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(54) Titre : PRAD1 CYCLINE (CYCLINE D1) ET SON ADNC
(54) Title: PRAD1 CYCLIN (CYCLIN D1) AND ITS CDNA

(57) **Abrégé/Abstract:**

A novel cyclin termed prad1 (cyclin D1), including human prad1 (cyclin D1); an antibody specific for prad1 (cyclin D1); a nucleic acid sequence which encodes prad1 (cyclin D1) or a portion of prad1 (cyclin D1); and methods of using such antibody or nucleic acid to diagnose a neoplastic condition characterized by overexpression of prad1 (cyclin D1).

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Abstract

A novel cyclin termed *prad1* (*cyclin D1*), including human *prad1* (*cyclin D1*); an antibody specific for *prad1* (*cyclin D1*); a nucleic acid sequence which encodes *prad1* (*cyclin D1*) or a portion of *prad1* (*cyclin D1*); and methods of using such antibody or nucleic acid to diagnose a neoplastic condition characterized by overexpression of *prad1* (*cyclin D1*).

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PRAD1 CYCLIN (CYCLIN D1) AND ITS CDNA

Background of the Invention

Partial funding for the work described herein was
5 provided by the U.S. Government, which has certain rights
in the invention.

This invention relates to the field of cyclins.

The cyclins are a class of eukaryotic proteins
that were originally identified by their cyclic
10 accumulation and destruction at defined points in
embryonic cell cycles (Evans et al., Cell 33:389-396,
1983). They bind to and are essential for activation of
cdc2 protein kinase (reviewed in Murray et al., Science
246:614-621, 1989; Nurse, Nature 344:503-508, 1990;
15 Draetta et al., Cell 56:829-838, 1989). At present, the
cyclins can be divided into three families on the basis
of their kinetics of oscillation across the cell cycle,
their amino acid sequences, and, in some cases, genetic
experiments in yeast that determine when their functions
20 are needed (reviewed in Nurse, 1990; Nasmyth, Cell
63:1117-1120, 1990; Westendorf, J. Cell Biol. 108:1431-
1444, 1989). The B-type "mitotic" cyclins drive cells
into mitosis; their sequences are conserved from yeast to
human (Nurse, 1990; Westendorf et al., 1989; and Pines et
25 al., Cell 58:833-846, 1989). The A-type cyclins, which
are less well understood, may act earlier in the cell
cycle (Minshull et al., EMBO J. 9:2865-2875, 1990; Pines
et al., Nature 346:760-763, 1990; Swenson et al., Cell
47:861-870, 1986). The recently described CLNs (or "G1
30 cyclins") of budding yeast are thought to perform
analogous functions by interacting with cdc2 homologues
at START, driving cells into S-phase (Nasmyth, 1990). A,
B, and CLN cyclins may act as stage-specific regulators
of progress across the cell cycle by conferring selective
35 substrate specificity upon cdc2 kinase (Minshull et al.,

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1990) or by selectively targeting *cdc2* to different intracellular compartments.

SUMMARY OF THE INVENTION

The invention features a novel cyclin, *prad1* (*cyclin D1*),
5 and an isolated DNA (termed PRAD1 or CYCLIN D1) which encodes it. CYCLIN D1 and *cyclin D1* are the currently accepted terms for PRAD1 and *prad1*, respectively (see examples of the nomenclature in Swerdlow et al., Human Pathology 33(1): 7-20, January 2002; Almadori et al., Head & Neck, 597-604, 2002;
10 Hsiang et al., Oncogene 21, 2212-2216, 2002). Both sets of terms, CYCLIN D1/*cyclin D1* and PRAD1/*prad1* are equivalent and are used here interchangeably.

PRAD1 DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, or synthetic DNA. It may be
15 identical to a naturally-occurring PRAD1 sequence (such as human PRAD1 cDNA, SEQ ID NO:1) or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides. By "isolated" is meant that the DNA is free of the coding sequences of genes that, in the naturally-
20 occurring genome of the organism from which the DNA of the invention is derived, immediately flank the gene encoding *prad1*. Included within the term *prad1* is human *prad1* and any homolog of human *prad1* (i.e., from another animal species, or

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a genetically altered version of a naturally-occurring *prad1* which exhibits a biological activity similar to that of the naturally-occurring protein) encoded by a DNA which is capable of hybridizing (1) under stringent hybridization conditions

5 (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor, NY, 1989) to a single-stranded probe consisting of a segment of at least eight (preferably 18-40) nucleotides of human PRAD1 cDNA (SEQ ID NO:1) or human PRAD1 genomic DNA, or (2) under less stringent conditions (e.g.,

10 washing at 2xSSC, at 40°C.) to a probe consisting of a segment of at least 40 (preferably 200-5000) nucleotides of human PRAD1 cDNA (SEQ ID NO:1) or human PRAD1 genomic DNA. Also within the invention are peptide fragments of a naturally-occurring *prad1*, which fragments are at least six amino acids

15 in length and preferably 10-50 amino acids; and single-stranded DNA or RNA probes (preferably radioactively labelled) containing at least 8 nucleotides

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of, but less than all of, human PRAD1-encoding RNA, human PRAD1 cDNA (SEQ ID NO:1) or human PRAD1 genomic DNA, and preferably between 10 and 5000 bases. Such DNA or RNA probes may be used in a diagnostic method which includes

5 the steps of obtaining a nucleic acid sample from an animal suspected of having a given neoplastic condition (or from a known tumor); contacting the nucleic acid sample with a single-stranded DNA or RNA probe capable of hybridizing to the PRAD1 homolog of the species to which

10 the animal belongs; and detecting the level of hybridization of the probe with the nucleic acid sample, such level being diagnostic for the neoplastic condition. Two examples of neoplastic conditions that may be

15 diagnosed by this method include centrocytic lymphomas, which appear to express abnormally high levels of PRAD1 mRNA, and those breast cancers which are characterized by a high degree of amplification of PRAD1 DNA.

The DNA sequence of the invention, which may be under the transcriptional control of a heterologous

20 promoter (defined as a promoter sequence other than the naturally-occurring promoter of the gene encoding *prad1*), may be incorporated into a vector (such as a phage) and thereby introduced into a cell. Included within the invention is a eukaryotic or prokaryotic cell (or an

25 essentially homogeneous population of such cells) containing (and preferably capable of expressing) a recombinant DNA molecule encoding *prad1*: that is, a cell into which (or into an ancestor of which) has been introduced, by means of genetic engineering, a DNA

30 molecule encoding *prad1*, resulting in that DNA molecule's being positioned adjacent to a DNA sequence to which it is not naturally adjacent (e.g., the *prad1*-encoding sequence is integrated into the genome of such cell).

The *prad1* protein of the invention may be produced by

35 culturing such cells and recovering *prad1* from the cells,

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or from their medium. Alternatively, DNA or mRNA encoding *prad1* may be combined with a standard *in vitro* expression system to produce *prad1*. *Prad1* so produced can be utilized in combination with a pharmacologically-
5 acceptable carrier to promote wound healing in an animal, or can be used to promote proliferation of an animal cell by treating the cell with a proliferation-inducing amount of the protein of the invention (for example, by transfecting the cell with DNA encoding *prad1* so that the
10 cell itself produces such a proliferation-inducing amount of *prad1*). Alternatively, the *prad1* (or an antigenic fragment thereof, determined by standard methodology) can be used to raise polyclonal or monoclonal antibodies capable of forming immune complexes with *prad1*, and thus
15 useful as a diagnostic for certain neoplastic conditions characterized by abnormally high levels of *prad1* expression. The method of using such an antibody as a diagnostic would include the steps of obtaining a sample of a tissue of an animal suspected of having a such a
20 neoplastic condition (e.g., certain lymphomas or breast cancers); contacting the sample with the antibody; and detecting the level of immune complexes formed by the antibody, such level being diagnostic for the neoplastic condition.

25 Also within the invention is a transgenic non-human vertebrate animal (preferably a mammal such as a rodent, e.g., a mouse) bearing a transgene (i.e., a piece of DNA which is artificially inserted into an embryonic cell, and becomes a part of the genome of the animal
30 which develops from that cell) which includes a DNA sequence encoding *prad1*, and any cells or cell lines derived from such an animal. A transgenic animal is an animal having cells that contain a transgene, which transgene was introduced into the animal, or an ancestor
35 of the animal, at an embryonic stage. If the embryonic

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stage is a single-cell stage, then all nucleated cells of the animal will carry the transgene. The particular *prad1* encoded by the transgene may be endogenous to the species of the transgenic animal, or may be that of a different species (e.g., human). By using a PRAD1 together with an appropriate promoter, a transgenic animal which readily develops neoplasias in a selected organ or tissue type will result, making such animal useful as a model for studying cancer in that organ or tissue.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The drawings are first described.

Drawings

Figure 1 is a Southern blot of *Msp*I-digested DNA probed with the 5' PTH gene probe (lanes 1, 2) and 3' PTH gene probe (lanes 3, 4).

Figure 2 is a diagrammatic representation of (a) the normal PTH gene, and (b) the two fragments resulting from the rearrangement in tumor M.

Figure 3 is diagrammatic representation of the D11S287 region, indicating known restriction sites and the locations of the 500 bp fragment, the 1.6 kb *Xho*I fragment, and Probe B.

Figure 4 is a Northern analysis of D11S287 expression in various cell types.

Figure 5 is a diagrammatic representation of PTH/D11S287 rearrangements in two parathyroid adenomas, and the relative locations of Probe B and a series of cloned cDNA segments.

Figure 6 is a representation of the nucleotide sequence and predicted amino acid sequence of human PRAD1 (SEQ ID NO:1) cDNA.

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Figure 7 is an illustration of sequence homology between the "cyclin box" region of human *prad1* and the corresponding regions of some A-type, B-type, and G1 cyclins.

5 Figure 8 is a Northern blot analysis of D11S287 [human PRAD1 (SEQ ID NO:1)] expression in various cell types.

Figure 9 is (a) a Northern blot analysis of HeLa cell RNA probed with a human PRAD1 cDNA (SEQ ID NO:1) probe, an H4 histone probe, and 28S rRNA; and (b) a graph depicting the results of the Northern blot.

Figure 10 is an analysis of the biological activity of recombinant human *prad1*.

Identification of Human PRAD1

15 Previous studies on DNA from cells of a benign parathyroid adenoma (reported in Arnold et al., J. Clin. Invest. 83:2034-2040, 1989) revealed evidence of a DNA rearrangement involving the parathyroid hormone (PTH) chromosomal locus (at chromosome 11, band p15) and a
20 segment of DNA (identified as Human Genome Database assignment D11S287) which normally maps to chromosome 11, band q13. It is now known that (a) although a number of previously-identified oncogenes (including INT-2 and HST-1), as well as the translocation breakpoint marker BCL-1
25 and possibly the gene for multiple endocrine neoplasia type I (MEN-I), map to the 11q13 region, the so-called D11S287 locus rearranged in at least some parathyroid adenomas is distinct from these previously-described markers; (b) D11S287 mRNA, while detectable in all
30 tissues analyzed, is significantly overexpressed in those parathyroid adenomas which have a 11q13/11p15 chromosomal rearrangement, and also in certain lymphomas (notably centrocytic lymphomas) characterized by rearrangement of the BCL-1 locus; and (c) the D11S287 locus is amplified
35 and expressed in many squamous cell and mammary

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carcinomas. This evidence suggests that D11S287 (also referred to herein as human PRAD1, for parathyroid adenoma) is a newly-identified oncogene which figures in a variety of types of neoplasms.

5 Cloning Human PRAD1 cDNA (SEQ ID NO:1)

Human PRAD1 cDNA (SEQ ID NO: 1) has been cloned and sequenced by the methods described in detail below, yielding the sequence shown in Figure 6. The longest open reading frame, starting at the first ATG codon,
10 encodes a predicted protein of 295 amino acids (M_r 33,729). Screening the Genbank peptide database with this sequence reveals significant homology only to members of the cyclin family, with greatest similarity in the region conserved among cyclins, ranging from 19.1% to
15 33.6% identity, and 44.1% to 59.2% similarity. The human PRAD1 (SEQ ID NO:1) protein (*prad1*) has significant sequence similarities to all three types of cyclins (A, B, and CLN cyclins), but cannot readily be assigned to any one type. This suggests that *prad1* may represent a
20 new and different cyclin family member.

PRAD1 Expression

PRAD1 mRNA is expressed in many tissues and is highly conserved across species (Figure 7). As with other cyclin mRNAs expressed in human cells (Pines et
25 al., Cell 58:833-846, 1989; Pines et al., Nature 346:760-763, 1990), human PRAD1 mRNA levels vary across the cell cycle (Figure 9), consistent with but not proving a role in cell cycle regulation. The peak in PRAD1 mRNA levels occurs late in the cell cycle or in G1.

30 Biological Activity of Recombinant Human *Prad1* Protein

Bacterially expressed recombinant human *prad1*, produced as described in detail below, was used to further investigate the link between human PRAD1 and the cyclins. Cyclins are known to form complexes with p34^{cdc2}
35 protein kinase, leading to its activation which can be

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assayed using exogenous histone H1 as a substrate. In addition, cyclin/p34^{cdc2} complexes can be purified by exploiting the ability of beads linked to p13^{suc1}, another cell cycle protein, to avidly bind p34^{cdc2} and, in turn, co-purify any proteins complexed with p34^{cdc2} (Draetta et al., Cell 56:829-838, 1989). When recombinant human *prad1* was added to clam embryo interphase cell lysates (which lack endogenous cyclins and contain inactive p34^{cdc2}), both p34^{cdc2} and *prad1* were bound by p13^{suc1}-beads (Figure 10). As *prad1* does not bind to protein A-Sepharose beads, its binding to p13^{suc1}-beads is most likely due to its interaction with p34^{cdc2} or a closely related protein. Furthermore, kinase activity was induced by the addition of the human PRAD1 (SEQ ID NO:1) in vitro translation product to interphase lysates (Figure 10). This kinase activity was lower than that seen with cyclin A. Cyclin B provided a negative control; for reasons not yet understood, our cyclin B translation product was not capable of activating p34^{cdc2} in this type of assay. The difference between the activities induced by cyclin A and human *prad1* may be specific to this clam assay system, or may reflect a genuine difference between the functions of, or the substrate specificities conferred by, cyclin A vs. human *prad1*.

