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(54) Title: β-CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER

(57) Abstract: A recombinant adenovirus (Ad-Mini-ME) which constitutively expresses the central third of APC includes all of the known β-catenin binding repeats. When expressed in colon cancer cells, Ad-Mini-ME blocked the nuclear translocation of β-catenin and inhibited β-catenin/Tcf-4-mediated transactivation. Accordingly, expression of endogenous targets of the APC/β-catenin/Tcf-4 pathway were down-regulated. Ad-Mini-ME infection of colorectal cancer cell lines with mutant *APC* but wild-type β-catenin resulted in substantial growth arrest followed by apoptosis. These findings suggest that the β-catenin binding domain in the central third of APC is sufficient for its tumor suppresion activity.

β-CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grant CA57345 awarded by the National Institutes of Health.

This application is a continuation-in-part of application Serial No. 09/003,687 filed January 6, 1998, which is a division of application 08/821,355 filed March 20, 1997, now U.S. Patent No. 5,851,775.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the field of cancer diagnostics and therapeutics. More particularly it relates to methods for diagnosing and treating cancers associated with APC or β-catenin mutations.

BACKGROUND OF THE INVENTION

Mutations of the adenomatous polyposis coli (*APC)* gene are the most common disease-causing genetic events in humans; approximately 50% of the population will develop colorectal polyps initiated by such mutations during a normal life span (*14*). Individuals who inherit *APC* mutations develop thousands of colorectal tumors, consistent with APC's tumor suppressor or "gatekeeping" role in colorectal tumorigenesis (*15,16*). APC homodimerizes through its amino-terminus (*17*), and interacts with at least six other proteins: β-catenin (18), γ-catenin (plakoglobin) (*19*), tubulin (*20*), EB1 (*21*), hDLG, a homologue of a *Drosophila* tumor suppressor protein (*22*), and ZW3/GSK3β kinase (*23*). Whether any of these interacting proteins communicate APC

growth-controlling signals is unknown. Thus there is a need in the art for a fuller understanding of how the tumor suppressor gene APC functions in cells.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide human nucleotide sequences encoding transcriptional activation proteins.

It is another object of the present invention to provide isolated preparations of transcriptional activation proteins.

It is an object of the present invention to provide methods of determining the presence or absence in a cell of wild-type APC or a downstream protein in the APC transcription regulatory pathway.

It is an object of the present invention to provide a fusion protein useful for binding to β-catenin.

It is an object of the invention to provide a polypeptide useful for binding to β-catenin.

It is an object of the invention to provide a method for inhibiting the growth of a tumor cell.

It is an object of the invention to provide a method for inducing apoptosis of a tumor cell.

It is an object of the invention to provide an adenovirus vector useful for binding to β-catenin.

Another object of the invention is to provide methods of identifying candidate drugs for use in Familial Adenomatous Polyposis (FAP) patients or patients with increased risk of developing cancer.

It is yet another object of the invention to provide methods of identifying candidate drugs for the treatment of cancer patients, in particular those with *APC* or β-catenin mutations.

Another object of the invention is to provide a method for diagnosing cancer in a sample suspected of being neoplastic.

Another object of the invention is to provide a method for treating a patient with colorectal cancer or other cancer associated with FAP.

These and other objects of the invention are achieved by providing one or more of the embodiments described below. In one embodiment of the invention an intron-free DNA molecule is provided which encodes Tcf-4 protein as shown in SEQ ID NO: 2 or 4.

According to another embodiment of the invention an isolated Tcf-4 protein is provided. The protein is substantially free of other human proteins, and has a sequence as shown in SEQ ID NO: 2 or 4.

In another embodiment of the invention a method is provided for determining the presence or absence in a cell of wild-type APC or a downstream protein in the APC transcription regulatory pathway. The method comprises the steps of:

introducing a Tcf-responsive reporter gene into the cell; and

measuring transcription of said reporter gene; wherein a cell which supports active transcription of said reporter gene does not have wild-type APC or does not have a wild-type downstream protein in the APC transcription regulatory pathway.

According to yet another embodiment of the invention a method is provided for determining the presence or absence in a cell of wild-type APC. The method comprises the steps of:

contacting a Tcf-responsive reporter gene with a lysate of the cell; and

measuring transcription of said reporter gene; wherein a lysate which inhibits said transcription has wild-type APC.

