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(54) Title: CELL CYCLE CONTROLLING COMPOSITIONS AND METHODS OF USING SAME

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A method of controlling cell cycle progression including into a cell to be controlled during the interphase portion of the cell cycle regulating composition. The composition is selected from the group consisting of a protein and a fragment of the protein to alter the cell cycle progression of the cell while maintaining the viability of the cell. The protein fragments have been found to have the unexpected and surprising characteristic of being soluble in low concentrations of glycerol, thereby enhancing their value in pharmaceutical applications.

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(57) Abstract

A method of controlling cell cycle progression including into a cell to be controlled during the interphase portion of the cell cycle regulating composition. The composition is selected from the group consisting of a protein and a fragment of the protein to alter the cell cycle progression of the cell while maintaining the viability of the cell. The protein fragments have been found to have the unexpected and surprising characteristic of being soluble in low concentrations of glycerol, thereby enhancing their value in pharmaceutical applications.

Description

CELL CYCLE CONTROLLING COMPOSITIONS AND METHODS OF USING SAME

Technical Field

This invention relates in general to the control of cell cycle progression and it more particularly relates to compositions and methods for regulating cell cycle progression to suppress tumorigenesis.

Background Art

It is well recognized that in the development of higher organisms, such as animals and humans, orderly cell cycle progression is a critical factor. Because of such progression, ordered and systematic cell differentiation occurs so that, ultimately from a single undifferentiated cell, a highly structured organism, having a variety of specialized tissues, develops. In general, physiologically normal cell cycle progression is very important to the organism, not only during the stages of early growth, but throughout the entire life of the organism. Thus, even after the organism has reached maturity, normal cycle progression is still a very important aspect of health. This, for example, can be clearly seen in the importance of proper cellular regulation in the blood forming and reproductive organs.

Under certain circumstances, normal cell cycle progression fails, often with catastrophic effects for the organism. Such a failure is seen, for example, in the various forms of cancer where, because of an unchecked, and uncontrolled, progression of cells through the cell cycle, from the completion of mitosis through interphase and back into mitosis, tumorigenesis becomes, in many cases, a life threatening event. Thus, a restraint on uncontrolled cell cycle progression is sometimes attempted in an effort to treat or control the tumorigenic condition.

Under current conditions, where tumorigenesis has developed, drastic treatment measures, such as radiation therapy and chemotherapy are employed. Both of these

modalities are very expensive and, in some cases, very damaging to the organism. Chemotherapy, for example, can cause the death of the patient and when chemotherapy is successful in controlling tumorigenesis, it may have long term adverse effects on the body. Such an adverse effect may result in premature heart problems for the patient. Thus, while conventional therapeutic methods, such as radiation and chemotherapy, serve a useful purpose in destroying certain uncontrolled cells, they also produce the unwanted effects of destroying useful cells and weakening organ systems.

In view of the foregoing, it would be highly desirable to have techniques and compositions for regulating unwanted cell progression, such as in tumorigenesis, while maintaining cell viability. In addition, it would be further very highly desirable if such techniques and compositions produce little or no adverse effects on the cell and, halt the development of tumorigenesis without causing irreversible changes at the cellular level.

A significant advantage of such novel techniques and compositions, in the case of tumorigenesis, would be the possibility of regulating unwanted cell proliferation, for enough time for other cancer treating procedures to be applied. Thus, the use of such techniques could reduce or eliminate the ravaging effects of conventional radiation and chemotherapeutic treatments, while arresting tumorigenesis. Hence, the patient would retain strength and vigor so that conventional techniques, such as surgery, could be more safely utilized.

Disclosure of Invention

It is an object of the present invention to provide a cell cycle controlling composition and method of using the same for controlling the progression of the cell cycle in living organisms.

It is a further object of the present invention to provide a safe and effective method and composition for reversibly arresting cell cycle progression in organisms.

It is a still further object of the present invention to provide a technique which may be used in combination with therapeutic methods to arrest tumorigenesis in organisms.

The present invention includes a method and compositions for controlling cell cycle progression, by introducing into a cell to be controlled, during the interphase of the cell, a cycle regulating composition. The composition is selected from the group consisting of a gene protein product and a fragment of the protein, to alter reversibly the cell cycle progression of the cell while maintaining its viability. The protein fragments have been found to have the unexpected and surprising characteristic of being soluble in low concentrations of glycerol, thereby enhancing their value in pharmaceutical applications.

An advantage of the present invention is that cell cycle progression can be reversibly arrested in a convenient and safe manner, without insult to the organism. Thus, tumorigenesis may, for example, be controlled.

A further advantage of the present invention is that the composition utilized for cell cycle progression control is relatively inexpensive and readily obtainable.

A still further advantage of the present invention is the fact that the composition therein utilized possesses little or no toxic effects on healthy cells, and may be used in conjunction with other methods of cancer treatment.

An even still further advantage of the present invention is the fact that the compositions and techniques are compatible for use with the regulatory regimens and are physiologically compatible with other methods and devices for regulating certain physiological processes of the body such as blood cell production and gamete production.

Brief Description of Drawings

The above mentioned and other objects and features of this invention and the manner of attaining them will become apparent, and the invention itself will be best understood by reference to the following description of the embodiment of the invention in conjunction with the accompanying drawings, wherein:

FIG. 1 depicts SDS-PAGE analysis of the concentration and purity of protein preparations used for microinjection;

FIGS. 2A-2F are photomicrographs depicting microinjection and immunostaining of labeled Saos-2 cells;

FIG. 3 graphically depicts the percentage of labeled cells up to four days after injection;

FIG. 4 graphically depicts the percentage of labeled cells as a function of $p56^{RB}$ concentration;

FIG. 5 graphically depicts the effect on cells when $p56^{RB}$ is injected in S phase;

FIG. 6A graphically depicts the dependence of cell cycle arrest on the time period of injection of $p56^{RB}$;

FIG. 6B graphically depicts the time of onset of S phase in Saos-2 cells after release from nocodazole arrest in mitosis;

FIG. 6C graphically depicts SAOS-2 cells coinjected with $p56^{RB}$ and R or G after release from nocodazole treatment;

FIG. 7 is a composite of FIGS. 7A, 7B, 7C, and 7D which depict the nucleotide sequence of the retinoblastoma gene and the amino acid sequence of the RB protein;

FIG. 8 depicts the amino acid sequence of the RB protein;

FIG. 9 depicts the nucleotide sequence of the retinoblastoma gene and the amino acid sequence of the RB gene product and, in addition, depicts the $p56^{RB}$ portion of the RB protein; and

FIG. 10 graphically depicts various stages of the cell cycle and the status of RB protein point in the cell cycle.

Best Mode for Carrying Out the Invention

It is generally recognized that, after mitosis of a somatic cell is completed, at the end of telophase, the cell enters into an interphase which, depending on a variety of factors, may be of short duration or last for a long period of time. Thus, for example, after cell differentiation and development of nerve tissue, the nerve cells may have a very long interphase. Conventionally, cellular interphase may be regarded as having three stages: G1 in which cell growth occurs without DNA replication, S phase, in which DNA replication occurs, and G2, in which DNA replication has been completed and the cell prepares for division. As

hereinafter described in greater detail, certain gene protein products, or fragments thereof, have the capacity for controlling progression through the cell cycle by stopping reversibly the progression at G1. According to the present invention, cell cycle controlling compositions were introduced into cells during the interphase portion of their cell cycles, to cause a reversible alteration of the cell cycle progression, while maintaining cell viability. After a certain time, when the compositions degraded sufficiently within the cell, the cell cycle has been observed to be reinstated with the cell progressing toward subsequent stages of interphase.

With regard to pathological conditions in an organism, such as tumorigenesis, there is a manifestation of unwanted cell cycle acceleration, in some cells. As more fully discussed below, cancer suppressor gene protein products, such as the RB protein, or a fragment thereof, were utilized to arrest Saos-2 osteosarcoma cells in the G1 stage of interphase. It has been found that the administration of the protein to the cells had no toxic effect on the cells, and was reversible.

In the examples discussed below, the following experimental procedures were followed.

Cell Culture

Saos-2 cells, an osteosarcoma cell line, were obtained from American Type Culture Collection. SR-40, which stably expresses exogenous, wild-type RB protein, was derived from Saos-2 by single cell cloning after infection with a retrovirus carrying the RB gene. Huang, H.-J. S.,

Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookstein, R., Friedmann, T., Lee, E.Y.-H.P. and Lee, W.-H. (1988) Science 242, 1563-1566. African Green monkey kidney cell lines CV-1 and COS-7 were also obtained from American Type Culture Collection. COS-7 was derived from CV-1 by transformation with an origin-defective SV-40. Gluzman, Y. (1981) Cell 23, 175-182. All the cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum as recommended.

Synchronization of Cells

Cells were synchronized at the G1/S boundary by isoleucine starvation for 36 hours and then incubated in complete medium supplemented with either aphidicolin or hydroxyurea (both from Sigma) for an additional 12 or 16 hours respectively. Heintz, H.H. and Hamlin, J.L. (1982) Proc. Natl. Acad. Sci. USA. 79, 4083-4087. Cells were released from G1/S block by washing three times with phosphate buffered saline (PBS), and re-feeding with complete media. Metaphase arrested cells were collected after an 8 to 12 hours treatment with 0.04 μg/ml nocodazole (sigma) as previously described. Zieve, G.W., Turnbull, D., Mullins, J.M. and McIntosh, J.R. (1980) Nocodazole Exp. Cell Res. 126, 397-405. To increase the yield of mitotic cells, cells were first arrested at the G1/S boundary by a 12-hour treatment with aphidicolin. Six hours after the removal of aphidicolin, nocodazole was added to the medium. Mitotic cells were collected by gentle washing and shaking and were then replated onto 35 mm dishes. Six hours after seeding,

the unattached cells were washed away and cells were injected at various times thereafter.

Protein Preparation

$p56^{RB}$ is the C-terminal half of the RB protein and contains both regions essential for SV40 T-antigen binding. It is produced in *E.coli* from a T7 RNA polymerase expression system. Huang, S., Lee, W.-H. and Lee, E.Y.-H.P. (1991) Nature 350, 160-162. $p110^{RB}$ is produced in insect cells by the recombinant baculovirus technique. Wang, N.P., Qian, Y., Chung, A.E., Lee, W.-H. and Lee, E.Y.-H.P. (1990b) Cell Growth & Differ. L, 429-437. Both proteins were purified to homogeneity by conventional chromatography. Histone H1 and Rabbit anti-goat IgG were purchased from Boehringer Mannheim and Vector laboratories, respectively. Antibodies .495, .47 and R2 were concentrated in microinjection buffer to an approximate concentration of 1 mg/ml. The T-antigen and p53 peptides were the gift of Nicholas Lin. Thése antibodies are described in Huang, S., Wang, N.-P., Tseng, B.Y., Lee, W.-H. and Lee, E.Y.-H.P. (1990) EMBO J.9, 1815-1822. The T peptide comprises amino acids 101-118, and was dissolved in microinjection buffer at 1mM or 5mM. Ludlow, J.W., deCaprio, J.A., Huang, C.-M., Lee, W.-H., Paucha, E. and Livingston, D.M. (1989) Cell. 56, 57-65. The mutant T peptide contains a lysine to aspartic acid substitution and was used at 5 mM. Ludlow, J.W., deCaprio, J.A., Huang, C.-M., Lee, W.-H., Paucha, E. and Livingston, D.M. (1989) Cell 56, 57-65. The p53 peptide was is dissolved in microinjection buffer at 5 mM. Protein preparations, except the full-length RB protein, were concentrated in an

injection buffer containing 20 mM Tris, pH 7.4; 0.1 mM EDTA; 10 mM KCl; 1 mM 2-mercaptoethanol and 2% glycerol using the Centricon 30 micro-concentrator (Amicon). $p110^{RB}$ was kept in a buffer containing 10% glycerol to reduce aggregation.

Protein preparations were analyzed by SDS-PAGE using standard techniques. Injections were performed directly on cells growing on 35mm culture dishes using an Eppendorf micromanipulator and microinjector with femtotip* capillary micropipets. Injection pressure was typically set between 50-100 hPa with an injection time of .3-.5 seconds. It was estimated that the protein preparations were diluted approximately 20 to 50-fold upon injection. Assuming a typical cell volume of 50-100 picoliters and a $p56^{RB}$ concentration of .5-1 milligram per milliliter, about 5-50 million molecules were injected per cell.