Use

Both *prad1* and a nucleotide encoding *prad1* are useful for the preparation of diagnostic tools for the classification and/or prognosis of lymphomas, breast cancers, and squamous cell cancers, as well as other cancers characterized by a high level of expression and/or amplification of the PRAD1 gene. For example, *prad1* or an antigenic peptide fragment of *prad1* could be used in accordance with standard methods (see, e.g., Harlow et al., Antibodies: A Laboratory Manual, Cold

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Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988; Yanaihara et al., U.S. Pat. No. 4,855,406; and Slamon et al., U.S. Pat. No. 4,918,162) to raise polyclonal or monoclonal antibodies capable of forming immune complexes
5 with *prad1*, and useful for detecting abnormally high levels of *prad1* in a given tissue sample. Similarly, a hybridization probe prepared from a segment of at least 12 (and preferably greater than 250) nucleotides of human PRAD1-encoding RNA, human PRAD1 cDNA (SEQ ID NO:1) or
10 human PRAD1 genomic DNA may be employed as a means for determining the number of copies of PRAD1 present in the genomic DNA of a given sample, or the level of PRAD1 mRNA expressed in cells of such sample.

The nucleic acids of the invention may also be used
15 therapeutically. Oligonucleotides which are antisense to human PRAD1 mRNA (or which express RNA that is antisense to human PRAD1 mRNA) may be synthesized to serve as an anticancer therapy in those cases diagnosed as having a rearrangement or amplification of human PRAD1: such
20 oligonucleotides would be introduced into tumor cells *in vivo* as a means to reduce production of *prad1* in such cells, and thereby to reduce neoplastic growth induced by an overabundance of *prad1*. (See, for example, Weinberg et al., U.S. Pat. No. 4,740,463). By linking a PRAD1
25 sequence to a selected tissue-specific promoter or enhancer and introducing by standard methods (e.g., as described by Leder et al., U.S. Pat. No. 4,736,866) the resultant hybrid gene into an animal embryo at an early developmental stage (e.g., the fertilized oocyte stage),
30 a transgenic animal which expresses elevated levels of *prad1* in the selected tissue (e.g., breast, squamous cell, B-lymphoid cell, parathyroid, and others) can be produced. The form

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of PRAD1 utilized can be one which encodes a *prad1* similar to that of the animal species used, or it can encode the *prad1* homolog of a different species (e.g., human). Such an animal would be useful as an *in vivo* model for neoplastic disease in the selected tissue. In addition, cells derived from such a transgenic animal may be used to establish an immortal cell line that retains at least some of its differentiated characteristics while proliferating indefinitely *in vitro*. Alternatively, one could stably transfect primary cells (e.g., a type that has proven difficult to maintain in culture, such as pituitary cells) with a PRAD1 gene linked to an appropriate promoter (e.g., the metallothionin promoter) which ensures high levels of expression of the gene, and thereby establish an immortal cell line derived from such primary cells. PRAD1 sequences may be particularly useful in this regard because overexpression of PRAD1 (at least in parathyroid tissues) appears to trigger the proliferation of normally quiescent cells without causing them to completely lose their differentiated phenotype.

Experimental Data

The DNA abnormality in parathyroid tumor M was initially characterized by Southern analysis of *MspI* digests using probes specific for the 5' and 3' regions in the PTH gene (see below), which revealed a unique, tumor-specific band. Figure 1 illustrates these Southern blots of tumor M (T) and peripheral blood leukocyte (L) DNA pairs. *MspI*- digested DNA was probed with the 5' PTH gene probe (lanes 1, 2) and 3' PTH gene probe (lanes 3, 4). Squares indicate the normal gene (6.3 kb); arrows indicate the rearranged allele (1.5 kb in lane 1, 5.4 kb in lane 3). There is an *MspI* site within the DNA to which the 3' probe hybridizes (see Figure 2a); therefore, a smaller band (2.2 kb) representing the most 3' section of the normal PTH gene is present in lanes 3 and 4. The

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intensities of the bands representing the abnormal allele were approximately equal to those representing the normal allele. Thus, in tumor M, as in tumor Y (Arnold et al., 1989), a clonal rearrangement of the PTH gene has occurred: in every tumor cell, one of the two alleles of the PTH gene remains normal but the other is disrupted. Figure 2(a) illustrates the normal PTH gene, with the positions of its three exons (Vasicek et al., Proc. Natl. Acad. Sci. USA 80:2127-2131, 1983), the 5' and 3' probes used in mapping and cloning, and the MspI sites indicated. In comparison, Figure 2(b) shows the two fragments resulting from the rearrangement in tumor M: one consists of the 5' PTH gene sequences plus juxtaposed non-PTH DNA (stippled area), while the other consists of 3' PTH gene sequences plus juxtaposed non-PTH DNA (cross-hatched area). In each fragment, the location of the breakpoint is shown by a diagonal line. The locations of several restriction enzyme sites, determined by Southern blot analysis of tumor DNA, are indicated: EcoRI (R), BamHI (B), HindIII (H), XhoI (X), SstI (S), MspI (M). The locations and sizes of the 1.5 kb and 5.4 kb rearranged MspI fragments, (shown in Figure 1) are indicated above each fragment. Below each fragment, lines ending in arrow tips depict the 1.5 kb and 16 kb cloned tumor DNA fragments. Analysis with multiple additional restriction enzymes indicated that the gene is separated into two parts, with the breakpoint located in the first intron (Figure 2b). Consequently, upstream regulatory elements and the first, non-coding exon in the 5' fragment are separated from the coding sequences in the 3' fragment. Each PTH gene fragment remains internally intact (within the limits of sensitivity of restriction mapping), but has become juxtaposed to non-PTH DNA.

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To identify the rearranged non-PTH DNA (shaded and cross-hatched areas in Figure 2b), two DNA fragments containing PTH gene sequences plus breakpoint-adjacent DNA were cloned from tumor M DNA. One was a 16 kb BamHI
5 fragment containing approximately 8 kb of non-PTH gene DNA adjacent to 8 kb of 3' PTH gene sequences (Figure 2b). Genomic Southern blots of normal DNA probed with subclones spanning most of the 8 kb of non-PTH DNA showed diffuse smears that did not yield to attempts at
10 competition with excess human DNA (Sealy et al., 1985). This indicated that the non-PTH DNA in the 16 kb fragment contained sequences highly repeated in the human genome, and precluded its chromosomal localization.

We also cloned a 1.5 kb EcoRI fragment containing
15 approximately 1 kb of the PTH gene's 5' region plus 500 bp of juxtaposed non-PTH DNA (Figure 2b). Probing normal human DNA blots with the subcloned 500 bp fragment demonstrated that it contained single-copy DNA; in situ hybridization and analysis of somatic cell hybrids
20 revealed that the 500 bp fragment's normal chromosomal location is 11q13.

Hybridization of the 500 bp breakpoint-adjacent DNA fragment to an RNA blot of six parathyroid adenomas, including two with PTH gene rearrangements, was negative.
25 To identify transcribed sequences near the breakpoint that could have been affected by the rearrangement, we walked along the chromosome by probing a normal human genomic library with the 500 bp subcloned fragment. We obtained a bacteriophage clone with a 14 kb insert, but
30 Northern blot analyses revealed no hybridization of subclones spanning the entire insert. Mapping of the 14 kb insert showed that the 500 bp fragment was at one end, and demonstrated that the adjacent cloned DNA had a restriction map identical to that of the genomic DNA
35 juxtaposed to tumor M's rearranged 5' PTH gene fragment.

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(Compare Figures 2b and 3). At the other end of the 14 kb insert was a 1.6 kb XhoI fragment (Figure 3) identical in size to an XhoI fragment 1 kb from tumor Y's D11S287 breakpoint (Arnold et al., 1989). We subcloned these two independent 1.6 kb XhoI fragments (one from the above normal phage clone and one from a tumor Y-derived clone) and used them sequentially to probe blots of normal human genomic DNA digested with 7 restriction enzymes. With every enzyme, the two probes hybridized to precisely comigrating fragments. In addition, restriction maps of the two 1.6 kb fragments themselves were identical for all 6 enzymes used. Thus, the 1.6 kb XhoI fragment linked tumor M's breakpoint-adjacent DNA with that of tumor Y (D11S287), confirming that the 11q13 breakpoints in the two adenomas are both in the D11S287 region, separated by 15 kb. The composite restriction map of the unrearranged D11S287 region is shown in Figure 3, in which restriction sites for the enzymes HindIII (H), BamHI (B), EcoRI (E), SacI (S), MspI (M) and XhoI (X) are indicated. The locations of the 500 bp fragment, the 1.6 kb XhoI fragment, and probe B are shown. This map is derived from the maps of the phage clones described above and by Arnold et al. (1989), and Southern blots of DNA from tumors M and Y.

The proximity of the 11q13 breakpoints suggested that the rearrangements could have similar functional consequences. Because none of the DNA between the two tumors' breakpoints is transcribed in parathyroid cells, we looked for transcribed sequences distal to tumor Y's breakpoint. We used fragment B (Figure 3), a breakpoint-adjacent DNA fragment from tumor Y, to probe a blot containing total RNA from human placenta, several parathyroid adenomas lacking PTH gene rearrangements, and tumors M and Y. We also hybridized probe B to another blot containing total RNA from placenta and from another

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parathyroid adenoma (tumor F) that was found recently to contain a clonal rearrangement of the PTH and D11S287 loci (Friedman et al., 1990); Southern blotting indicated that tumor F's rearrangement closely resembled tumor Y's.

5 Figure 4 presents the results of the Northern blots, in which 10 micrograms of total RNA was probed with Probe B (top panels), and with a 28S rRNA probe (bottom panels). Size determination was based on the migration of 28S rRNA. Lanes contain the following samples: lanes 1, 7: 10 placenta; lanes 2, 3, 4: parathyroid adenomas without PTH gene or D11S287 rearrangements; lanes 5, 6, 8: tumors Y, M, and F, respectively; lanes 7 and 8 are a separate Northern filter. The middle panel is a longer exposure of lanes 1-6 in the top panel. In lanes 5 and 8 15 (tumors Y and F) a faint band was visible, larger than the highly-overexpressed 4.5 kb band, which was not seen in lane 6 (tumor M) (data not shown). Exposure times: top row (probe B): lanes 1-6, 17h; lanes 7 and 8, 12h; Middle row (probe B): all lanes, 52h; Bottom row (28S 20 rRNA): all lanes, 1.5h. An approximately 4.5 kb transcript (slightly smaller than the 28S rRNA band) was seen in all lanes of Figure 4. However, the intensity of the 4.5 kb band in tumors M, Y and F was roughly 15-fold greater than that in any of the other specimens. We 25 demonstrated that the 4.5 kb band represents polyadenylated RNA by finding its intensity amplified in poly A+ RNA (data not shown).

Parathyroid adenoma M initially was identified as having an abnormal PTH gene during studies of the 30 monoclonality of parathyroid adenomas (tumor 1 in Arnold et al., N. Eng. J. Med. 318:658-662, 1988). All tumor specimens were frozen in liquid nitrogen shortly after surgical removal. Extraction of high molecular weight DNA, restriction enzyme digestion and Southern blotting 35 were performed as previously described (Arnold et al., N.

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Eng. J. Med., 309:1593-1599, 1983). Total RNA was isolated by the guanidinium thiocyanate/cesium chloride method, electrophoresed on a denaturing formaldehyde-agarose gel, and transferred to nitrocellulose or nylon filters (Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 7.19-7.22, 7.37-7.39, 11.31-11.32, 1989). Hybridization conditions were similar to those used for Southern filters. Blots were washed at high stringency (0.1 x SSC, 65°).

PTH gene fragments used as hybridization probes were the 775 bp BglII fragment (5' PTH probe) and the 2.6 kb SstI-EcoRI fragment (3' PTH probe) from pPTHg108 (Igarashi et al., Mol. Cell. Biol. 6:1830-1833, 1986) (Figure 2a). The 500 bp fragment and probe B (Figure 3) were subcloned into pUC-18 from the breakpoint-adjacent DNA of the phage clones containing the rearranged PTH gene fragment plus juxtaposed DNA from tumor M (see above), and tumor Y (Arnold et al., 1989), respectively. The 1.6 kb XhoI fragment from the 14 kb insert cloned from the normal human genomic library was also sub-cloned into pUC-18. The 1.6 kb XhoI fragment from tumor Y was subcloned from a λ phage 2001 clone containing the 17 kb HindIII fragment of tumor Y's unrearranged D11S287 allele (Arnold et al., 1989). All the above probes were random-primed and labelled with [32 P]dATP (Feinberg & Vogelstein, Anal. Biochem. 132:6-13, 1983). The 28S RNA oligonucleotide was end-labelled with [32 P]dATP (Sambrook et al., 1989) and used to probe the Northern filters to control for the amount of high molecular weight RNA present in each lane.

To clone the rearranged 5' PTH gene fragment (Figure 2b), an EcoRI library of tumor genomic DNA was constructed using the λ ZapII vector (Stratagene). This library was screened with the 5' PTH gene probe, and the

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rearranged allele was distinguished from the normal allele by size, as DNA blots predicted that the rearranged EcoRI fragment would be 1.5 kb in size, and the normal fragment 3.5 kb. One clone containing the
5 rearranged gene was identified in 1×10^6 plaques that were screened.

To clone the rearranged 3' PTH gene fragment (Figure 2b), a BamHI library of tumor genomic DNA was constructed in EMBL-3. Because restriction mapping
10 indicated that both the normal and rearranged 3' PTH BamHI fragments were 16 kb in size, the library was screened with the 3' PTH probe (expected to hybridize to both the normal and rearranged PTH alleles) and then with the 5' PTH probe (expected to hybridize only to the
15 normal allele). One clone containing the rearranged allele was identified in 6.5×10^3 plaques screened. As predicted, it contained 8 kb of 3' PTH gene sequences and 8 kb of newly-juxtaposed DNA. Most of this 8 kb was subcloned in roughly 2 kb units into pUC-18, and used to
20 probe Southern filters of normal genomic DNA.

Prereassociation was performed by sonicating 1 mg of human placental genomic DNA and incubating it for 10-60 min with 50-100 ng of labelled repeat-containing subcloned DNA. This mix was then hybridized to a
25 Southern filter containing normal human DNA using standard conditions.

The genomic library used to obtain the 14 kb insert was a partial Sau-3a digest of normal human DNA cloned into an EMBL-3 like vector (Clontech).

30 Chromosomal mapping using human-mouse somatic cell hybrids (Shows et al., Adv. Hum. Genet. 12:341-452, 1982; Shows et al., Somatic Cell Mol. Genet. 10:315-318, 1984); Southern blotting (Naylor et al., J. Exp. Med. 57:1020-1027, 1983); and in situ hybridization (Zabel et al.,
35 Cytogenet. Cell Genet. 39:200-205, 1985; Nakai et al.,

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Cytogenet. Cell Genet. 43:215-217, 1986) was performed as previously described.