In still another embodiment of the invention a method of identifying candidate drugs is provided. The drugs may be useful for treatment of FAP or other cancer patients or patients with increased risk of developing cancer. The method comprises the steps of:

contacting a cell having no wild-type APC or a mutant β-catenin with a test compound;

measuring transcription of a Tcf-responsive reporter gene, wherein a test compound which inhibits the transcription of the reporter gene is a candidate drug for cancer therapy.

According to yet another aspect of the invention another method is provided for identifying candidate drugs for use in for use in FAP patients, colon cancer patients, patients with mutations in β-catenin or *APC*, or patients with increased risk of developing cancer. The method, comprises the steps of:

contacting a Tcf-responsive reporter gene with a test compound under conditions in which the reporter gene is transcribed in the absence of the test compound; and

measuring transcription of the Tcf-responsive reporter gene; wherein a test compound which inhibits said transcription is a candidate drug for cancer therapy.

According to another aspect of the invention a method is provided for identifying candidate drugs for use in FAP patients or patients with increased risk of developing cancer. The method comprises the steps of:

contacting a test compound with β-catenin and Tcf-4 under conditions in which β-catenin and Tcf-4 bind to each other; and

determining whether the test compound inhibits the binding of β-catenin and Tcf-4, a test compound which inhibits the binding being a candidate for cancer therapy or prophylaxis.

According to still another embodiment of the invention a method is provided for diagnosing cancer in a sample suspected of being neoplastic. the method comprises the steps of:

comparing a CTNNB sequence found in the sample to a second CTNNB sequence found in a normal tissue, wherein a difference between the first and second sequence is an indicator of cancer.

According to one aspect of the invention a fusion protein is provided. It comprises an enzyme covalently linked to a portion of APC which comprises its β-catenin binding domain. A polynucleotide encoding the fusion protein is also provided.

According to another embodiment a polypeptide is provided. The polypeptide comprises a portion of APC which comprises its β-catenin binding domain. An isolated and purified polynucleotide encoding the polypeptide is also provided.

According to another aspect of the invention a method is provided for inhibiting the growth of a tumor cell. A polynucleotide which encodes a polypeptide comprising the β-catenin binding domain of APC is administered to the tumor cell, whereby growth of the tumor cell is inhibited.

According to another aspect of the invention a method is provided for inducing apoptosis of a tumor cell. A polynucleotide which encodes a polypeptide comprising the β-catenin binding domain of APC is administered to the tumor cell, whereby apoptosis of tumor cells is induced.

Also provided by the present invention is an adenovirus vector which comprises a polynucleotide encoding the β-catenin binding domain of APC.

According to another aspect of the invention a method is provided for treating a patient with colorectal cancer or other cancer associated with FAP. The method comprises the step of:

administering to the patient a nucleotide sequence comprising a portion of the APC coding sequence, said portion consisting of the β-catenin binding site.

According to another aspect of the invention a method is provided for treating a patient with colorectal cancer or other cancer associated with FAP. The method comprises the step of:

administering to the patient a polypeptide comprising a portion of the APC coding sequence, said portion consisting of the β-catenin binding site.

The present invention thus provides the art with diagnostic, therapeutic and drug discovery methods especially useful for FAP and other cancers with *APC* or β-catenin mutations.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Sequence comparison of hTcf-4 and hTcf-1.

Two alternative splice forms of *hTcf-4* were identified, each encoding a different COOH-terminus. One form (hTcf-4E; SEQ ID NO:6) was homologous to hTCF-lE (Fig. 1A) (7); the other form (hTcf-4B; SEQ ID NO: 5) was homologous to hTcf-1B (Fig. 1B). The highly conserved NH_2-terminal interaction domain and the High Mobility Group (HMG) box DNA-binding region are boxed. Abbreviations for the amino acids are: A. Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; IC, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; P, Ar g; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence has been deposited in GenBank (accession number:)

Figs. 2A, 2B, and 2C. Analysis of *hTcf-4* expression in colonic epithelium.

(Fig. 2A) Northern blot analysis of *hTcf-4, hTcf-1, hLef-I* expression in Jurkat T cells (lane 1); colonic mucosa (lane 2); colon carcinoma cell lines DLD-1 (lane 3), HCT116 (lane 4); SW480 (lane 5); SW620 (lane 6); HT29 (lane 7). Lane 2 contains 5 μg total RNA; all others contain 15 μg total RNA. The positions of 18S and 28S ribosomal RNAs are shown. EtBr, ethidium bromide stain. (Fig. 2B) In situ hybridization of healthy human colon tissue to an *hTcf-4* probe. (Fig. 2C) In situ hybridization to a negative control probe (a fragment of the *E. coli* neomycin resistance gene).