After injection, the growth media was supplemented with BrdU (Amersham) according to manufacturer's recommendations. Following the appropriate labeling period, media was removed and cells were washed 3x with PBS. Cells were then fixed by incubation with ice-cold, absolute methanol for 30 minutes. After re-hydration by washing with PBS, specimens were incubated with a mouse monoclonal antibody directed against BrdU (Amersham) for one hour at room temperature. After washing 3x with PBS, a fluorescein conjugated anti-mouse antibody (Amersham) was added and incubation was continued for an additional hour at room temperature. The anti-mouse antibody was removed by washing 3x with PBS, and the specimens were incubated with Texas Red conjugated streptavidin (Amersham). The streptavidin bound to the

* Trade-mark

biotinylated RαG co-injected with all protein preparations and thus served as a cytoplasmic marker for injected cells. After a final wash, a solution composed of equal volumes of glycerol and PBS was added and the specimens were covered with a glass coverslip. Specimens were examined under a fluorescent microscope with Texas Red and Fluorescein filters.

The following examples demonstrate the experimental results achieved after introduction of gene product proteins into tumor cell lines. The capacity of the proteins, or fragments thereof to arrest cell cycle progression in G1 has been demonstrated. In addition, the examples demonstrate that the effect of the blocking occurs only when the protein is administered during the G phase and that the blocked cell cycle progression can be relieved by SV40 T antigen.

In certain specific examples, the RB gene product was used to arrest the cell cycle progression wherein an Saos-2 osteosarcoma cell line was treated with the RB protein or a fragment thereof. It was discovered that cell cycle progression was arrested in G1 and that this progression is reversible.

In the following examples, when the retinoblastoma gene is referred to, it is intended to mean the gene having the nucleotide sequence depicted in FIGS. 7 and 9. When reference is made to the RB protein (pRB^{110}) the protein having the amino acid sequence depicted in FIGS. 7, 8 and 9 is intended. With reference to FIG. 8, the truncated protein fragment, $p56^{RB}$ is illustrated. In addition, the $p56^{RB}$, fragment is depicted in FIG. 9 as commencing at

arrow A and terminating at arrow B. With further reference to FIG. 9, the C terminal peptide is illustrated, commencing at amino acid 917 (arrow C) and terminating at amino acid 928 (arrow B).

Single-letter abbreviations in the drawings for the amino acid residues are: A, Alanine; C, Cysteine; D, Aspartic acid; E, Glutamic acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutanine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophane; and Y, Tyrosine.

EXAMPLE I

Introduction of RB Proteins into Human Osteosarcoma Saos-2 Cells by Microinjection

With reference now to FIG. 1, there is shown the concentration and purity of protein preparations used in the microinjection techniques discussed below. The protein preparations used for microinjection were analyzed by SDS-PAGE. Lane 1: $p110^{RB}$ from insect cells infected with recombinant RB baculovirus; lane 2: biotinylated rabbit anti-goat antibody; lane 3: anti-RB .495 antibody; lane 4: anti-RB R2 antibody; lane 5: anti-RB .47 antibody; lane 6: histone H1; lane 7: $p56^{RB}$ from E. coli. One microliter of each sample was loaded on a 15% acrylamide gel. The gel was stained with Coomassie brilliant blue. The positions of molecular weight standards, in kiloDaltons, are indicated.

Two forms of RB protein were prepared for the microinjection experiments. Wild type $p110^{RB}$, both

hypophosphorylated and unphosphorylated forms, was purified to near homogeneity from baculovirus infected insect cells. At concentrations approaching 1 mg/ml, however, the protein aggregated into a form which could not be injected. To partially alleviate this problem, $p110^{RB}$ was purified, stored, and injected in buffers containing 10% glycerol. An unphosphorylated, amino-truncated 56 kDa RB protein ($p56^{RB}$), containing an intact T-antigen binding domain, was expressed in E. coli and purified to near homogeneity. Since $p56^{RB}$ could be concentrated to 1 mg/ml and injected in a standard buffer containing 2% glycerol, it was used in most of the following experiments.

Human osteosarcoma cell line Saos-2 was chosen as the recipient cell line. This cell line lacked expression of wild-type $p110^{RB}$, but contained a cytoplasmic, carboxy-truncated 95 kDa protein which can not bind T-antigen. Saos-2 cells responded to exogenous expression of RB, introduced by retrovirus mediated gene transfer, by an initial enlargement of cell size and loss of tumorigenicity in nude mice. Hence, it was determined that these cells might be particularly sensitive to injection of RB protein.

In FIG. 2, there is depicted the results of microinjection and immunostaining of Saos-2 cells labeled with BrdU. Saos-2 cells were injected as described herein. Panel A. shows cells injected with $p56^{RB}$, immediately fixed, and indirectly immunostained for RB protein with Texas Red. Panel B contains uninjected cells labeled with BrdU for 4 hours, and then fixed and immunostained with fluorescein conjugated anti-BrdU antibody. Panels C and D are a single

field of synchronized cells co-injected in early G1 with $p56^{RB}$ and RαG, incubated with BrdU for 24 hours, then fixed and stained. The cells stained with Texas Red mark injected cells, while the cells stained with fluorescein indicate cells which have incorporated BrdU. Panels E and F are a single field of cells also injected in early G1 with RαG alone.

To determine if the proteins could be translocated to the nucleus, sub-confluent, asynchronously growing Saos-2 cells were cytoplasmically microinjected with $p56^{RB}$ or $p110^{RB}$ and fixed 5 to 15 minutes later. The cells were then immunostained with rabbit anti-RB antibody .47 and a Texas Red conjugated anti-rabbit antibody. Staining was mainly observed in the nucleus (FIG. 2A), although there was some staining observed in the cytoplasm of some injected cells. Both $p110^{RB}$ and $p56^{RB}$ proteins were capable of being transported to the nucleus within 15 minutes.

For subsequent experiments, cells were typically co-injected with an RB protein and a biotinylated, polyclonal rabbit anti-goat antibody (RαG) that served as a cytoplasmic marker for injected cells. It was estimated that 5-50 million molecules of RB protein were injected per cell. The number of endogenous RB protein molecules per cell was estimated to be approximately 1 million. Hence the injected protein represented at least a 5 to 50-fold excess over endogenous levels. Following injection the cells were incubated in growth media containing bromodeoxyuridine (BrdU). Cells progressing through S phase during the labeling period will incorporate BrdU into their DNA. After

fixation, cells were immunostained for BrdU with a fluorescein-conjugated antibody and for RαG with Texas Red conjugated streptavidin (FIG. 2). The percentage of injected cells that incorporated BrdU could be determined under a fluorescence microscope as the fraction of Texas red-positive cells that were also fluorescein-positive.

BrdU incorporation in RB-injected, asynchronously growing cells was only slightly less than that of uninjected cells or cells injected with RαG alone over a four hour labeling period (Table 1). These results indicated that the RB protein preparations used were not generally toxic to cells as determined by their continued viability, DNA incorporation, and cell adherence.

EXAMPLE II

RB Proteins Injected in Early G1 Block Incorporation of BrdU

Since only a small effect on BrdU incorporation was observed upon injection of RB protein, the cells might only be sensitive to RB protein at a particular point in the cell cycle. The effect of RB protein could be more readily observed by injecting synchronized cells at this point. Cells were synchronized by treatment with nocodazole, which arrests cells in mitosis, and were then released and incubated for an additional 6 hours at which time non-adherent cells were removed and the remaining cells injected. Following a 24 hour incubation in the presence of BrdU, cells were fixed and stained for BrdU and RαG. At least 80-90% of uninjected cells could be stained for BrdU. Cells injected with $p56^{RB}$, however, were almost completely inhibited from progressing through G1 and into S phase over

the labeling period. In this regard, please see Table 2 and FIGS. 2C and 2D. Similar inhibition was observed upon injection with $p110^{RB}$. In contrast, 60-70% of cells injected with histone and RαG, or RαG alone were able to enter S phase during the labeling period (FIGS. 2E and 2F). Although $p110^{RB}$ inhibited BrdU incorporation under these conditions, results were somewhat variable. Over time, $p110^{RB}$ preparations tended to aggregate which led to difficulty in injection. Relative to preparations of $p110^{RB}$, $p56^{RB}$ activity was very consistent.

To extend this observation to cells which already expressed wild-type $p110^{RB}$, $p56^{RB}$ was injected into cell line SR-40 which was derived from Saos-2 and stably expressed $p110^{RB}$. The effect of RB protein injection at early G1 phase on synchronized SR-40 cells was identical to the effect on Saos-2 cells (Table 2); very few cells injected with $p56^{RB}$ entered S phase over the 24 hour labeling period. Thus, the presence of endogenous wild-type $p110^{RB}$ did not interfere with the effect of $p56^{RB}$ on cell cycle progression.

To determine how long the $p56^{RB}$ block to entry of S phase lasted, the labeling period in the presence of BrdU was extended from one day to 2 or 4 days. FIG. 3 graphically shows the number of injected Saos-2 cells which incorporated BrdU during the labeling period. Saos-2 cells were co-injected with $p56^{RB}$ and RαG 6-7 hours after release from nocodazole treatment. After injection, cells were incubated in media supplemented with BrdU for the indicated number of days before fixing and staining. The percentage

value indicates the number of injected cells which had incorporated BrdU during the labeling period. The values represent at least 100 injected cells. Inhibition of cell cycle progression was still observed after 2 days of incubation with BrdU prior to fixing and staining. Four days after injection, however, 80% of $p56^{RB}$ injected cells were able to incorporate BrdU. These observations indicated that the block of cell cycle progression by RB protein was reversible, and lasted between 2 and 4 days.

The dose dependence of the $p56^{RB}$ effect was also measured. Diluted aliquots of $p56^{RB}$ were injected into synchronously growing cells in early G1 phase. FIG. 4 graphically shows the dependence of cell cycle arrest on the dose of $p56^{RB}$. Saos-2 cells were co-injected 6-8 hours after release from nocodazole treatment with the indicated concentrations of $p56^{RB}$ and 1 mg/ml of RαG. After 24 hours of incubation in growth medium with BrdU, cells were fixed and stained for BrdU and RαG. The histogram indicates the percentage of injected cells which incorporated BrdU. Each value represents at least 150 injected cells. As shown in FIG. 4, 5-fold dilution of $p56^{RB}$ from the original concentration diminished the inhibitory activity while aliquots diluted by up to 2-fold still retained the inhibitory effect. Therefore, the block of entry into S phase by $p56^{RB}$ was dependent on the amount of protein injected. The threshold for block of BrdU incorporation was between 0.2 and 0.5 mg/ml of injected $p56^{RB}$.

To demonstrate that the inhibition of entry into S phase was caused by RB protein, we attempted to alleviate

the block with reagents that bound specifically to $p56^{RB}$. The $p56^{RB}$ was mixed with 1 mg/ml solutions of rabbit polyclonal antibodies .495, .47, or R2 (FIG. 1). These antibodies were raised against unique RB fusion proteins, and recognized $p56^{RB}$ on Western blots (31, 49, 56). Injection of the mixture of $p56^{RB}$ and .495 resulted in BrdU incorporation in about 30% of injected cells, compared to an almost total lack of incorporation in cells injected with $p56^{RB}$ alone (Table 2). Cells co-injected with $p56^{RB}$ and antibody .47 incorporated BrdU about 16% of the time. Co-injection of $p56^{RB}$ and antibody R2 also exhibited a lessened inhibitory activity although the effect was not as dramatic as with antibodies .47 or .495. Because antibodies that bind RB protein diminished the inhibitory effect of $p56^{RB}$, the block to BrdU incorporation was due specifically to RB protein.

EXAMPLE III

BrdU Incorporation is not Inhibited by $p56^{RB}$ if Injected in S phase

The lack of BrdU incorporation observed after injection of RB protein in early G1 phase could be explained by inhibition of DNA synthesis. To test this, Saos-2 cells were arrested in early S phase by treatment with aphidicolin and then injected with $p56^{RB}$. As shown graphically in FIG. 5, BrdU incorporation is not inhibited by $p56^{RB}$ if it is injected in the S phase. Saos-2 cells were arrested at G1/S by treatment with hydroxyurea or aphidicolin and were then injected.