A λ gt11 placental cDNA library (Clontech) was screened with radiolabeled Probe B. A clone denominated λ P1-4 and another similar phage clone were isolated. Probe B and the insert of λ P1-4 were sequenced. The region of genomic and cDNA overlap was followed in Probe B by a GT splice donor sequence in only one orientation, confirming hybridization data which had suggested transcription in the left to right orientation, as shown in Figure 5. The next probe was made by polymerase chain reaction amplification of the 3' region of the λ P1-4 cDNA insert, and used to rescreen the same library. From 5×10^5 pfu of this library, one of 16 positive clones, λ P1-5, had an insert extending further downstream. The PstI/EcoRI fragment of λ P1-5 was then used to rescreen the library, and 12 similar clones, the longest of which was λ P2-3, were obtained. The sequence of the insert of λ P2-3 revealed polyadenylation signals and a polyA stretch of 16 nucleotides in an appropriate position, consistent with the expected orientation. Standard methods for library screening and probe labeling were used (Davis et al., *Basic Methods in Molecular Biology* (Elsevier, New York, Amsterdam, London, 1986). These clones are illustrated in Figure 5, together with a schematic representation of PTH/D11S287 rearrangements in two parathyroid adenomas. The 5' PTH region (11p15, thick lines) was juxtaposed to the D11S287 region (11q13, thin lines) in each of these adenomas. The breakpoints in the D11S287 region are 15 kb apart. Genomic Probe B is shown as a darkened box, whose open area represents the first exon of PRAD1. Also shown are restriction maps of the inserts of representative overlapping PRAD1 cDNA clones, λ P1-4, λ P1-5, and λ P2-3; and the deduced restriction map of the PRAD1 cDNA. The coding region is

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shown as a crosshatched box. Scale of 1 kb is shown as arrows. Symbols used for restriction sites are: B, BamHI; E, EcoRI; H, HindIII; P, PstI.

The inserts of the clones λ P1-4, λ P1-5, and λ P2-3 shown in Figure 5, and of other independent clones, were subcloned into pGEM7Zf(+) (Promega). Sequences were obtained using the double-stranded DNA sequencing technique (dideoxy method) with modified T7 DNA polymerase (Sequenase (TM); U.S. Biochemical Corporation), as described by the manufacturer. Several oligonucleotides were synthesized as internal primers to facilitate sequencing. The coding region was sequenced in both orientations and in at least two independent clones. Set forth in Figure 6 are the nucleotide sequence and predicted amino acid sequence of human PRAD1 cDNA (SEQ ID NO:1). Nucleotide numbers are on the right. Nucleotide 3495, shown as W, indicates A or T because the sequences of two independent clones did not agree. Nucleotide 4017 is shown as R, meaning A or G, for the same reason.

Figure 7 illustrates sequence homology between the "cyclin box" region of the predicted PRAD1 protein (*prad1*) and that of A-type cyclins (human and clam cyclin A) (Swenson et al., Cell 47:861-870, 1986, and Wang et al., Nature 343:555-557, 1990); B-type cyclins (human cyclin B and *S. pombe cdc13*) (Pines et al., Cell 58:833-846, 1989; and Booher et al., EMBO J. 7:2321-2327, 1988), and one *S. cerevisiae* G1 cyclin (*cln3*) (Nash et al., EMBO J. 7:4335-4346, 1988; Cross et al., Mol. Cell. Biol. 8:4675-4684, 1988). Clam cyclin A and *S. pombe cdc13* homologies with *prad1* are representative of those found in their families; *cln3* aligns with *prad1* more closely than does *cln1* or 2. Identical amino acids are shown as |. Conservative substitutions are shown as *. Alignment was made with the assistance of the BESTFIT program (Devereux et al., Nucl. Acids Res. 12:387-395, 1984) and

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conservative amino acids are grouped as follows: D, E, N, Q; H, K, R; A, G, P, S, T; I, L, M, V; F, W, Y. Amino acid numbers are on the right in this Figure.

5 RNAs were prepared for Northern blot analysis from the indicated tissues by standard procedures (Davis et al., 1986). 10 μ g total RNA was loaded and separated on an agarose-formaldehyde gel, blotted onto nitrocellulose, and hybridized with Probe B or the 28S rRNA oligonucleotide. The filters were washed at high
10 stringency (0.1x SSC, 60°C) and autoradiographed. Figure 8 illustrates a Northern blot analysis of total RNA from human thyroid (lane 1), human placenta (lane 2), bovine parathyroid (lane 3), bovine thyroid (lane 4), bovine lymph node (lane 5), bovine skeletal muscle (lane 6),
15 murine heart (lane 7), and murine liver (lane 8). PRAD1 mRNA (shown in the upper panel) is approximately 4.5 kb in size, slightly smaller than the 28S rRNA; 28S rRNA hybridization is shown in the lower panel. Figure 9(a) shows a Northern blot analysis of total RNA from HeLa S3
20 cells after release from G1/S block. HeLa S3 cells (American Type Culture Collection), maintained in Dulbecco Modified Eagle Medium (DMEM, GIBCO) with 7% fetal bovine serum (FBS), were synchronized at the G1/S boundary by sequential thymidine-aphidicolin treatment
25 (Heintz, et al., Mol. Cell. Biol. 3:539-550, 1983) with a slight modification. Log-phase cells were incubated in complete medium (DMEM with 7% FBS, penicillin G, and streptomycin) with addition of 2mM thymidine (Sigma) for 12 h. After release from thymidine block by 3 washes
30 with PBS, the cells were incubated for 10 h with 24 μ M deoxycytidine (Sigma) and 24 μ M thymidine, recovered by trypsinization, counted, and aliquoted equally (5.0×10^4 cells/cm²). Incubation with 5 μ g/ml aphidicolin (Sigma) for 14 h was followed by release from G1/S block with 4
35 DMEM washes and incubation in complete medium.

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[³H]Thymidine (NEN) was added to an aliquot 15 min before each indicated time point; a 30 min incubation and harvesting for trichloroacetic acid (TCA) precipitation followed. RNAs from parallel aliquots were extracted
5 (Chomczynski et al., Anal. Biochem. 162:156-159, 1987) at the indicated times; time zero was just before release from aphidicolin. RNAs (5 µg per lane) were blotted onto nitrocellulose and sequentially hybridized with the PRAD1 λp1-4 cDNA insert, human H4 histone pF0108X (Pauli et
10 al., Science 236:1308-1311, 1987), and a 28S rRNA oligonucleotide as described above. Human PRAD1 mRNA is shown in the upper panel of Figure 9(a); H4 histone mRNA in the middle panel shows the pattern expected in well-synchronized cells (Heintz et al., 1983); and 28S rRNA is
15 shown in the lower panel as a control for RNA loading. In Figure 9(b) are compared the relative amounts of human PRAD1 mRNA (-●-), H4 histone mRNA (-○-), and
[³H]thymidine incorporation (-□-) of HeLa S3 cells after release from G1/S block. The signals of the blot shown
20 in Figure 9(a) were measured by densitometry and normalized to the 28S rRNA to produce the graph of Figure 9(b).

Clam embryo interphase cell lysates lacking endogenous cyclins were prepared by adding 100 µM emetine
25 during first mitosis, as described previously (Luca et al., J. Cell Biol. 109:1895-1909, 1989), followed by homogenization and centrifugation at 150,000 x g. Aliquots of the supernatant were frozen in liquid nitrogen. [³⁵S]methionine-labeled *pradl* was produced in a
30 reticulocyte lysate *in vitro* translation system (Promega) according to manufacturer's instructions, by using a plasmid (denominated pP1-8) containing the λP1-4 insert in pGEM7Zf(+) (Promega). To produce *pradl* in *E. coli*, pT4R-1 was constructed by insertion of the λP1-4 insert
35 into the NcoI and BamHI sites of pET-3d (Studier et al.,

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Meth. Enzym. 108:60-89, 1990). BL21(DE3) cells were transformed with pT4R-1, cultured, and treated with 0.4 mM isopropylthio-beta-galactosidase (IPTG) for 3 h to induce *prad1* expression. The induced product was
5 purified from cell culture as inclusion bodies (Gardella et al., J. Biol. Chem 265:15854-15859, 1990). On SDS-polyacrylamide gels, the apparent sizes of the *in vitro* translation product and the bacterially-expressed product were the same (M_r 35 kD). Rabbit anti-*prad1* antisera were
10 raised against a synthetic peptide corresponding to amino acids 9-37 of *prad1*. Antisera were assayed by immunoprecipitation of the *in vitro* translation product. Antisera specificity was shown by comparison with normal rabbit serum and by successful competition with the (9-
15 37) peptide (data not shown).

Thawed clam embryo lysate (16.5 μ l) and bacterially-expressed *prad1* (5.5 μ l) were mixed and incubated at 18°C. for 30 min before transfer to 4°C, dilution with 4 volumes of buffer A (50 mM Tris pH 7.4, 150 mM NaCl, 5mM
20 EDTA, 5mM EGTA, 1 mM ammonium molybdate) and addition of p13^{suc1} - or protein A-Sepharose (TM), followed by mixing for 1 h. Beads were then pelleted and washed in buffer A + 0.5% Tween-20 (TM); in buffer B (50 mM Tris pH 7.4, 1.0 M NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM molybdate, 0.5%
25 Tween-20 (TM)); and finally in buffer A without Tween-20 (TM), all at 4°C. Washed beads were boiled in SDS sample buffer for 3 min and the supernatant split into three samples for electrophoresis. Gels were silver stained or blotted onto nitrocellulose filters and reacted with
30 rabbit antibodies generated against bacterially-expressed, full-length *S. pombe cdc2* protein or *prad1* peptide as above. Antibody binding was visualized by alkaline phosphatase-linked secondary antibodies, according to the manufacturer's directions (Promega).
35 Figure 10 demonstrates that *prad1* protein added to clam

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embryo cell lysates binds to p13^{suc1}-Sepharose beads and activates histone H1 kinase activity. Bacterially expressed *prad1* was incubated with a clam embryo interphase lysate lacking endogenous cyclins A and B.

5 The lysates were then mixed with p13^{suc1}- or protein A-Sepharose beads. The bound material was eluted, electrophoresed and either silver stained (a) or immunoblotted with anti-*prad1* antiserum (b) or anti-*cdc2* antiserum (c). Lane M shows molecular weight markers

10 (from top to bottom) of 116, 94, 68, 56, 40, and 31 kD. Lane 1 shows whole clam embryo interphase lysate plus 18 ng *prad1* protein. Lanes 2, 3, 4, 5, and 6 represent clam embryo lysate to which 0, 18, 45, 225, or 18 ng of *prad1*, respectively, were added; these mixes were then assayed

15 for material binding to p13^{suc1}-Sepharose (lanes 2-5) or protein A-Sepharose (lane 6) beads. Lane 7 shows bacterially-expressed *prad1*. Arrows indicate the positions of *prad1* and *cdc2* marker proteins.

Equal volumes of clam embryo interphase lysate and

20 reticulocyte lysate containing [³²P]-labeled kinase products were then examined by SDS-PAGE, followed by autoradiography. Synthetic clam cyclins A and B (Westendorf et al., J. Cell Biol. 108:1431-1444; Swenson et al., Cell 47:861-870, 1986) and *prad1* mRNAs were

25 transcribed and translated as described above. Translation product (3 μ l) and clam embryo lysate (3 μ l) were mixed. Samples were frozen immediately in liquid nitrogen. The remainder was incubated for 30 min at 18°C and then frozen. Samples were diluted with 1 volume of

30 ice-cold buffer A, thawed on ice, and mixed with an equal volume of kinase mix (40 mM Hepes pH 7.3, 20 mM MgCl₂, 10 mM EGTA, 0.2 mg/ml histone H1, 10 μ M cAMP-dependent kinase inhibitor (Sigma), 0.5 mCi/ml [γ -³²P]ATP and incubated at 23°C for 10 min. Double-strength SDS sample

35 buffer was then added and the entire mix was analyzed by

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SDS-PAGE followed by autoradiography, as shown in Figure 10(d).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Arnold, Andrew

(ii) TITLE OF INVENTION: PRAD1 CYCLIN AND ITS CDNA

(iii) NUMBER OF SEQUENCES: 1

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(D) STATE: Massachusetts
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(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 3.30)
(D) SOFTWARE: WordPerfect (Version 5.0)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: 07/667,711
(B) FILING DATE: March 11, 1991

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(2) INFORMATION FOR SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4244
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION : 1:

GGCGCAGTAG CAGCGAGCAG CAGAGTCCGC ACGCTCCGGC GAGGGGCAGA AGAGCGCGAG 60
GGAGCGCGGG GCAGCAGAAG CGAGAGCCGA GCGCGGACCC AGCCAGGACC CACAGCCCTC 120
CCCAGCTGCC CAGGAAGAGC CCCAGCC ATG GAA CAC CAG CTC CTG TGC TGC GAA 174
Met Glu His Gln Leu Leu Cys Cys Glu
1 5
GTG GAA ACC ATC CGC CGC GCG TAC CCC GAT GCC AAC CTC CTC AAC GAC 222
Val Glu Thr Ile Arg Arg Ala Tyr Pro Asp Ala Asn Leu Leu Asn Asp
10 15 20 25
CGG GTG CTG CGG GCC ATG CTG AAG GCG GAG GAG ACC TGC GCG CCC TCG 270
Arg Val Leu Arg Ala Met Leu Lys Ala Glu Glu Thr Cys Ala Pro Ser
30 35 40
GTG TCC TAC TTC AAA TGT GTG CAG AAG GAG GTC CTG CCG TCC ATG CGG 318
Val Ser Tyr Phe Lys Cys Val Gln Lys Glu Val Leu Pro Ser Met Arg
45 50 55
AAG ATC GTC GCC ACC TGG ATG CTG GAG GTC TGC GAG GAA CAG AAG TGC 366
Lys Ile Val Ala Thr Trp Met Leu Glu Val Cys Glu Glu Gln Lys Cys
60 65 70
GAG GAG GAG GTC TTC CCG CTG GCC ATG AAC TAC CTG GAC CGC TTC CTG 414
Glu Glu Glu Val Phe Pro Leu Ala Met Asn Tyr Leu Asp Arg Phe Leu
75 80 85
TCG CTG GAG CCC GTG AAA AAG AGC CGC CTG CAG CTG CTG GGG GCC ACT 462
Ser Leu Glu Pro Val Lys Lys Ser Arg Leu Gln Leu Leu Gly Ala Thr
90 95 100 105
TGC ATG TTC GTG GCC TCT AAG ATG AAG GAG ACC ATC CCC CTG ACG GCC 510
Cys Met Phe Val Ala Ser Lys Met Lys Glu Thr Ile Pro Leu Thr Ala
110 115 120
GAG AAG CTG TGC ATC TAC ACC GAC AAC TCC ATC CGG CCC GAG GAG CTG 558
Glu Lys Leu Cys Ile Tyr Thr Asp Asn Ser Ile Arg Pro Glu Glu Leu
125 130 135

