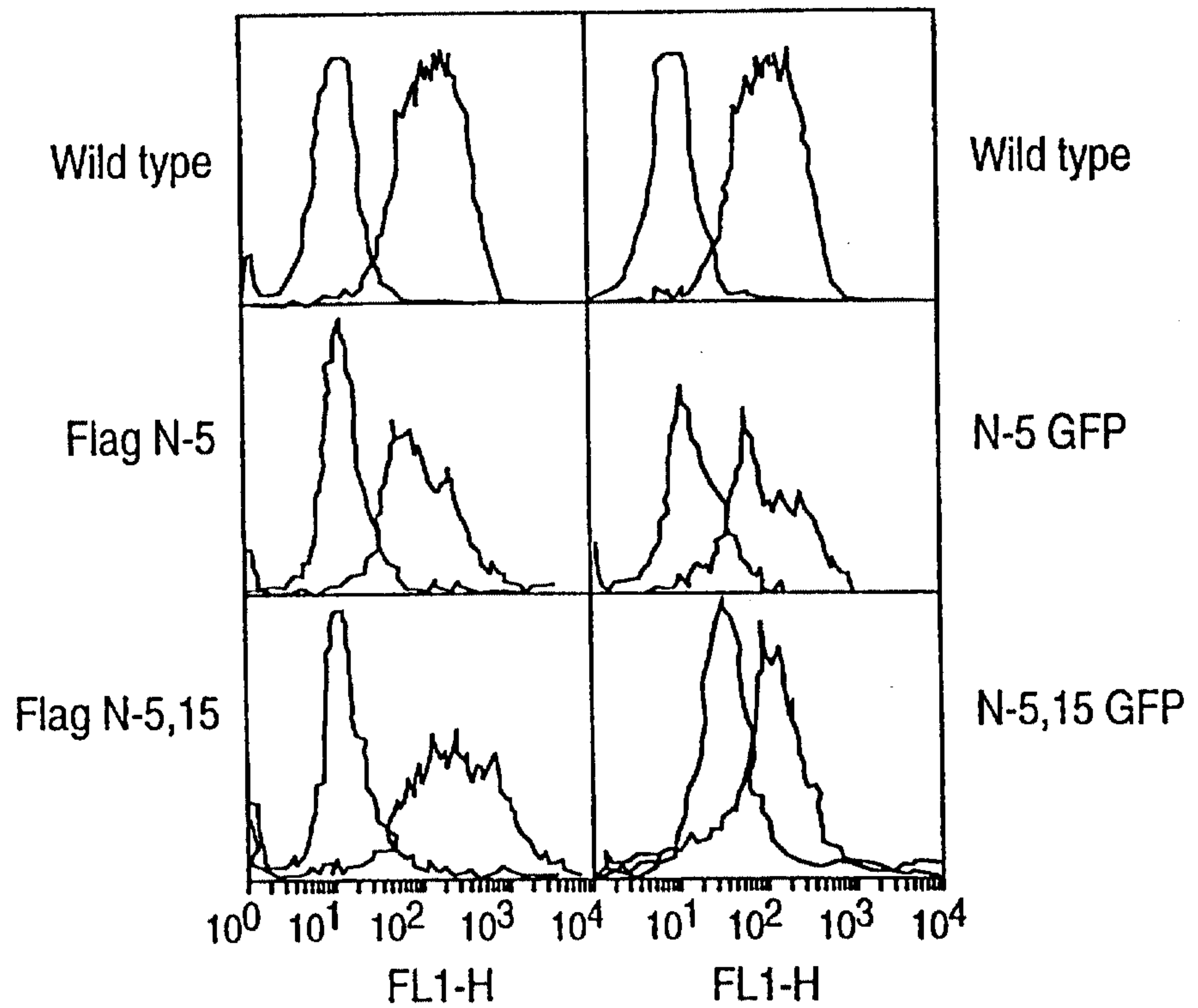
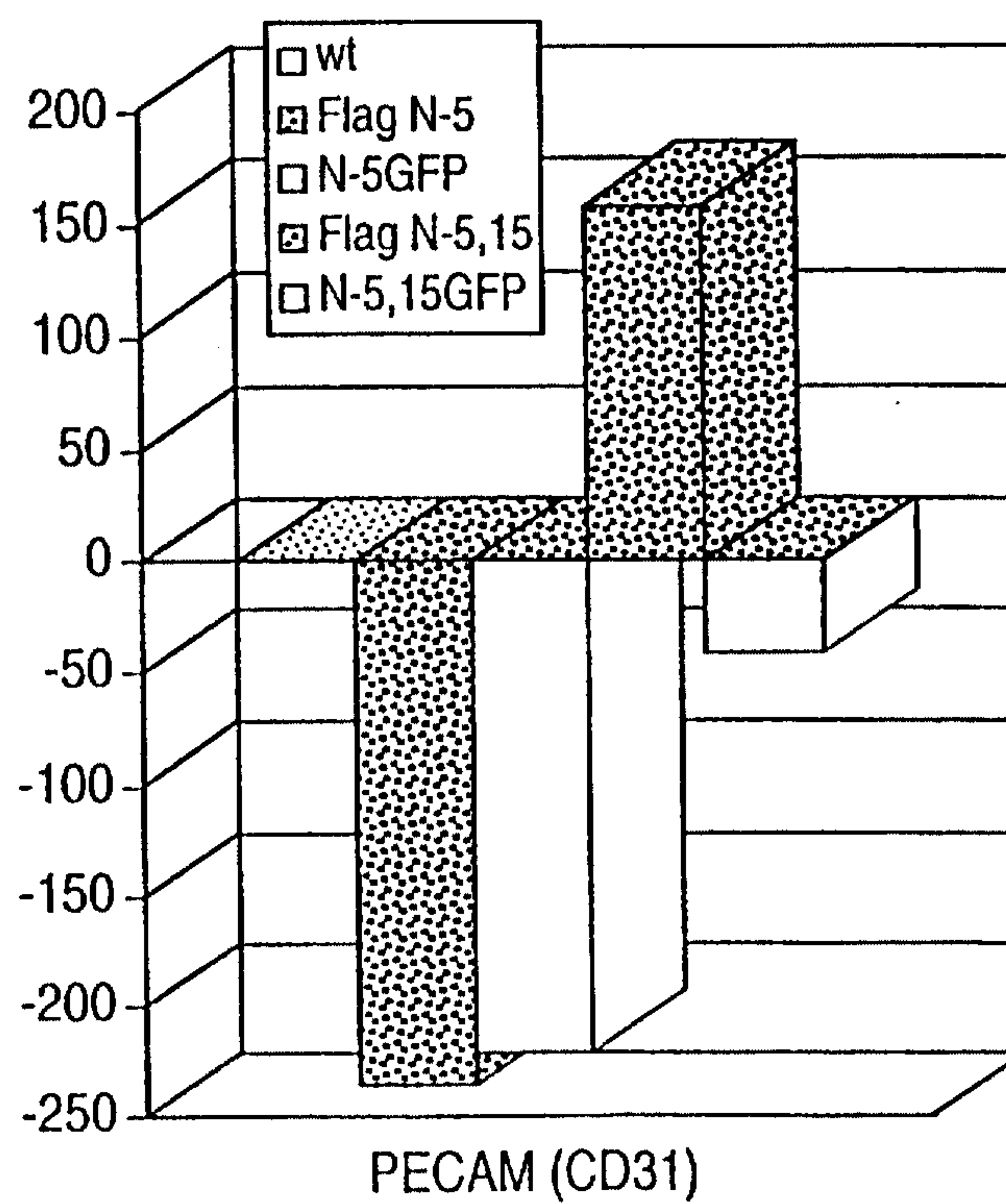


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FIG. 14D



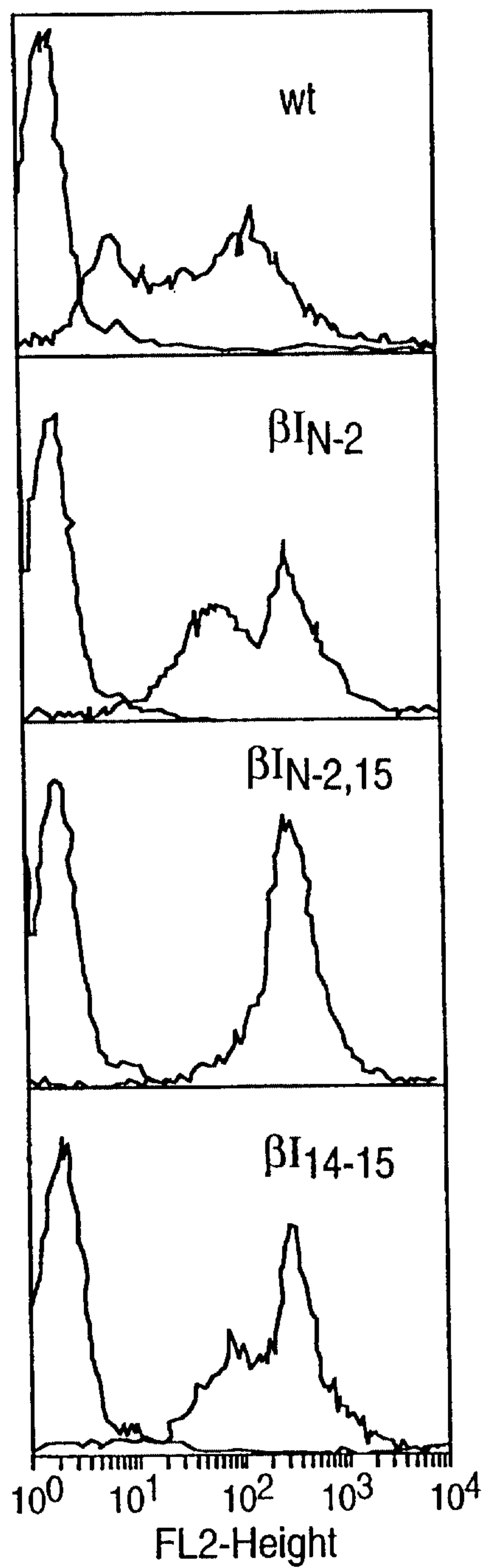
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FIG. 15A**FIG. 15B**

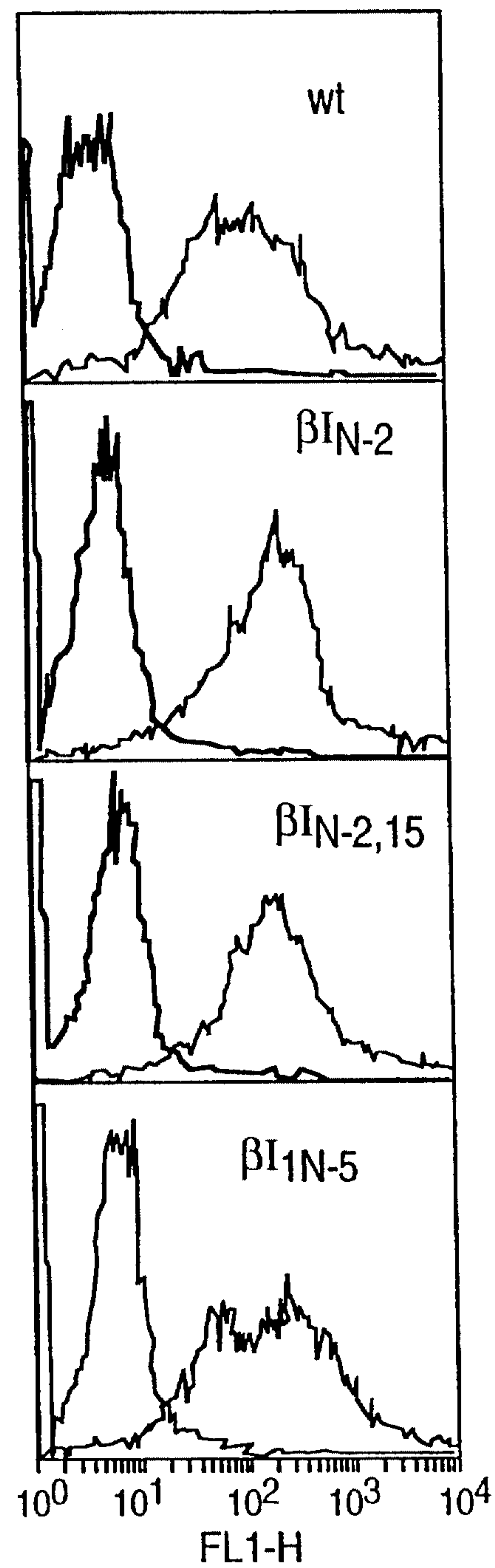
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FIG. 16A

CD45

**FIG. 16B**

TNFR-1



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FIG. 16C

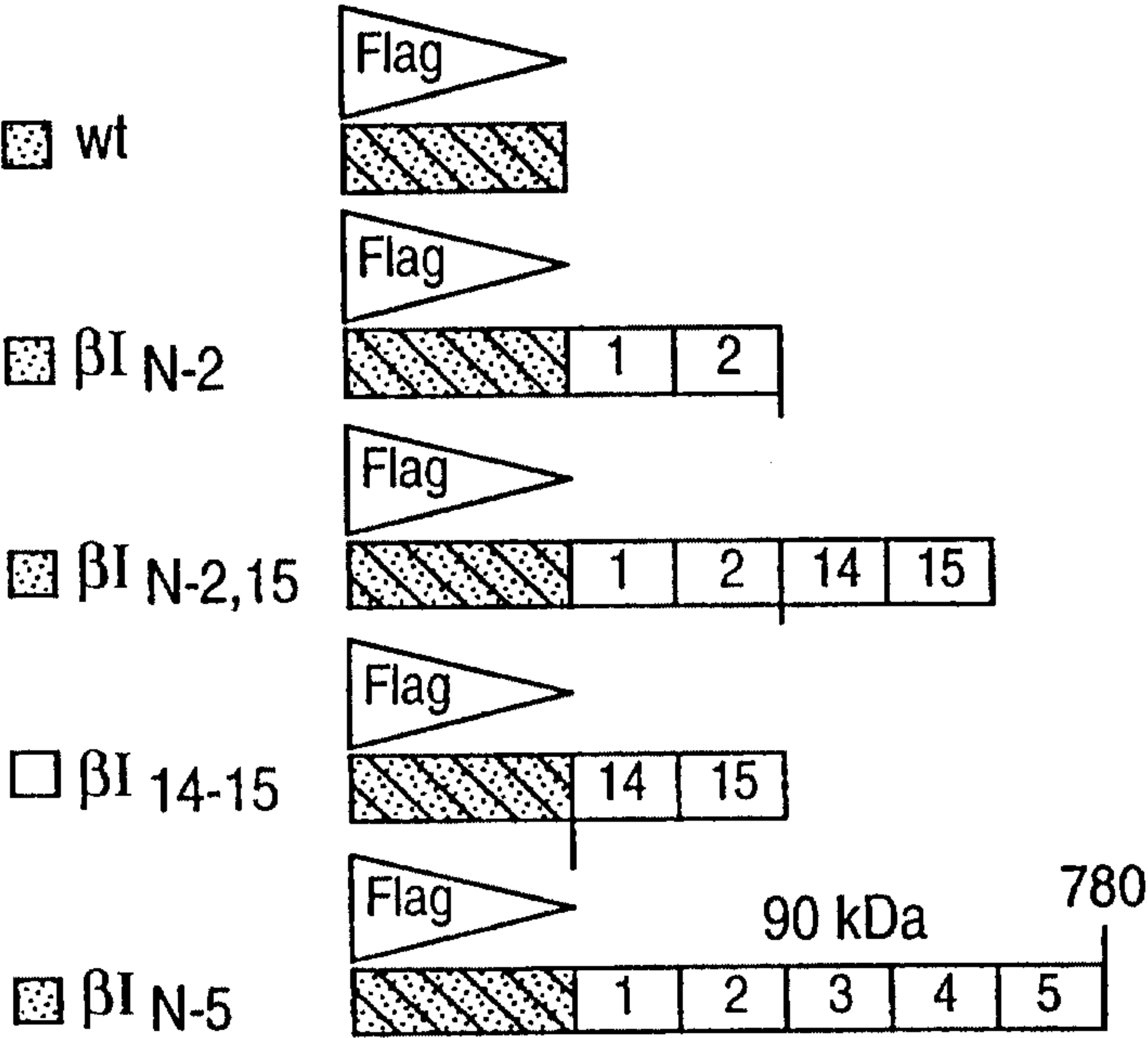
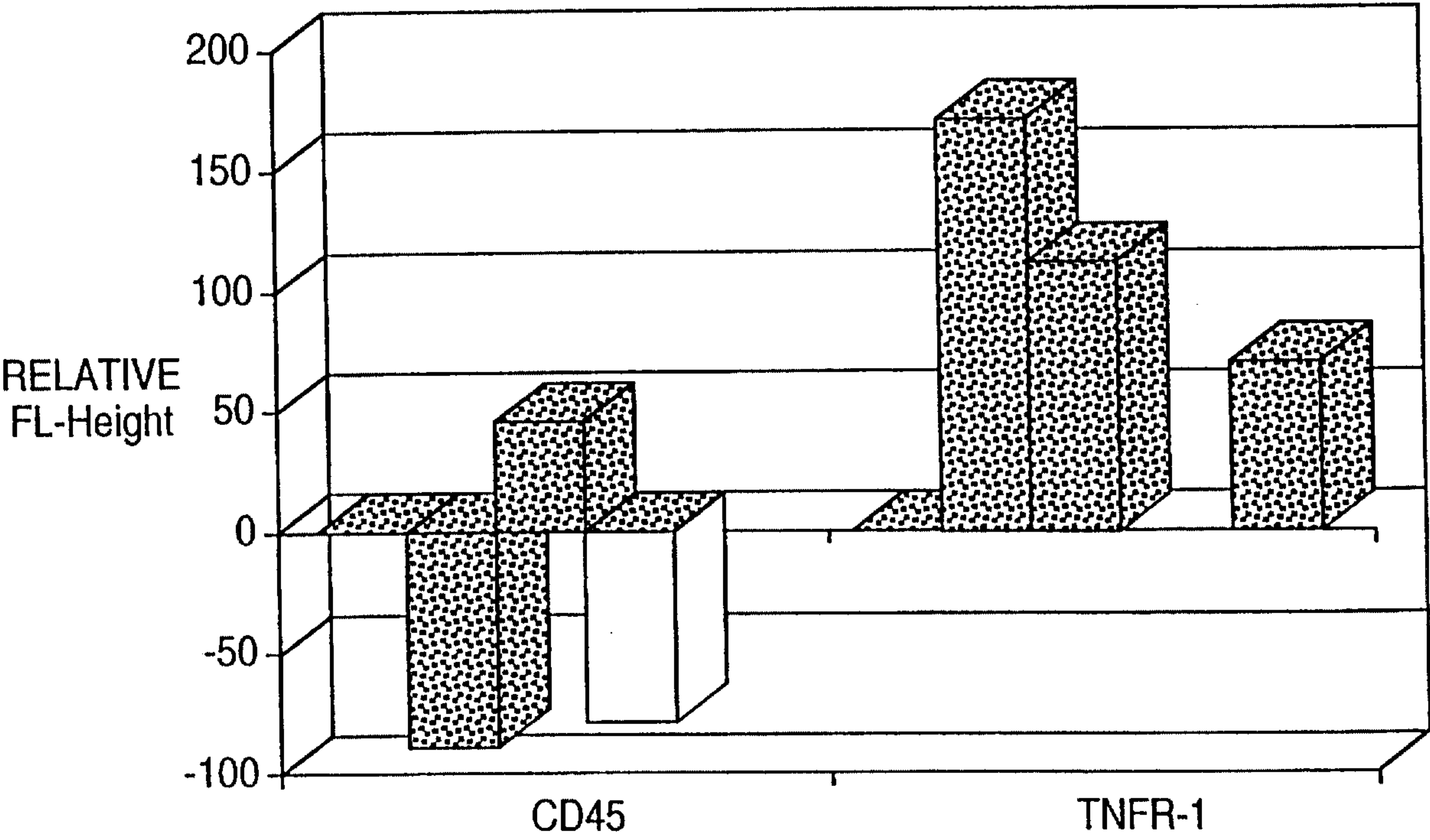