After injection, cells were released from arrest and incubated with BrdU for 6-8 hours (hydroxyurea) or 4-6 hours

(aphidicolin) at which time the cells were fixed and stained. Cells were also arrested in mitosis by treatment with nocodazole. After release, cells were collected and injected about 6 hours later. Following injection, cells were incubated with BrdU for 24 hours and then fixed and stained.

The protein preparations used for injection are indicated. The percentage of injected cells that stained for BrdU after the respective labeling period is shown. After injection, cells were released from aphidicolin treatment and incubated with BrdU for 4-6 hours, fixed, and immunostained for BrdU and RαG as described. In contrast to cells injected in early G1, approximately 60% of $p56^{RB}$ injected, aphidicolin arrested cells stained positively for BrdU incorporation. The percentage of cells entering S phase and the intensity of BrdU staining was similar to cells injected with histone and RαG, or RαG alone. To control for any possible lag period of the function of RB protein after injection, aphidicolin arrested cells injected with $p56^{RB}$ were incubated for an additional 6 hours prior to release and labeling with BrdU. Despite a somewhat lower intensity of BrdU staining, which was probably caused by the prolonged incubation with aphidicolin itself, the percentage of $p56^{RB}$ injected cells that incorporated BrdU was comparable to that of cells injected with RαG alone.

Similar experiments were performed by arresting cells with hydroxyurea rather than aphidicolin. Hydroxyurea arrests cells near the G1/S boundary at a point distinct from aphidicolin arrest. Saos-2 cells were arrested near

G1/S by treatment with hydroxyurea and then were injected with $p56^{RB}$. After injection, cells were released form hydroxyurea treatment and labeled for 6 to 8 hours with BrdU. Again, injection of $p56^{RB}$ had no detectable inhibitory effect on BrdU incorporation (FIG. 5). The percentage of cells synthesizing DNA was the same whether injecting with $p56^{RB}$ and RαG, histone and RαG, or RαG alone.

The results were consistent with observations of asynchronously growing cells injected with RB protein, and implied that DNA synthesis, per se, was not blocked significantly by $p56^{RB}$. Therefore, inhibition of DNA synthesis could not explain the complete lack of BrdU incorporation seen upon injection of $p56^{RB}$ into synchronized cells in early G1 phase.

EXAMPLE IV

RB Protein Blocks Progression Through the G1 Phase at a Specific Point

Given the results of the above experiments, the inhibitory effect of $p56^{RB}$ must have been due to a block in progression through the G1 phase. To define the position of this arrest within the G1 phase, a careful calibration of the time of onset of S phase in the cell cycle of Saos-2 cells was first determined. FIGS. 6A, 6B and 6DC graphically depict the dependence of cell cycle arrest on the time period of injection for $p56^{RB}$. With regard to FIG. 6A, Saos-2 cells were arrested in mitosis by treatment with nocodazole. After release from arrest, the cells were incubated for the indicated number of hours in the presence of BrdU, then fixed and stained with an anti-BrdU antibody.

The histogram indicates the percentage of counted cells that stained for BrdU. Each value represents at least 200 cells.

As shown in FIG. 6A, uninjected cells began to stain for BrdU at approximately 20 hours after the release. By 30 hours virtually 80 to 90 percent of cells had incorporated BrdU. Based on this, a time-course experiment in which nocodazole treated cells were injected with $p56^{RB}$ at various times after release from arrest was then performed. FIG. 6B depicts the time of onset of S phase in Saos-2 cells, in hours, after release from nocodazole arrest in mitosis. The length of G1 phase is about 22 to 24 hours. The length of S phase is about 7 to 8 hours. The length of the G2 phase has not been precisely determined. The arrows indicate the time points of injection that are used for the experiment described in 6C.

With regard to FIG. 6C, Saos-2 cells were co-injected with 1 mg/ml $p56^{RB}$ and RαG at the indicated times, in hours, after release from nocodazole treatment. Incubation was continued after injection in growth medium with BrdU until 30 hours after the original release from nocodazole. After BrdU labeling, cells were fixed and stained for BrdU and RαG. The histogram shows the percentage of $p56^{RB}$ injected cells which incorporated BrdU. Each value represents at least 200 injected cells.

Very few cells entered S phase at 30 hours after release if injected with $p56^{RB}$ 5-10 hours after release from nocodazole (FIG. 6C). A significant number of cells incorporated BrdU if injected with $p56^{RB}$ after 14-16 hours

post-arrest. If injected 17-19 hours after release, $p56^{RB}$ had no detectable inhibitory effect on entry into S phase.

Given that S phase began between 22 and 24 hours after release from nocodazole, the results suggested that cells become refractory to the inhibitory effect of injected RB protein about 6-10 hours prior to the G1/S transition. This confirmed the notion that the inhibitory effect of RB protein on the cell cycle was due to a block in progression through the G1 phase rather than a block of DNA synthesis itself. Furthermore, the observations implied that RB may function at a specific, relatively early time point within the G1 phase.

EXAMPLE V

SV40 T Antigen Relieves the Blocked Cell Cycle Progression by p56 RB

As a test of the hypothesis that SV40 T-antigen can functionally inactivate RB protein upon binding, we compared the effect of $p56^{RB}$ injection on G1 progression in the presence or absence of T-antigen. A 1mM T peptide solution which was capable of binding to $p56^{RB}$, was mixed with an equal volume of $p56^{RB}$ and injected into synchronized cells in early G1 phase. At this concentration, the peptide was at a 100 to 200-fold molar excess over the RB protein. About 28% of synchronized cells injected with the $p56^{RB}$/T peptide mixture entered S during the labeling period (Table 3). If the T peptide concentration was increased to 5 mM, or a 500 to 1000-fold molar excess, approximately 70% of injected cells could incorporate BrdU. By contrast, almost none of the cells progressed into S phase after injection

with $p56^{RB}$ and a 500 to 1000-fold molar excess of a carboxy-terminal p53 peptide that did not bind $p56^{RB}$.

The effects of injection of $p56^{RB}$ on African Green monkey kidney cell lines CV-1 and COS-7 were also compared. COS-7 cells were chosen since they were derived from CV-1 cells by transformation with an origin-defective SV-40 mutant, and they expressed a high level of T-antigen. Therefore, they can serve as a good system for testing the effect, if any, of the presence of endogenous T-antigen on the activity of the injected RB protein. The percentage of synchronized CV-1 cells injected with $p56^{RB}$ in early G1 phase that incorporated BrdU was at least 5-fold lower than cells injected with RαG alone (Table 3). Synchronized COS-7 cells, however, were not inhibited at all from progressing into S phase. These results indicated that the presence of endogenous T-antigen, or co-injection with a T-antigen peptide, could neutralize the inhibitory activity of RB protein. Thus, those skilled in the art can utilize this system to screen RB protein fragments to identify those fragments capable of blocking cell cycle progression by comparing the percentage of CV-1 to COS-7 cells entering S phase in the presence of the RB protein fragment.

The foregoing results provide functional evidence which indicates that RB protein can act by inhibiting progression through the cell cycle. Injection of $p56^{RB}$ does not detectably inhibit DNA synthesis per se, since aphidicolin or hydroxyurea arrested cells are not affected, but rather blocks progression through the G1 phase. This inhibition of cycle progression is dose-dependent, is not due to general

toxicity to the cells, and is specific for RB protein because antibodies that bind specifically to $p56^{RB}$ can attenuate its activity. The cycling cell is sensitive to RB protein inhibition until roughly 6-10 hours before the onset of DNA replication; after this time, exogenously added $p56^{RB}$ no longer inhibits progression into S phase. Assuming there is a small lag period between $p56^{RB}$ injection and its appearance in an active form in the nucleus, it may be concluded that RB protein inhibits progression to S phase at a critical point in G1 phase. The 6-10 hour time window prior to DNA synthesis may be analogous to the time period following the G1 restriction point, a point of irreversible commitment to S phase in mammalian cell lines 47.

The time window defined may not correspond precisely to the time point when RB protein functions under normal physiological conditions. The time it takes for injected $p110^{RB}$ or $p56^{RB}$ to appear in an active configuration and/or location is not clear. However, RB proteins are transported to the nucleus within 15 minutes after cytoplasmic injection, and continued incubation of $p56^{RB}$ injected cells with aphidicolin for several hours prior to release and labeling does not decrease the percentage of cells which incorporate BrdU. These observations suggest that the time it takes RB protein to become functional after injection may be short.

The transition from an active form of $p110^{RB}$ to an inactive form has been assumed to occur at the G1/S boundary since the protein undergoes extensive phosphorylation at this point. However, because of the foregoing, an earlier

time period, which seems to be critical for $p56^{RB}$ activity has been defined. Thus, RB may be involved in a regulatory decision which the cell makes at a point about 6-10 hours prior to the G1/S transition. The RB protein can be phosphorylated at multiple sites both in vivo and in vitro, yet it is not yet clear which phosphorylation sites are important for RB function. Subtle increases in phosphorylation of $p110^{RB}$ during G1 phase may be responsible for the cells' transition from an RB-responsive to a non-responsive state. Some phosphorylation of $p110^{RB}$ has been observed in the G1 phase, although this may have been due to incompletely synchronized cells.

The carboxy-terminal 56 kiloDaltons of RB is sufficient to inhibit progression through the G1 phase. This indicates that the carboxy terminal half of $p110^{RB}$ is, in fact, a functional domain with respect to its effect on the cell cycle. Two biochemical activities have been ascribed to the carboxy-terminal half of $p110^{RB}$. DNA binding and protein binding. Based on findings to date, the sequence specificity of RB binding to DNA is low; although RB binds with slightly higher affinity to DNA with high G/C content, no particular sequence is strongly preferred. On the other hand, specificity has been observed in binding of RB to proteins. The transforming proteins of several DNA transforming viruses as well as a subset of cellular proteins bind to the same domain of $p110^{RB}$ and therefore can compete with one another for RB protein binding. This region is where the majority of naturally occurring inactivating mutations of RB are located. It seems likely,

then, that the block to progression of G1 phase by RB protein is dependent on specific protein-protein interactions.

Mixing $p56^{RB}$ with a T-antigen derived peptide, which can bind $p56^{RB}$, eliminates its inhibitory consequences. Co-injection of $p56^{RB}$ with a T peptide containing a single amino acid substitution at a 500 to 1000-fold molar excess can also attenuate, to some extent, the block to cell cycle progression. Although the amino acid substitution lowers its affinity for RB protein in vitro, this mutant T peptide may still bind $p56^{RB}$ in vivo when present in vast molar excess. In contrast, the injection of a totally irrelevant p53 peptide had no effect on the inhibitory activity of RB protein, which thus substantiated the specificity of the interaction observed between RB protein and T-antigen peptide. CV-1 cells injected in early G1 with $p56^{RB}$ are arrested before S phase while COS-7 cells, which express high levels of T-antigen, are not arrested, however. These observations, taken together, indicate that T-antigen binding does have functional consequences for RB protein. Since the injected $p56^{RB}$ is unphosphorylated, the test results are consistent with the hypothesis that the active form of RB protein, with respect to its inhibition of cell proliferation, is the unphosphorylated form. Therefore, the transforming proteins of some DNA tumor viruses, including SV40 T-antigen and adenovirus E1A, may promote cell growth, at least in part, by binding and inactivating underphosphorylated $p110^{RB}$. The immortalization of cells and the induced escape from quiescence upon expression of

these transforming proteins are phenotypes consistent with deregulation of the cell cycle.

Given that the carboxy-terminal half of $p110^{RB}$ is biologically active, the question remains as to the function of the amino terminal half of the protein. Sequences within this region may be required for the proper phosphorylation of $p110^{RB}$. In murine cells, polypeptides similar to $p56^{RB}$ are not hyperphosphorylated. Also, several consensus sites for the cdc2/MPF kinase are present within this region. It follows that the amino terminal half of $p110^{RB}$ may contain a regulatory domain which can modulate RB function. Release of $p56^{RB}$ block to G1 progression does not occur until 3-4 days after injection; this lengthy arrest period may be due to the inability of the cell to regulate RB protein in a normal manner, or to the relatively large amount of RB protein injected. A comparison of the activity in this assay of different RB proteins with mutations in phosphorylation sites would aid in resolving this issue. If $p56^{RB}$is constitutively active, mutations creating such polypeptides would be expected to inhibit cell proliferation and thus place cells at a selective disadvantage. This may explain the lack of naturally occurring mutants within the amino terminal half of RB.