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CTG CAA ATG GAG CTG CTC CTG GTG AAC AAG CTC AAG TGG AAC CTG GCC Leu Gln Met Glu Leu Leu Leu Val Asn Lys Leu Lys Trp Asn Leu Ala 140 145 150	606
GCA ATG ACC CCG CAC GAT TTC ATT GAA CAC TTC CTC TCC AAA ATGACCA Ala Met Thr Pro His Asp Phe Ile Glu His Phe Leu Ser Lys Met Pro 155 160 165	654
GAG GCG GAG GAG AAC AAA CAG ATC ATC CGC AAA CAC GCG CAG ACC TTC Glu Ala Glu Glu Asn Lys Gln Ile Ile Arg Lys His Ala Gln Thr Phe 170 175 180 185	702
GTT GCC CTC TGT GCC ACA GAT GTG AAG TTC ATT TCC AAT CCG CCC TCC Val Ala Leu Cys Ala Thr Asp Val Lys Phe Ile Ser Asn Pro Pro Ser 190 195 200	750
ATG GTG GCA GCG GGG AGC GTG GTG GCC GCA GTG CAA GGC CTG AAC CTG Met Val Ala Ala Gly Ser Val Val Ala Ala Val Gln Gly Leu Asn Leu 205 210 215	798
AGG AGC CCC AAC AAC TTC CTG TCC TAC TAC CGC CTC ACA CGC TTC CTC Arg Ser Pro Asn Asn Phe Leu Ser Tyr Tyr Arg Leu Thr Arg Phe Leu 220 225 230	846
TCC AGA GTG ATC AAG TGT GAC CCA GAC TGC CTC CGG GCC TGC CAG GAG Ser Arg Val Ile Lys Cys Asp Pro Asp Cys Leu Arg Ala Cys Gln Glu 235 240 245	894
CAG ATC GAA GCC CTG CTG GAG TCA AGC CTG CGC CAG GCC CAG CAG AAC Gln Ile Glu Ala Leu Leu Glu Ser Ser Leu Arg Gln Ala Gln Gln Asn 250 255 260 265	942
ATG GAC CCC AAG GCC GCC GAG GAG GAG GAA GAG GAG GAG GAG GAG GTG Met Asp Pro Lys Ala Ala Glu Glu Glu Glu Glu Glu Glu Glu Val 270 275 280	990
GAC CTG GCT TGC ACA CCC ACC GAC GTG CGG GAC GTG GAC ATC TGA Asp Leu Ala Cys Thr Pro Thr Asp Val Arg Asp Val Asp Ile 285 290 295	1035
GGGCGCCAGG CAGGCGGGCG CCACCGCCAC CCGCAGCGAG GGCGGAGCCG GCCCCAGGTG	1095
CTCCACTGAC AGTCCCTCCT CTCCGGAGCA TTTTGATACC AGAAGGGAAA GCTTCATTCT	1155
CCTTGTTGTT GGTTGTTTTT TCCTTTGCTC TTTCCCCCTT CCATCTCTGA CTTAAGCAAA	1215
AGAAAAAGAT TACCCAAAAA CTGTCTTTAA AAGAGAGAGA GAGAAAAAAA AAATAGTATT	1275
TGCATAACCC TGAGCGGTGG GGGAGGAGGG TTGTGCTACA GATGATAGAG GATTTTATAC	1335
CCCAATAATC AACTCGTTTT TATATTAATG TACTTGTTTC TCTGTTGTAA GAATAGGCAT	1395
TAACACAAAG GAGGCGTCTC GGGAGAGGAT TAGGTTCCAT CCTTTACGTG TTAAAAAAA	1455

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AGCATAAAAA CATTTTAAAA ACATAGAAAA ATTCAGCAAA CCATTTTTAA AGTAGAAGAG	1515
GGTTTTAGGT AGAAAAACAT ATTCTGTGC TTTTCCTGAT AAAGCACAGC TGTAGTGGGG	1575
TTCTAGGCAT CTCTGTACTT TGCTTGCTCA TATGCATGTA GTCACTTTAT AAGTCATTGT	1635
ATGTTATTAT ATTCCGTAGG TAGATGTGTA ACCTCTTCAC CTTATTCATG GCTGAAGTCA	1695
CCTCTTGGTT ACAGTAGCGT AGCGTGGCCG TGTGCATGTC CTTTGCGCCT GTGACCACCA	1755
CCCCAACAAA CCATCCAGTG ACAAACCATC CAGTGGAGGT TTGTCGGGCA CCAGCCAGCG	1815
TAGCAGGGTC GGGAAAGGCC ACCTGTCCCA CTCCTACGAT ACGCTACTAT AAAGAGAAGA	1875
CGAAATAGTG ACATAATATA TTCTATTTTT ATACTCTTCC TATTTTGTGA GTGACCTGTT	1935
TATGAGATGC TGGTTTTCTA CCCAACGGCC CTGCAGCCAG CTCACGTCCA GGTTCACCC	1995
ACAGCTACTT GGTTTGTGTT CTTCTTCATA TTCTAAAACC ATTCCATTTC CAAGCACTTT	2055
CAGTCCAATA GGTGTAGGAA ATAGCGCTGT TTTTGTGTG TGTGCAGGGA GGGCAGTTTT	2115
CTAATGGAAT GGTTTGGGAA TATCCATGTA CTGTGTTGCA AGCAGGACTT TGAGGCAAGT	2175
GTGGGCCACT GTGGTGGCAG TGGAGGTGGG GTGTTTGGGA GGCTGCGTGC CAGTCAAGAA	2235
GAAAAAGGTT TGCATTCTCA CATTGCCAGG ATGATAAGTT CCTTTCCTTT TCTTTAAAGA	2295
AGTTGAAGTT TAGGAATCCT TTGGTGCCAA CTGGTGTTTG AAAGTAGGGA CCTCAGAGGT	2355
TTACCTAGAG AACAGGTGGT TTTTAAGGGT TATCTTAGAT GTTTCACACC GGAAGGTTTT	2415
TAAACACTAA AATATATAAT TTATAGTTAA GGCTAAAAAG TATATTTATT GCAGAGGATG	2475
TTCATAAGGC CAGTATGATT TATAAATGCA ATCTCCCCTT GATTTAAACA CACAGATACA	2535
CACACACACA CACACACACA CACAAACCTT CTGCCTTTGA TGTTACAGAT TTAATACAGT	2595
TTATTTTTAA AGATAGATCC TTTTATAGGT GAGAAAAAA CAATCTGGAA GAAAAAACC	2655
ACACAAAGAC ATTGATTCAG CCTGTTTGGC GTTTCACAGA GTCATCTGAT TGGACAGGCA	2715
TGGGTGCAAG GAAATTAGG GTACTCAACC TAAGTTCGGT TCCGATGAAT TCTTATCCCC	2775
TGCCCCCTTC TTTAAAAAAC TTAGTGACAA AATAGACAAT TTGCACATCT TGGCTATGTA	2835
ATTCTTGTA TTTTATTTA GGAAGTGTTG AAGGGAGGTG GCAAGAGTGT GGAGGCTGAC	2895
GTGTGAGGGA GGACAGGCGG GAGGAGGTGT GAGGAGGAGG CTCCCGAGGG GAAGGGGCGG	2955
TGCCACACC GGGGACAGGC CGCAGCTCCA TTTCTTATT GCGCTGCTAC CGTTGACTTC	3015

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CAGGCACGGT TTGGAAATAT TCACATCGCT TCTGTGTATC TCTTTCACAT TGTTTGCTGC	3075
TATTGGAGGA TCAGTTTTTT GTTTTACAAT GTCATATACT GCCATGTACT AGTTTTAGTT	3135
TTCTCTTAGA ACATTGTATT ACAGATGCCT TTTTGTAGT TTTTTTTTTT TTTATGTGAT	3195
CAATTTTGAC TTAATGTGAT TACTGCTCTA TTCCAAAAG GTTGCTGTTT CACAATACCT	3255
CATGCTTCAC TTAGCCATGG TGGACCCAGC GGGCAGGTTT TGCCTGCTTT GGCGGGCAGA	3315
CACGCGGGCG CGATCCCACA CAGGCTGGCG GGGGCCGGCC CCGAGGCCGC GTGCGTGAGA	3375
ACCGCGCCGG TGTCCCCAGA GACCAGGCTG TGTCCCTCTT CTCTTCCCTG CGCCTGTGAT	3435
GCTGGGCACT TCATCTGATC GGGGGCGTAG CATCATAGTA GTTTTACAG CTGTGTTATW	3495
CTTTGCGTGT AGCTATGGAA GTTGATAAT TATTATTATT ATTATTATAA CAAGTGTGTC	3555
TTACGTGCCA CCACGGCGTT GTACCTGTAG GACTCTCATT CGGGATGATT GGAATAGCTT	3615
CTGGAATTTG TTCAAGTTTT GGGTATGTTT AATCTGTTAT GTACTAGTGT TCTGTTTGTT	3675
ATTGTTTTGT TAATTACACC ATAATGCTAA TTTAAAGAGA CTCCAAATCT CAATGAAGCC	3735
AGCTCACAGT GCTGTGTGCC CCGGTCACCT AGCAAGCTGC CGAACCAGAA GAATTTGCAC	3795
CCCGCTGCGG GCCCAGGTGG TTGGGGCCCT GCCCTGGCAG GGTCATCCTG TGCTCGGAGG	3855
CCATCTCGGG CACAGGCCCA CCCCGCCCCA CCCCTCCAGA ACACGGCTCA CGCTTACCTC	3915
AACCATCCTG GCTGCGGCGT CTGTCTGAAC CACGCGGGGG CCTTGAGGGA CGCTTTGTCT	3975
GTCGTGATGG GGCAAGGGCA CAAGTCCTGG ATGTTGTGTG TRTCGAGAGG CCAAAGGCTG	4035
GTGGCAAGTG CACGGGGCAC AGCGGAGTCT GTCCTGTGAC GCGCAAGTCT GAGGGTCTGG	4095
GCGGCGGGCG GCTGGGTCTG TGCATTTCTG GTTGACCCGC GCGCTTCCC AGCACCAACA	4155
TGTAACCGGC ATGTTTCCAG CAGAAGACAA AAAGACAAAC ATGAAAGTCT AGAAATAAAA	4215
CTGGTAAAC CCCAAAAAA AAAAAAAA	4244

Claims:

1. An isolated DNA molecule comprising a sequence encoding prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, wherein said DNA molecule hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.
2. The DNA molecule of claim 1, wherein said prad1 (cyclin D1) has the same amino acid sequence as the polypeptide encoded by SEQ ID NO:1.
3. The DNA molecule of claim 1, wherein said molecule hybridizes to the complement of the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.
4. A vector comprising an isolated DNA molecule comprising a sequence encoding prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, wherein said DNA molecule hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.
5. The vector of claim 4, wherein said DNA sequence encoding prad1 (cyclin D1) is under the transcriptional control of a heterologous promoter.
6. A cultured cell comprising an isolated DNA molecule comprising a sequence encoding prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, wherein said DNA molecule hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.
7. The cell of claim 6, wherein said isolated DNA molecule is integrated into the genome of said cell.

8. The cell of claim 6, wherein said cell is a eukaryotic cell.
9. The cell of claim 6, wherein said cell is capable of expressing prad1 (cyclin D1) from said isolated DNA molecule.
10. The cell of claim 6, wherein said cell expresses said prad1 (cyclin D1) from said isolated DNA molecule.
11. A method for making prad1 (cyclin D1), comprising culturing the cell of claim 6 in a medium under conditions suitable for expression of prad1 (cyclin D1).
12. The method of claim 11, further comprising recovering prad1 (cyclin D1) from said cell or said medium.
13. The method of claim 12, wherein said prad1 (cyclin D1) has the same amino acid sequence as the polypeptide encoded by SEQ ID NO:1.
14. The cell of claim 6, wherein said prad1 (cyclin D1) has the same amino acid sequence as the polypeptide encoded by SEQ ID NO:1.
15. Prad1 (cyclin D1) produced by expression of an isolated DNA molecule encoding prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, wherein said DNA molecule hybridizes under stringent conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.
16. Prad1 (cyclin D1) synthesized by the cell of claim 6.
17. A purified prad1 (cyclin D1) of claim 15 or 16.
18. A method for making prad1 (cyclin D1), which method comprises

growing the cell of claim 6 in a medium to form a population of cells which express said prad1 (cyclin D1), and

recovering said prad1 (cyclin D1) from said population of cells or said medium.

19. A method for making prad1 (cyclin D1), which method comprises
combining the isolated DNA of claim 1 with an expression system to produce and
recovering said prad1 (cyclin D1); and
recovering said prad1 (cyclin D1) from said expression system.

20. Prad1 (cyclin D1) for the use in promoting wound healing in an animal, wherein said prad1 (cyclin D1), a cyclin which induces kinase activity clam embryo interphase cell lysate, is encoded by an isolated DNA molecule that hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) of SEQ ID NO:1.

21. The use of a proliferation-inducing amount of prad1 (cyclin D1) to promote proliferation of an animal cell, wherein said prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, is encoded by an isolated DNA molecule that hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.

22. An antibody that specifically binds prad1 (cyclin D1) and which does not cross-react with the *Xenopus laevis* cyclin A1, wherein said prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, is encoded by an isolated DNA molecule that hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.

23. The antibody of claim 22, wherein said prad1 (cyclin D1) is a human prad1 (cyclin D1).

24. A purified antibody of claim 23.
25. The purified antibody of claim 24, wherein said prad1 (cyclin D1) is a human prad1 (cyclin D1).
26. An isolated RNA molecule comprising a sequence encoding prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, wherein said RNA molecule hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of at least 40 nucleotides of the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.
27. The RNA molecule of claim 26, wherein said sequence is 40-3000 nucleotides in length.
28. The RNA molecule of claim 26, wherein said prad1 (cyclin D1) is a human prad1 (cyclin D1).
29. An RNA molecule transcribed from the isolated DNA of claim 1.
30. A nucleic acid probe comprising an RNA molecule of any of claims 26 to 28.
31. A nucleic acid primer comprising an RNA molecule of any of claims 26 to 28.
32. An isolated DNA molecule comprising a sequence encoding prad1 (cyclin D1), wherein said sequence encoding prad1 (cyclin D1) consists of the sequence of SEQ ID NO: 1.
33. Prad1 (cyclin D1) for use in the manufacture of a medicament for promoting wound healing in an animal, wherein said prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, is encoded by an isolated DNA molecule that hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.