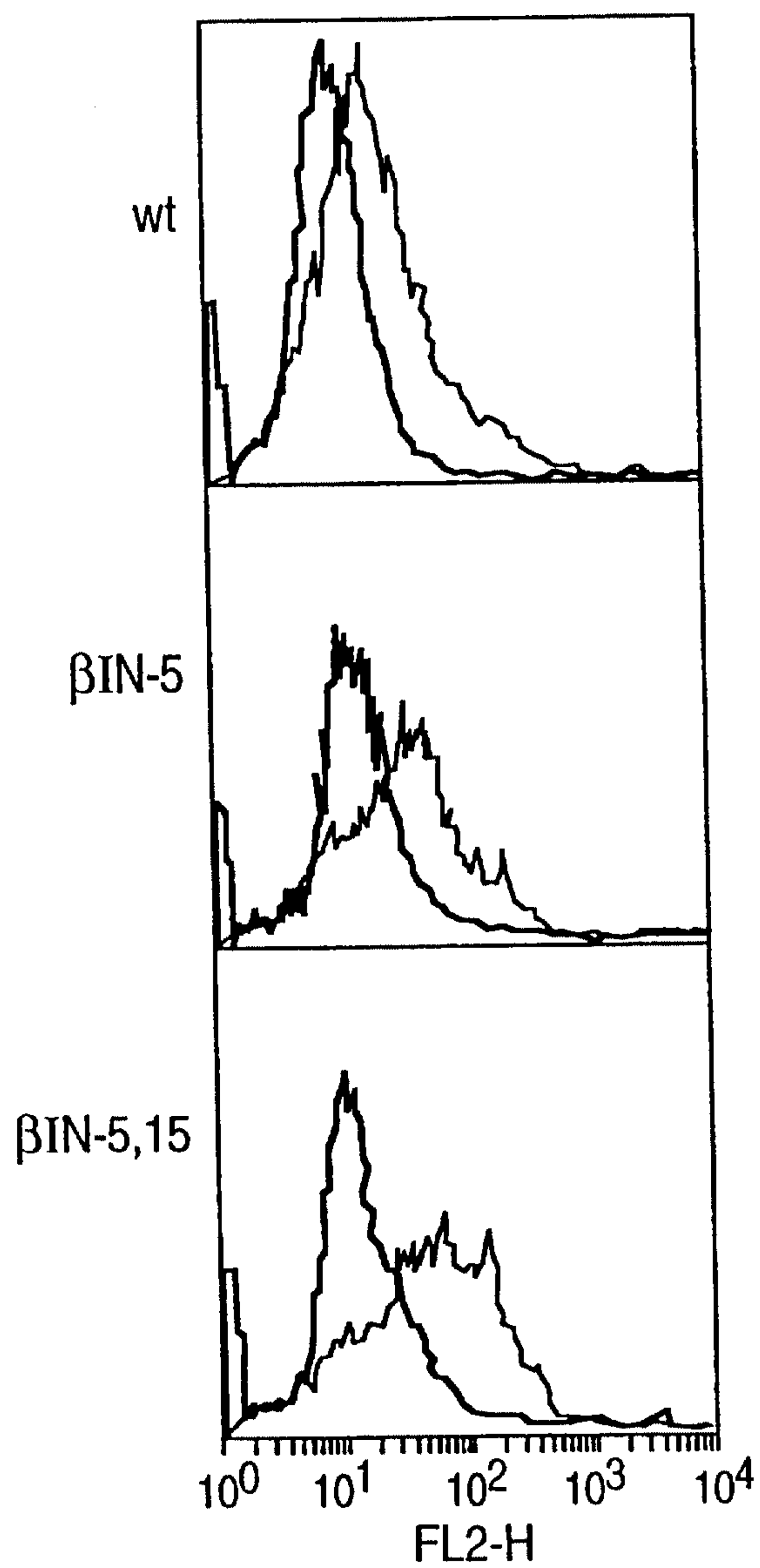
FIG. 16D



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FIG. 17A

Fas

**FIG. 17B**

Fas-L

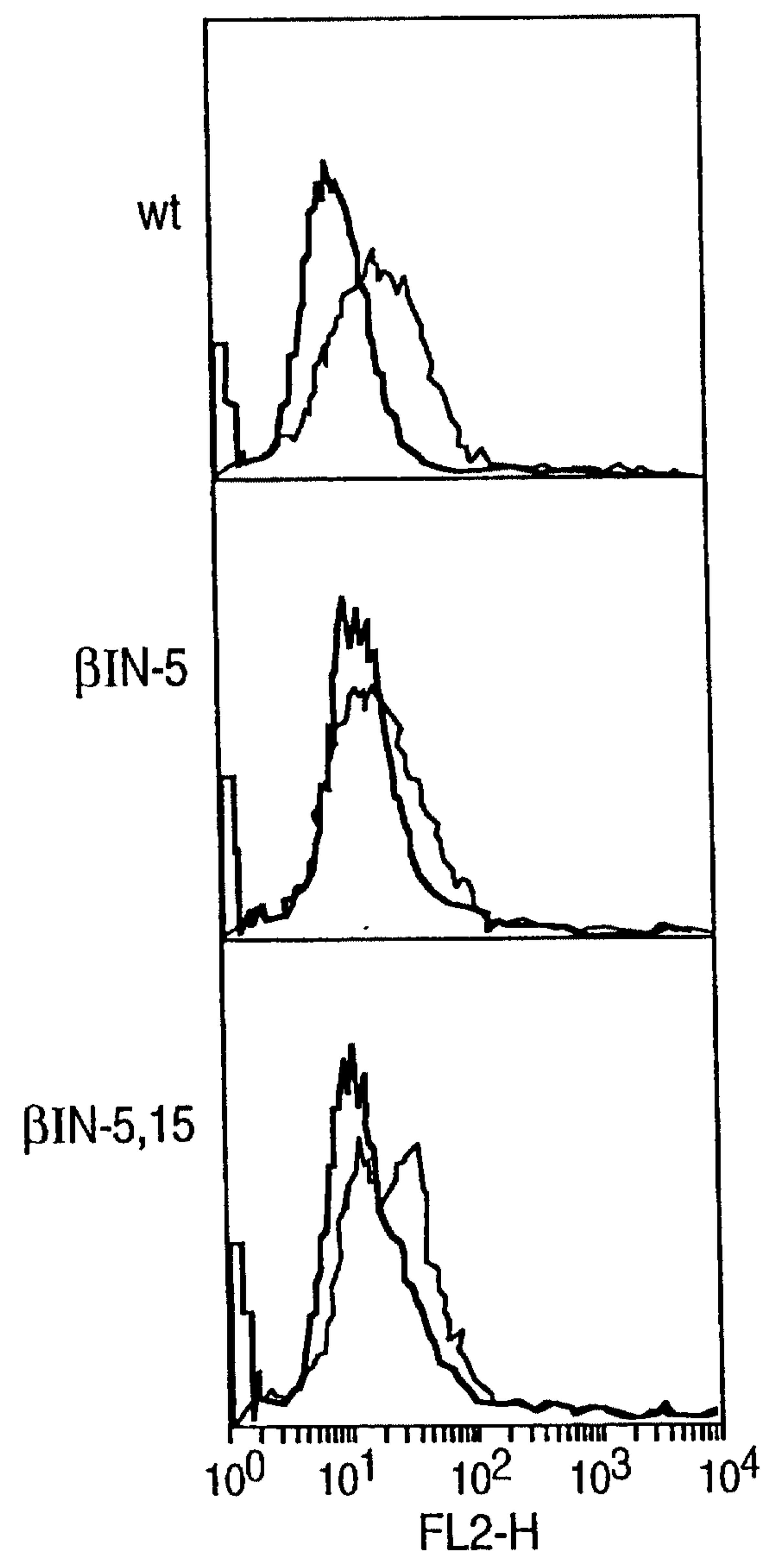


FIG. 17C

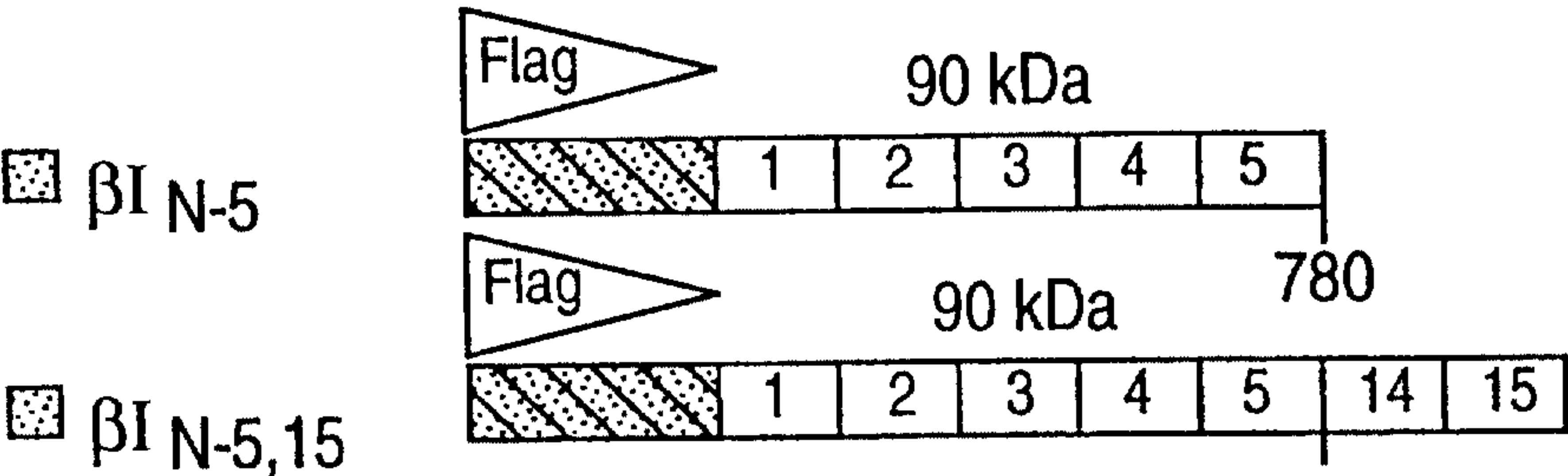
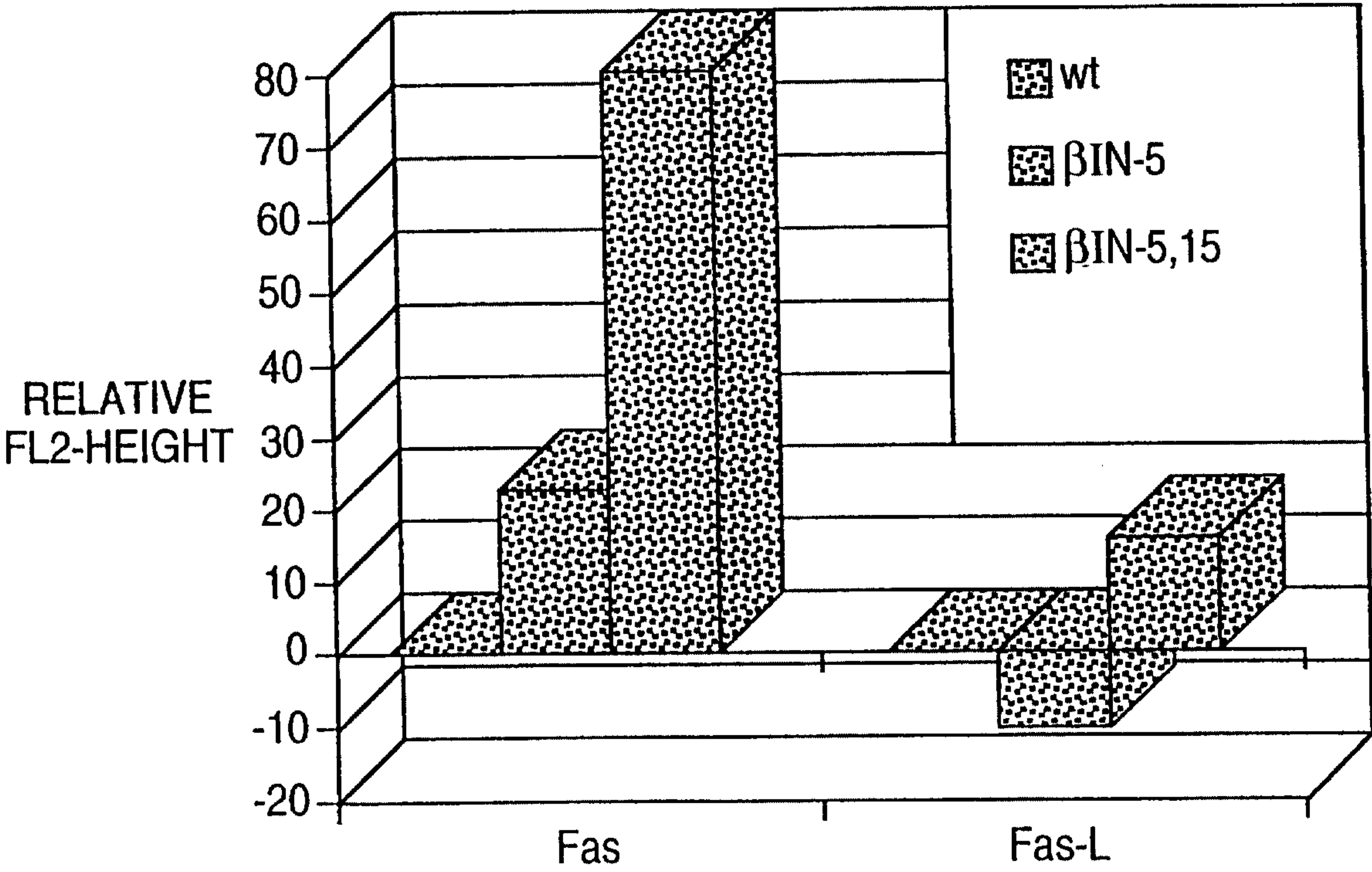
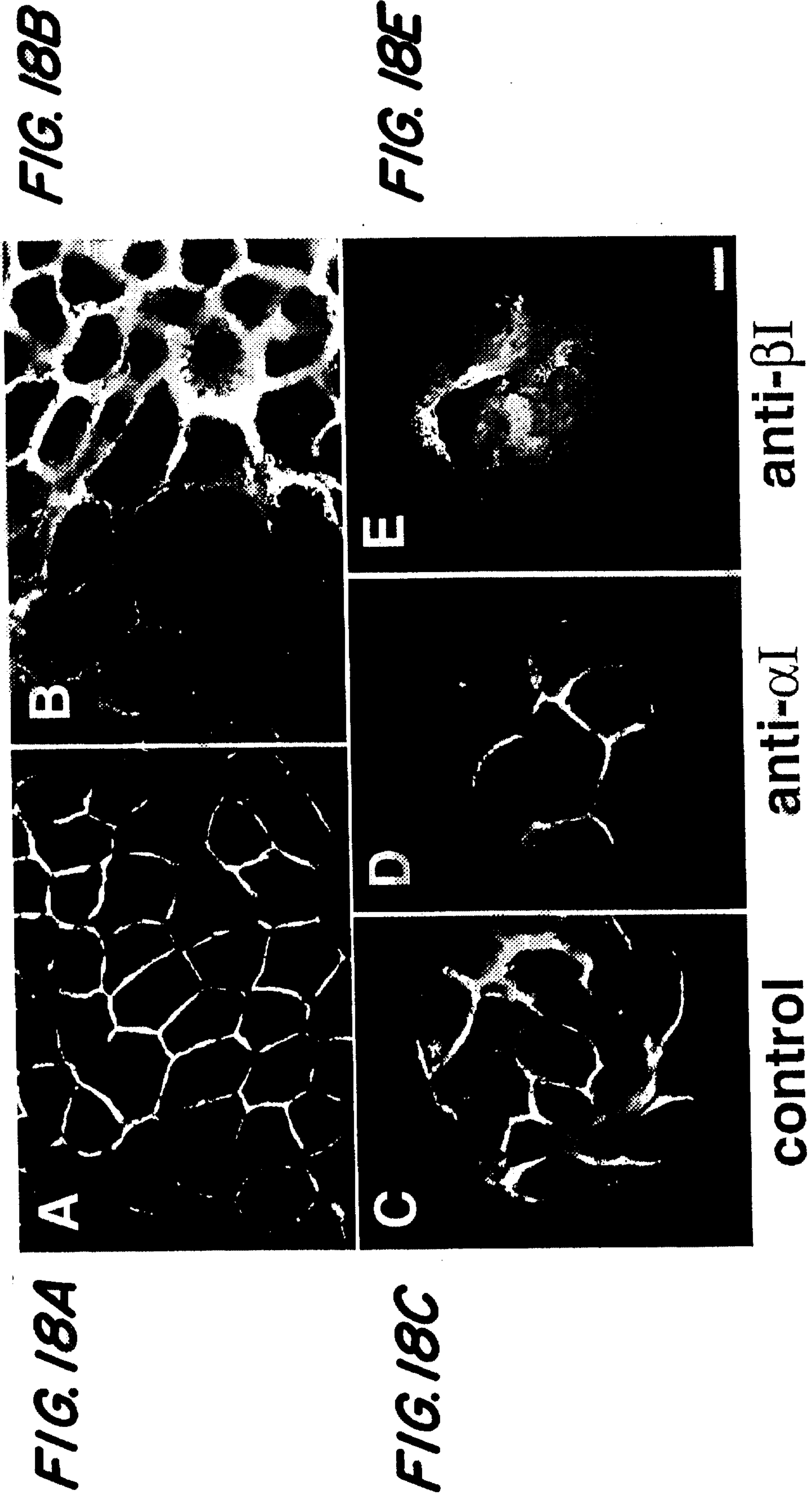