The results described here indicate that RB participates in control of cell cycle progression by acting at a control point within the G1 phase. This point is significantly earlier than the observed hyperphosphorylation of $p110^{RB}$ as cells enter S phase. Thus, it is reasonable to propose a model which incorporates the test results as well

as other known properties of $p110^{RB}$ (FIG. 10). Since RB has been shown to associate with several cellular proteins, and the protein binding domains of RB are sufficient to block cell cycle progression, it seem highly possible that the binding of cellular proteins is critical for the observed RB function. The model suggests that RB participates in regulation of the cell cycle by sequestering cellular proteins vital for DNA synthesis and/or cycle progression at a particular time period during the G1 phase. Inactivation of $p110^{RB}$ by mutation, by phosphorylation at key sites, or by binding with transforming proteins is hypothesized to release these cellular proteins, permitting them to provide functions that are necessary for continuation of the cell cycle. Once cells commit themselves to continuation of the cell cycle by release of these proteins, $p110^{RB}$ can no longer effectively inhibit their function and therefore the block to cell cycle progression is relieved.

Sub-nuclear localization of RB may be crucial to its ability to sequester other proteins in an inactive form. RB protein segregates with cellular replication proteins to sites of Herpes virus DNA replication upon infection, a situation where normal regulation of the cell cycle is subverted. In G1, the underphosphorylated form of $p110^{RB}$ is tightly associated with a particular nuclear locale. Purified $p110^{RB}$ also tends to polymerize, perhaps explaining the difficulty in maintaining its solubility at high concentrations, and it has limited homology to a certain class of intermediate filament proteins. These observations suggest that $p110^{RB}$ may contribute to some structural

component of the nucleus, and that association of $p110^{RB}$ with this component is necessary for its inhibition of cell cycle progression. The hyperphosphorylation would presumably free $p110^{RB}$ from its sub-nuclear location allowing RB to provide some other function, or to reset its ability to sequester other proteins during the subsequent cell cycle. The model predicts that regulation of RB activity could be accomplished by specifying its nuclear location and/or the cellular proteins to which it binds.

The biological consequence of complete loss of RB function is generation of retinoblastoma and perhaps some other tumors. involvement of RB in the cell cycle provides a means to explain this phenomenon. RB may act to halt progression through G1 phase until the cell receives proper signals for commitment to continuation of the cell cycle. Thus, loss of RB function may contribute to tumorigenesis in different tissues by permitting unscheduled cell proliferation.

In view of the foregoing, it can be seen that the RB gene product is a nuclear phosphoprotein which undergoes changes in phosphorylation status in synchrony with the cell cycle. To test whether RB regulates cell cycle progression, purified RB proteins, either full-length or a truncated form containing the T-antigen binding region, were injected into cells and the effect on entry into S phase determined. Synchronized cells injected early in G1 with either protein are inhibited from progressing into S phase. This effect is antagonized by co-injection with antibodies directed against RB. Injection of RB protein into cells arrested at the G1/S

boundary or 6-10 hours before the end of G1 has no effect on BrdU incorporation suggesting that RB protein does not apparently perturb DNA synthesis in S phase. These results provide direct evidence that RB may regulate cell proliferation by restricting cell cycle progression at a specific point in G1, and they also establish a biological assay for the activity of RB protein. Co-injection of RB protein with a T-antigen peptide, or injection into cells expressing T-antigen does not prevent cells from progressing into S phase. This experiment substantiates the hypothesis that T-antigen binding has functional consequences for RB protein.

Table 1. Microinjection of Asynchronously Growing Cells

Protein Sample	Total Number of Injected Cells[a]	Total Number of BrdU Stained Cells[b]	% Injected Cells Entering S Phase
pp110^{RB}	1094	416	38
P56^{RB}	1315	408	31
RαG	1725	707	41
Histone	106	42	40
Uninjected			41

[a] The total number of injected cells (Texas Red positive) counted from at least five independent experiments, except for the Histone injection which represents one experiment.

[b] The number of injected cells which incorporated BrdU as evidenced by uniform staining with a fluorescein linked anti-BrdU antibody. Cells were incubated in growth media with BrdU for 4 hours prior to staining.

Table 2. Microinjection of Synchronously Growing Cells in Early G1

Cell Line	Protein	Number of Injected Cells[a]	Number of BrdU Stained Cells[b]	% of Injected Cells Entering S phase
Saos-2				
	$p110^{RB}$ [c]	256	8	3
	RαG[c]	270	173	64
	$p56^{RB}$	388	12	3
	RαG	295	202	68
	Histone	595	333	56
	Uninjected			84
	$p56^{RBd}$	247	10	4
	$p56^{RBd}$+.495	80	24	30
	$p56^{RBd}$+.47	596	95	16
	$p56^{RB}$d+R2	712	43	6
SR-40				
	$p56^{RB}$	89	3	3
	Histone	201	105	52
	RαG	134	74	55

[a] The total number of injected cells (Texas Red positive) counted from at least three independent experiments.

[b] The number of injected cells which incorporated BrdU as evidenced by uniform staining with a fluorescein linked anti-BrdU antibody. Cells were incubated in growth media with BrdU for 24 hours after injection and prior to staining.

[c] Injections were performed in a buffer containing 10% glycerol.
[d] p56RB was injected at a concentration of about .3-.5 mg/ml, rather than 1 mg/ml, in these experiments.

Table 3. SV40 T-antigen relieves the block to G1 progression by $p56^{RB}$

Cell Line	Protein[a]	Number of Injected Cells[b]	Number of BrdU Stained Cells[c]	% of Injected Cells Entering S phase
Saos-2				
	$p56^{RB}$	247	10	4
	$p56^{RB}$+T	724	203	28
	$p56^{RB}$+p53	727	7	1
CV-1				
	$p56^{RB}$	459	46	10
	Histone	471	259	55
	RαG	332	176	53
COS-7				
	$p56^{RB}$	257	224	87
	Histone	191	159	83

[a] All proteins were injected at concentrations of about 1 mg/ml, except for $p56^{RB}$ in Saos-2 cells which was injected at about .3-.5 mg/ml.

[b] Cell lines are microinjected 6-8 hours after release from nocodazole treatment. The total number of injected cells (Texas Red positive) is indicated.

[c] Cells were incubated in growth media with BrdU for 24 hours after injection and prior to staining.

While particular embodiments of the present invention have been disclosed, it is to be understood that various different modifications are possible and are contemplated within the true spirit and scope of the appended claims. There is no intention, therefore, of limitations to the exact abstract or disclosure herein presented.

CLAIMS:

1. Use of a carboxy-terminal biologically active fragment of $p110^{RB}$ of 56 kDa to alter cell cycle progression while maintaining the cell viable, during the interphase of the cell cycle, for controlling cell cycle progression in a cell that lacks a functional retinoblastoma tumor suppressor protein.

2. The use of claim 1 during the G1 portion of the cell cycle.

3. The use of claim 1, wherein said use is between about 6 and about 10 hours prior to the G1/S transition.

4. The use of claim 1, wherein said fragment comprises amino acid 379 to amino acid 928 of the amino acid sequence of Figure 8.

5. An isolated polypeptide for controlling cell cycle progression, consisting of a carboxy-terminal biological active fragment of $p110^{RB}$ of 56 kDa.

6. The polypeptide of claim 5, wherein said fragment consists of amino acid 379 to amino acid 928 of the amino acid sequence of Figure 8.

7. A pharmaceutical composition for controlling cell cycle progression in a cell that lacks a functional retinoblastoma tumor suppressor protein, comprising a carboxy-terminal biologically active fragment of $p110^{RB}$ of 56 kDa and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition of claim 7, wherein the fragment comprises amino acid 379 to amino acid 928 of the amino acid sequence of Figure 8.

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Figures: fig 1- 2A TO 2F

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Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)

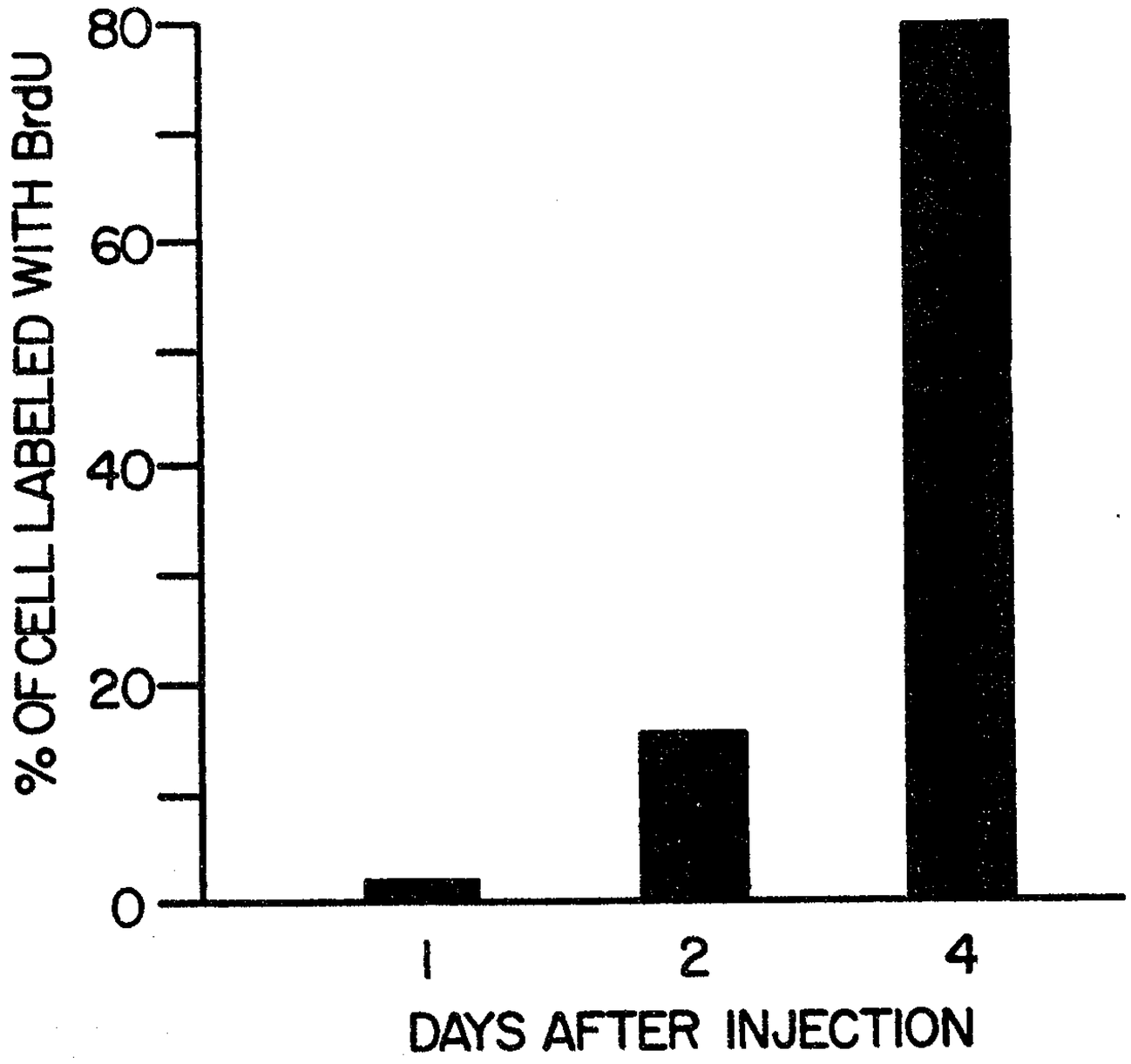
% OF CELL LABELED WITH BrdU
80
60
40
20
0
1
2
4
DAYS AFTER INJECTION