34. PRAD1 (CYCLIN D1) DNA for use in preparing prad1 (cyclin D1), wherein said prad1 (cyclin D1), a cyclin which induces kinase activity in a clam embryo interphase cell lysate, is produced by expression of an isolated DNA molecule encoding prad1 (cyclin D1), and wherein said DNA molecule hybridizes under stringent hybridization conditions to a single-stranded probe consisting of a segment of 40 nucleotides complementary to the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.

35. The PRAD1 (CYCLIN D1) of claim 34, wherein said DNA molecule hybridizes to the complement of the coding region of the human PRAD1 (CYCLIN D1) cDNA of SEQ ID NO:1.

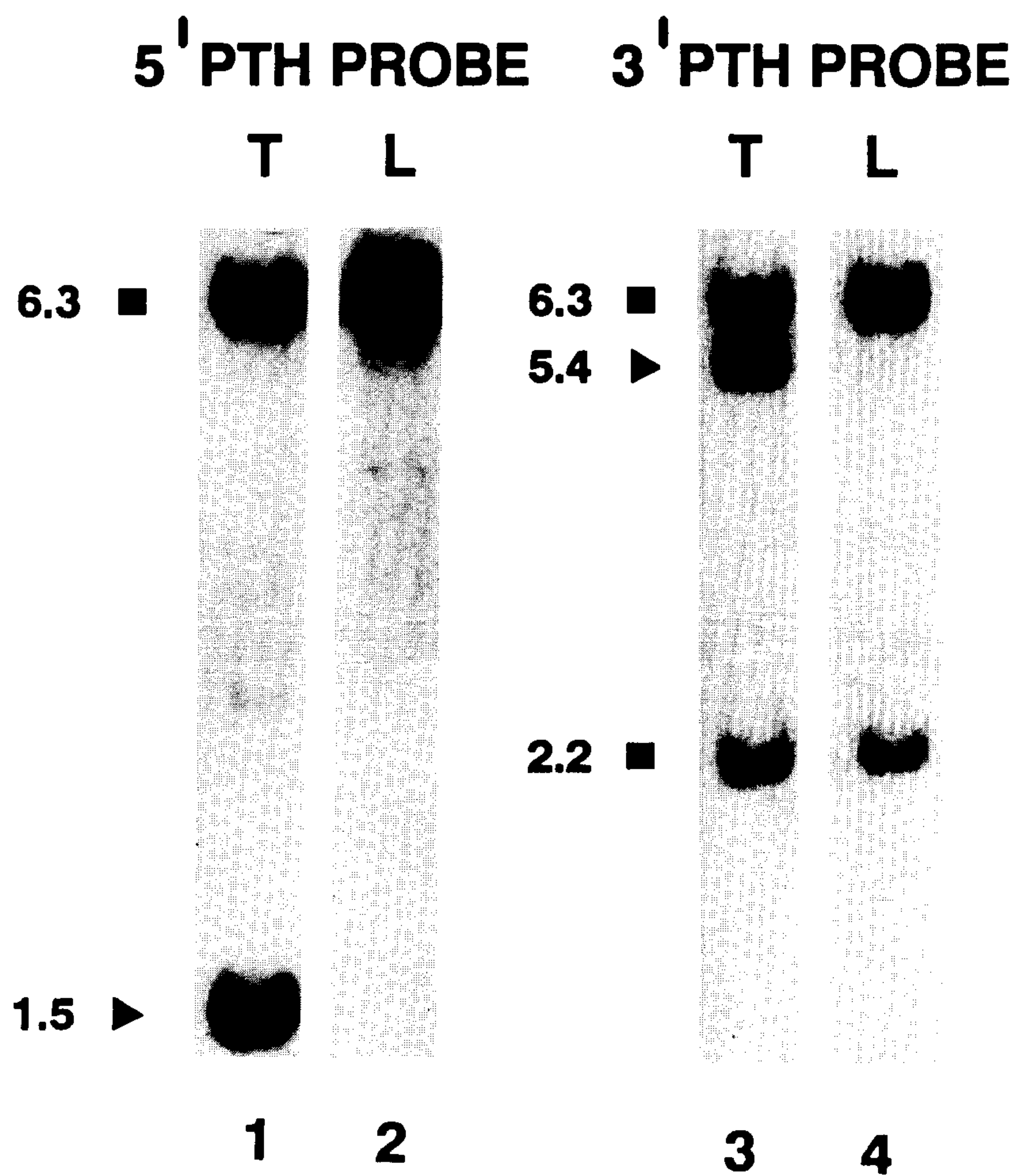


FIG. 1

NORMAL PTH ALLELE

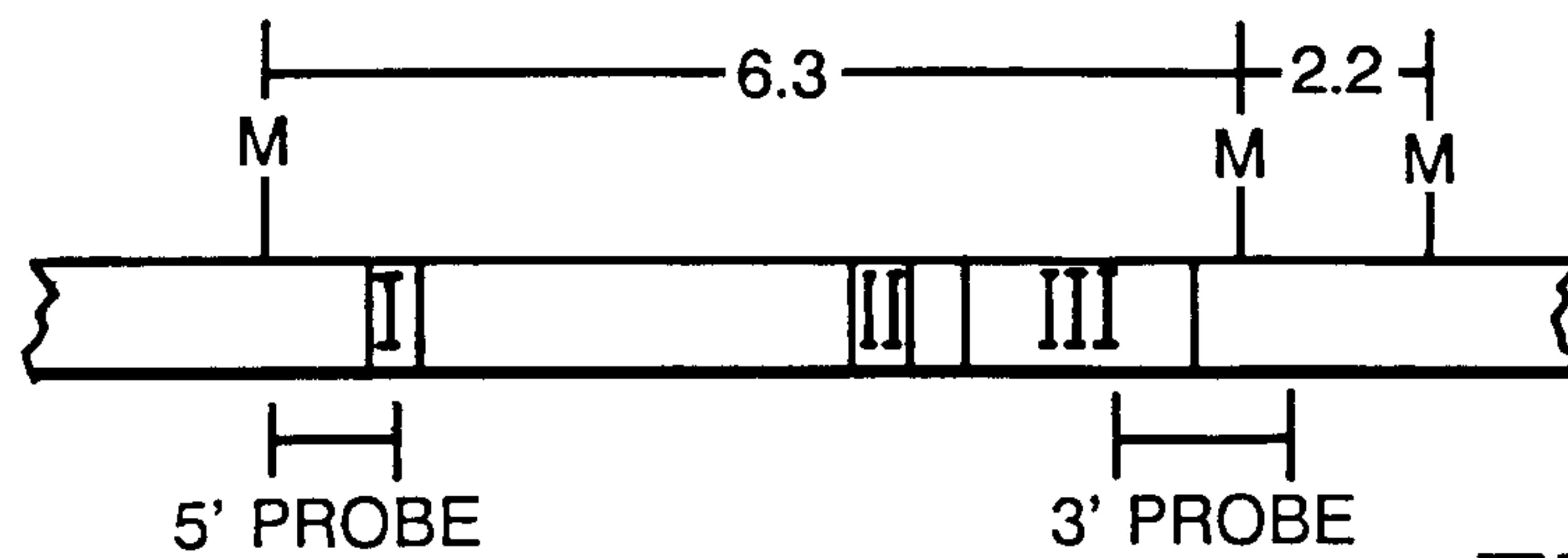


FIG. 2A

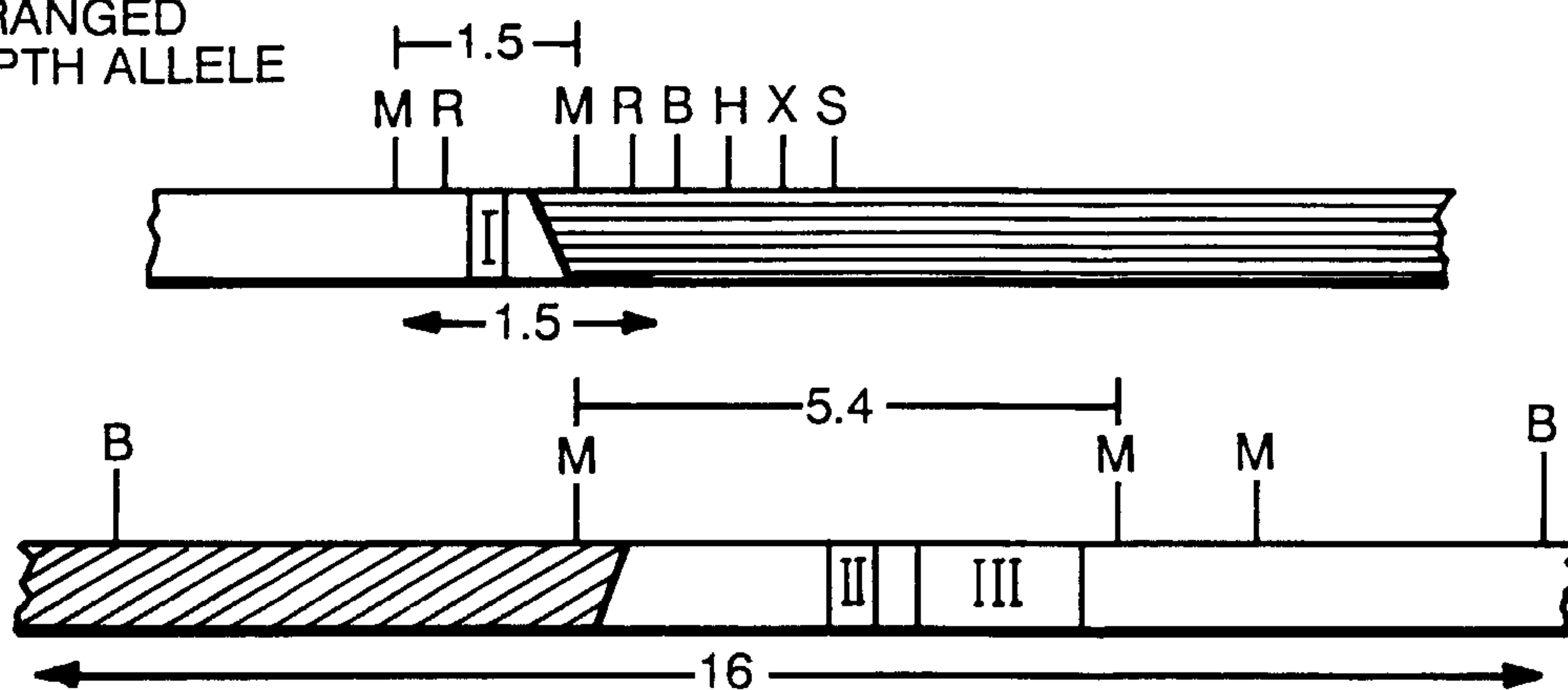
REARRANGED
PTH ALLELE

FIG. 2B

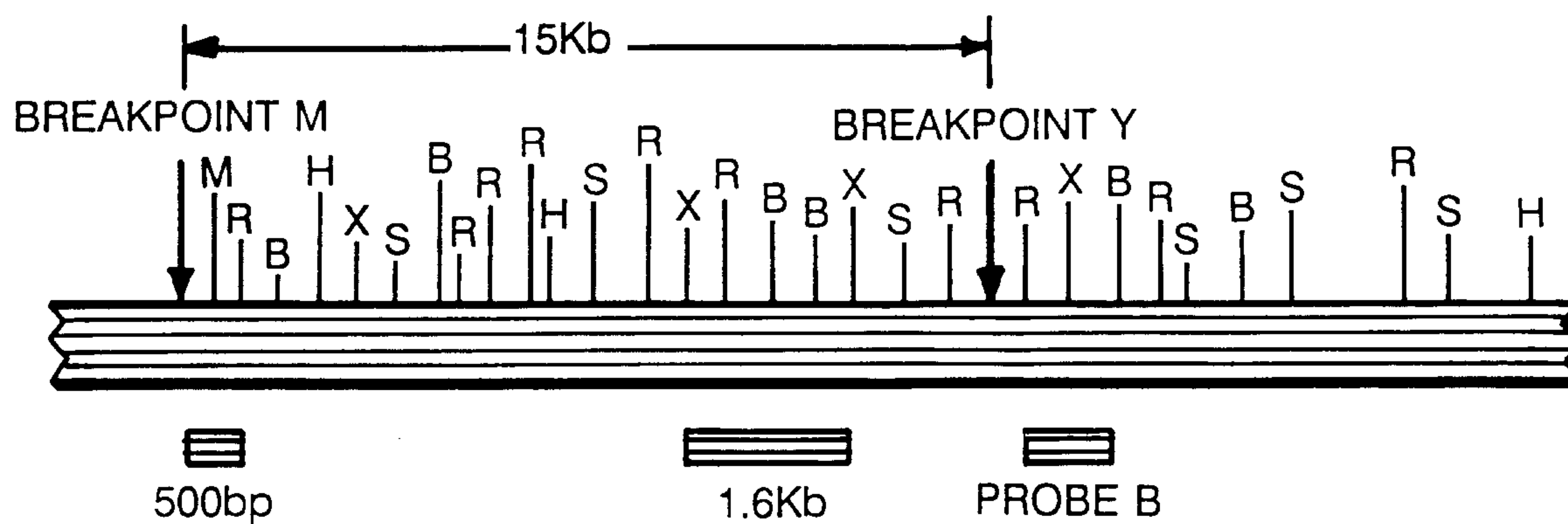


FIG. 3

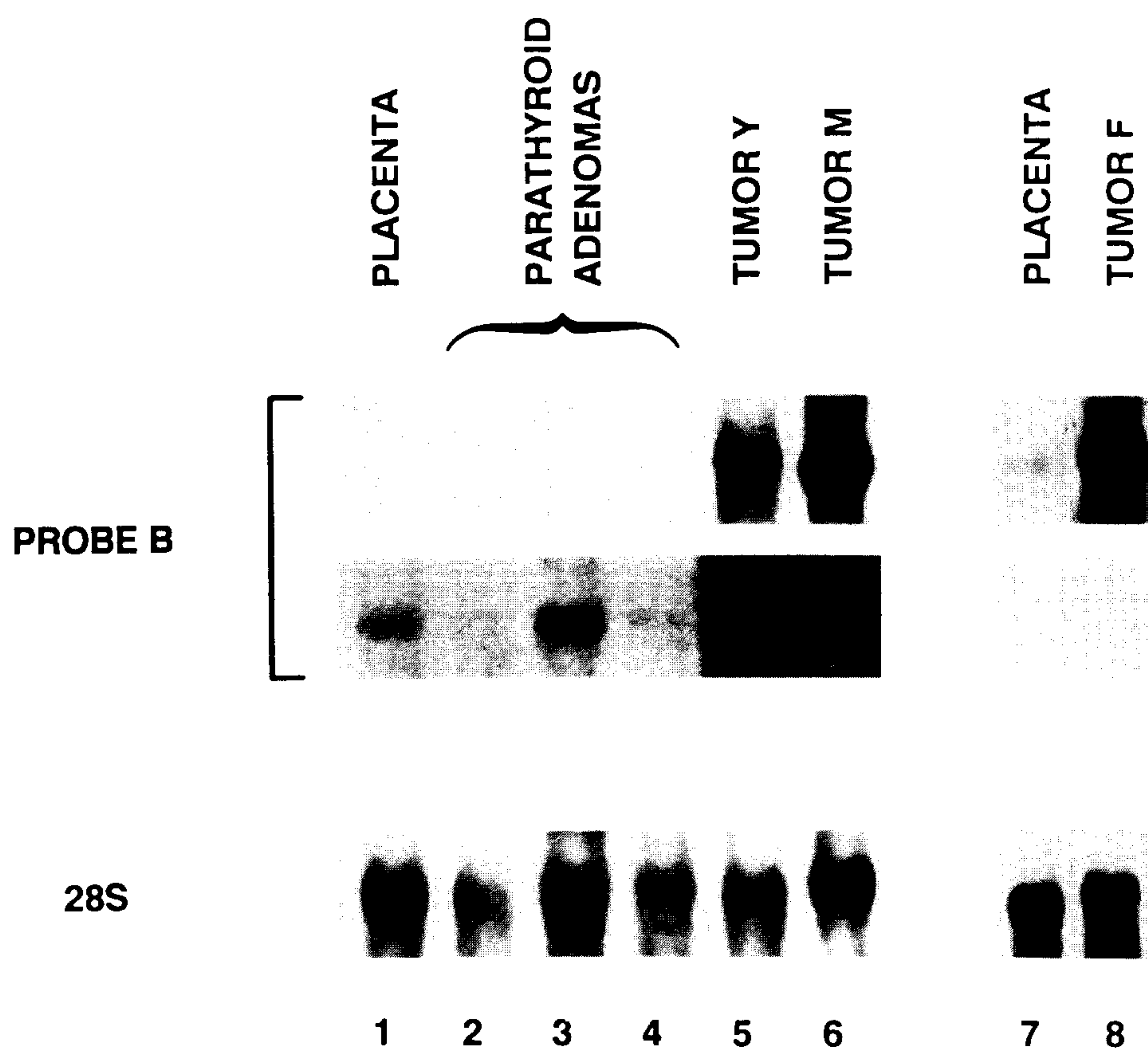


FIG. 4

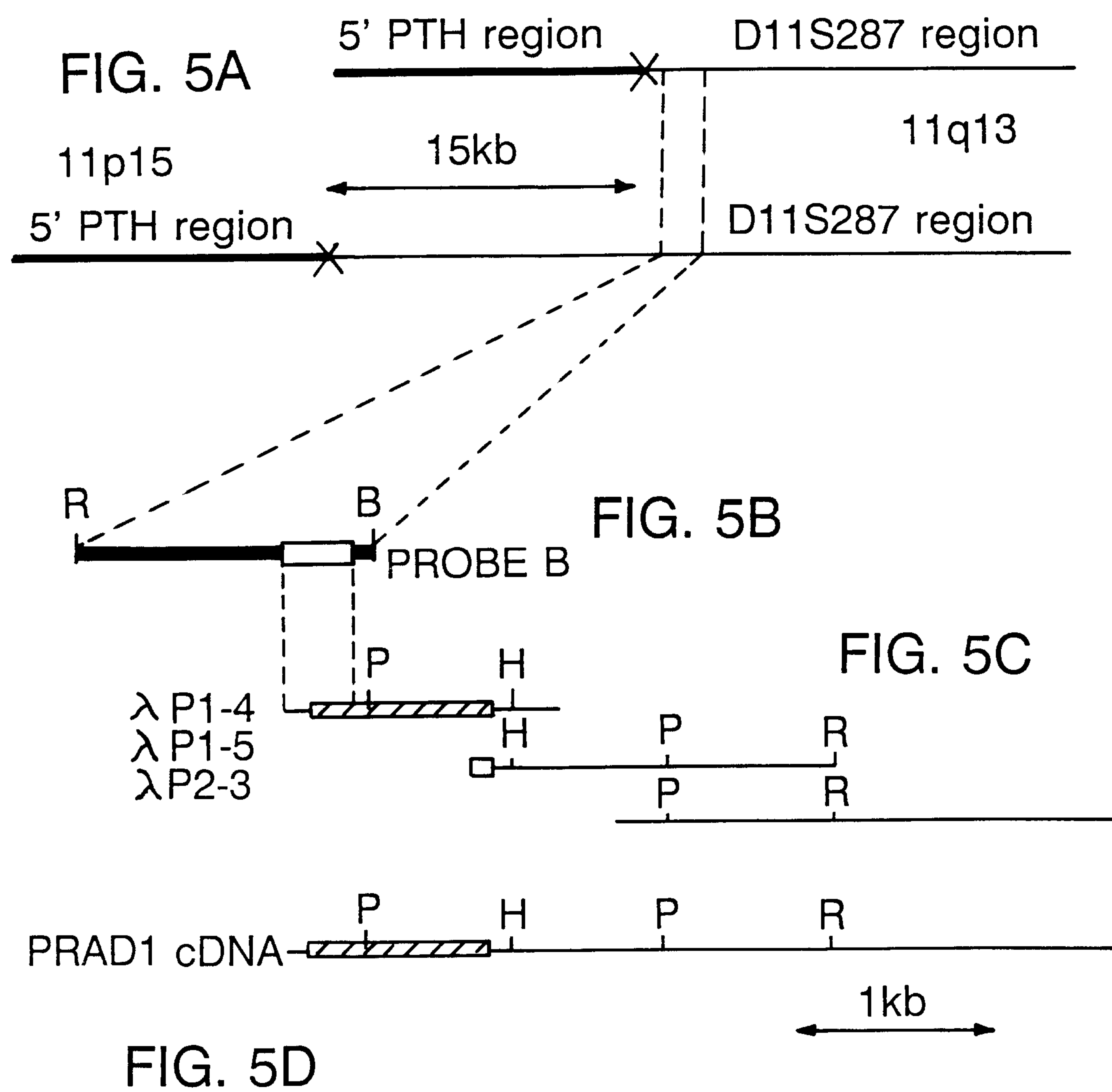


FIG.6-1

GGCGCAGTAG	CAGCGAGCAG	CAGAGTCCGC	ACGCTCCGC	GAGGGGCAGA	AGAGCGCGAG	60
GGAGCGCGGG	GCAGCAGAAG	CGAGAGCCGA	GCGCGGACCC	AGCCAGGACC	CACAGCCCTC	120
CCCAGCTGCC	CAGGAAGAGC	CCCAGCC	ATG GAA	CAC CAG	CTC CTG TGC GAA	174
			Met	Glu	His Gln Leu Leu Cys Cys Glu	
GTG GAA	ACC ATC	CGC CGC	GCG TAC	CCC GAT	GCC AAC CTC CTC AAC GAC	222
Val Glu Thr Ile	Arg Arg	Ala Arg	Tyr Pro	Asp Ala	Asn Leu Leu Asn Asp	
10	15	20	25	30	35	
CGG GTG	CTG CGG	GCC ATG	CTG AAG	GCG GAG	GAG ACC TGC GCG CCC TCG	270
Arg Val Leu Arg	Ala Met	Leu Leu	Lys Ala	Glu Glu	Thr Cys Ala Pro Ser	
	30	35	40	45	50	
GTG TCC	TAC TTC	AAA TGT	GTG CAG	AAG GAG	GTC CTG CCG TCC ATG CGG	318
Val Ser Tyr Phe	Lys Cys	Val Gln	Lys Glu	Val Leu	Pro Ser Met Arg	
	45	50	55	60	65	
AAG ATC	GTC GCC	ACC TGG	ATG CTG	GAG GTC	TGC GAG GAA CAG AAG TGC	366
Lys Ile Val Ala	Thr Thr	Trp Met	Leu Glu	Val Cys	Glu Glu Gln Lys Cys	
	60	65	70	75	80	
GAG GAG	GAG GTC	TTC CCG	CTG GCC	ATG AAC	TAC CTG GAC CGC TTC CTG	414
Glu Glu Glu Val	Phe Phe	Pro Pro	Leu Ala	Met Asn	Tyr Leu Asp Arg Phe Leu	
	75	80	85	90	95	
TCG CTG	GAG CCC	GTG AAA	AAG AGC	CGC CTG	CAG CTG GGG GCC ACT	462
Ser Leu Glu Pro	Val Val	Lys Lys	Ser Arg	Leu Leu	Gln Leu Gly Ala Thr	
	90	95	100	105	110	
TGC ATG	TTC GTG	GCC TCT	AAG ATG	AAG GAG	ACC ATC CCC CTG ACG GCC	510
Cys Met Phe Val	Ala Val	Ser Ser	Lys Met	Lys Glu	Ile Pro Leu Thr Ala	
	110	115	120	125	130	

FIG.6-2

GAG AAG CTG TGC ATC TAC ACC GAC AAC TCC ATC CGG CCC GAG GAG CTG	558
Glu Lys Leu Cys Ile Tyr Thr Asp Asn Ser Ile Arg Pro Glu Glu Leu	
125 130 135	
CTG CAA ATG GAG CTG CTG CTG CTG AAG AAG CTC AAG TGG AAC CTG GCC	606
Leu Gln Met Glu Leu Leu Val 145	
140 150	
GCA ATG ACC CCG CAC GAT TTC ATT GAA CAC TTC CTC TCC AAA ATGáCCA	654
Ala Met Thr Pro His Asp Phe Ile Glu His Phe Leu Ser Lys Met Pro	
155 160 165	
GAG GCG GAG GAG AAC AAA CAG ATC ATC CGC AAA CAC GCG CAG ACC TTC	702
Glu Ala Glu Glu Asn Lys Gln Ile Arg Lys His Ala Gln Thr Phe	
170 175 180 185	
GTT GCC CTC TGT GCC ACA GAT GTG AAG TTC ATT TCC AAT CCG CCC TCC	750