FIG. 17D



Microinjection of β IN-4 or Mab VIII C7 (anti- β I) blocks Na,K-ATPase assembly at the plasma membrane



gggaacggaggctgctcggaacacctgctcggcagttatcattagaggaagatcgcatatctcgaggatccgqatttt
ggagqaaagcagaatagacaaggaagcaggaagccgctaccaccATGAGCAGCACGCTGTCAACCCACAGACTTTTGACA
GC TTGGAAATCCAGGGCCAGTACAGTGACATCAACAACCGCTGGGACC TTCTGACTCGGACTGGGACAATGACAGCAGC
TCGGCCCGCCTCTTTGAGAGGCTCTCGCATTAAGGCTCTGGCAGATGAACGAGAAGCTGTGCAGAGAAACCTTCACCAA
GTGGGTAAACTCGCACCTGGCCCGGTCACGTGCCGGGTGGGGACCTGTACAGCGACCTCGGGACGACGCAACCTGC
TGAGGCTCCTCGAGGTGCTCTCGGGAGAGATACTGCCAAAGCCTACAAAGGCCGCATCGGATCCACTGCCCTGGAGAAC
GTGGACAAGGCACCTGCAGTTCCTCAAGGAGCAGAAAGTGCACCTTGGAACAATGGGCTCCCATGACATTTGTGACGGAAA
CCACCGACTGACCC TTGGGCTGGTCTGGACCATCATCCTTCGATTCCAGATCCAAGACATCAGTGTGGAGACAGAAAGACA
ACAAGGAGAAGTACCCAAGGATGCCCTGCTTCTGTGTGCCAGATGAAGACTGCAGGTTATCCCAACGTCAATGTA
CACAACTTCAACCACTGGAGAGATGGACTAGCTTTCAACGCCATCGTGCATAAACACCGCCAGACCTGCTGGATTT
TGAGTCTCTGAAGAAGTGAATGCACACTATAATCTGCAGATGCATTTCAATCTGGCTGAAAGGAACCTGGACTTACCA
AGCTGCTGGATCCCGAAGACGTGAATGTGGACCAGCCAGATGAGAAGTCAATCATTACCTATGTGGCTACTTACTACCAT
TACTTCTCAAGATGAAGGCCCTGGCCGTGGAAGGCAAGAA TTGGCAAGGTGCTGGACCATGCCATGGAGGCAGAGCG
CCTGGTGGAGAAATACGAGTCCCTGGCCTCGGAGCTGCTGCAGTCGAGCAAAACGATCGTGACCTGAGGAGCAGAGCG
AGTTGGCCAACTCCCTTAGCGGGTCCAGAACCACTGCAGTCCCTCAACTCCTACCGCACCGTGGAGAGCCGCCAAG
TTTACCGAGAAAGGAAC TTGGAAGTGCTGCTTCACCATCCAGAGCAAGCTTCGGGCCAACCAACAGAGGTCTACAC
GCCCGGAGGGCCGCTCATCTCGGACATCAACAAGGCTTGGAGCGGCTGGAGAGCGGAGCACGAGCGTGAGCTGG
CCCTGCGCACCGAGCTCATCCGCCAGGAGAGCTGGCCGCTGAGCAGCTGGCCCGCTTCGACCGCAAGCTGCCATGCGGGAG
ACCTGGCTCAGCGAGAACCAAGCGCTCGTGTCCAGGACAAC TTTGGGCTGGAGCTGGCAGCTGTGAGGCAGCAGTACG
GAAGCACGAAGCCATTGAGACGGACATCGTGGCCTACAGCGGCCGGTGCAGGCAGTGACCGCTCTGGACTTCTTGCGGCAG
CCGCCGAGCGCTACCACGACATCAAGCGCATCGCCGCTCGGCTCGGCAGCACAACTGGCACGGCTCGGACTTCTTGCGGCAG
ATGGTGGCCGCCCGGAGCGGCTCCTCCTCAACCTGGAGCTGCAGAAAGGTGTTCCAGGACCTGCTACCTCATGGA
CTGGATGGAAGAGATGAAGGGCCGGCTGCAGTCTCAGGACCTGGGCAGCACCTAGCAGGAGTGAGGACCTGCTGCAGC
TGCACGAGCTGGTGAGGCAGACATCGCCGTGAGGCCGAGAGG GTCGGGCGGTGCGGCGCTCAGCGCTTCTGCGCTTCTGC
AACCAGGGAAGAGTATAGACCTTGCAACCGCGGCTGGAGCGG GTGGCCAGCTAGAGCAGAGCTATGAGGC
ACTGTGCGAGTTGGCAGCGGCGGCGGCTGGAGGAA TCAGCGGCTGAGGAA TCAGCGGCGGACCTGACCGGTGCCCTCCGC
AAGCTGAGGCTGGTGCGGAGCAGCACCTCTGGCTCAGCCGACACGGGCCGAGACCTGACCGGTGCCCTCCGC
CTGCTCAACAAGCACACGCCCTGCGGGCGAGATGAGCGGCCGTGGGCCCTGAAGCTCACCTGAGCAGGGCCCA
GCAGTTGGTGGCCGAGGTCACCTGGGGCAAGCCAGGCCCTCTGCCGTGCACTGAAC TCAAGCCAGTGGGAGCGGC
TAGAGGCCCTGGCCGAGGAGCGTGCCAGCGGCTGGCCCAAGCCGACGCTTACCAGTTCAGGCCGATGCAACGAC

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FIG. 19B

ATGGAGGCCTGGTTGACGCACTGGCCCTGGTGTCAGCCCCGAGCTGGGACGACGAGTTCTCCACGCAGGCTCT
AGCCAGGCAGCATCGGGCCCTGGAGGAGGAGATTGGAAGCCACCGGCAACCTGGACGCCCTTGAAGGAACAGGCAGCAG
CCCTGCCCCACACTGAGCCGCACGCCCGAGGTGCAGGGCCGGTGCCACCCCTGGAGCGGCACTACGAGGAGCTGCAG
GCCGGCAGGCAGCGAGCGCGGCTTGAGGCGAGCCCTGGCGCTCTACACCATGCTCAGCGAGGCCCGGGCCTGTG
G
ACTCTGGGTGGAGGAGAAGGAGCAGTGGCTAACGGGCTGGCCCTGCCTGAACGCCCTGGAGGACCTGGAGGTCGTGCAGC
AGAGGTTGAGACCCCTGGAGCCTGAAATGAACACCCCTTGACGACAAATCACCGCGGTGAATGACATTTGCCGAGCAGTTA
CTGAAGGCCAACCCCCAGGCAAGACCCGATTGTCAACACCCAGGAGCAGCTCAACACAGGTGGCAGCAGTTTCGGCG
TCTGGCAGACGGCAAGAAGGAGCTCTACCTCAGCCCTGAGCATCCAGAACTACCACTTAGAGTGCACGGAGACCCAGG
CCTGGATGAGAGAGAAGACCAAGTCAATCGAGTCCACCCAGGGCTAGGCAACGATCTGGCTGGGTGCTGGCCCTGCAG
CGCAAGCTGGCCGGCACGAGCGGACCTGGAGGCCATCGCCGCCGGGTGGGCACTGACTCGAGAGGCAAAATGCCCT
GGCTGCCGGCCATCCCGCTCAGGCAGTGGCCATCAACGCCCGGCTGAGAGAGGTGCAGACCGGCTGGAGGACCTCAGGG
CCACCATGCGGCGTCGAGAAGATCGCTGGGGAGCGCGGCTGCAGGACTTCTTGCGCAGCTTGATGACTTCCAG
GCCTGGCTAGGCCGCACTCAGACTGCTGTGGCCTCTGAAGAAGGGCCGCCACCCCTGCCTGAGGCAGAGCCCTCCTGGC
CCAACATGCAGCCCTGCGGGAGAGGTGGAGCGGCCAGAGCGAGTATAGCCGGCTGCAGCCCTGGCGGAGGAGGTGA
CCCGGACCAAGCTGACCCCAAGTGCCCTCTCCTACGACAGCGACTGGAGCCCTGGAACTGGCTGGAGAGCTGGGC
CGAATGTGGAGAGCCGGCAAGTCCGCTGGCCCAAGGCTCCAGGATTCCTCGGGATTCCTCGTCAGGCTGA
GGCGTGTCTCAGCAGCCAGGAATATGTTCTGTCTCACACGGAGATGCCAGGACACTCCAGGCTGCTGATGCTGCCATTA
AAAACCTGGAGGACTTCATGAGCACCATGGACGCCAATGGGAACGGATCCACGGCTCCTGGAGGCTGGCCGCCAGCTG
GTATCTGAAGGCAACATCCACGCCGACAAAGATTGCGGAAAGGCAGACTCCATTGAGAGGACACAAAGAATCAAGA
CGCAGCGCAGCAATTTCTGGGCCGTCTTCGGGACAACCGGAGCAGCAGCATTTCTGCAAGATTGTCACGAGCTGAAGC
TCTGGATCGACGAGAAGATGCTGACAGCCAGGACGTGCTCTATGACGAGGCCCGCAACCTGCATACCTAAGTGCGAGAAG
CACCAGGCAATTCATGGCCGAGCTGGCTGCCAACAAAGACTGGCTGGACAAGGTGGACAAGGAAGGCAAGAGCTCACCCCT
TGAGAAGCCAGAGCTGAAGCCCTGGTGTGCGAGAAGCTGAGAGACCTGCACAGGCGCTGGGACGAGCTGGAGACCA
CCCAAGCCAAAGGCCCGCAGCCTCTTTGATGCCAACCGAGCTGAGCTGTTTGCCCAAGAGCTGTGTGCCCTGGAGAGCTGG
CTGGAGAGCCTGCAGGCCCGCAGCTCGATGACTACGGCAAGGACCTCACCAGCGTCAACATCCTGCTCAAGAAGCA
GCAGATGCTGGAATGGGAGATGGCTGTGAGAGAGAGGTGGAGGCAATCCAGGCCCAAGCAAGCACTGGCCAGG

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FIG. 19C

AGGACCAGGGTGCAGGGAGGTGGAGAGAACCTCGAGGGCCGTGGAGGAGAAGTTCAGGGCCTTGTCAGGCCCATGCGG
GAACGCTGCCGGCCTGCAGGCTTCTCGGAGCAGCACCAAGTTCACCCGCGATGTGAAGATGAGATTTTGTGGTGAC
AGAGCGCTGCCCATGGCCAGCTCCATGGAGCATGGCAAGGACCTGCCAGCGTCCAGCTTCTCATGAAGAAACCAGA
CCCTGCAGAAAGAGATTCAAGGCCATGAGCCCGGATCGCGGACCTGAGGGAGCGGACGTGAACTTCGAGGAAGCGACTGGA
GCAGTCCAGAGCTGGCTGAGCTGCAGGAATGTGAAACGCCCTGGGCCACGAGCTGGAATTCGAGGAAGCGACTGGA
GGATGCCCTCGAGCCAGCAGTTCTACCGGATGCCGCCGAGGCGGAGGCCCTGGATGGCGAGCAGGAATTACACATGA
TGGCCAGGAAAGGCCAAGGATGAGTGAAGTCCAGGAGGTGAAGAACCAAGGTGCTGGAGCAAGCCCTGGCC
GACTACGCGAGACCATCCACCAGCTGGCGCCAGCAGCAGGACATGATTGACCAGGACCCAGAGCACTCGGAT
ATCCATCCGCCAAGCCAGGTGGACAAGCTGTATGCCGGCTGAAGGAGCTGGCTGGAGCGCGGAGCGCCTGCAGG
AGCACCTCCGGCTGTGCCAGCTCCGCCGAGCTGGATGACCTGGAACAGTGGATCCAGGCGGAGGTGGTGGCGGCC
TCCACGAGCTGGGCCAGGACTACGAGCATGTGACTATGCTCCGAGACAAATTCGAGAGTTCTCCGGGACACAAAGCAC
CATCGGTACGAGCGCGTAGATAGCGCCAATGCGCTGGCCAAATGGGCTCATTGCTGGGGCCATGCTGCACGGGCCACCG
TGGCCGAGTGAAGGACAGTCTCAACGAGGCCCTGGCTGACCTGCTTGAAGTGGTGGACACACGGGGTCAGGTGCTGGCC
GGGGCTACGAGCTGCAGCGCTTCTGCACGGGCAAGCCCTGGCGGGTGCAGCACAAAGCAGCAGCATTCAGGCC
GGACGGGACTGGCCGCGACCTCAACGCTGCCAGGCGCTGCAGCGCCGACACTGTGCTACGAGCATGACATTCAGGCC
TCAGCCCCAGGTCCAGCAGGTGCAGGACGACCGGCTCCAGAAAGGCTACGCTGGAGACAAGGCTGAGGAGATC
GGCGCCACATGCAGGCCGTGGCCGAGGCTGGGCCAGCTTCAGGGAAGCTCTGCCGCCCGCCGAGCTGCTGGA
CACCACAGACAAGTTCGGCTTCTCAAGGCTGTCCGGAACTGATGCTGGATGGATGAGGTCAACCTGCAGATGGATG
CCAGGAGCGTCCCCGGGATGTCTCCGGATCTAGTCATCAAGAACCAAGCATCAAGGCAGAGATAGAGGCC
CGGCAGACCGCTTCTCCTCCTGCATCGACATGGGAAGGAGCTGCTGCCAGGAGCCACTATGCCGCCGAGGATCTC
AGAGAAGCTGTCTCAGCTGCAGGCACGGCCAGGACAGCTGAGAAGTGGCAGGAGAAGATGGACTGGCTTCAGCTGG
TTTTGGAGGTGCTTGTTGGAAGAGATGCAGGGATGGCAGAGGCCCTGGCTCTGCAGCCAGGACCACTGGTGGCAGC
GCTGAGCTGGGTTGCACGGTGCAGCAAGTTGAGAGCCTCATCAAGCGGCCACGAGGCCCTCCAGAAATCAGAGTGGCCTG
GGAGGAGCGATTCTGTGCGCTGGAGAGCTTACTGCGCTAGAGGAGCGGGAGAGGAGCAAGAGAGGAGGAGG
AGGAGCGCGGAACAGCCGCTGCTCCGAACCCACAGCCAGTGTGCTCCAGGGACCTGCTGGCGGCCAGACAGCT
TCTGACACCACCTGGACGGAACCCAGCCACCATCCACACAGCACCCAGTGTAAATGGAGTCTGCACAGA

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FIG. 19D

TGGAGAGCCCTCACAGCCCTGCTGGACAACAGAGACTTGAGCACAGCAGCTTCCCCGAAGGGCCGGACCTGGCTCAG
GGGACGAAGCCAATGGGCCCCGGGAGAGAGAGCCGACTCGGGCCCGCCCATCTGCAATGCCCCAGAGCAGG
TCTACCGAGTCAGCCCATGCTGCCACCTCGAGGCCAGAGCCATCTGCCCAGGAGCAGATGGAGGGATGCT
GTCCCGCAAGCAGAGATGGAGGCCCTTCGGGAAGAGGCTGCCAACAGGTCTCTGGCAGAACGTGTACTGTCTCTGCGGC
GTGGAGCCTCGGCTTTACAAGATGCCAAGCAGCCAGCGGGAGTGCCATACCAAGGAGAGTGCTGTCAAGCCTG
GCCAGGCCCAGGGCAGCGTCGCCCTTTGATTACCGAAAGCGCAACATGTCTCAAGCTGGCTTACAGGATGGAAGA
ATATTTATCCAGGCCAAGATGAGGCAGAGATGAGCTCGTGCTACGGGTGTTGAATGCAGCCATTGCCACAGCGTCTT
CTGCCCTCTGGAGAGCCTGAAGAGCCGGTGTGCCCAGCACCCCGGGCATGACCCGGGCCATGACCATGCCCCAGTG
TCACCCGTCGGGCTGAGGGCCTGTTGTGCTCCGACGCAAGACGGCAGAGAACGAGAGCGGAGAAACGCTTCAGCTT
CTTTAAGAAACAAGTAGttggggcaaggctccaggccactccctccgttcaggaaactgccaggacagtcg
acaggaccgacctgtgtaggacaactgcctgctgtagggctgtgttgccaaggtaaccccatcaccaggaaactgtca
ctgggacgagtcattcccaagggcagccctctctctgtgttaattccagactgggtggaccaggtaac
ccctctccacccccgcgaactctcccttccagcctgtgcctctgtccctcaccacgggtgtggacagtgccgc
acctcaacatagggcatgtgggagtggtgctgccccctgcctcagggctattctctgcatgcgagggcactgcctct
gcctctggttctcaccctcagaccagccaggaaacctctagagctgaagcagggccctgggggcagaaagtgtccagatg
acagtcagaggcgagagccctccctcccccacccctgaactccagctgccactccatccagctgctctcaa
tggctccagggtgtgttctggggacagccaccgcttgagctgtggccaaggagggtgattaaacagctcagctctc