Figs. 3A, 3B. Transactivational properties of β-catenin/hTcf-4.

All reporter assays were performed as duplicate transfections. For each condition, both values are shown. (**Fig.** 3A) Reporter gene assays in IIA1.6 B cells. Cells were transfected by electroporation with 1 μg luciferase reporter plasmid, 5 **μg** β-catenin expression plasmid, and 3 II- hTcf-4 expression plasmids. Empty pCDNA was added to a total of 10 μg, plasmid DNA. (**Fig.** 3B) Reporter gene assays in SW480 colon carcinoma cells. Cells were transfected with 0.3 **μg,** of the indicated luciferase reporter gene, 0.7 **μg** pCATCONTROL as internal control, the indicated amounts of pCMVNeo-APC, and empty PCDNA to a total of 2.5 μg plasmid DNA. Control CAT values are given in the right panel.

Fig. 4. Constitutive presence of β-catenin-hTcf-4 complexes in $APC^{-/-}$ cells. Gel retardation assays were performed on nuclear extracts from the indicated cell lines before and after a 20-hour exposure to Zn^{++}. Samples in lanes 1, 4, 7, 10 were incubated under standard conditions. To the samples in lanes 2, 5, 8, 11, 0.25 μg, anti β-catenin was added. To the samples in lanes 3, *6,* 9, 12, 0.25 μg of a control (human CD4) antibody was added. N.S., nonspecific band also observed with mutant (nonbinding) probe (lane Mt).

Figs. 5A and 5B. Effects of *APC* mutations on catenin regulated transcription (CRT). (**Fig. 5A**) Schematics of wild-type (WT) and mutant APC. APC is a 2843-amino-acid (AA) protein (*32*) with contains armadillo (ARM) repeats in the amino-terminus (*33*), 15 and 20 AA β-catenin-binding repeats in the central region (*18,19*), and a basic region in the carboxyl-terminus (*32*). The carboxyl-terminus also contains a TXV sequence which mediates DLG binding (*22*). (**Fig. 5B**) Effects of WT and mutant APC on CRT. SW480 cells containing endogenous mutant *APC* were transfected with the APC expression vectors shown in (**Fig. 5A**) and CRT was measured. Cells were transfected with increasing amounts of WT *APC* (0, 0.15 and 0.5 μg) or 0.5 μg mutant *APC*. CRT reporter activities are

expressed relative to assays containing no WT APC and are the means of three replicates. Error bars represent standard deviations.

Lipofectamine was used to cotransfect SW480 cells with an internal control (0.5 μg pCMV-βgal), a reporter construct (0.5 μg pTOPFLASH or pFOPFLASH) and the indicated amount of the various APC expression vectors. The pTOPFLASH reporter contained an optimized Tcf-binding site 5' of a luciferase reporter gene, whereas pFOPFLASH contained a mutated site that does not bind Tcf. The amount of DNA in each transfection was kept constant by addition of an appropriate amount of empty expression vector (pCEP4). Luciferase and β-galactosidase activities were determined 16 hours after transfection. Luciferase activity was corrected for transfection efficiency (using the control β-galactosidase activity) and nonspecific transcription (using the pFOPFLASH control).

Figs. 6A and 6B. Evaluation of CRT in colorectal cancer cell lines with WT APC. (**Fig. 6A**) Immunoblot of endogenous APC in the DLD1, SW480, HCT116, SW48 and 293 cell lines, developed with APC monoclonal antibody FE9 (*34*). (**Fig. 6B**) Effects of exogenous WT APC on CRT in cell lines with endogenous mutated or WT APC. Cells were transfected with increasing amounts (0, 0.15 μg, 0.5 μg for DLD1 and SW48; 0, 0.5 μg, 5 μg for HCT116) of WT APC or APC1309Δ mutant (0.5 μg for DLD1 and SW48; 5 μg for HCT116) and CRT was assessed as in Fig. 5. CRT reporter activities are expressed relative to activity in extracts without exogenous APC and are the means of three replicates. Error bars represent standard deviations.