Val Ala Leu Cys Ala Thr Asp Val Lys Phe Ile Ser Asn Pro Pro Ser	
190 195 200	
ATG GTG GCA GCG GCG AGC GTG GTG GCG CAA GTG CAA GGC CTG AAC CTG	798
Met Val Ala Ala Gly Ser Val Val Ala Ala Val Gln Gly Leu Asn Leu	
205 210 215	
AGG AGC CCC AAC AAC TTC CTG TCC TAC CGC CTC ACA CGC TTC CTC	846
Arg Ser Pro Asn Asn Phe Leu Ser Tyr Arg Leu Thr Arg Phe Leu	
220 225 230	
TCC AGA GTG ATC AAG TGT GAC CCA GAC TGC CTC CGG GCC TGC CAG GAG	894
Ser Arg Val Ile Lys Cys Asp Pro Asp Cys Leu Arg Ala Cys Gln Glu	
235 240 245	

FIG.6-3

CAG ATC GAA GCC CTG CTG GAG TCA AGC CTG CGC CAG GCC CAG CAG AAC 942
 Gln Ile Glu Ala Leu Leu Leu Ser Ser Leu Arg Gln Ala Gln Gln Asn 265
 250
 ATG GAC CCC AAG GCC GCG GAG GAG GAA GAG GAG GAG GAG GTG 990
 Met Asp Pro Lys Ala Ala Glu Glu Glu Glu Glu Glu Glu Val 280
 270
 GAC CTG GCT TGC ACA CCC ACC GAC GTG CGG GAC GTG GAC ATC TGA 1035
 Asp Leu Ala Cys Thr Pro Thr Asp Val Arg Asp Val Asp Ile 295
 285
 GGGCGCCAGG CAGGCGGGCG CCACCGCCAC CCGCAGCGAG GCGGAGCCG GCCCCAGGTG 1095

 CTCCACTGAC AGTCCCTCCT CTCCGGAGCA TTTGATACC AGAAGGAAA GCTTCATTCT 1155

 CCTTGTTGTT GGTGTTT TCCCTTGCTC TTCCCCCTT CCATCTCTGA CTTAAGCAAA 1215

 AGAAAAAGAT TACCCAAAAA CTGCTTTTAA AAGAGAGAGA GAGAAAAAA AAATAGTATT 1275

 TGCATAACCC TGAGCGGTGG GGGAGGAGGG TTGTGCTACA GATGATAGAG GATTTATAC 1335

 CCCAATAATC AACTCGTTT TATATTAAATG TACTTGTTTC TCTGTTGTAA GAATAGGCAT 1395

 TAACACAAAG GAGGCGTCTC GGGAGAGGAT TAGGTTCCAT CCTTTACGTG TTTAAAAAAA 1455

 AGCATAAAAA CATTTTAAAA ACATAGAAAA ATTCAGCAA CCATTTTAA AGTAGAAGAG 1515

 GGTTTtaggt AGAAAAACAT ATTCTTGTC TTTTCTGAT AAAGCACAGC TGtagtggg 1575

 TTCTAGGCAT CTCTGTACTT TGCTTGCTCA TAGCATGTA GTCACCTTAT AAGTCATTGT 1635

 ATGTTATTAT ATTCCGTAGG TAGATGTGTA ACCTCTTAC CTTATTCATG GCTGAAGTCA 1695

 CCTCTTGgTT ACAGTAGCGT AGCGTGGCCG TGTGCATGTC CTTTGGCCT GTGACCACCA 1755

CCCCAACAAA	CCATCCAGTG	ACAAACCATC	CAGTGGAGGT	TTGTCGGGCA	CCAGCCAGCG	1815
TAGCAGGGTC	GGGAAAGGCC	ACCTGTCCCA	CTCCTACGAT	ACGCTACTAT	AAAGAGAAGA	1875
CGAAATAGTG	ACATAATATA	TTCTATTTTT	ATACTCTTCC	TATTTTGTGA	GTGACCTGTT	1935
TATGAGATGC	TGGTTTCTA	CCCAACGGCC	CTGCAGCCAG	CTCACGTCCA	GGTCAACCC	1995
ACAGCTACTT	GGTTTGTGTT	CTTCTTCATA	TTCTAAAACC	ATCCATTTC	CAAGCACTTT	2055
CAGTCCAATA	GGTGTAGGAA	ATAGCGCTGT	TTTTGTGTG	TGTGCAGGGA	GGGCAGTTTT	2115
CTAATGGAAT	GGTTTGGGAA	TATCCATGTA	CTTGTTTGCA	AGCAGGACTT	TGAGGCAAGT	2175
GTGGGCCACT	GTGGTGGCAG	TGAGGTGGG	GTGTTTGGGA	GGCTGCGTGC	CAGTCAAGAA	2235
GAAAAAGGTT	TGCATTCTCA	CATTGCCAGG	ATGATAAGTT	CCTTTCCTTT	TCTTTAAAGA	2295
AGTTGAAGTT	TAGGAATCCT	TTGGTGCCAA	CTGGTGTTTG	AAAGTAGGGA	CCTCAGAGGT	2355
TTACCCTAGAG	AACAGGTGGT	TTTTAAGGGT	TATCTTAGAT	GTTTCACACC	GGAAGGTTTT	2415
TAAACACTAA	AATATATAAT	TTATAGTTAA	GGCTAAAAAG	TATATTTATT	GCAGAGGATG	2475
TTCATAAGGC	CAGTATGATT	TATAAATGCA	ATCTCCCCCT	GATTTAAACA	CACAGATACA	2535
CACACACACA	CACACACACA	CACAAACCTT	CTGCCCTTGA	TGTTACAGAT	TTAATACAGT	2595
TTATTTTTAA	AGATAGATCC	TTTTATAGGT	GAGAAAAAAA	CAATCTGGAA	GAAAAAAACC	2655
ACACAAAGAC	ATTGATTCAG	CCTGTTTGGC	GTTTCCCAGA	GTCATCTGAT	TGGACAGGCA	2715
TGGGTGCAAG	GAAAAATTAGG	GTACTCAACC	TAAGTTCGGT	TCCGATGAAT	TCTTATCCCC	2775