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FIG. 19E

MSSTLSPTDFDSLEIQGQYSDINNRRWDLPSDWDNDSSARLFFERSRIKALADEREAVQKKTFTKWNSHLARVTCRVGD
LYSDLRDGRNLLRLLVLSGEILPKPTKGRMRIHCLENVDKALQFLKEQKVHLENMGSHDIVDGNHRLTLGLVWTILRF
QIQDISVETEDNKEKSAKDALLWCQMKTAGYPNVNVHNFTTSWRDGLAFNAIVHKHRPDLLDFESLKKCNAHYNLQNA
FNLAEEKELGLTKLLDPEDVNVDPDEKSIITYVATYYHYFSKMKALAVEGKRIGKVLDHAMEAERLVEKYESLASSELLQW
IEQTIVTLNDRQLANSLSGVQNQLQSFNSYRTVEKPPKFTTEKGNLEVLFTIQSKLRANNQKVYTPREGRLISDINKAWE
RLEKAEHERELALRTELIRQEKLEQLAARFDRKAAMRETWLSNQRLVSQDNFGLELAAVEAAVRKHEAIEDIVAYSGR
VQAVDAVAELAERYHDIKRIAARQHNVARLWDFLRQMVAARRERLLNLELQKVFDLLYLMDWMEEMKGRQLSQDGLG
RHLAGVEDLLQLHELVEADIAVQAERVRAVSASALRFCNPGKEYRPCDPQLVSEKLEQSYEALCELAARRARLEES
RRLWRFLWEVGEAEAWVREQQHLLASADTGRDLTGALRLNKHHTALRGEMSGRLGPLKLTLEQGGQLVAEGHPGASQASA
RAAELQAQWERLEALAEERAQRLAQAASLYQFQADANDMEAWLVDALRLVSSPELGHDEFSTQALARQHRALEEEIRSHR
PTLDALREQAAALPPTLSRTPEVQGRVPTLERHYEELQARAGERARALEAALAYTMLSEAGACGLWEEKQWLNGLAL
PERLEDLEVQQRFFETLEPEMNTLAAQITAVNDIAEQLLKANPPGKDRIVNTQEQLNHRWQQFRRLADGKKAALTSALSI
QNYHLECTETQAWMREKTKVIESTQGLGNDLAGVLALQRKLAGTERDLEAJARVGETREANALAAAGHPAQAVAINARL
REVQGTWEDLRATMRRREESLGEARRLQDFLRSDDFQAWLGRQTAVASEEGPATLPEAEALLAQHAALRGEVERAQSE
YSRLRALGEEVTRDQADPQCLFLRQRLEALGTGWEEELGRMWESRQGRLAQAHGFGFLRDARQAEGVLSSEQEYVLSHTEM
PGTLQAADAAIKKLEDFMSTMDANGERIHGGLLEAGRQLVSEGNIHADKIREKADSIERRHKKNQDAQQFLGRLDNREQ
QHFLQDCHELKLWIDEKMLTAQDVSYDEARNLHTKWQKHQAFMAELAANKDWLDKVDKEGQELTLEKPELKALVSEKL RD
LHRRWDELETTTQAKARSLFDANRAELFAQSCCALESWLESQAQLHSDDYGKDLTSVNILLKKQQLMEWEMAVREKEVE
AIQAQAKALAQEDQGAGEVERTSRAVEEKFRALCQPMRECRRLQASREQHQFHFRDVEDEILWTERLPMASMEHGKDL
PSVQLLMKKNQTLQKEIQGHEPRIADLRERQRALGAAAGPELAELQEMWKRLGHELELRGKRLEDALRAQQFYRDAAEA
EAWMGEQELHMMGQEKAKDEL SAQAEVKKHQVLEQALADYATIHQLAASSQDMIDHEHPSTRISIRQAQVDKLYAGLK
ELAGERRERLQEHRLCQLRRELDLLEQWQEREWAAASHELGQDYEHVTMLRDKFREFSRDTSTIGQERVDSANALANG
LIAGGHAARATVAEWKDSLNEAWADLLELDDTRGQVLAAYELQRFHGAARQALARVQHKKQQQLPDGTGRDLNAAEALQR
RHCA YEHDICALSPQVQVQDDGHRLQKAYAGDKAEIEGRHMQAAEAWAQLQGSSAARRQLLDDTTDKFRFFKAVRELM
LWMDEVNLQMDAQERPRDVSSADLVKNQQGIKAEIARADRFSSCIDMGKELLARSHYAAEIESEKLSQLQARRQETAE
KWQEKMDWLQLVLELVFGRDAGMAEAWLCSQEPLVRSaelGCTVDEVESLIKREHAFQKS AVAWEERFCALEKLTAL EE
REKERKRKREEEERKQPPAPEPTASVPPGDLVGGQTASD TTWDGTQPRPPSTQAPSVNGVCTDGEPSQPLLGQQRLEH
SSFPEGPGSGDEANGPRGERQTRTRGPAPSAMPQSRSTESAHAATLPPRGPEPSAQEQMEGMLCRKQEMEAFGKKAAN
RSWQNVYCVLRRGSLGFYKDAKAASAGVPYHGEVPVSLARAQGSVAFDYRK RKHVFKLGLQDGKEYLFQAKDEAEMSSWL
RVVNAIA TASSASGEPEEPVVPSTTRGMTRAMTMPVSPVGAEGPVLRSKDGREREREKRF SFFKKNK.

FIG. 19F

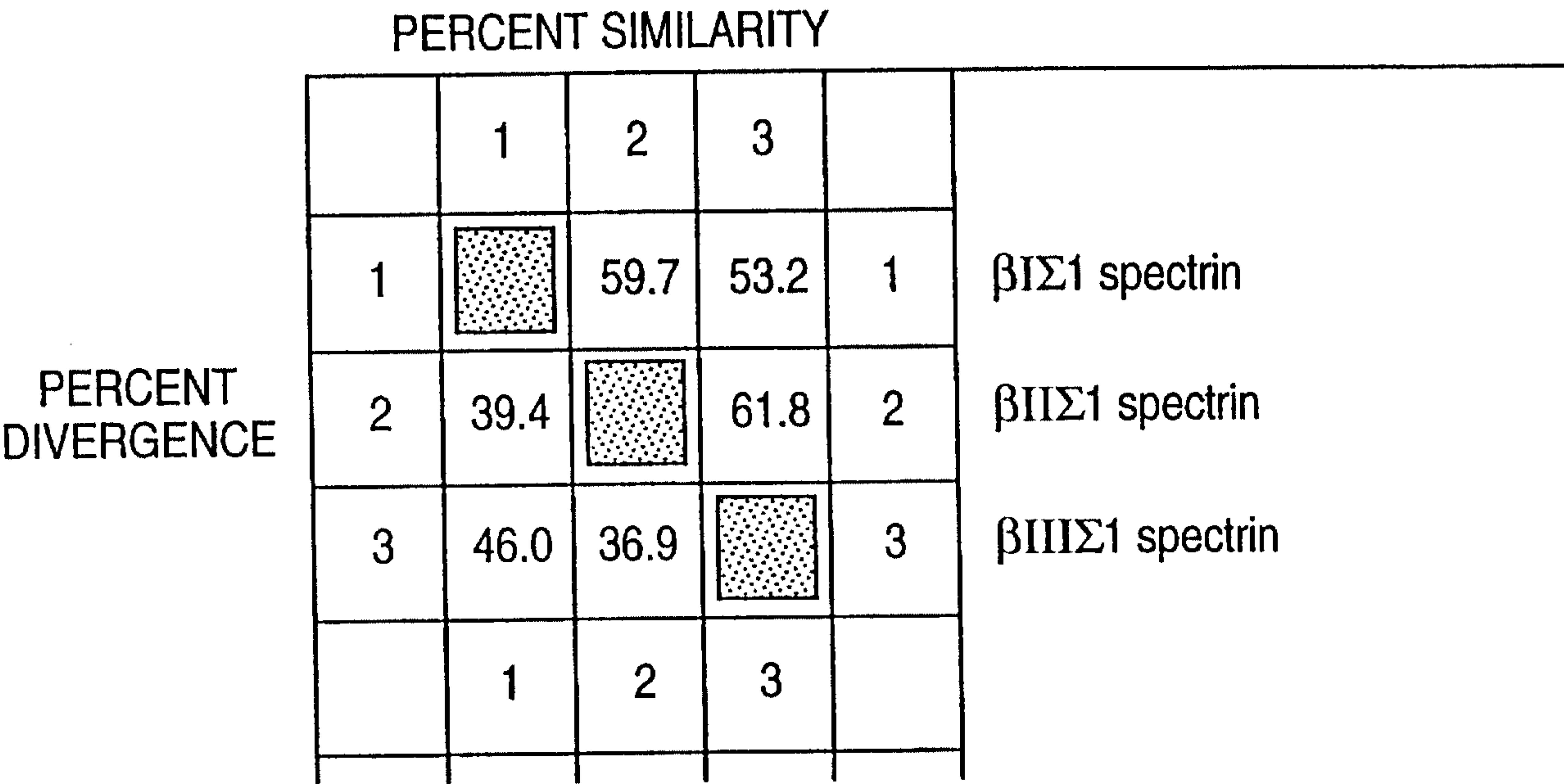


FIG. 19G

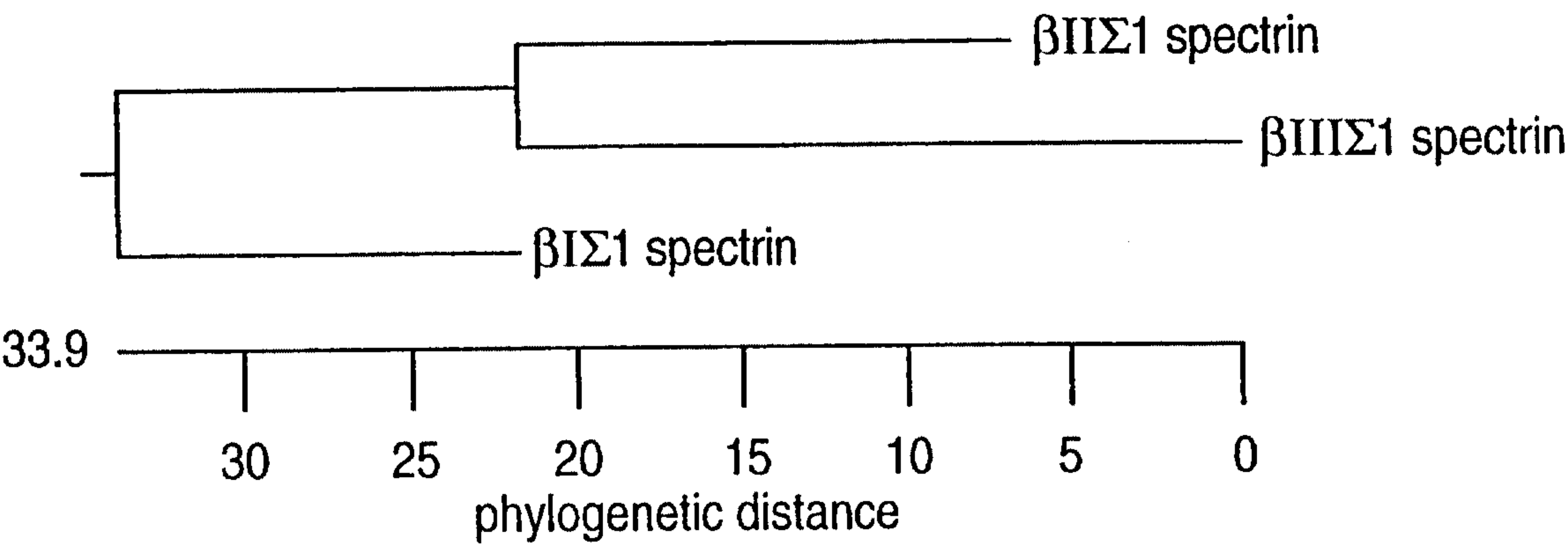


FIG. 20A

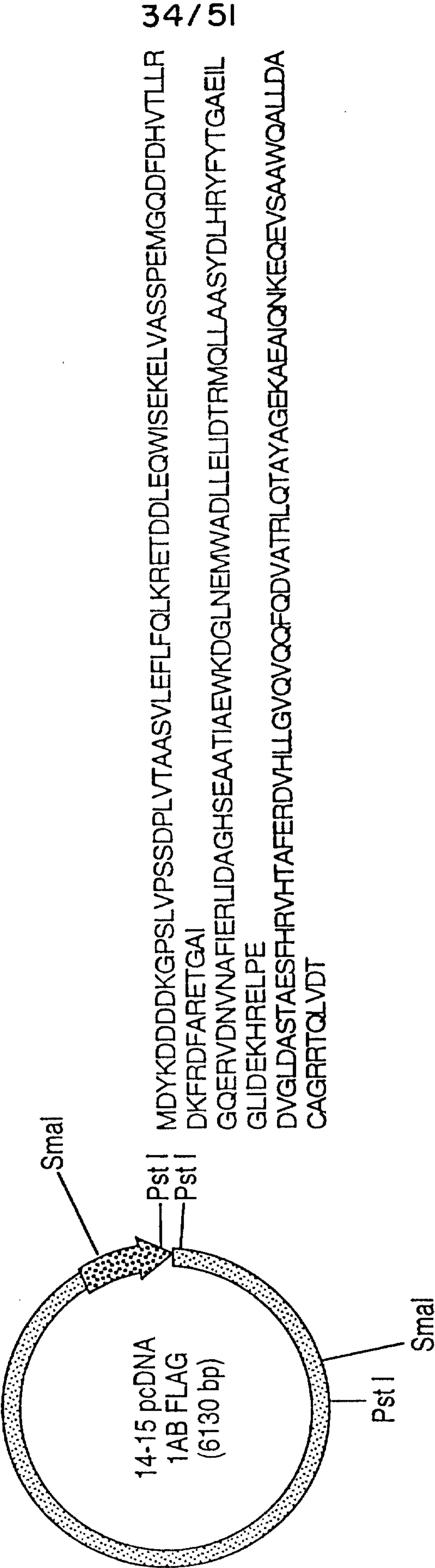
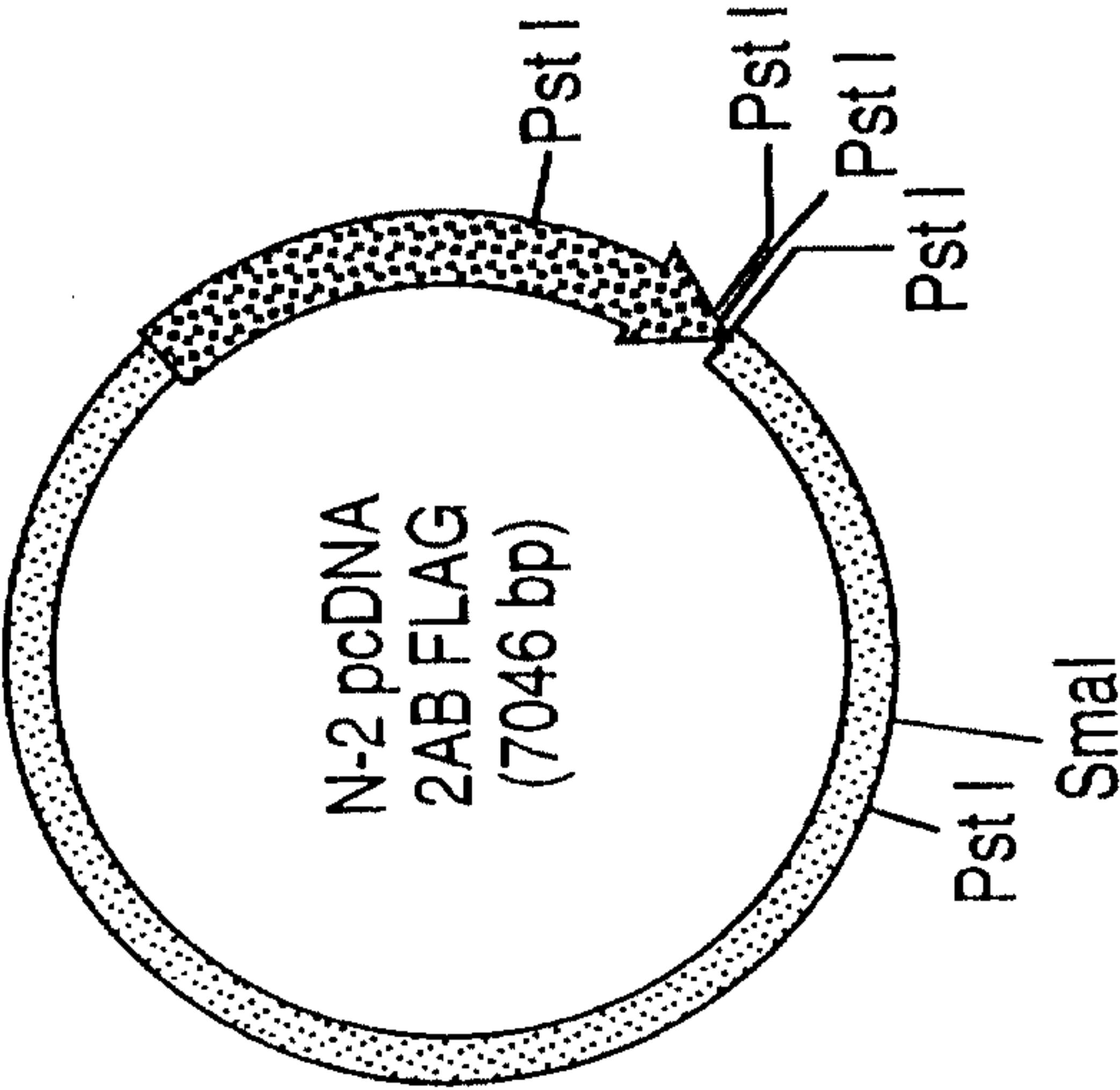