Figs. 7A, 7B, and 7C. Evaluation of β-catenin in colorectal cancer cell lines with WT APC. (**Fig. 7A**) Immunoblot of the cell lines used in this study, developed with β-catenin monoclonal C19220 (Transduction Laboratories, Lexington, KY)(*31*). (**Fig. 7B**) Sequence of *CTNNB1* in HCT116 and SW48. Overlapping segments constituting the entire *CTNNB1* were amplified by RT-PCR from SW480, DLD1, HCT116, and SW48 cells, and sequenced directly with ThermoSequenase (Amersham). In the case of

HCT116, a PCR product containing the deleted region was also cloned into pCI-neo (Promega, Madison) and multiple clones corresponding to each allele were individually sequenced.

The left panel (nts 121 to 143 from HCT116) reveals the presence of a deletion in addition to the WT sequence. The middle panel (antisense strand 156 to 113 of the WT and deleted alleles of HCT116) reveals the 3-bp deletion (ΔTCT) that removed codon 45 in half the clones. The right panel (nts 80 to 113 from SW48) reveals a C to A transition affecting codon 33 (TCT to TAT). (**Fig. 7C**) Schematic of β-catenin illustrating the armadillo repeats (*33*) in human (SEQ ID NO: 10), xenopus (SEQ ID NO: 10), and drosophila (SEQ ID NO: 11), and negative regulatory domain. The residues in larger type fit the consensus sequence for GSK3β phosphorylation (*29*) and those in bold have been demonstrated to affect down regulation of β-catenin through GSK3β phosphorylation in Xenopus embryos (*27*). The five mutations found in human colon cancers are indicated at the top.

Figs. 8A and 8B. Functional evaluation of β-catenin mutants. (**Fig. 8A**) Constitutive nuclear complex of β-catenin and Tcf in HCT116 cells. The presence of nuclear β-catenin-Tcf complexes was assessed by gel shift assays. Lanes 1 to 3, optimal Tcf retardation probe shifted with nuclear extract from HCT116 cells with addition of no antibody (lane 1), anti β-catenin (0.25 μg, lane 2), or an irrelevant antibody (0.25 μg, lane 3). Lane 4, mutant Tcf retardation probe shifted with nuclear extract from HCT116 cells. n.s., nonspecific shifting seen with the mutant probe. (**Fig. 8B**) Effects of the β-catenin mutations on CRT. 293 cells were transfected with WT (WT) or mutant (Δ45, S33Y) β-catenin and CRT was assessed. CRT reporter activities are expressed relative to WT β-catenin and are the means of three replicates. Error bars represent standard deviations. β-catenin expression constructs were prepared as follows. WT *CTNNB1* was amplified by RT-PCR from SW480 cells and cloned into the mammalian expression vector pCI-neo

(Promega) to produce pCI-neo-β-cat. The pCI-neo-β-cat Δ45 and S33Y were generated by replacing codons 1 to 89 in pCI-neo-β-cat with a PCR product encoding the equivalent region from HCT116 or SW48 cDNA, respectively. The structures of all constructs were verified by sequence analysis. Lipofectamine was used to cotransfect 293 cells with an internal control (0.1 μg CMV-βgal), a reporter (0.5 μg pTOPFLASH or pFOPFLASH), a Tcf-4 expression vector (0.5 μg pCDNA-TCF4), and β-catenin (0.5 μg) or dominant negative hTcf-4 1.0 μg) expression vectors. CRT was determined as described above.

Figs. 9A, 9B and 9C. Characterization of the Ad-Mini-ME. **Fig. 9A.** Map of GFP/cAPC incorporated into Ad-Mini-ME. Linear representation of different domains in APC including oligomerization domains, armadillo repeats, 15-aa repeats, 20-aa repeats, basic domain, and EB-1 binding site are shown. The β-catenin binding and degradation domain which comprises 15-aa repeats, 20-aa repeats and SAMP repeats was fused with the carboxyl-terminal of GFP. Expression of this cassette is driven by a CMV promoter in Ad-Mini-ME adenovirus vector. **Fig. 9B.** Western blot analysis with an anti-GFP antibody. Infection of SW480 cells with Ad-Mini-ME resulted in production of a fusion protein of the expected size (150 kD) whereas the Ad-GFP infected cells generated the expected 17 kD GFP. **Fig. 9C.** Fluorescence microscopy revealed that the GFP/cAPC (green fluorescence protein/central region of APC) fusion protein was diffusely localized to the cytoplasm in DLD1 and HCT116 cells.