FIG. 3

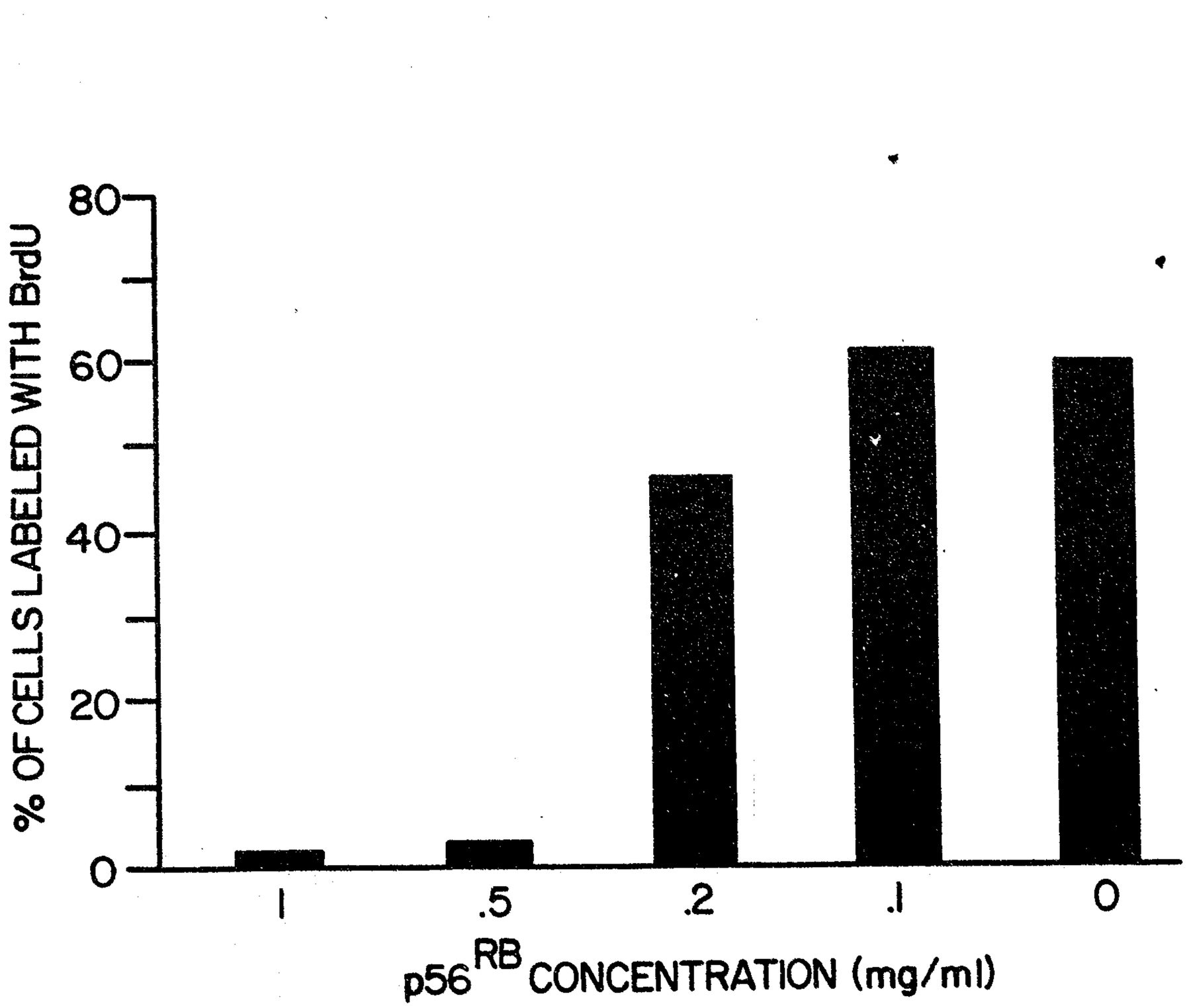
% OF CELLS LABELED WITH BrdU
80
60
40
20
0
1
.5
.2
.1
0
p56RB CONCENTRATION (mg/ml)

FIG. 4

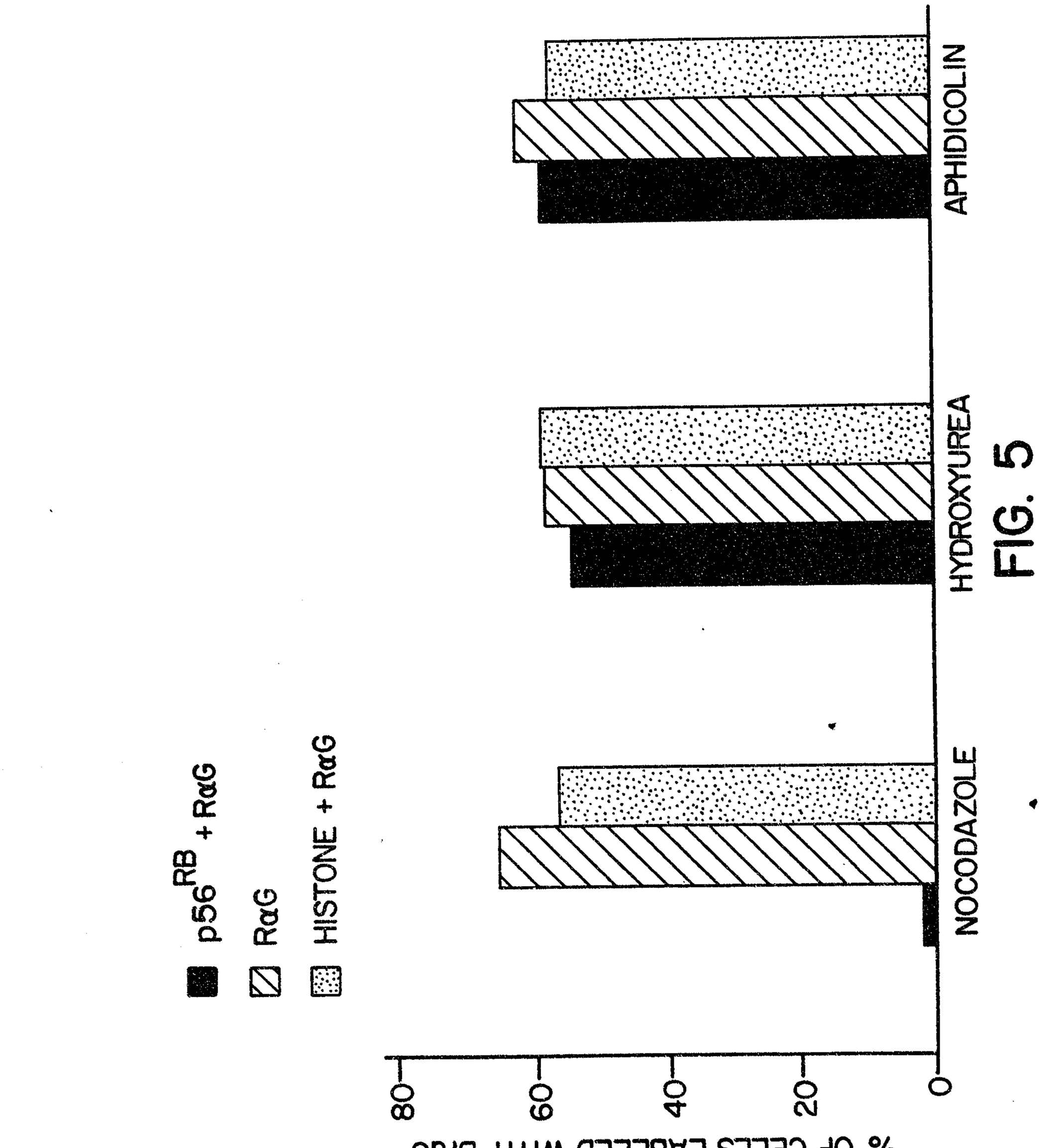
% OF CELLS LABELED WITH BrdU
80
60
40
20
0
NOCODAZOLE
HYDROXYUREA
APHIDICOLIN
p56RB + RαG
RαG
HISTONE + RαG