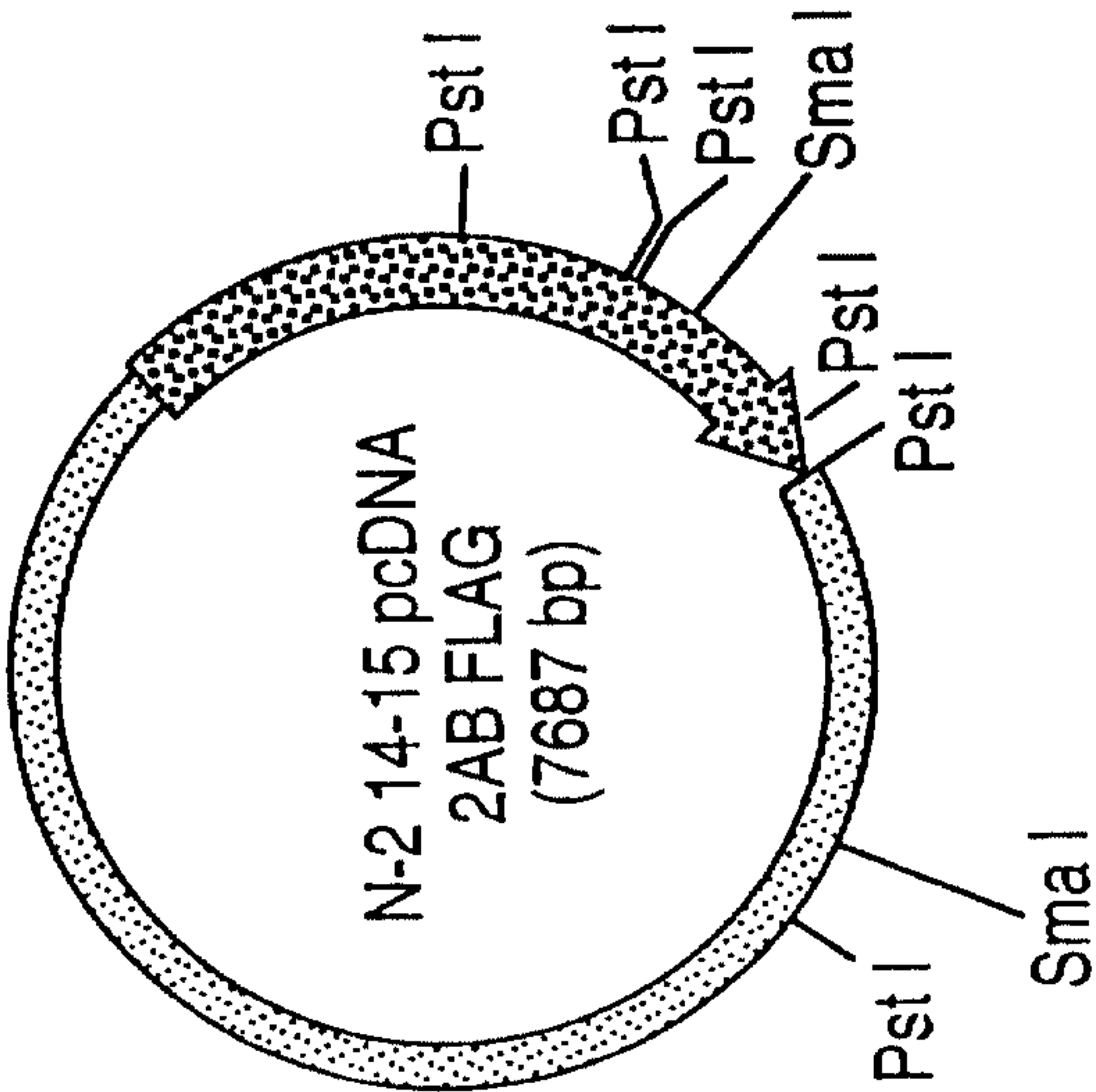
FIG. 20B



MDYKDDDDKGPKLGTGLGSMTSATEFENVGNQPPYSRINARWDAPDDELNDNDSSARLERSRIKA
LADEREVQKKTFT
KWVNSHLARVSCRITDLYKDLRDGRMLIKLLEVLSGEMLPKPTKGKMRHCLENVDKALQFLKEQR
VHLENMGSHDIVDG
NHRVLGLIWTILRFQIQDIVQTQEGRETRSAKDALLWCQMKTAGYPHVNVNTFTSSWKDGLAFN
ALHKHRPDLID
FDKLDKSNARHNLEHAFNVAERQLGIIPLLDPEDVFTENPDEKSIITYVAFYHYFSKMKVLAVEGKR
VGKVIDHAIEIE
KMKIYSGLASDLLTWIEQTTVLNSRKFANSLTGVCQQQLQAFSTYRTVEKPPKFEKGNLEVLFTIQ
SRMRANNQKVY
TPHDGKL VSDINRAWESLEEAGYRRELALRNELIRQEKLQARRFDRKAAMRETWLNENQRLVAQD
NFGYDLAAVEAAK
KKHEAIEDTAAYEERVRALEDLAQELENYHDQKRITARKDNILRLWSYLQELLQSRQRLETTEF
CRYPShWRPLEH
ASRGPSIVSPKC.

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FIG. 20C

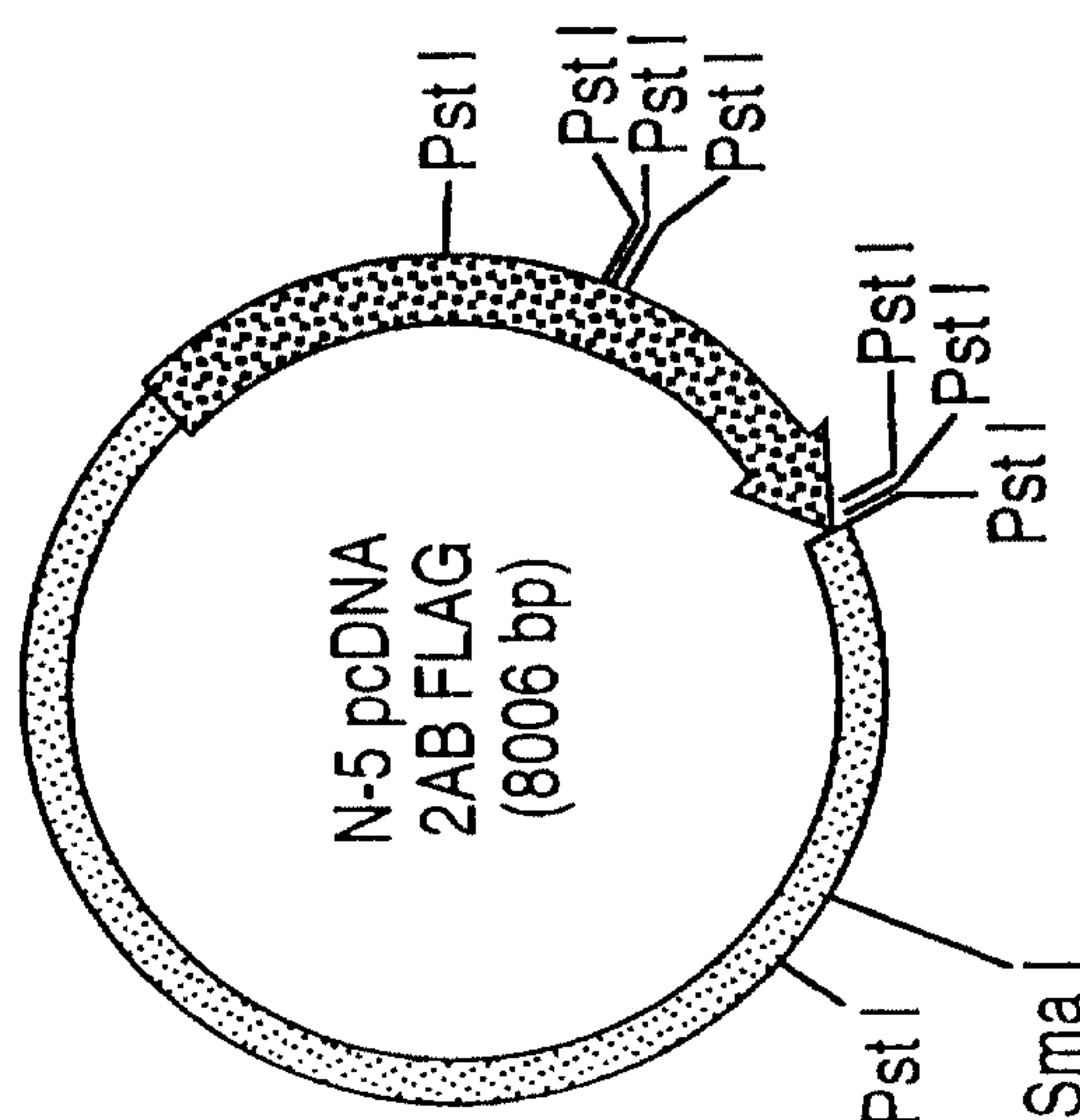


MDYKDDDDKGPKLGTGLGSMTSATEFENVGNQPPYSRINARWDAPDDELDNDNSSARLFERSRIKA
LADEREVQKKTFT
KWVNSHLARVSCRITDLYKDLRDGRMLIKLLEVLSGEMLPKPTKGKMRIHCLENVDKALQFLKEQR
VHLENMGSHDIVDG
NHRLVGLIWTIILRFQIQDIVQTQEGRETRSAKDALLWCQMKTAGYPHVNVNTNFTSSWKDGLAFN
ALHKHRPDLID
FDKLDKSNARHNLEHAFNVAERQLGIPLDPEDVFTENPDEKSITYVAFYHYFSKMKVLAVEGKR
VGKVIDHAETE
KMKIYSGLASDLLTWIEQTITVLNSRKFANSLTGVQQQLQAFSTYRTVEKPPKFQEKGNLEVLFTIQ
SRMRANNQKVY
TPHDGKLVSDINPAWESLEEAGYRRELALRNELIRQEKLEQLARRFDRKAAMRETWLNENQRLVAQD
NFGYDLAAVEAAK
KKHEAIEDTAAAYEERVRALEDLAQELEKENYHDQKRITARKDNILRLWSYLQELLQSRQRLETTEF
LFQLKRETDDLE
QWISEKELVASSPEMGQDFDHVTLLRDKFRDFARETGAIGQERVNDVNAFIERLIDAGHSEAAATIAEW
KDGLNEMWADLL
ELIDTRMQLLAASYDLHRYFYTGAELGLIDEKHRELPEDVGLDASTAESFHRVHTAFERDVHLLGVQV
QQFQDVATRLQ
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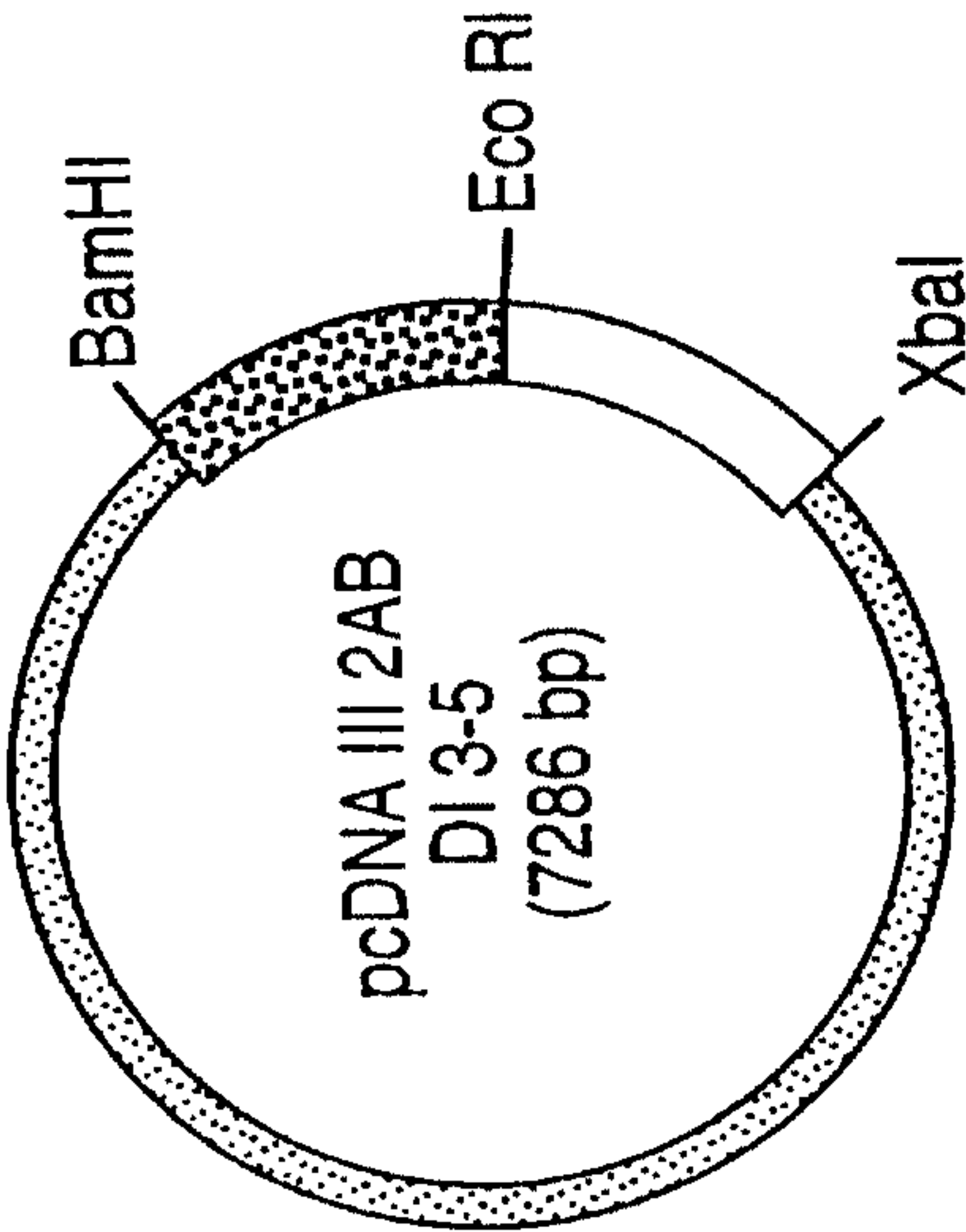
FIG. 20D



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MDYKDDDDKGPKLGTGLGSMTSATEFENVGNQPPYSRINARWDAPDDELNDNDNSSARLFFERSRIKA
LADEREWQKKTFT
KWNNSHLARVSCRITDLYKDLRDGRMLIKLLEVLSGEMLPKPTKGKMRIHCLENVDKALQFLKEQR
VHLENMGSHDIVDG
NHRLVLGLIWTIILRFQIQDIVQQTQEGRETRSAKDALLWCQMKTAGYPHVNVNTNFTSSWKDGLAFN
ALIHKHRPDLID
FDKLDKSNARHNLEHAFNVAERQLGIPLDPEDVFTENPDEKSITYVAFYHYFSKMKVLAVEGKR
VGKVIDHALETE
EFLQKLFQDMLHSIDWMDEIKAHLLSAEFGKHILLEVEDLLQKHKLMEADIAIQGDKVKAITAATLKFT
EGKGYQPCDPQV
IQDRMSHLEQCFFEELSNMAAGARTQLEQSKRLWKFFWEMDEAESWIKKEQYSSLDYGKDLTSLI
LQRKHKA FEDEL R
GLDAHLEQIFQEAHGMVARKQFGHPQIEARIKEVSAQWDQLKDLAFCCKNLQDAENFFQFQGDADDL
KAWLQDAHRLLS
GEDVGQDEGATRALGKKHKDFLEEESRGVMEHLEQQAQGFPEEFRDSPDVTHRLQALRELYQQQWA
QADLRQQRLOEA