Figs. 10A and 10B. Ad-Mini-ME suppresses CRT. **Fig. 10A.** The luciferase activity was dramatically inhibited in Ad-Mini-ME infected DOT cells as compared with control and Ad-GFP infected DOT cells. **Fig. 10B.** No significant differences in luciferase activity among control, Ad-GFP infected, and Ad-Mini-ME infected Dluc cells were observed at day 1 and 2. Values are the average of three experiments.

Fig. 11. Western blot analysis of c-MYC and cyclin D1 in DLD1, SW480, and HCT116. As compared with the control and Ad-GFP infected cells, c-MYC expression is strongly repressed in both Ad-Mini-ME infected DLD1 and SW480 cells but is only partially inhibited in Ad-Mini-ME infected HCT116. Similarly, repression of cyclin D1 expression by Ad-Mini-ME is observed in DLD1and SW480 but not in HCT116. Similar expression levels of α-tubulin in each lane indicate that similar amounts of protein are loaded.

Figs. 12A and 12B. Immunofluorescence staining of β-catenin in DLD1 (**Fig. 12A**) and SW480 (**Fig. 12B**).

Cells were infected with Ad-Mini-ME or Ad-GFP as indicated. Cells were stained for β-catenin and counterstained with DAPI as indicated. Ad-GFP infected cells demonstrate a predominant nuclear staining of β-catenin. In contrast, almost all the Ad-Mini-ME infected cells exhibited cytoplasmic and membrane β-catenin staining with minimal nuclear staining.

Figs. 13A and 13B. Cell growth and colony formation assays. **Fig. 13A**. Growth kinetics in DLD1, SW480, and HCT116 cells after mock-, Ad-GFP- or Ad-Mini-ME infections, as indicated. **Fig. 13B.** Colony formation in collagen gel after mock-, Ad-GFP- or Ad-Mini-ME infections, as indicated. The number of colonies is indicated below each well.

Fig. 14. Apoptosis in virus-infected cells. Cells were stained with Hoechst33258 dye and at least 500 cells were counted and the results are expressed as the fold increase in the percentage of apoptotic cells in each sample.

Fig. 15. Proliferation and apoptosis in DLD1 induced tumors. Subcutaenous tumors in nude mice were generated by injecting Ad-Mini-ME or Ad-GFP infected DLD cells as indicated. Hematoxylen

and eosion (H&E) staining revealed extensive apoptosis in Ad-Mini-ME infected cells. Immunoperoxidase staining with the M30 antibody (M30) which recognizes apoptotic cells revealed that the majority of Ad-Mini-ME infected cells were positive while only a few Ad-GFP infected cells were positive for this antibody. Immunoperoxidase staining with an antibody specific for the proliferation marker Ki-67 (Ki-67) revealed a remarkable decrease in Ki-67 nuclear labeling in Ad-Mini-ME infected cells. In contrast, the Ad-GFP infected cells were diffusely positive.

DETAILED DESCRIPTION

It is a discovery of the present invention that hTcf-4 binds to β-catenin and activates transcription in colorectal epithelial cells. Moreover, it has now been found that APC regulates this transcriptional activation, at least in part by binding to β-catenin. In colorectal cancer cells this regulation is frequently abrogated, either by mutation of APC or by mutation of β-catenin.

Two alternative splice forms of human Tcf-4 have been found. One form (hTcf-4E) is homologous to hTcf-1E and the other (hTcf-4B) is homologous to hTcf-1B. The sequence of the nucleotide and amino acid sequences are shown in SEQ ID NOs: 1-4. The coding sequences and proteins can be used in assays as described below. Intron-free DNA molecules are provided which are originally made by reverse transcription of a mRNA molecule. They can be propagated in cells or amplified as is desired. Isolated Tcf-4 proteins can be provided substantially free of other human proteins if, for example, the nucleotide sequences are expressed in non-human cells. Methods and vectors for achieving such expression are well known in the art. Choice of such expression means is made by the skilled artisan according to the desired usage and convenience.

Cells can be tested to determine if they have a wild-type APC or a wild-type downstream protein in the APC transcription regulatory pathway, called

herein the CRT pathway (β-catenin/Tcf-regulated transcription). One protein within the CRT pathway which has been identified as a target of mutations in human cancers is β-catenin (encoded by the *CTNNB1* gene). Other parts of the pathway are also likely to be targets. Although the target genes of the CRT pathway have not been identified, they can be readily identified using the system disclosed here. Genes which are differentially transcribed in the presence of wild-type and mutant *CTNNB1*, for example, can be identified.