FIG. 5

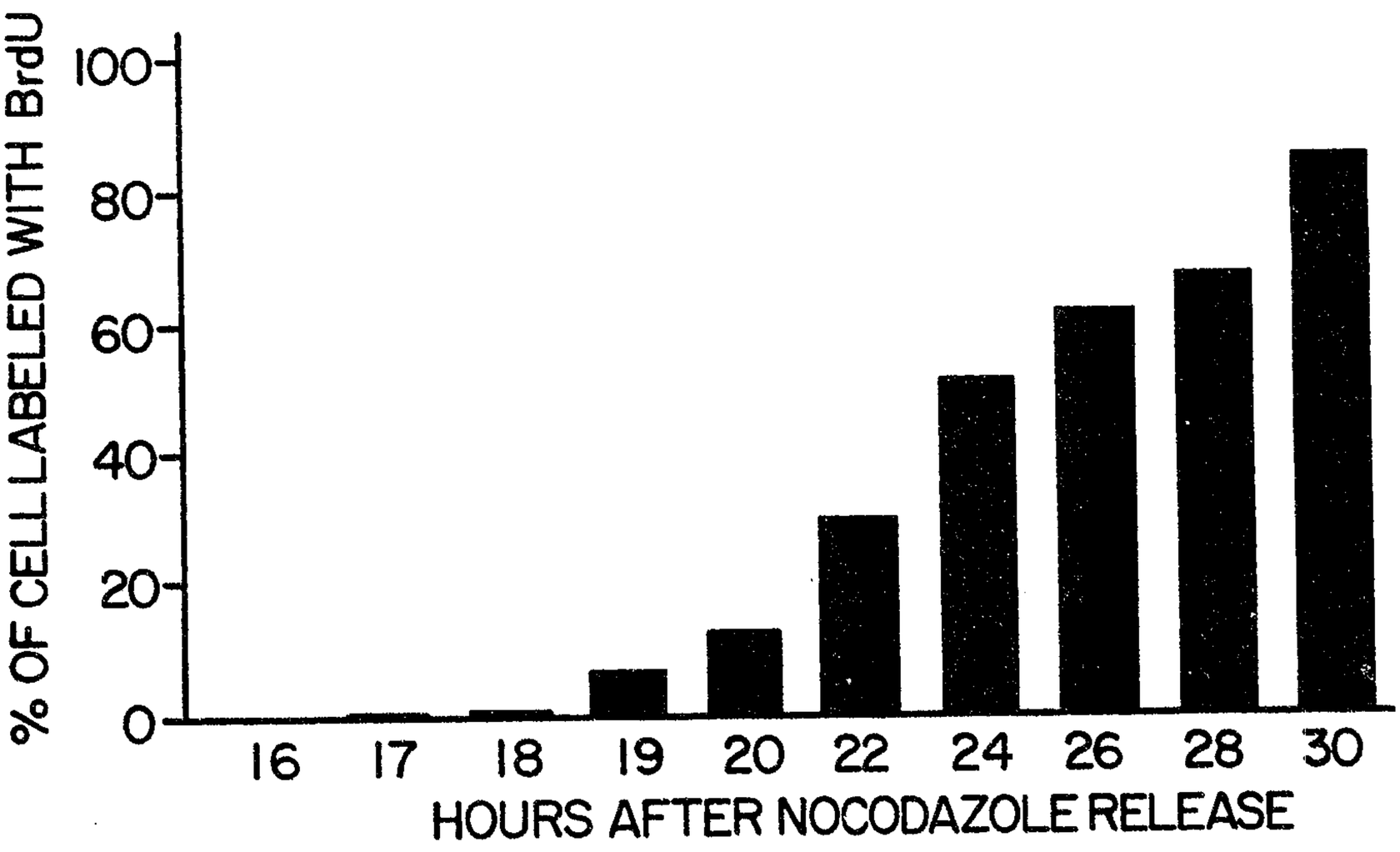

FIG. 6A

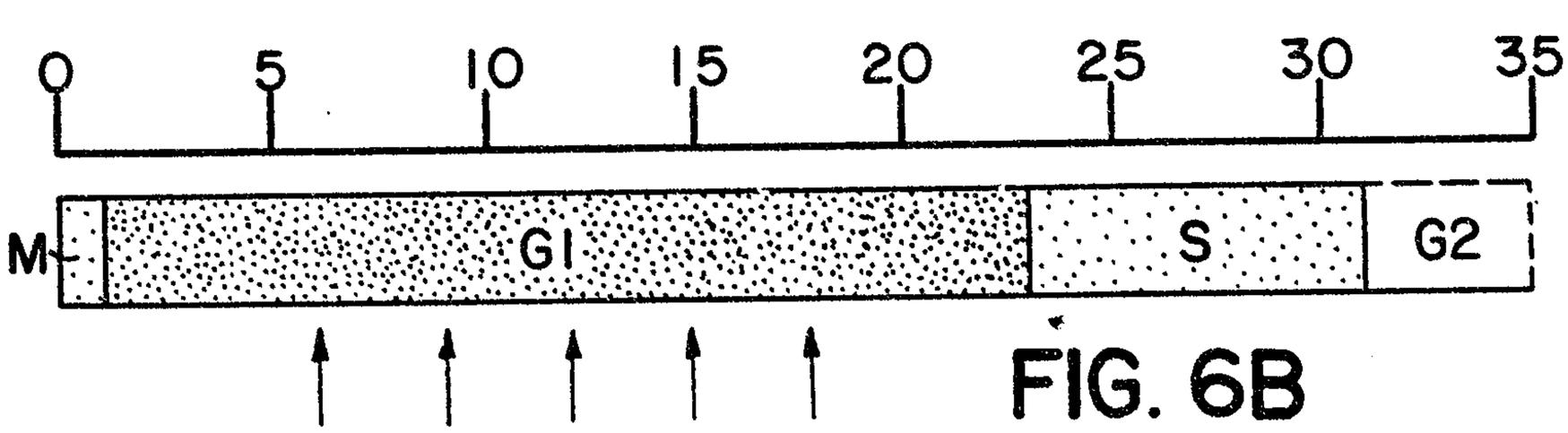

FIG. 6B

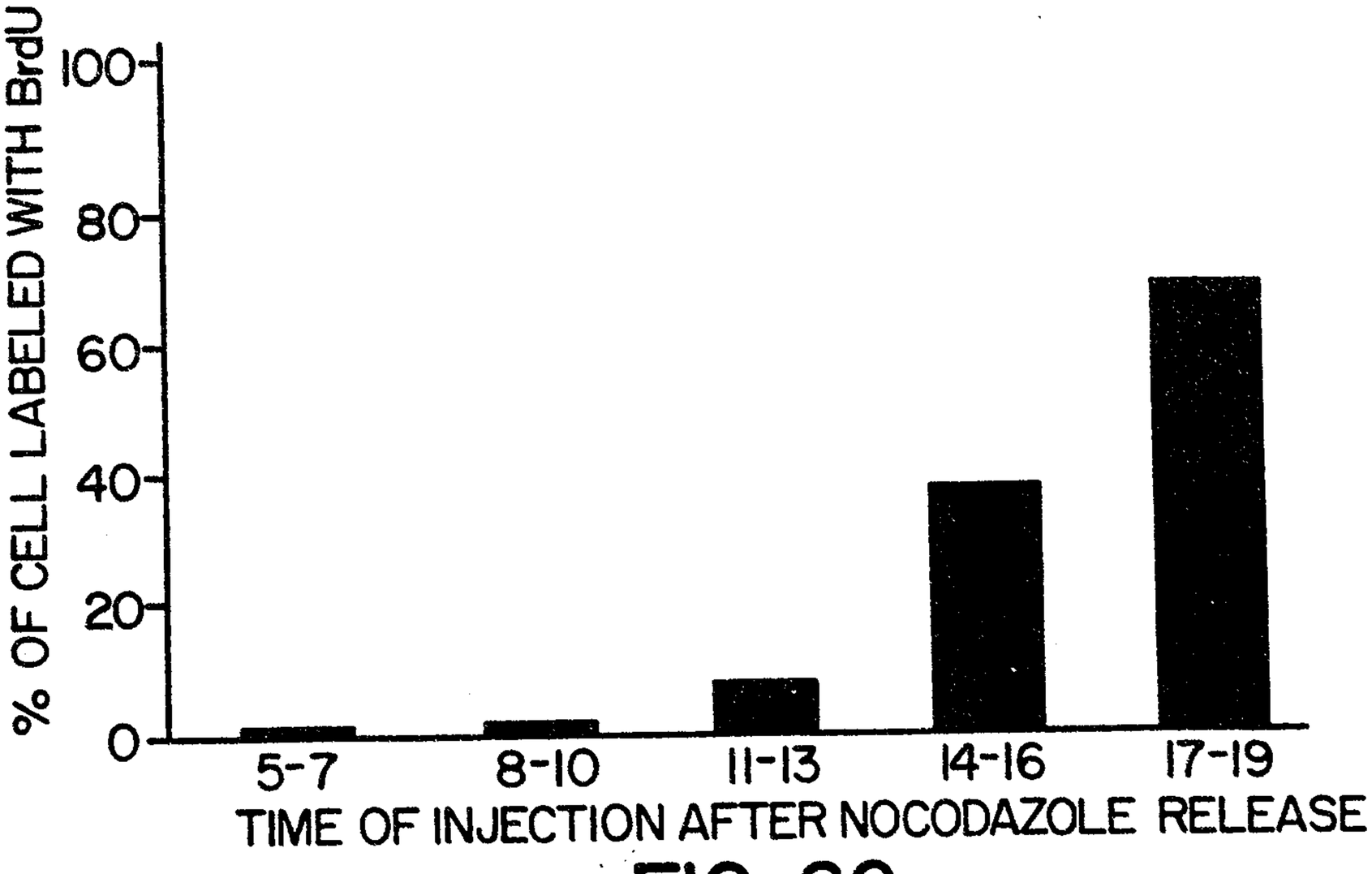

FIG. 6C

```
TTCCGGTTTTTCTCAGGGGACGTTGAAATTATTTTTGTAACGGGAGTCGGGAGAGGACGG

CCGCCGCGGAAAGGCGTCATGCCGCCCAAAACCCCCCGAAAAACGGCCGCCACCGCCGCC
                      M  P  P  K  T  P  R  K  T  A  A  T  A  A

CAGGACAGCGGCCCCGGAGGACCTGCCTCTCGTCAGGCTTGAGTTTGAAGAAACAGAAGAA
 Q  D  S  G  P  E  D  L  P  L  V  R  L  E  F  E  E  T  E  E

TGGTTAACTTGGGAGAAAGTTTCATCTGTGGATGGAGTATTGGGAGGTTATATTCAAAAG
 W  L  T  W  E  K  V  S  S  V  D  G  V  L  G  G  Y  I  Q  K

TTCACTTTTACTGAGCTACAGAAAAACATAGAAATCAGTGTCCATAAATTCTTTAACTTA
 F  T  F  T  E  L  Q  K  N  I  E  I  S  V  H  K  F  F  N  L

TATGATGTATTGTTTGCACTCTTCAGCAAATTGGAAAGGACATGGGAACTTATATATTTG
 Y  D  V  L  F  A  L  F  S  K  L  E  P  T  C  E  L  I  Y  L

TGGATCACATTTTTATTAGCTAAAGGGGAAGTATTACAAATGGAAGATGATCTGGTGATT
 W  I  T  F  L  L  A  K  G  E  V  L  Q  M  E  D  D  L  V  I
```

FIG. 7A-1

```
CTCAAAGAACCATATAAAACAGCTGTTATACCCATTAATGGTTCACCTCGAACACCCAGG
 L  K  E  P  Y  K  T  A  V  I  P  I  N  G  S  P  R  T  P  R

GAAGTTCTCTGTAAAGAACATGAATGTAATATAGATGAGGTGAAAAATGTTTATTTCAAA
 E  V  L  C  K  E  H  E  C  N  I  D  E  V  K  N  V  Y  F  K

GAAAATCTTTCTAAACGATACGAAGAAATTTATCTTAAAAATAAAGATCTAGATGCAAGA
 E  N  L  S  K  R  Y  E  E  I  Y  L  K  N  K  D  L  D  A  R

AGAACACCACGAAAAAGTAACCTTGATGAAGAGGTGAATGTAATTCCTCCACACACTCCA
 R  T  P  R  K  S  N  L  D  E  E  V  N  V  I  P  P  H  T  P

CAACCTTCAGAAAATCTGATTTCCTATTTTAACAACTGCACAGTGAATCCAAAAGAAAGT
 Q  P  S  E  N  L  I  S  Y  F  N  N  C  T  V  N  P  K  E  S

GGACAGGGTTGTGTCGAAATTGGATCACAGCGATACAAACTTGGAGTTCGCTTGTATTAC
 G  Q  G  C  V  E  I  G  S  Q  R  Y  K  L  G  V  R  L  Y  Y
```

FIG. 7A-2

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```
GGCGTGCCCCGCGTGCGCGCGCGTCGTCCTCCCCGGCGCTCCTCCACAGCTCGCTGGCTC  120

GCTGCCGCCGCGGAACCCCCGGCACCGCCGCCGCCGCCCCCTCCGTAGGAGGACCCAGAG  240
 A  A  A  A  E  P  P  A  P  P  P  P  P  P  P  E  E  D  P  E  (34)

CCTGATTTTACTGCATTATGTCAGAAATTAAAGATACCAGATCATGTCAGAGAGAGAGCT  360
 P  D  F  T  A  L  C  Q  K  L  K  I  P  D  H  V  R  E  R  A  (74)

AAAAAGGAACTGTGGGGAATCTGTATCTTTATTGCAGCAGTTGACCTAGATGAGATGTCG  480
 K  K  E  L  W  G  I  C  I  F  I  A  A  V  D  L  D  E  M  S  (114)

CTAAAAGAAATTGATACCAGTACCAAAGTTGATAATGCTATGTCAAGACTGTTGAAGAAG  600
 L  K  E  I  D  T  S  T  K  V  D  N  A  M  S  R  L  L  K  K  (154)

ACACAACCCAGCAGTTCGATATCTACTGAAATAAATTCTGCATTGGTGCTAAAAGTTTCT  720
 T  Q  P  S  S  S  I  S  T  E  I  N  S  A  L  V  L  K  V  S  (194)

TCATTTCAGTTAATGCTATGTGTCCTTGACTATTTTATTAAACTCTCACCTCCCATGTTG  840
 S  F  Q  L  M  L  C  V  L  D  Y  F  I  K  L  S  P  P  M  L  (234)
```

FIG. 7B-1

```
CGAGGTCAGAACAGGAGTGCACGGATAGCAAAACAACTAGAAAATGATACAAGAATTATT  960
 R  G  Q  N  R  S  A  R  I  A  K  Q  L  E  N  D  T  R  I  I  (274)

AATTTTATACCTTTTATGAATTCTCTTGGACTTGTAACATCTAATGGACTTCCAGAGGTT 1080
 N  F  I  P  F  M  N  S  L  G  L  V  T  S  N  G  L  P  E  V  (314)

TTATTTTTGGATCATGATAAAACTCTTCAGACTGATTCTATAGACAGTTTTGAAACACAG 1200
 L  F  L  D  H  D  K  T  L  Q  T  D  S  I  D  S  F  E  T  Q  (354)

GTTAGGACTGTTATGAACACTATCCAACAATTAATGATGATTTTAAATTCAGCAAGTGAT 1320
 V  R  T  V  M  N  T  I  Q  Q  L  M  M  I  L  N  S  A  S  D  (394)

ATACTGAAAAGAGTGAAGGATATAGGATACATCTTTAAAGAGAAATTTGCTAAAGCTGTG 1440
 I  L  K  R  V  K  D  I  G  Y  I  F  K  E  K  F  A  K  A  V  (434)

CGAGTAATGGAATCCATGCTTAAATCAGAAGAAGAACGATTATCCATTCAAAATTTTAGC 1360
 R  V  M  E  S  M  L  K  S  E  E  E  R  L  S  I  Q  N  F  S  (474)
```

FIG. 7B-2

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```
AAACTTCTGAATGACAACATTTTTCATATGTCTTTATTGGCGTGCGCTCTTGAGGTTGTA
 K  L  L  N  D  N  I  F  H  M  S  L  L  A  C  A  L  E  V  V

CCATGGATTCTGAATGTGCTTAATTTAAAAGCCTTTGATTTTTACAAAGTGATCGAAAGT
 P  W  I  L  N  V  L  N  L  K  A  F  D  F  Y  K  V  I  E  S

CATCGAATCATGGAATCCCTTGCATGGCTCTCAGATTCACCTTTATTTGATCTTATTAAA
 H  R  I  M  E  S  L  A  W  L  S  D  S  P  L  F  D  L  I  K

CCTCTCCAGAATAATCACACTGCAGCAGATATGTATCTTTCTCCTGTAAGATCTCCAAAG
 P  L  Q  N  M  H  T  A  A  D  M  Y  L  S  P  V  R  S  P  K

GCCTTCCAGACCCAGAAGCCATTGAAATCTACCTCTCTTTCACTGTTTTATAAAAAAGTG
 A  F  Q  T  Q  K  P  L  K  S  T  S  L  S  L  F  Y  K  K  V

GAATTAGAACATATCATCTGGACCCTTTTCCAGCACACCCTGCAGAATGAGTATGAACTC
 E  L  E  H  I  I  W  T  L  F  Q  H  T  L  Q  N  E  Y  E  L
```