FIG. 20F



MDYKDDDDKGPKLGTGLGSMTSATEFENVGNQPPYSRINARWDAPDDELNDNDNSSARLFERSRIKA
LADEREWQKKTFT
KWVNSHLARVSCRITDLYKDLRDGRMLIKLLEVLSGEMLPKPTTKGKMRIHCLENVDKALQFLKEQR
VHLENMGSHDNDG
NHRLVLGLIWTIILRFQIQDIWQTQEGRETRSAKDALLWCQMKTAGYPHVNVNTNFTSSWKDGLAFN
ALHKHRPDLID
FDKLKDSNARHNLEHAFNVAERQLGIPLLDPEDVFTENPDEKSIITYVAFYHYFSKMVKVLAVEGKR
VGKVIDHAIEIE
EFLQKLFQDMLHSIDWMDEIKAHLLSAEFGKHILLEVEDLLQKHKLMEADIAIQGDKVKAITAATLKFT
EGKGYQPCDPQV
IQDRMSHLEQCFEELSNNMAAGARTQLEQSKRLWKFFWEMDEAESWIKKEQYSSLDYGKDLTSVLI
LQRKHKA FEDEL R
GLDAHLEQIFQEAHGMVARKQFGHPQIEARIKEVSAQWDQLKDLAAFCCKNLQDAENFFQFGDADDL
KAWLQDAHRLLS
GEDVGQDEGATRALGKKHKDFLEELEESRGVMEHLEQQAQGFPEEFRDSPDVTHRLQALRELYQQVVA
QADLRQQRLOEA
LDLYTVFGETDACEWLMGEKEKWLAEMEMPDLTLEDLVQHRFDILDQEMKTLMTQIDGVNLAANS
LVESGHPRSRVVKQ
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KLSGLERDVAAI

FIG. 20G-1

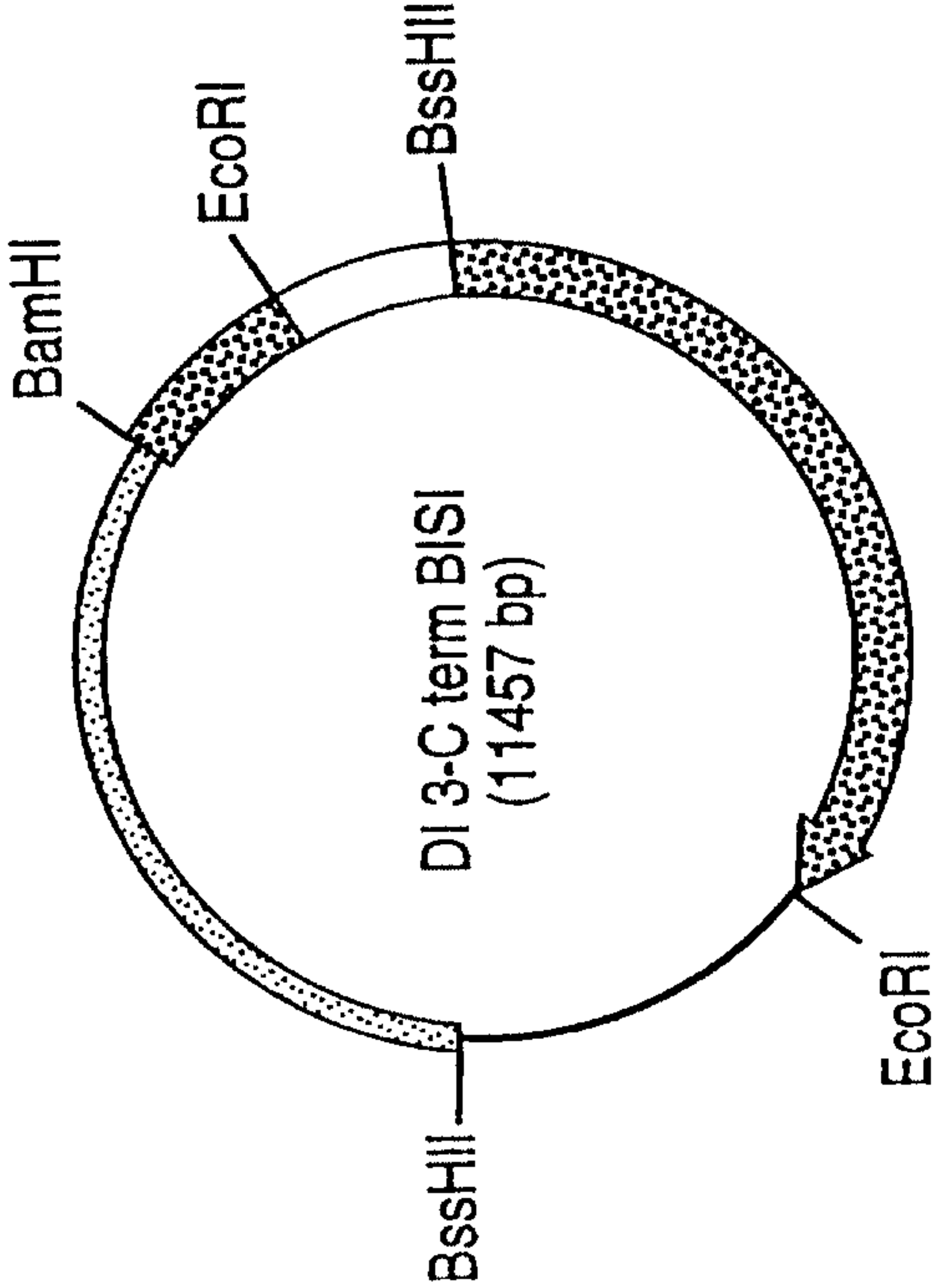


FIG. 20G-2

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WLSITQKAVASE
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MWESRSHTLAQC
LGFQEFQKDAKQAEAILSNQEYTLAHLEPPDSLEAAEAGIRKFEDFLGSMENNRDKVLSPVDSGNKLV
AEGNLYSDKIKE
KVQLIEDRRHRKNNEKAQASVLLRDNLELQNFLQNCQELTLWINDKLLTSQDVSYDEARNLHNKWLK
HQARVAELASHEG
WLENIDAEQKQMDKPKQFTALVSQKLEALHRLWDELQATTKEKTQHLSAARSSDLRLQTHADLNK
WISAMEDQLRSDDP
GKDLTSVNRMLAKLKRVEDQVNRKEELGELFAQVPSMGEEGGDADLSIEKRFLDILLEPLGRRKKQL
ESSRAKLQISRDL
EDELWVEERLPLAQSDYGTNLQTVQLFMKKNQTLQNEILGHTPRVEDVLQRGQQLVEAAEIDCQDL
EERLGHLOQSSWD
RLREAAAGRLQRLRDANEAAQQYYLDADAEAWIGEQLYISDEIPKDEEGAIVMLKRHLRQQRAVEDY
GRNIKQLASRA
QGILLSAGHPEGEQIRLQGQVDKHYAGLKDVAEERKRKLENMYYHLFQLKRETDDEQWISEKELVASS
PEMGQDFDHVTL
LRDKFRDFARETGAIGQERVDNVNAFIERLIDAGHSEAAATIAEWKDGLENMWADLLELIDTRMQLLAA
SYDLHRYFYTGA
EILGLIDEKHRELPEVDGLDASTAESFHRVHTAFERDVHLLGVQVQQFQDVATRLQTAYAGEKAEAIQNK
EQEVSAAWQA
LLDACAGRRTQLVDTADKFRFFSMARDLLSWMESIRQIETQERPRDVSSVELLMKYHQGINAEIETR
SKNFSACLELGE
SLLRQHQHQAEEEEIREKLQQVMSRRKEMNEKWEARWERLRLMLEVCQFSRDASVAEAWLJAQEPYLA
SGDFGHTVDSVEKL
IKRHEAFEKSTASWAERFAALEKPTTLELKERQIAERPAEETGPQEEEEGETAGEAPVSHHAATERTSPV
SLWSRLSSSWE
SLOPEPSHPY.

MDYKDDDDKGPKLGTGLGSMTSATEFENVGNQPPYSRINARWDAPDDELNDNDSSARLFFERSRIKA
LADEREWQKKTFT
KWVNSHLARVSCRITDLYKDLRDGRMLIKLLEVLSGEMLPKPTKGKMRIHCLENVDKALQFLKEQR
VHLENMGSHDIVDG
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ALHKHRPDLID
FDKLDKSNARHNLEHAFNVAERQLGIPLDPEDVFTENPDEKSIITYVVAFYHYFSKMKVLAVEGKR
VGKVIDHAETE
EFLQKLFQDMLHSIDWMDEIKAHLLSAEFGKHLLLEVEDLLQKHKLMEADIAIQGDKVKAITAATLKFT
EGKGYQPCDPQV
IQDRMSHLEQCFEELSNMAAGARTQLEQSKRLWKFFWEMDEAESWIKKEQYSSLDYGKDLTSVLI⁴₂
LQRKHKAFEDEL⁵
GLDAHLEQIFQEAHGMVARKQFGHPQIEARIKEVSAQWDQLKDLAFCCKNQLQDAENFFQFQGDADDL⁵
KAWLQDAHRLLS
GEDVGQDEGATRALGKKHKDFLEELSESRGVMHELEQQAQGFPEEFRDSPDVTHRLQALRELYQQWA
QADLRQQRLOEA
LDLYTVFGETDACE⁵LMGEKEKWLAE⁵MEMPTLEDLEVQHRFDILDQEMKTLMTQIDGVNLAANS
LVESGHPPRSREV⁵KQ
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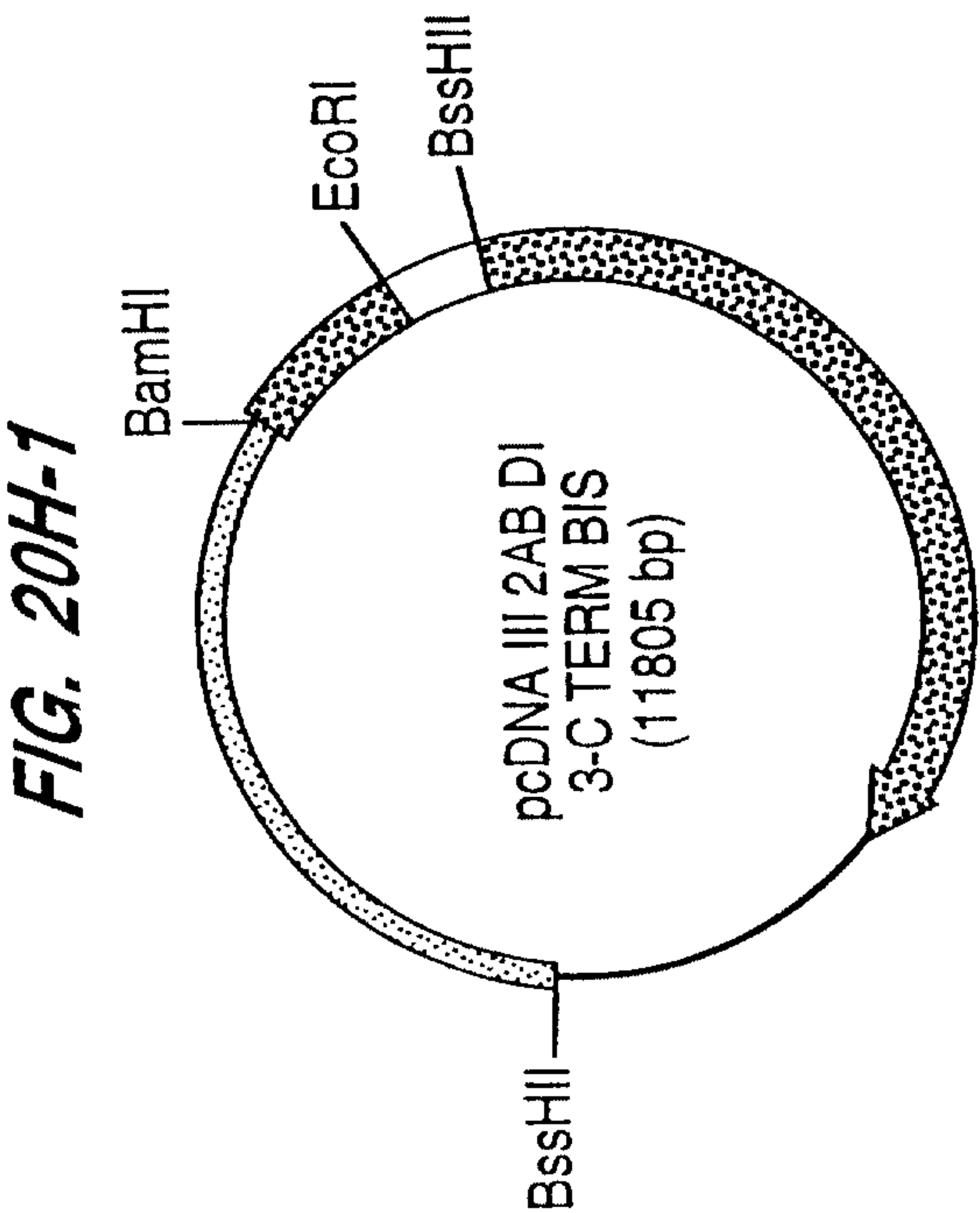
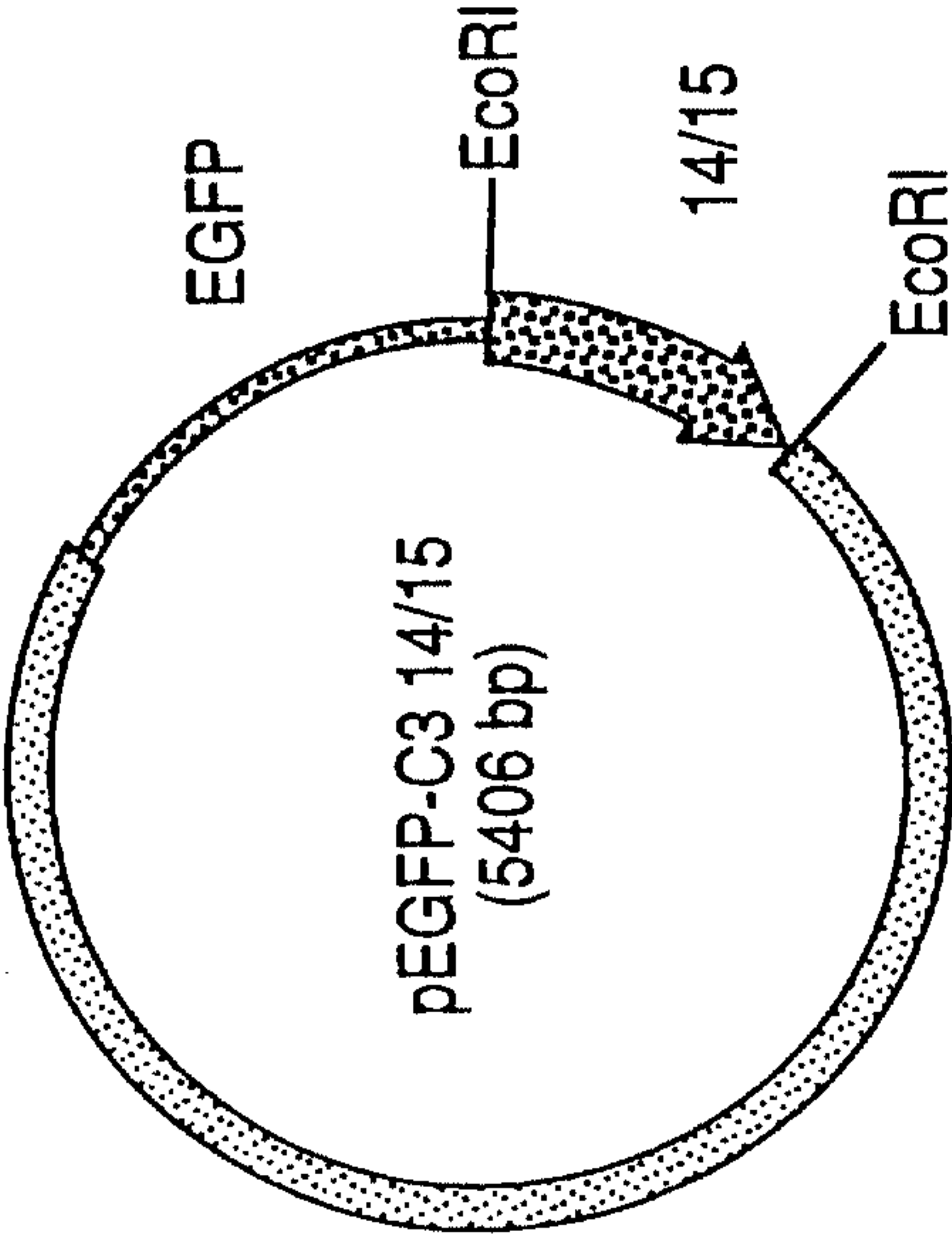


FIG. 20H-2

QARVDALERESQQLMDSHPQKENIGQRQKHLEELWQGLQQSLQGGQEDLLGEVSQQLQAFLOLDLDDFOA
WLSITQKAVASE
DMPESLPEAEQLQQHAGIKDEIDGHQDSYQRVKESGEKVIQQQTDPEYLLGQRLEGLDTGWDALGR
MWESRSHTLAQC
LGFQEFQKDAKQAEAILSNQEYTLAHLEPPDSLEAAEAGIRKFEEDFLGSMENNRDKVLSPVDSGNKL
AEGNLYSDKIKE
KVQLIEDRHRKNNNEKAQEASVLLRDNLQNLQNCQELTLWINDKLLTSQDVSYDEARNLHNKWLK
HQAFAELASHEG
WLENIDAEKGQLMDEKPOFTALVSQKLEALHRLWDELQATTKEKTQHLSAARSSDLRLQTHADLNK
WISAMEDQLRSDDP
GKDLTSVNRMLAKLKRVEDQVNVKKEELGELFAQVPSMGEEGGADLSIEKRFLDLEPLGRRKKQL
ESSRAKLQISRDL
EDET LWEEERLPLAQSDYGTNLQTVQLFMKKNQTLQNEILGHTPRVEDVLQRGQQLVEAAEIDCQDL
EERLGHLOQSSWD
PLREAAAGRQLRLRDANEAAQQYYLDADAEAEAWIGEQLYVISDEIPKDEEGAINMLKRHLRQQRAVEDY
GRNIKQLASRA
QGLLSAGHPEGEQIRLQGGQVDKHYAGLKDVAEERKRKLENMYHLFOLKRETDDLEQWISEKELVASS
PEMGQDFDHVTL
LRDKFRDFARETGAIGQERVNDVNAFIERLIDAGHSEAAATIAEWKDGLENMWADLLELIDTRMQLLAA
SYDLHRYFYTGA
EILGLIDEKHRELPEVDGLDASTAESFHRVHTAFERDVHLLGVQVQFQDVATRLQTAYAGEKAEIQNK
EQEVSAAWQA
LLDACAGRRTQLVDTADKFRFFSMARDLLSWMESIIRQIETQERPRDVSSVELLMKYHQGINAEIETR
SKNFSACLELGE
SLLRQHQHQASEEIREKLQQVMSRRKEMNEKWEARWERLRLMILLEVCQFSRDASVAEAWLJAQEPYLA
SGDFGHTVDSVEKL
IKRIHEAFEKSTASWAERFAALEKPTTLELKERQIAERPAEETGPQEEEEGETAGEAPVSHHAATERTSPG
EEEGTWPNLQ
QPPPPGQHKDGQKSTGDERPTTEPLFKVLDTPLSEGDEPATLPAPRDHGQSVQMEGYLGRKHDLEGP
NKKASNRSWNNLY
CVLRNSELTFYKDAKNLALGMPYHGEEPLALRHAICEIAANYKKKKHVFKLRLSNGSEWLFHGKDEE
EMLSWLQGVSTAI
NESQSIRVKAQSLPLPSLSGPDASLGKKDKERFSFFPKKDYKDDDDK.