FIG.6--4

FIG.6--5

TGCCCCCTTCC	TTTAAAAAAC	TTAGTGACAA	AATAGACAAT	TTGCACATCT	TGGCTATGTA	2835
ATTCTTGTA	TTTTTATTTA	GGAAGTGTTG	AAGGAGGTG	GCAAGAGTGT	GGAGGCTGAC	2895
GTGTGAGGA	GGACAGGCGG	GAGGAGGTGT	GAGGAGGAGG	CTCCCGAGGG	GAAGGGGCGG	2955
TGCCCACACC	GGGGACAGGC	CGCAGCTCCA	TTTTCTTATT	GCGCTGCTAC	CGTTGACTTC	3015
CAGGCACGGT	TTGGAAATAT	TCACATCGCT	TCTGTGTATC	TCTTTCACAT	TGTTTGCTGC	3075
TATTGGAGGA	TCAGTTTTTT	GTTTACAAT	GTCATATACT	GCCATGTACT	AGTTTTAGTT	3135
TTCTCTTAGA	ACATTGTATT	ACAGATGCCT	TTTTTGTAGT	TTTTTTTTTT	TTTATGTGAT	3195
CAATTTTGAC	TTAATGTGAT	TACTGCTCTA	TTCCAAAAG	GTTGCTGTTT	CACAATACCT	3255
CATGCTTCAC	TTAGCCATGG	TGGACCCAGC	GGCAGGTTC	TGCCCTGCTT	GGCGGGCAGA	3315
CACGCGGGCG	CGATCCCACA	CAGGCTGGCG	GGGGCCGGCC	CCGAGGCCGC	GTGCGTGAGA	3375
ACCGCGCCCG	TGTCCCCAGA	GACCAGGCTG	TGTCCCTCTT	CTCTTCCCTG	CGCCTGTGAT	3435
GCTGGGCACT	TCATCTGATC	GGGGCGGTAG	CATCATAGTA	GTTTTTACAG	CTGTGTTATW	3495
CTTTGCGTGT	AGCTATGGAA	GTTGCATAAT	TATTATTATT	ATTATTATAA	CAAGTGTGTC	3555

FIG.6-6

TTACGTGCCA CCACGGCGTT GTACCTGTAG GACTCTCATT CGGGATGATT GGAATAGCTT 3615
CTGGAATTG TTCAAGTTT GGGTATGTT AATCTGTTAT GTAGTAGTGT TCTGTTTGTT 3675
ATTGTTTGT TAATTACACC ATAATGCTAA TTAAAGAGA CTCCAAATCT CAATGAAGCC 3735
AGCTCACAGT GCTGTGTGCC CCGGTCACCT AGCAAGCTGC CGAACCAAAA GAATTGAC 3795
CCCGCTGCGG GCCCACGTGG TTGGGGCCCT GCCCTGGCAG GGTCATCCTG TGCTCGGAGG 3855
CCATCTCGG CACAGGCCCA CCCCGCCCCA CCCCTCCAGA ACACGGCTCA CGCTTACCTC 3915
AACCATCCTG GCTGCGGCGT CTGTCTGAAC CACGCGGGG CCTTGAGGGA CGCTTTGTCT 3975
GTCGTGATGG GGCAAGGGCA CAAGTCCTGG ATGTTGTGTG TRTCGAGAGG CCAAAGGCTG 4035
GTGGCAAGTG CACGGGGCAC AGCGGAGTCT GTCCGTGTGAC GCGCAAGTCT GAGGGTCTGG 4095
GCGGCGGCG GCTGGGTCTG TGCATTCTG GTTGCACCGC GCGCTTCCC AGCACCAACA 4155
TGTAACCGGC ATGTTTCCAG CAGAAGACAA AAAGACAAAC ATGAAAGTCT AGAAATAAAA 4215
CTGGTAAAC CCCAAAAAA AAAAAAAA 4244

FIG. 7

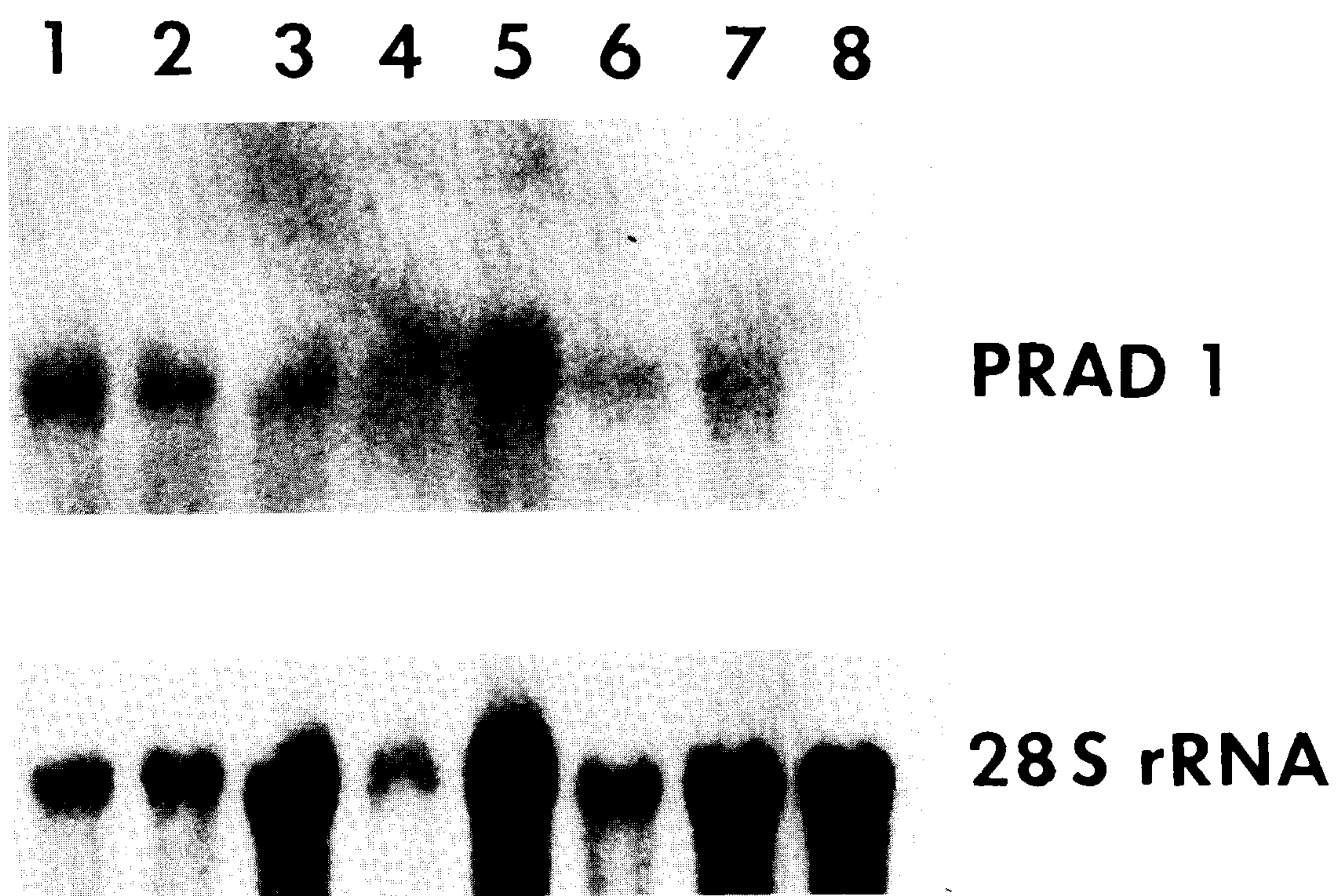


FIG. 8

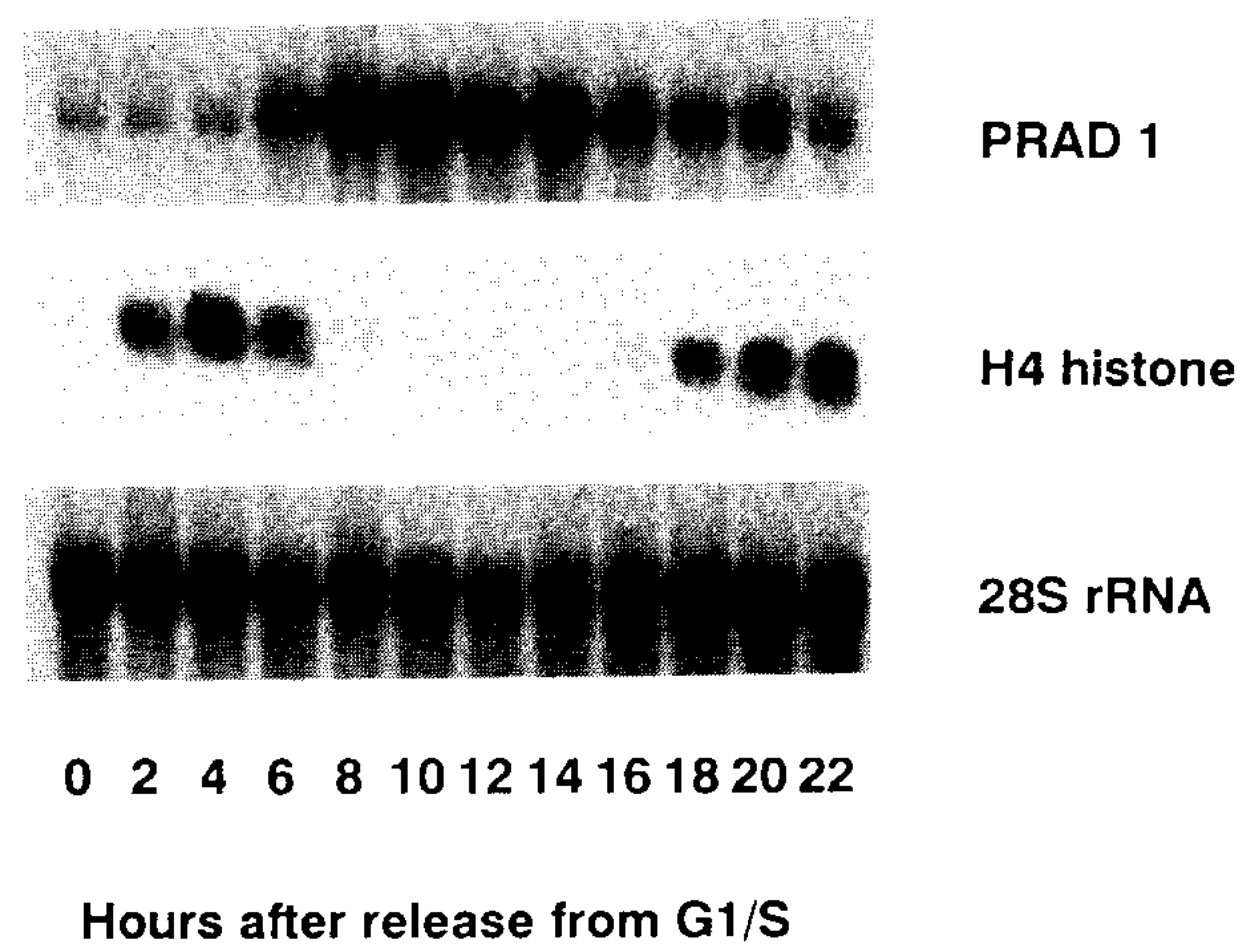
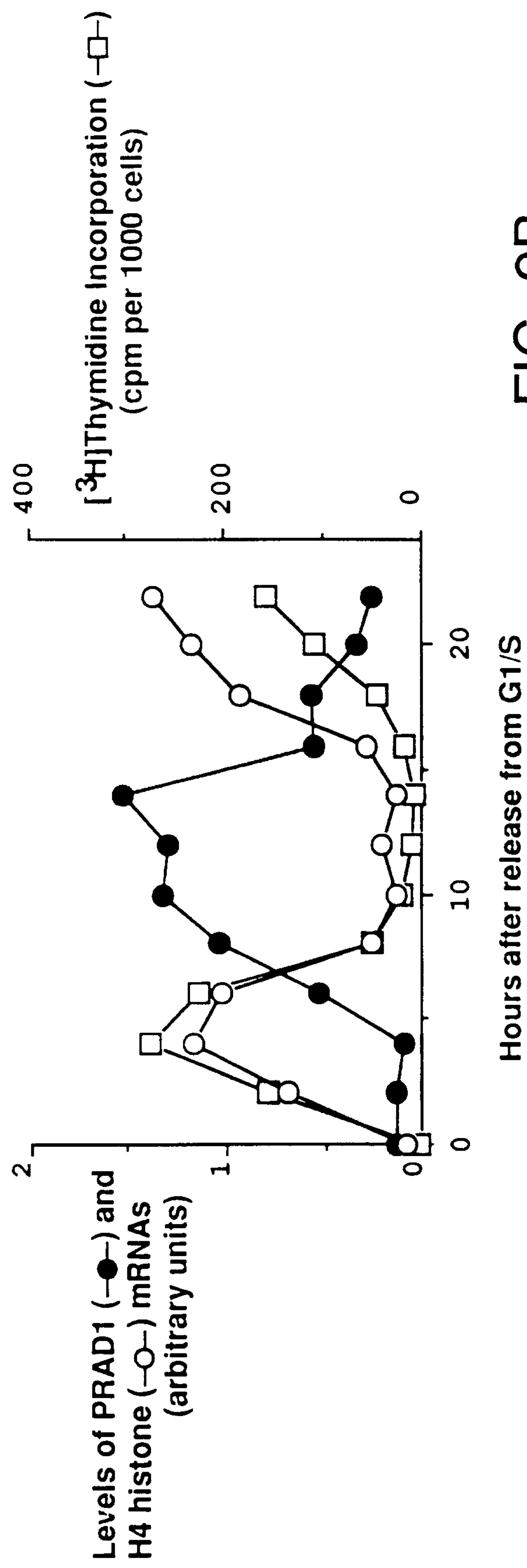