FIG. 7C-1

```
AAGAATATAGACCTTAAATTCAAAATCATTGTAACAGCATACAAGGATCTTCCTCATGCT
 K  N  I  D  L  K  F  K  I  I  V  T  A  Y  K  D  L  P  H  A

TTCTATAACTCGGTCTTCATGCAGAGACTGAAAACAAATATTTTGCAGTATGCTTCCACC
 F  Y  N  S  V  F  M  Q  R  L  K  T  N  I  L  Q  Y  A  S  T

TCACCCTTACGGATTCCTGGAGGGAACATCTATATTTCACCCCTGAAGAGTCCATATAAA
 S  P  L  R  I  P  G  G  N  I  Y  I  S  P  L  K  S  P  Y  K

ATTGGTGAATCATTCGGGACTTCTGAGAAGTTCCAGAAAATAAATCAGATGGTATGTAAC
 I  G  E  S  F  G  T  S  E  K  F  Q  K  I  N  Q  M  V  C  N

CTACGCTTTGATATTGAAGGATCAGATGAAGCAGATGGAAGTAAACATCTCCCAGGAGAG
 L  R  F  D  I  E  G  S  D  E  A  D  G  S  K  H  L  P  G  E

AAAATGAATGATAGCATGGATACCTCAAACAAGGAAGAGAAATGAGGATCTCAGGACCTT
 K  M  N  D  S  M  D  T  S  N  K  E  E  K
```

FIG. 7C-2

2120353

PCT/US92/08918

```
ATGGCCACATATAGCAGAAGTACATCTCAGAATCTTGATTCTGGAACAGATTTGTCTTTC 1680
 M  A  T  Y  S  R  S  T  S  Q  N  L  D  S  G  T  D  L  S  F  (514)

TTTATCAAAGCAGAAGGCAACTTGACAAGAGAAATGATAAAACATTTAGAACGATGTGAA 1800
 F  I  K  A  E  G  N  L  T  R  E  M  I  K  H  L  E  R  C  E  (554)

CAATCAAAGGACCGAGAAGGACCAACTGATCACCTTGAATCTGCTTGTCCTCTTAATCTT 1920
 Q  S  K  D  R  E  G  P  T  D  H  L  E  S  A  C  P  L  N  L  (594)

AAAAAAGGTTCAACTACGCGTGTAAATTCTACTGCAAATGCAGAGACACAAGCAACCTCA 2040
 K  K  G  S  T  T  R  V  N  S  T  A  N  A  E  T  Q  A  T  S  (634)

TATCGGCTAGCCTATCTCCGGCTAAATACACTTTGTGAACGCCTTCTGTCTGAGCACCCA 2160
 Y  R  L  A  Y  L  R  L  N  T  L  C  E  R  L  L  S  E  H  P  (674)

ATGAGAGACAGGCATTTGGACCAAATTATGATGTGTTCCATGTATGGCATATGCAAAGTG 2280
 M  R  D  R  H  L  D  Q  I  M  M  C  S  M  Y  G  I  C  K  V  (714)
```

FIG. 7D-1

2120353

PCT/US92/08918

```
GTTCAGGAGACATTCAAACGTGTTTTGATCAAAGAAGAGGAGTATGATTCTATTATAGTA 2400
 V  Q  E  T  F  K  R  V  L  I  K  E  E  E  Y  D  S  I  I  V  (754)

AGGCCCCCTACCTTGTCACCAATACCTCACATTCCTCGAAGCCCTTACAAGTTTCCTAGT 2520
 R  P  P  T  L  S  P  I  P  H  I  P  R  S  P  Y  K  F  P  S  (794)

ATTTCAGAAGGTCTGCCAACACCAACAAAAATGACTCCAAGATCAAGAATCTTAGTATCA 2640
 I  S  E  G  L  P  T  P  T  K  M  T  P  R  S  R  I  L  V  S  (834)

AGCGACCGTGTGCTCAAAAGAAGTGCTGAAGGAAGCAACCCTCCTAAACCACTGAAAAAA 2760
 S  D  R  V  L  K  R  S  A  E  G  S  N  P  P  K  P  L  K  K  (874)

TCCAAATTTCAGCAGAAACTGGCAGAAATGACTTCTACTCGAACACGAATGCAAAAGCAG 2880
 S  K  F  Q  Q  K  L  A  E  M  T  S  T  R  T  R  M  Q  K  Q  (914)

GGTGGACACTGTGTACACCTCTGGATTCATTGTCTCTCACAGATGTGACTGTAT... to 4757
                                                             (928)
```

FIG. 7D-2

```
                                M  P  P  K  T  P  R  K  T  A  A  T  A  A  A  A  A  A  E  P  P  A  P  P  P  P  P  P  P  E  E  D  P  E  ( 34)
Q  D  S  G  P  E  D  L  P  L  V  R  L  E  F  E  E  T  E  E  P  D  F  T  A  L  C  Q  K  L  K  I  P  D  M  V  R  E  R  A  ( 74)
W  L  T  W  E  K  V  S  S  V  D  G  V  L  G  G  Y  I  Q  K  K  K  E  L  W  G  I  C  I  F  I  A  A  V  D  L  D  E  M  S  (114)
F  T  F  T  E  L  Q  K  N  I  E  I  S  V  H  K  F  F  N  L  L  K  E  I  D  T  S  T  K  V  D  N  A  M  S  R  L  L  K  K  (154)
Y  D  V  L  F  A  L  F  S  K  L  E  R  T  C  E  L  I  Y  L  T  Q  P  S  S  S  I  S  T  E  I  N  S  A  L  V  L  K  V  S  (194)
W  I  T  F  L  L  A  K  G  E  V  L  Q  M  E  D  D  L  V  I  S  F  Q  L  M  L  C  V  L  D  Y  F  I  K  L  S  P  P  M  L  (234)
L  K  E  P  Y  K  T  A  V  I  P  I  N  G  S  P  R  T  P  R  R  G  Q  M  R  S  A  R  I  A  K  Q  L  E  N  D  T  R  I  I  (274)
E  V  L  C  K  E  H  E  C  N  I  D  E  V  K  N  V  Y  F  K  N  F  I  P  F  M  N  S  L  G  L  V  T  S  N  G  L  P  E  V  (314)
E  N  L  S  K  R  Y  E  E  I  Y  L  K  N  K  D  L  D  A  R  L  F  L  D  H  D  K  T  L  Q  T  D  S  I  D  S  F  E  T  Q  (354)
R  T  P  R  K  S  N  L  D  E  E  V  N  V  I  P  P  H  T  P  V  R  T  V  M  N  T  I  Q  Q  L  M  M  I  L  N  S  A  S  D  (394)
Q  P  S  E  N  L  I  S  Y  F  N  N  C  T  V  N  P  K  E  S  I  L  K  R  V  K  D  I  G  Y  I  F  K  E  K  F  A  K  A  V  (434)
G  Q  G  C  V  E  I  G  S  Q  R  Y  K  L  G  V  R  L  Y  Y  R  V  M  E  S  M  L  K  S  E  E  E  R  L  S  I  Q  N  F  S  (474)
K  L  L  N  D  N  I  F  N  M  S  L  L  A  C  A  L  E  V  V  M  A  T  Y  S  R  S  T  S  Q  N  L  D  S  G  T  D  L  S  F  (514)
P  W  I  L  N  V  L  N  L  K  A  F  D  F  Y  K  V  I  E  S  F  I  K  A  E  G  N  L  T  R  E  M  I  K  H  L  E  R  C  E  (554)
H  R  I  M  E  S  L  A  W  L  S  D  S  P  L  F  D  L  I  K  Q  S  K  D  R  E  G  P  T  D  H  L  E  S  A  C  P  L  N  L  (594)
P  L  Q  N  N  H  T  A  A  D  M  Y  L  S  P  V  R  S  P  K  K  K  G  S  T  T  R  V  N  S  T  A  N  A  E  T  Q  A  T  S  (634)
A  F  Q  T  Q  K  P  L  K  S  T  S  L  S  L  F  Y  K  K  V  Y  R  L  A  Y  L  R  L  N  T  L  C  E  R  L  L  S  E  N  P  (674)
E  L  E  M  I  I  W  T  L  F  Q  H  T  L  Q  N  E  Y  E  L  M  R  D  R  H  L  D  Q  I  M  M  C  S  M  Y  G  I  C  K  V  (714)
K  N  I  D  L  K  F  K  I  I  V  T  A  Y  K  D  L  P  H  A  V  Q  E  T  F  K  R  V  L  I  K  E  E  E  Y  D  S  I  I  V  (754)
F  Y  N  S  V  F  M  Q  R  L  K  T  N  I  L  Q  Y  A  S  T  R  P  P  T  L  S  P  I  P  H  I  P  R  S  P  Y  K  F  P  S  (794)
S  P  L  R  I  P  G  G  N  I  Y  I  S  P  L  K  S  P  Y  K  I  S  E  G  L  P  T  P  T  K  N  T  P  R  S  R  I  L  V  S  (834)
I  G  E  S  F  G  T  S  E  K  F  Q  K  I  N  Q  M  V  C  N  S  D  R  V  L  K  R  S  A  E  G  S  N  P  P  K  P  L  K  K  (874)
L  R  F  D  I  E  G  S  D  E  A  D  G  S  K  N  L  P  G  E  S  K  F  Q  Q  K  L  A  E  M  T  S  T  R  T  R  M  Q  K  Q  (914)
K  M  N  D  S  M  D  T  S  N  K  E  E  K                                                                                (928)
                                   |
                                   FRAGMENT (p56RB)
```

FIG. 8

```
TTCCGGTTTT TCTCAGGGGA CGTTGAAATT ATTTTTGTAA CGGGAGTCGG GAGAGGACGG      60

GGCGTGCCCC GCGTGCGCGC GCGTCGTCCT CCCCGGCGCT CCTCCACAGC TCGCTGGCTC     120

CCGCCGCGGA AAGGCGTC ATG CCG CCC AAA ACC CCC CGA AAA ACG GCC GCC        171
                       Met Pro Pro Lys Thr Pro Arg Lys Thr Ala Ala
                         1               5                  10

ACC GCC GCC GCT GCC GCC GCG GAA CCC CCG GCA CCG CCG CCG CCG CCC        219
Thr Ala Ala Ala Ala Ala Ala Glu Pro Pro Ala Pro Pro Pro Pro Pro
               15                  20                  25

CCT CCG TAG GAG GAC CCA GAG CAG GAC AGC GGC CCG GAG GAC CTG CCT        267
Pro Pro Glu Glu Asp Pro Glu Gln Asp Ser Gly Pro Glu Asp Leu Pro
         30                  35                  40

CTC GTC AGG CTT GAG TTT GAA GAA ACA GAA GAA CCT GAT TTT ACT GCA        315
Leu Val Arg Leu Glu Phe Glu Glu Thr Glu Glu Pro Asp Phe Thr Ala
      45                  50                  55

TTA TGT CAG AAA TTA AAG ATA CCA GAT CAT GTC AGA GAG AGA GCT TGG        363
Leu Cys Gln Lys Leu Lys Ile Pro Asp His Val Arg Glu Arg Ala Trp
  60                  65                  70                  75
```

FIG. 9-I

```
TTA ACT TGG GAG AAA GTT TCA TCT GTG GAT GGA GTA TTG GGA GGT TAT      411
Leu Thr Trp Glu Lys Val Ser Ser Val Asp Gly Val Leu Gly Gly Tyr
                    80                  85                  90

ATT CAA AAG AAA AAG GAA CTG TGG GGA ATC TGT ATC TTT ATT GCA GCA      459
Ile Gln Lys Lys Lys Glu Leu Trp Gly Ile Cys Ile Phe Ile Ala Ala
                95                 100                 105

GTT GAC CTA GAT GAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA AAC      507
Val Asp Leu Asp Glu Met Ser Phe Thr Phe Thr Glu Leu Gln Lys Asn
        110                 115                 120

ATA GAA ATC AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT GAT      555
Ile Glu Ile Ser Val His Lys Phe Phe Asn Leu Leu Lys Glu Ile Asp
    125                 130                 135

ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG TAT      603
Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys Lys Tyr
140                 145                 150                 155

GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA CTT      651
Asp Val Leu Phe Ala Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu Leu
                160                 165                 170
```