FIG. 20I



MVSKGEELFTGWPIILVELDGDVNGHKFSVSGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTLTYGV
QCFSRYPDHMK
QHDFFKSAMPEGVYQERTIFFKDDGNNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNNS
HNYYIMADKQKN
GIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHIMVLEFVTAA
GITLGMDELYKY
SDPLVTAASVLEFLFQLKRETDDLEQWISEKELVASSPEMGQDFDHVTLTRDKFRDFARETGAIGQER
VDNVNAFIERLI
DAGHSEAATIAEWKDGLENMWADLLELIDTRMQLLAASYDLHRYFYTGAEILGLIDEKHRELPEDEVGL
DASTAESFHRVH
TAFERDVHLLGVQVQQFQDVATRLQTAYAGEKAEAIQNKKEQEVSAAWQALLDACAGRRTQLVDT.

MTSATEFENVGNQPPYSRINARWDAPDDELDNDNSSARLFERSRIKALADEREVQKKTFTTKWVNS
HLARVSCRITDLYK
DLRDGRMLIKLLEVLSGEMLPKPTKGKMRHICLENVDKALQFLKEQRVHLENMGSHDIVDGNHRLV
LGLIWTIILRFQIQ
DIVVQTQEGRETRSAKDALLWCQMIKTAGYPHVNVNTFTSSWKDGLAFNALIHKHRPDLIDFDKDKD
SNARHNLEHAFNV
AERQLGIPLDPEDEVFTENPDEKSIITYVAFYHYFSKMKVLAVEGKRVGKVIDHAIEKEMIEKYSGL
ASDLLTWIEQ
TTVLNSRKFANSLTGVQQQLQAFSTYRTVEKPPKFQEKGNLEVLLFTIQSRMRANNQKVYTPHDGKL
VSDINRAWESLE
EAGYRRELALRNELRQEKLEQLARRFDRKAAMRETWLNENQRLVAQDNFGYDLAAVEAAKKKHEAI
ETDTAAYEERVRA
LEDLAQELEKENYHDQKRITARKDNILRLWSYLQELLQSRQRLETTLALQKLFQDMLHSIDWMDEI
KAHLLSAEFGKHL
LEVEDLLQKHKLMEADIAIQGDVKAITAATLKFTGKGYQPCDPQVIQDRMSHLEQCFFEELSNMAAGA
RTQLEQSKRLW
KFFWEMDEAESWIKKEQIYSSLDYGKDLTSVLILQRKHKAFEDELRLGLDAHLEQIFQEAHGMVARKQ
FGHPQIEARIKE
VSAQWDQLKDIAAFCKNILQDAENFFQFGDADDLKAWLQDAHRLLSGEDVGQDEGATRALGKKHK
DFLEELEESRGVME
HLEQQAQGFPEEFRDSPDVTHRLQALRELYQQVWAQADLRQQRLQEADPPVATMVSKGEELFTGVVPIL
VELDGDVNGHK
FSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTLYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ
ERTIFFKDDGNY
KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNIGIKVNFKIRHNIEDGS
VOLADHYQQNT
PIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYK.

FIG. 20J

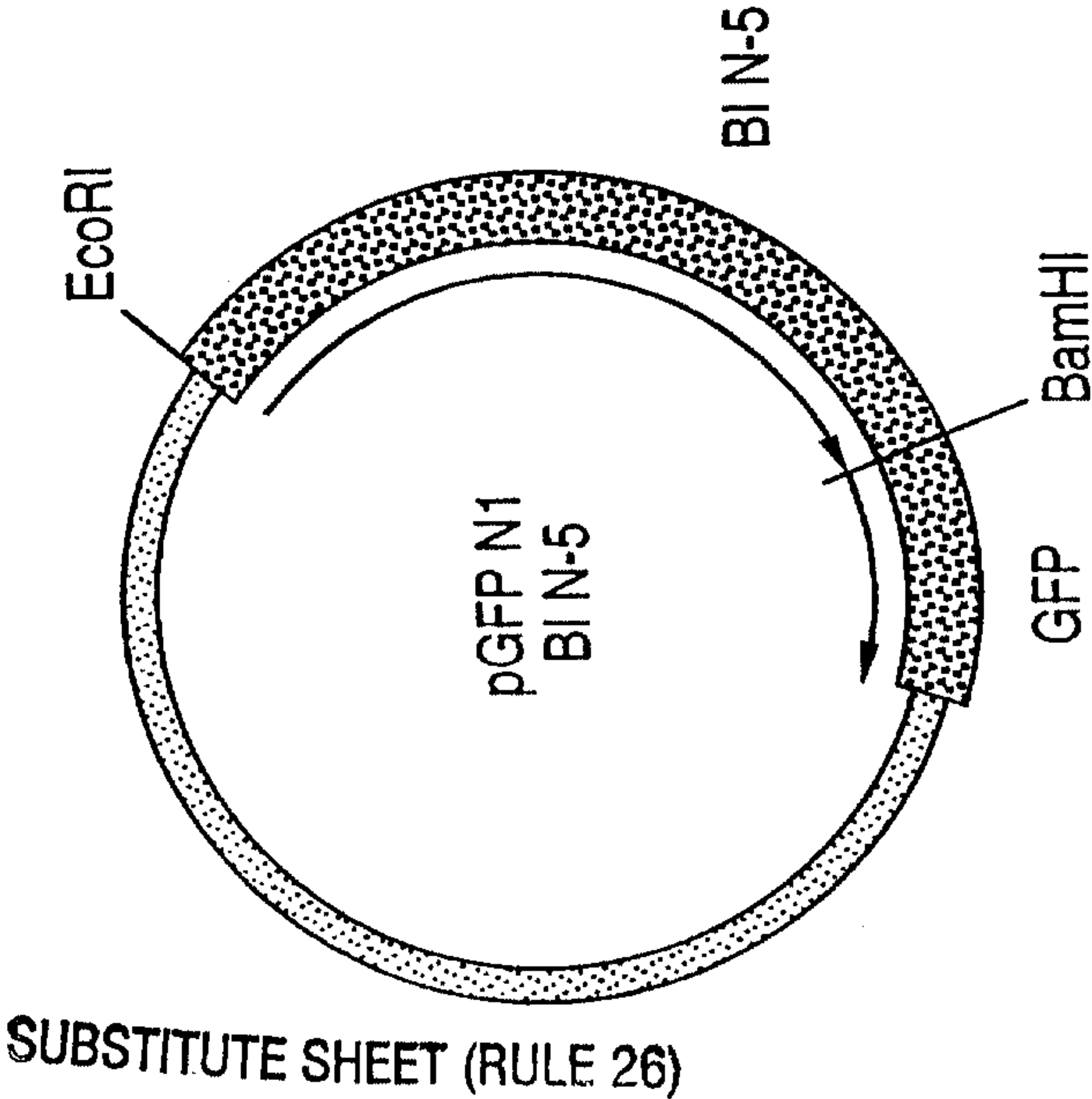
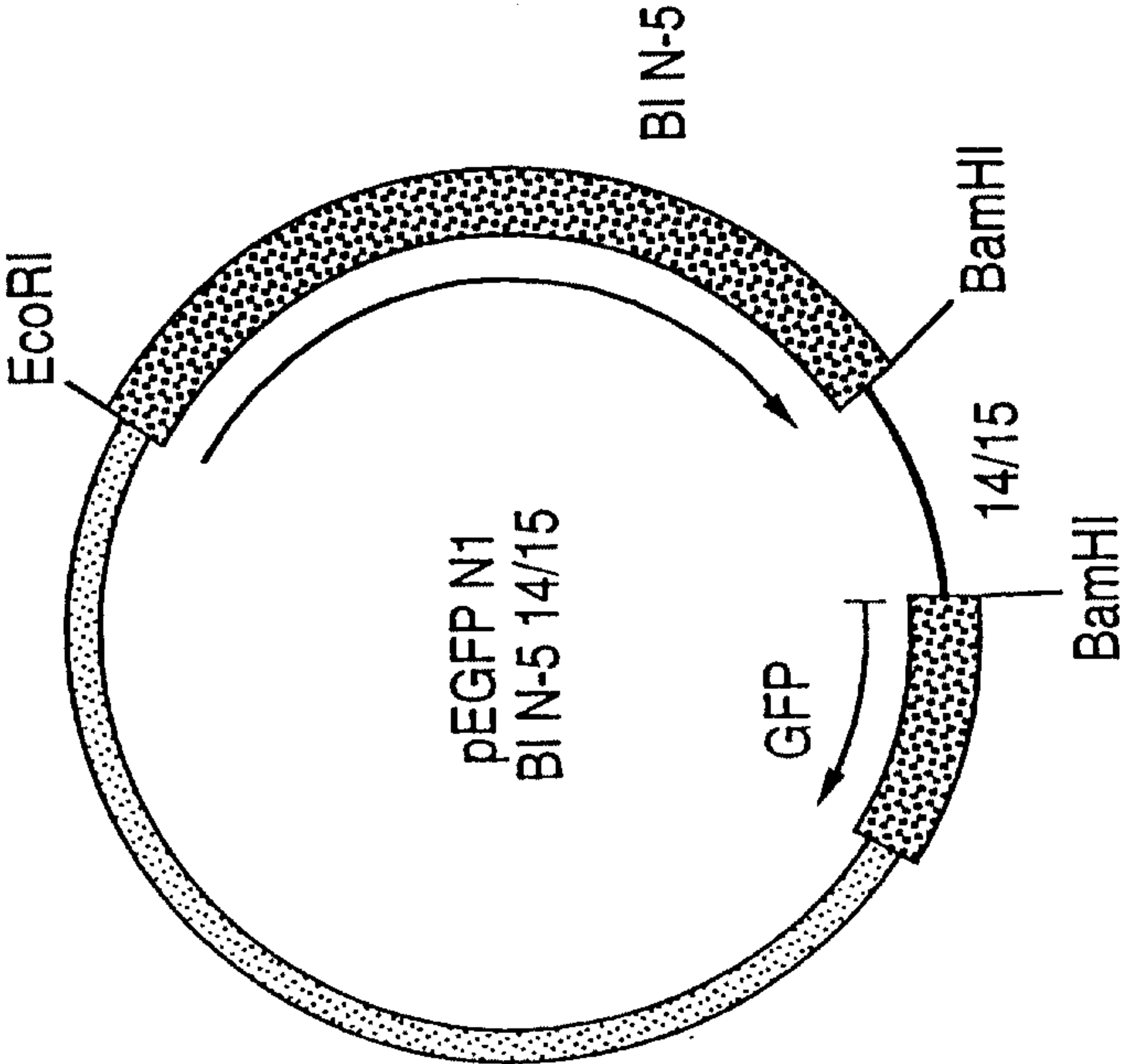


FIG. 20K-1



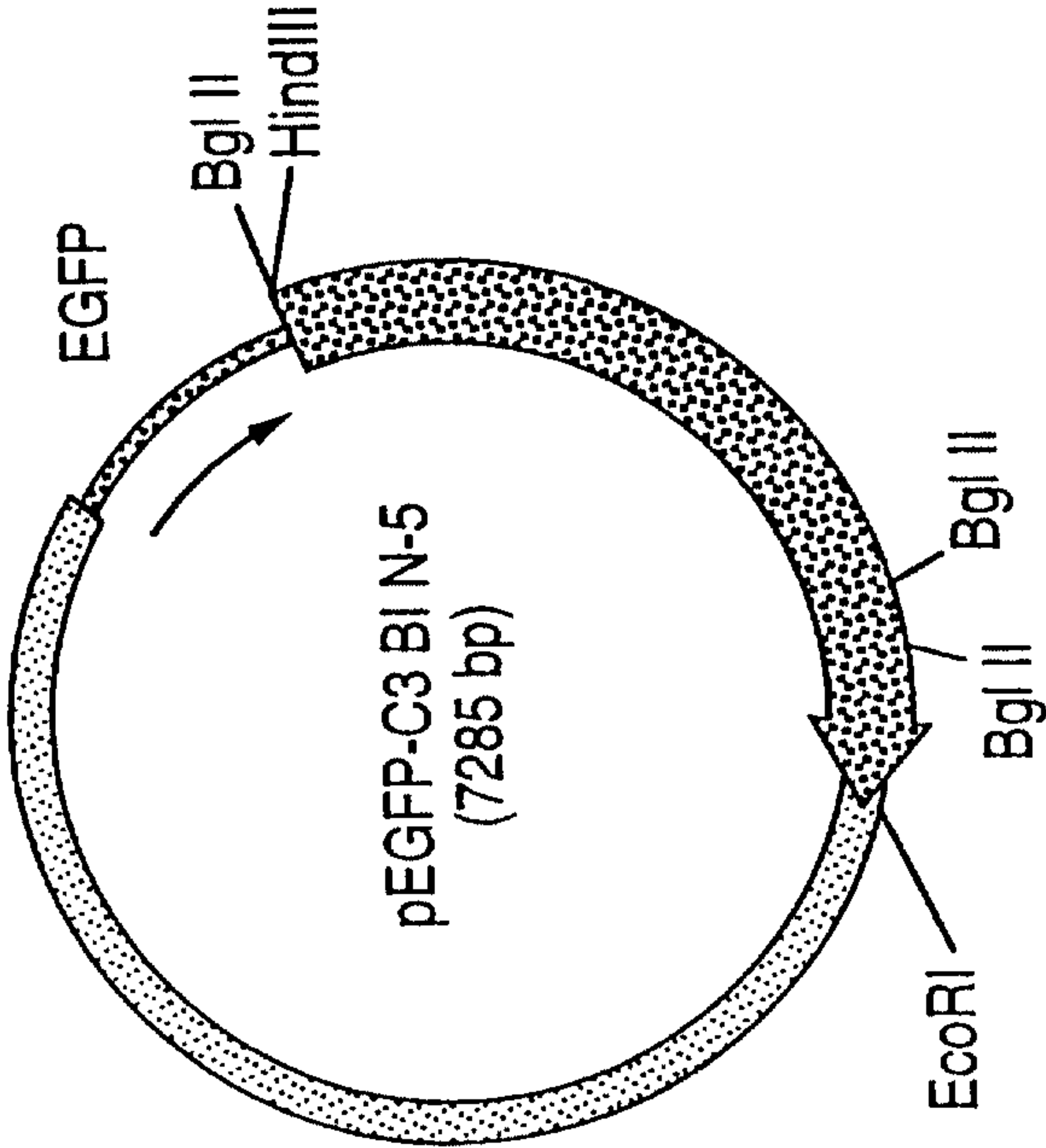
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LGLIWTIILRFQIQ
DIVVQTQEGRETRSAKDALLWCQMKTAGYPHVNVNTFTSSWKDGLAFNALIHKHRPDLIDFDKDKD
SNARHNLEHAFNV
AERQLGIPLDPEDVFTENPDEKSIITYVAFYHYFSKMVKVLAVEGKRVGKVIDHAIEKTEKMEKYSGL
ASDLLTWIEQ
TITVLSRKFANSLTGVCQQQLQAFSTYRTVEKPPKFQEKGNLEVLFTIQSRMRANNQKVYTPHDGKL
VSDINRAWESLE
EAGYRRELALRNELIRQEKLEQLARRFDRKAAMRETWLNENQRLVAQDNFGYDLAAVEAAKKHEAI
ETDTAAAYEERVRA
LEDLAQELENYHDQKRITARKDNILRLWSYLQELLQSRQRLETTALQKLFQDMLHSIDWMDEI
KAHLLSAEFGKHL
LEVEDLLQKHKLMEADIAIQGDVKKAITAATLKFTGKGYQPCDPQVIQDRMSHLEQCFFELSNMAAGA
RTQLEQSKRLW
KFFWEMDEAESWIKEKEQYSSLDYGKDLTSLILQRKHKAFEDELRLGLDAHLEQIFQEAHGMVARKQ
FGHPQIEARIKE
VSAQWDQLKDLAFCCKNLQDAENFFQFGDADDLKAWLQDAHRLLSGEDVGGDEGATRALGKKHK
DFLEEESRGVME

FIG. 20K-2

HLEQQAQGFPEEFRDSPDVTHRLQALRELYQQVVAQADLRQQRLQEQADPLFQLKRETDDLEQWISEKE
LVASSPEMGQDF
DHVTLLRDKFRDFARETGAIGQERVDNVNAFIERLIDAGHSEAAATIAEWKDGLENMWADLLELIDTR
MQLLAASYDLHRY
FYTGAELGLIDEKHRELPEDVGLDASTAESFHRVHTAFERDVHLLGVQVQQFQDVATRLQTAYAGEKAE
AQNKEQEV
AAWQALLDACAGRRTQLVDTDPPVATMVSKGEELFTGVPIVVELDGDVNGHKFSVSGEGEGDATY GK
LTLKFICTTGKL
PVPWPPTLVTTLTYGVCFSRYPDHMKQHDFKFSAMPEGVQERTIFFKDDGNYKTRAEVKFEGDTLV
NRIELKGIDFKED
GNILGHKLEYNYNSHNVIMADKQKNGIKVNFKIRHNIEDGVSQADHYQQNTPIGDPVLLPDNHYL
STQSALS KDPNE
KRDHIMVLLFEVTAAGITLGMDELYK.