FIG. 9A



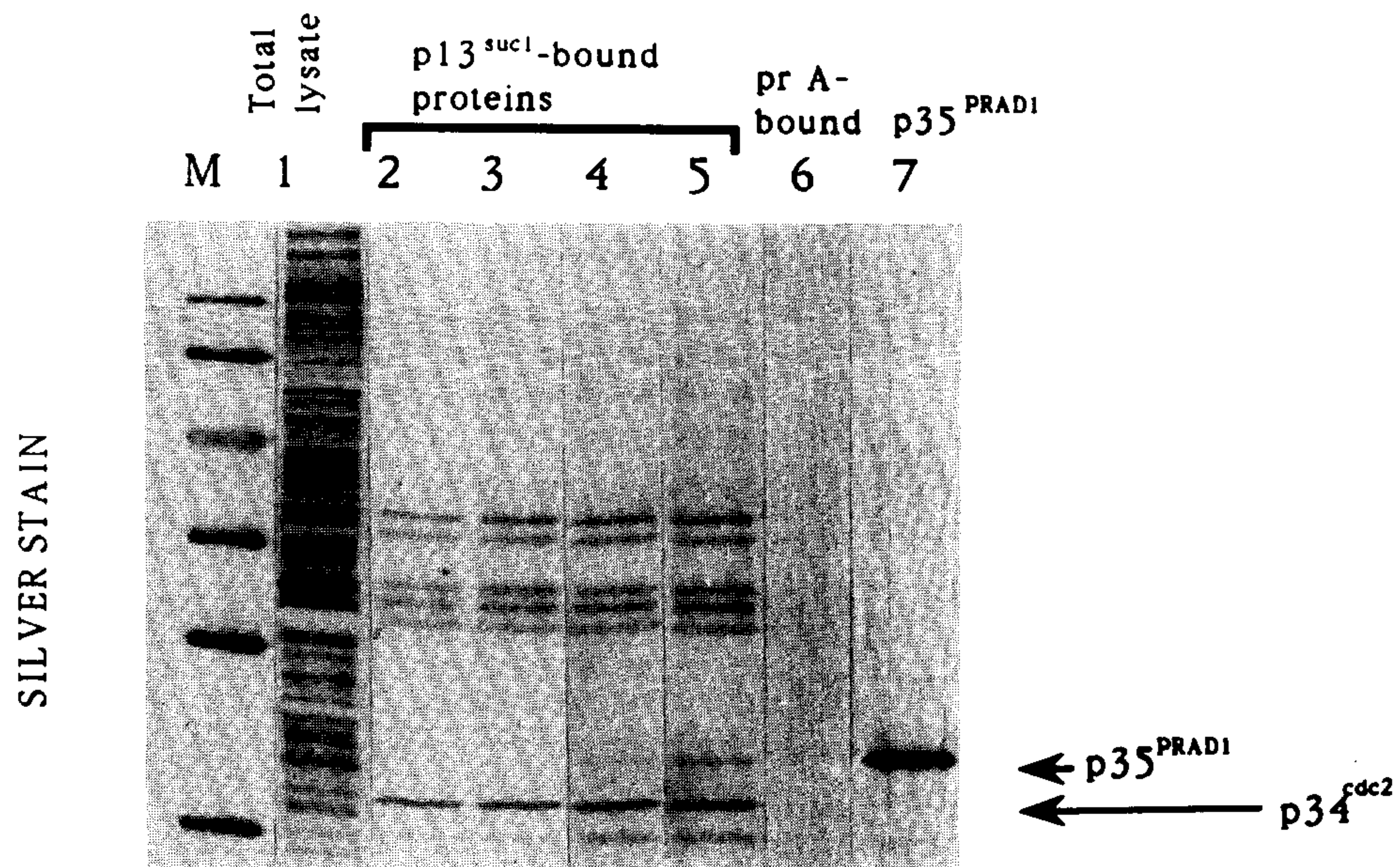


FIG. 10A

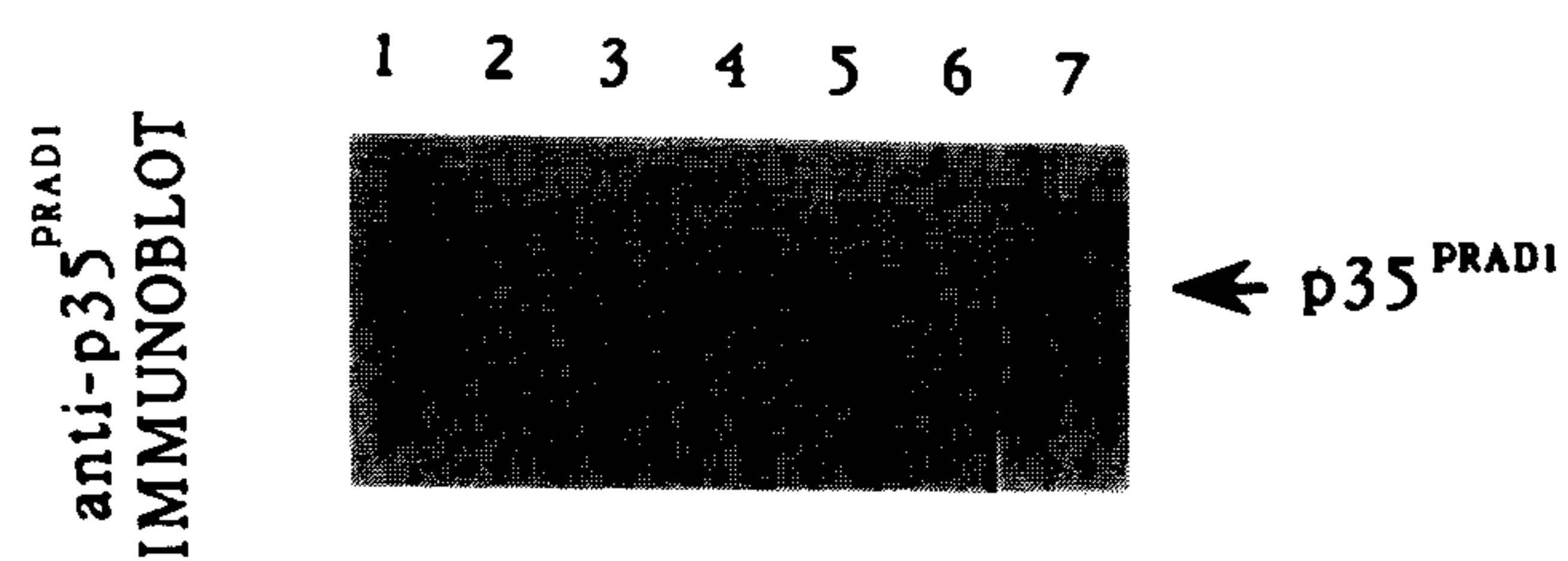


FIG. 10B

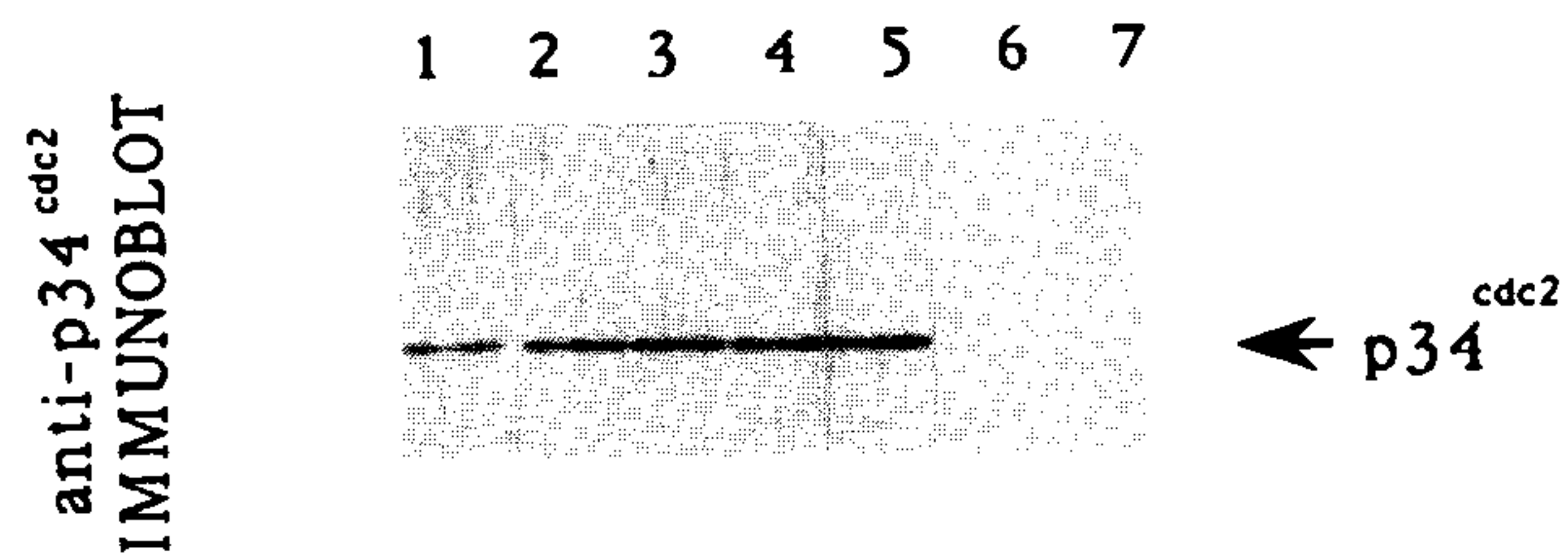


FIG. 10C

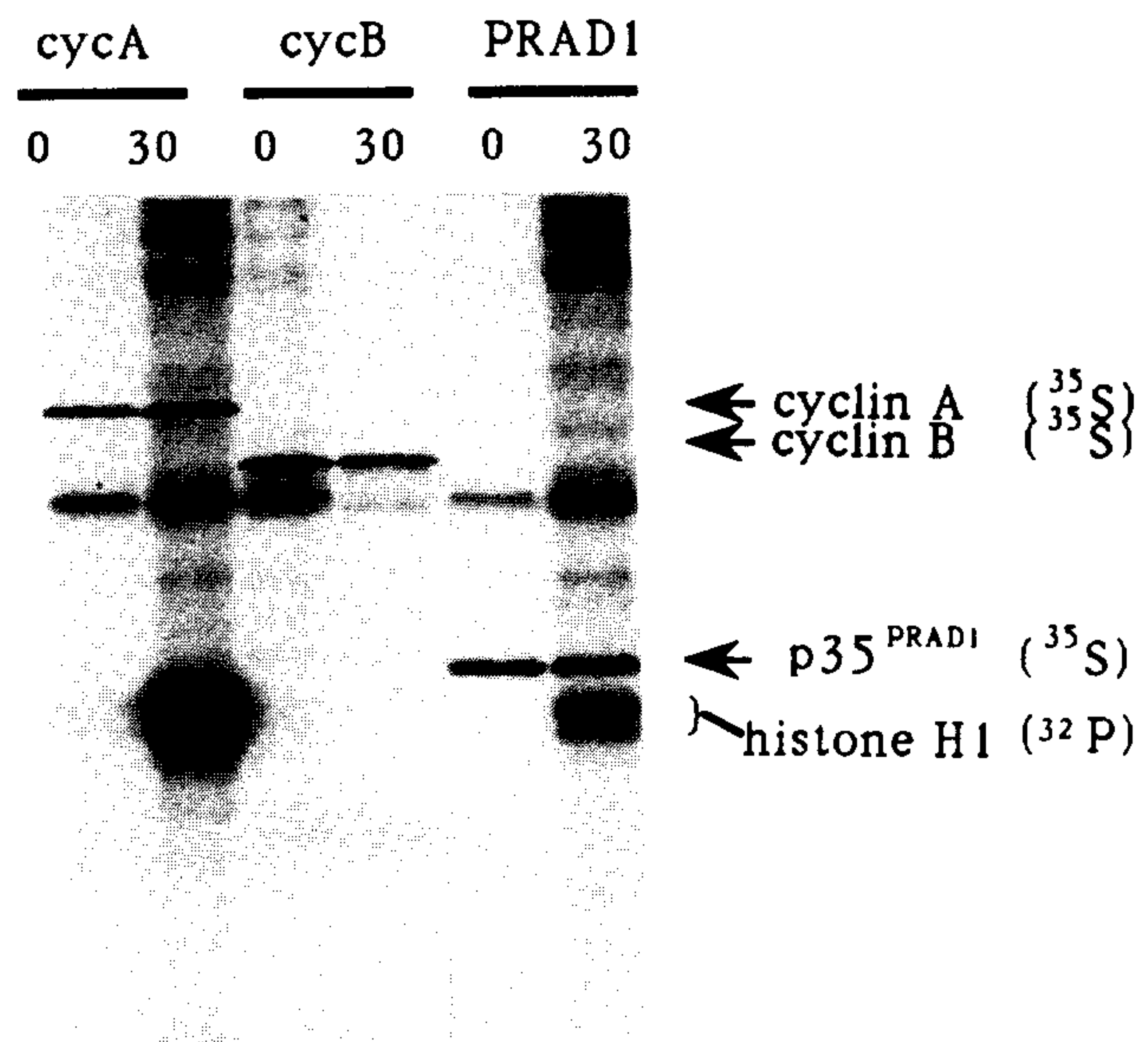


FIG. 10D