FIG. 9-2

```
ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT TCT        699
Ile Tyr Leu Thr Gln Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn Ser
            175                 180                 185

GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA GGG        747
Ala Leu Val Leu Lys Val Ser Trp Ile Thr Phe Leu Leu Ala Lys Gly
        190                 195                 200

GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA ATG        795
Glu Val Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln Leu Met
    205                 210                 215

CTA TGT GTC CTT GAC TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC        843
Leu Cys Val Leu Asp Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu Leu
220                 225                 230                 235

AAA GAA CCA TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA        891
Lys Glu Pro Tyr Lys Thr Ala Val Ile Pro Ile Asn Gly Ser Pro Arg
                240                 245                 250

ACA CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA CTA        939
Thr Pro Arg Arg Gly Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln Leu
            255                 260                 265
```

FIG. 9-3

2120353

```
GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA TGT          987
Glu Asn Asp Thr Arg Ile Ile Glu Val Leu Cys Lys Glu His Glu Cys
        270                 275                 280

AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT TTT         1035
Asn Ile Asp Glu Val Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro Phe
    285                 290                 295

ATG AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA GAG GTT GAA         1083
Met Asn Ser Leu Gly Leu Val Thr Ser Asn Gly Leu Pro Glu Val Glu
300                 305                 310                 315

AAT CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT CTA         1131
Asn Leu Ser Lys Arg Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp Leu
                320                 325                 330

GAT GCA AGA TTA TTT TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT TCT         1179
Asp Ala Arg Leu Phe Leu Asp His Asp Lys Thr Leu Gln Thr Asp Ser
            335                 340                 345

ATA GAC AGT TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT GAT         1227
Ile Asp Ser Phe Glu Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu Asp
        350                 355                 360
```

FIG. 9-4

```
GAA GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT ATG      1275
Glu Glu Val Asn Val Ile Pro Pro His Thr Pro Val Arg Thr Val Met
    365                 370                 375

AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA AGT GAT CAA      1323
Asn Thr Ile Gln Gln Leu Met Met Ile Leu Asn Ser Ala Ser Asp Gln
380                 385                 390                 395

CCT TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC TGC ACA GTG AAT CCA      1371
Pro Ser Glu Asn Leu Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn Pro
                400                 405                 410

AAA GAA AGT ATA CTG AAA AGA GTG AAG GAT ATA GGA TAC ATC TTT AAA      1419
Lys Glu Ser Ile Leu Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe Lys
            415                 420                 425

GAG AAA TTT GCT AAA GCT GTG GGA CAG GGT TGT GTC GAA ATT GGA TCA      1467
Glu Lys Phe Ala Lys Ala Val Gly Gln Gly Cys Val Glu Ile Gly Ser
        430                 435                 440
```

FIG.9-5

2120353

PCT/US92/08918

```
CAG CGA TAC AAA CTT GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC      1515
Gln Arg Tyr Lys Leu Gly Val Arg Leu Tyr Tyr Arg Val Met Glu Ser
    445                 450                 455

ATG CTT AAA TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA      1563
Met Leu Lys Ser Glu Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser Lys
460                 465                 470                 475

CTT CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT CTT      1611
Leu Leu Asn Asp Asn Ile Phe His Met Ser Leu Leu Ala Cys Ala Leu
                480                 485                 490

GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG AAT CTT GAT      1659
Glu Val Val Met Ala Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu Asp
            495                 500                 505

TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT TTA      1707
Ser Gly Thr Asp Leu Ser Phe Pro Trp Ile Leu Asn Val Leu Asn Leu
        510                 515                 520
```

FIG. 9-6

2120353

PCT/US92/08918

```
AAA GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA GAA      1755
Lys Ala Phe Asp Phe Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala Glu
    525                 530                 535

GGC AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA CAT      1803
Gly Asn Leu Thr Arg Glu Met Ile Lys His Leu Glu Arg Cys Glu His
540                 545                 550                 555

CGA ATC ATG GAA TCC CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT      1851
Arg Ile Met Glu Ser Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe Asp
                560                 565                 570

CTT ATT AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA      1899
Leu Ile Lys Gln Ser Lys Asp Arg Glu Gly Pro Thr Asp His Leu Glu
            575                 580                 585

TCT GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA GCA      1947
Ser Ala Cys Pro Leu Asn Leu Pro Leu Gln Asn Asn His Thr Ala Ala
        590                 595                 600

GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA GGT TCA ACT      1995
Asp Met Tyr Leu Ser Pro Val Arg Ser Pro Lys Lys Lys Gly Ser Thr
    605                 610                 615
```

FIG. 9-7

2120353

```
ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA GCC      2043
Thr Arg Val Asn Ser Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser Ala
620                 625                 630                 635

TTC CAG ACC CAG AAG CCA TTG AAA TCT ACC TCT CTT TCA CTG TTT TAT      2091
Phe Gln Thr Gln Lys Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe Tyr
                640                 645                 650

AAA AAA GTG TAT CGG CTA GCC TAT CTC CGG CTA AAT ACA CTT TGT GAA      2139
Lys Lys Val Tyr Arg Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys Glu
            655                 660                 665

CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT      2187
Arg Leu Leu Ser Glu His Pro Glu Leu Glu His Ile Ile Trp Thr Leu
        670                 675                 680

TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT      2235
Phe Gln His Thr Leu Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His
    685                 690                 695
```

FIG. 9-8

```
TTG GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG AAG          2283
Leu Asp Gln Ile Met Met Cys Ser Met Tyr Gly Ile Cys Lys Val Lys
700                 705                 710                 715

AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT CTT          2331
Asn Ile Asp Leu Lys Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp Leu
                720                 725                 730

CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT TTG ATC AAA GAA GAG          2379
Pro His Ala Val Gln Glu Thr Phe Lys Arg Val Leu Ile Lys Glu Glu
            735                 740                 745

GAG TAT GAT TCT ATT ATA GTA TTC TAT AAC TCG GTC TTC ATG CAG AGA          2427
Glu Tyr Asp Ser Ile Ile Val Phe Tyr Asn Ser Val Phe Met Gln Arg
        750                 755                 760

CTG AAA ACA AAT ATT TTG CAG TAT GCT TCC ACC AGG CCC CCT ACC TTG          2475
Leu Lys Thr Asn Ile Leu Gln Tyr Ala Ser Thr Arg Pro Pro Thr Leu
    765                 770                 775
```

FIG. 9-9

2120353

PCT/US92/08918

```
TCA CCA ATA CCT CAC ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA      2523
Ser Pro Ile Pro His Ile Pro Arg Ser Pro Tyr Lys Phe Pro Ser Ser
780                 785                 790                 795

CCC TTA CGG ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT      2571
Pro Leu Arg Ile Pro Gly Gly Asn Ile Tyr Ile Ser Pro Leu Lys Ser
                800                 805                 810

CCA TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT CCA      2619
Pro Tyr Lys Ile Ser Glu Gly Leu Pro Thr Pro Thr Lys Met Thr Pro
            815                 820                 825

AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG ACT TCT GAG      2667
Arg Ser Arg Ile Leu Val Ser Ile Gly Glu Ser Phe Gly Thr Ser Glu
        830                 835                 840

AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC AGC GAC CGT GTG CTC      2715
Lys Phe Gln Lys Ile Asn Gln Met Val Cys Asn Ser Asp Arg Val Leu
    845                 850                 855

AAA AGA AGT GCT GAA GGA AGC AAC CCT CCT AAA CCA CTG AAA AAA CTA      2763
Lys Arg Ser Ala Glu Gly Ser Asn Pro Pro Lys Pro Leu Lys Lys Leu
860                 865                 870                 875
```

FIG. 9-10

```
CGC TTT GAT ATT GAA GGA TCA GAT GAA GCA GAT GGA AGT AAA CAT CTC          2811
Arg Phe Asp Ile Glu Gly Ser Asp Glu Ala Asp Gly Ser Lys His Leu
                880                 885                 890

CCA GGA GAG TCC AAA TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT          2859
Pro Gly Glu Ser Lys Phe Gln Gln Lys Leu Ala Glu Met Thr Ser Thr
            895                 900                 905

CGA ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA          2907
Arg Thr Arg Met Gln Lys Gln Lys Met Asn Asp Ser Met Asp Thr Ser
        910                 915                 920

AAC AAG GAA GAG AAA TGAGGATCTC AGGACCTTGG TGGACACTGT GTACACCTCT          2962
Asn Lys Glu Glu Lys
    925

GGATTCATTG TCTCTCACAG ATGTGACTGT AT                                      2994
```

FIG. 9-11

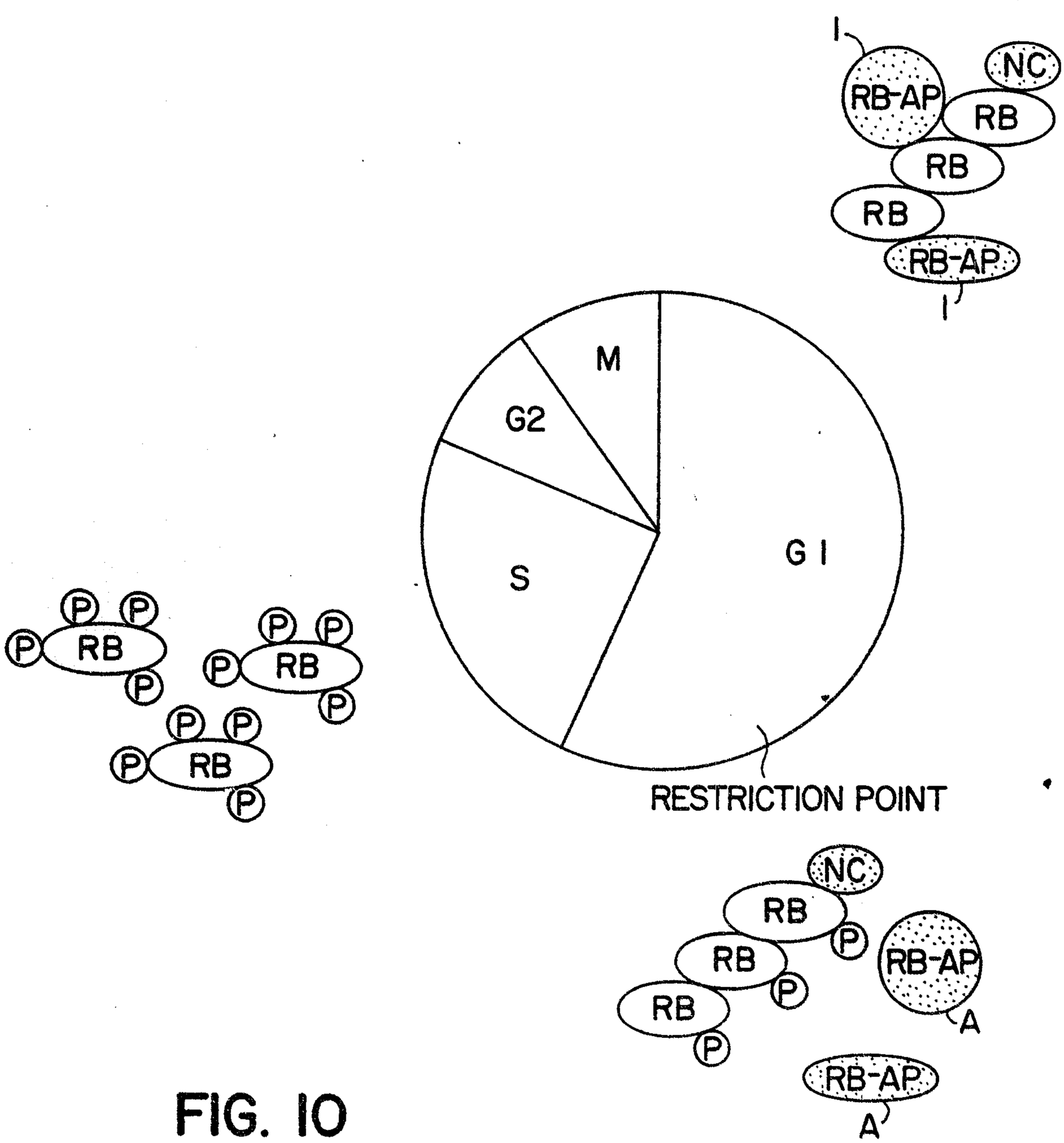
NC
RB-AP
RB
RB
RB
RB-AP
M
G2
S
G1
RESTRICTION POINT
P
RB
NC
RB
RB
RB
RB-AP
A
RB-AP
A

FIG. 10