MVSKGEELFTGWPILVELDQDVNGHKFSVSGEGEGDATYGKLTkFICTTGKLPVPWPtLVTLTYGV
QCFSRYPDHMK
QHDFFKSAMPEGVYQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKIDFKEDGNILGHKLEYNYS
HNVIYIMADKQKN
GIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHVLTQSALS KDPNEKRDHMMVLEFVTAA
GITLGMDELYKY
SDLELKLGTELGSMTSATEFENVGNQPPYSRINARWDAPDDELNDNSSARLFERSRIKALADEREV
VQKKTFTKWVNSH
LARVSCRITDLYKDLRDGRMLIKLLEVLSGEMLPKPTKGKMRIHCLENVDKALQFLKEQRVHLENMG
SHDIVDGNHRLVL
GLWTLIRFQIQDIVQTQEGRETRSAKDALLWCQMKTAGYPHVNVNTFTSSWKDGLAFNALIHKH
RPDLIDFDKDKD
SNARHNLEHAFNVAERQLGIPLDPEDVFTENPDEKSITYVAFYHYFSKMKVLAVEGKRVGKVIDH
AIETEKMIKY
SGLASDLLTWIEQTITVLSNRKFANSLTGVQQQLQAFSTYRTVEKPPKFQEKGNILEVLLFTIQSRMPAN
NQKVYTPHDGK
LVSDINRAWESLEEAGYRRELALRNELIRQEKLEQLARRFDRKAAMRETWLNENQRLVAQDNFGYDL
AAVEAAKKKHEAI
ETDTAA YEERVRALEDLAQELEKENYHDQKRITARKDNILRLWSYLQELLQSRQRLETTALQKL FQ
DMLHSIDWMDEI
KAHLLSAEFGKHILLEVEDLLQKHKLMEADIAQGDKVKAITAATLKFTGKGYQPCDPQVIQDRMSHLE
QCFEELSNMAA
GARTQLEQSKRLWKFFWEMDEAESWIKKEQYSSLDY GKD L TSVLILQRKHKA FEDEL RGLDAHLE
QIFQEAHGMVARK
QFGHPQIEARIKEVSAQWDQLDLAFCCKNLQDAENFFQFGDADDLKAWLQDAHRLLSGEDVGGD
EGATRALGKKHKD
FLEEESRGVMEHLEQQAQGFPEEFRDSPDVTHRLQALRELYQQVWAQADLRQQRLOEAEFCRRYR
GPGIHRIL

FIG. 20L



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FIG. 21B

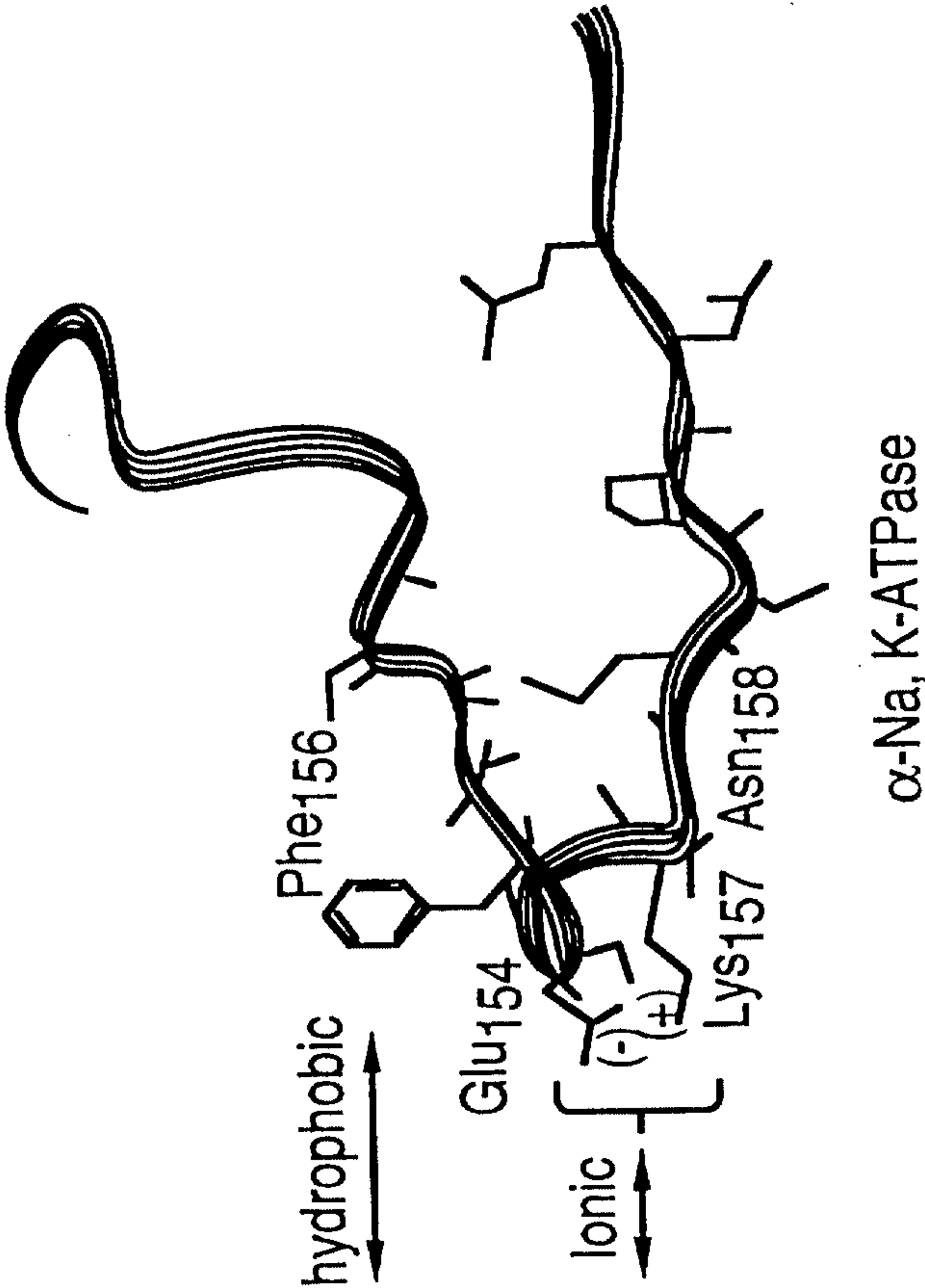
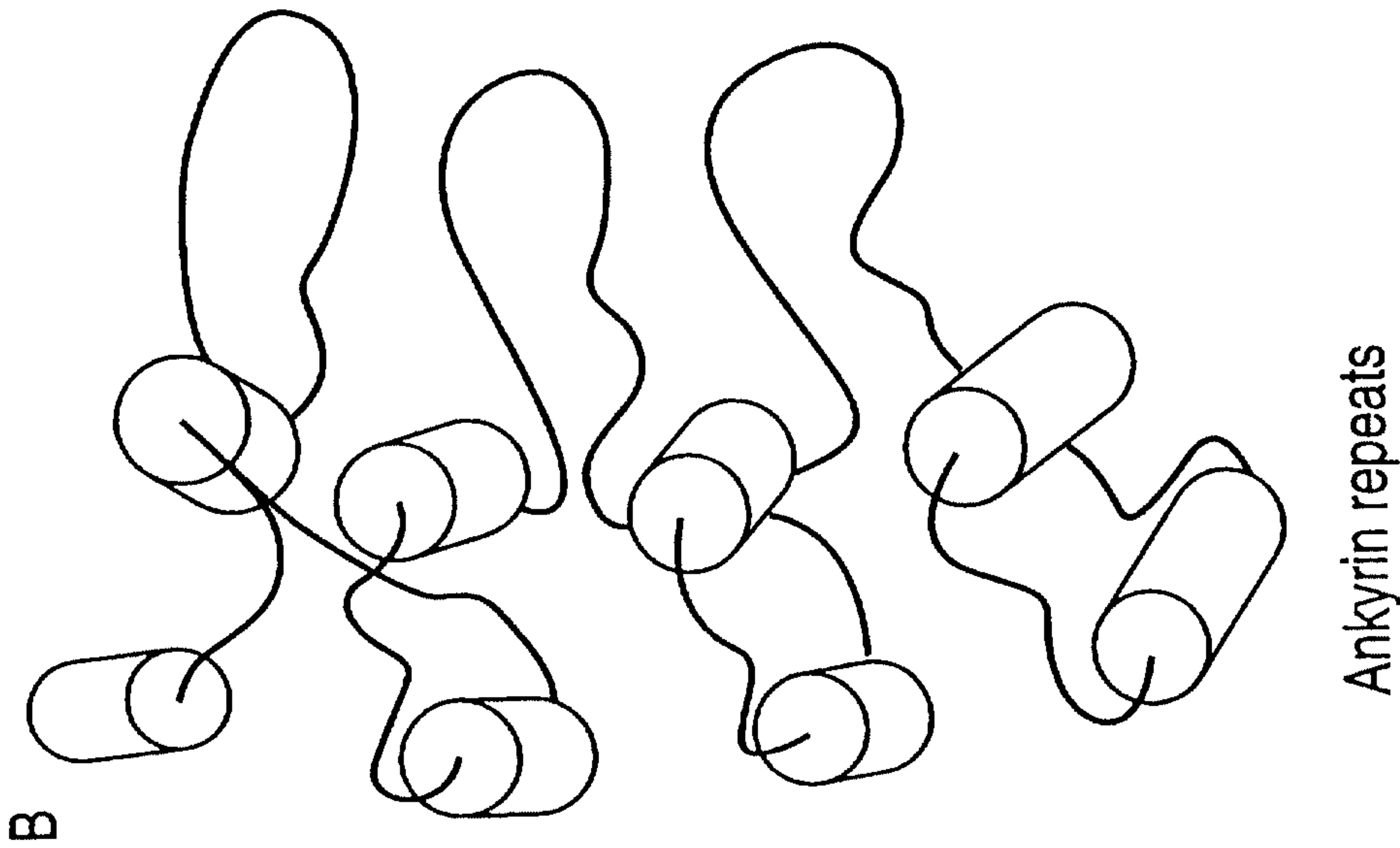
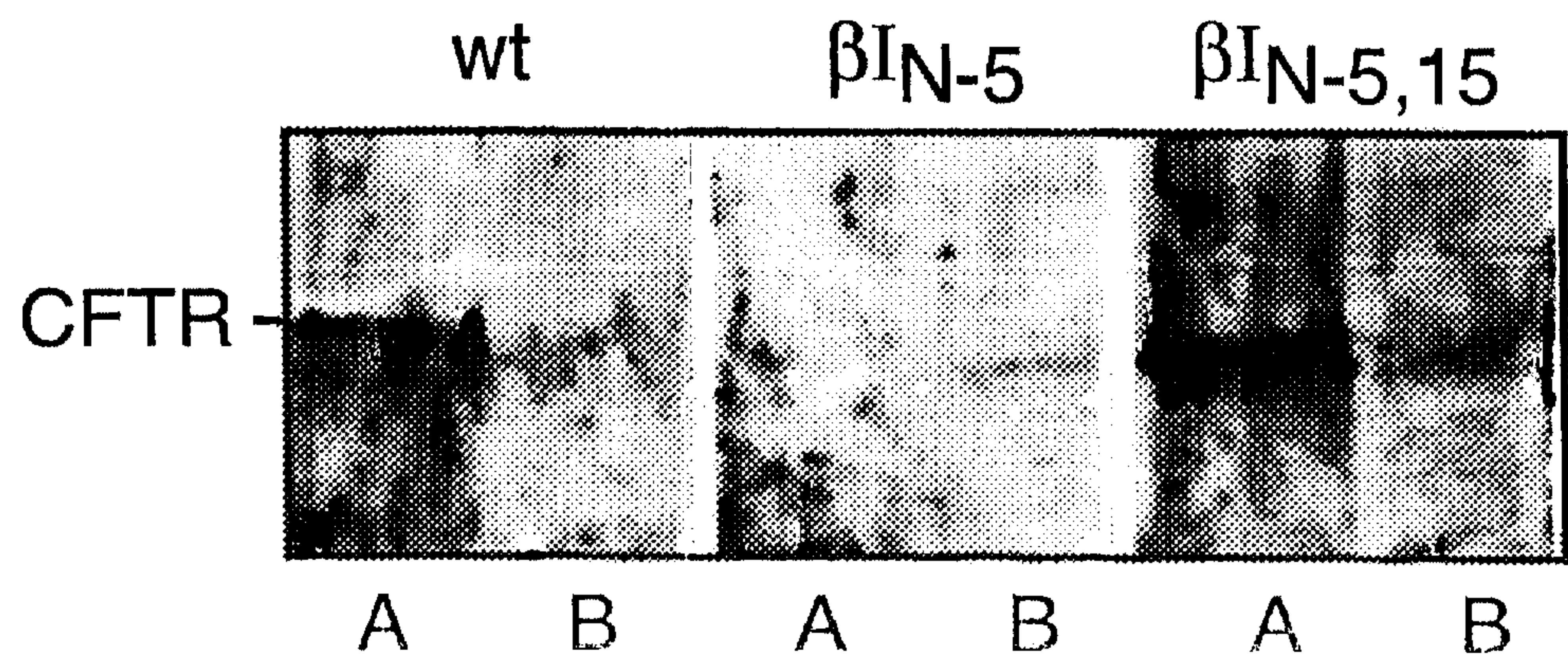
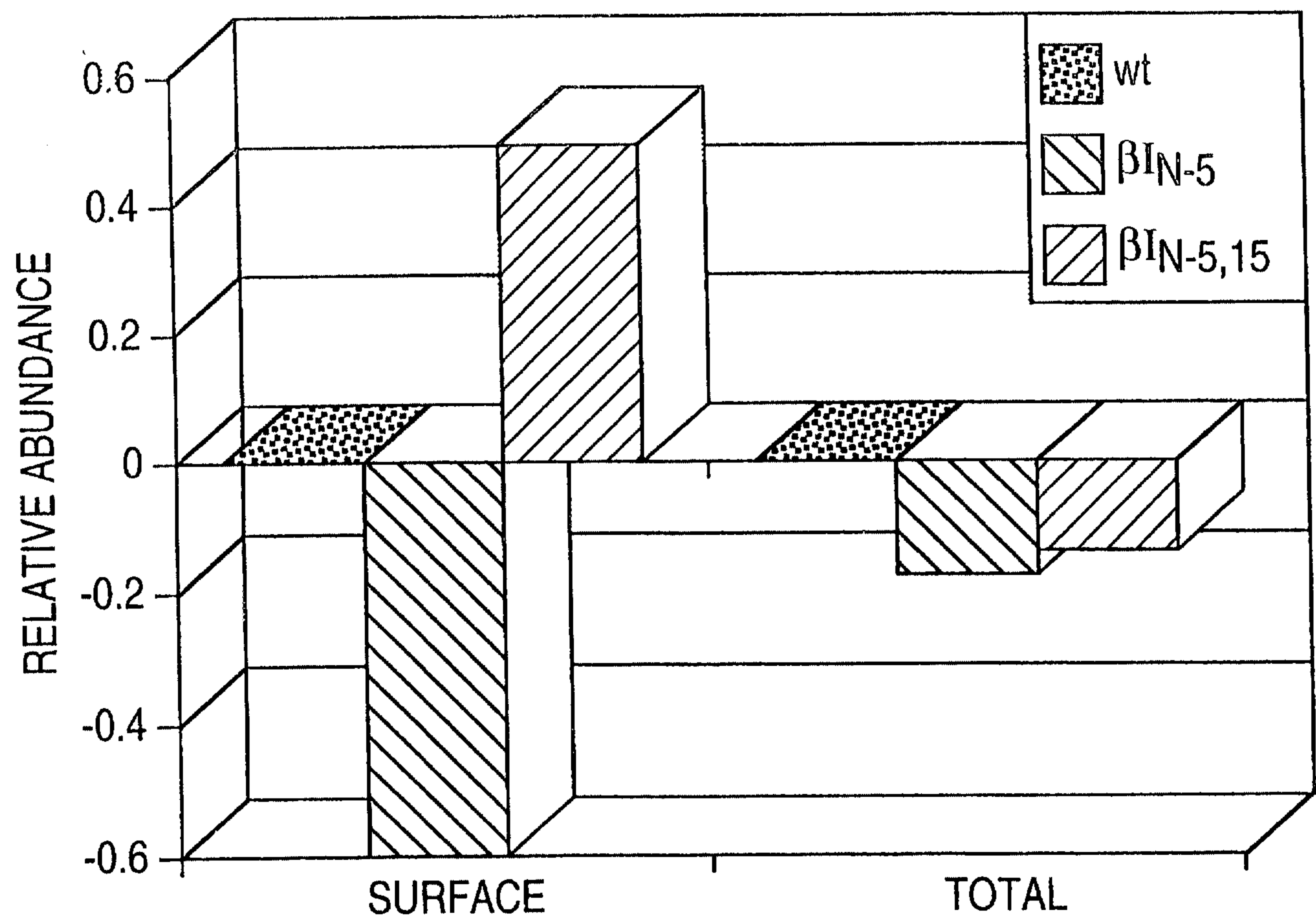


FIG. 21A



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FIG. 22A**FIG. 22B**

MODULATION OF SURFACE DISPLAY OF CFTR
IN MDCK CELLS BY SAATS