Tcf-responsive reporter genes are those constructs which comprise a readily detectable or assayable gene (such as luciferase, β- galactosidase, chloramphenicol acetyltransferase) linked *in cis* to a Tcf-responsive element. Such responsive elements are known in the art (7) and any such elements can be used. An optimal Tcf motif contains the sequence CCTTTGATC. From one to twenty copies, and preferably from three to six copies, of the motif may be used. Mutation of the sequence to CCTTTGGCC abrogates responsiveness. Another necessary part of such constructs is a minimal promoter, such as the *c-Fos* or the Herpes virus thymidine kinase promoter. Transcription of the reporter gene may be performed by any means known in the art, usually by assaying for the activity of the encoded gene, although immunological detection methods can also be used. In addition, transcription can be monitored by measuring the transcribed mRNA directly, typically using oligonucleotide probes.

As shown below, a cell which has a wild-type APC protein will inhibit CRT. However, most mutations in *APC* render APC unable to inhibit CRT. Similarly, certain mutations in *CTNNB1* render β-catenin super-active and/or refractory to the inhibition by APC. Thus measuring Tcf-responsive reporter gene transcription is an indication of the status of *APC* and *CTNNB1*. Mutations in both of these genes are associated with cancers and therefore provides diagnostic and prognostic information.

Assays for CRT can be accomplished *in vitro* or in cells. If the assay is to be accomplished in cells, then a Tcf-responsive reporter gene must be introduced into the cell. Any means for introducing genetic material into cells can be used, including but not limited to infection, transfection, electroporation. If the assay is to be performed *in vitro* then the components for transcription must be present. These include suitable buffers, RNA polymerase, as well as ribonucleotides. If the protein product is to be assayed, then the components for translation must also be present, such as ribosomes, and amino acids.

These assays can also be used to screen compounds for potential as anti-cancer therapeutic agents. Using either the *in vitro* or cell form of the assay, test compounds can be introduced to determine whether they are able to mimic the effect of wild-type APC or to convert a mutant APC into a form which is able to inhibit CRT or a mutant β-catenin into a form which is regulatable by APC. In addition, compounds can be tested for the ability to inhibit the binding of β-catenin and Tcf-4, thus mimicking the action of APC. Such a test can be conducted *in vitro* or *in vivo*, for example using a two hybrid assay.

A means for diagnosis of cancers is the result of the observation that *CTNNB1* mutations are found in tumor cells, especially those which have wild-type APC. Such mutations can be found, *inter alia,* by sequencing either the gene or the protein found in a sample. Functional assays can also be used, such as whether β-catenin binds to APC or Tcf-4, or whether it is capable of mediating CRT. Sequences can be compared to those found in a normal tissue of a human, especially the same human who provided the sample being tested. Suitable tumors for testing include, but are not limited to those which are associated with FAP. Suitable tumors include colorectal cancer, thyroid cancer, brain cancer, medulloblastoma, desmoid tumor, osteoma, breast cancer, and head and neck cancer. Because APC mutations

are so frequent, and because it appears that APC mutations do not occur in the same tumors as CTNNB1 mutations, one can prescreen samples for APC mutations before performing a CTNNB1 determination.

Fusion proteins are known in the art and are the product of recombinant technology. At least two polypeptides or proteins which derive from different naturally occurring proteins are joined together as a single polyamino acid sequence. Typically this is accomplished by engineering two polynucleotides which encode the polypeptides or proteins so that they form a single message-encoding unit which contains a single open reading frame. One of the polypeptides can be an enzyme or other readily detectable protein. One of the polypeptides is a portion of APC which comprises the β-catenin binding site. The APC-derived polypeptide may be the N-terminal or the C-terminal portion of the fusion protein, or if more than two polypeptides are joined together, the APC-derived polypeptide can be internal. One particularly useful fusion partner is green fluorescent protein (GFP) which is readily detectable. Other useful enzymes, such as luciferase, β-galactosidase, and chloramphenicol acetyltransferase can also be used as is convenient. Other polypeptide moieties, such as epitope tags, can also be engineered into the fusion protein. These can be used, for example, to facilitate detection and purification of the fusion protein. The fusion protein is advantageous because it permits an independent means of verification that the APC-derived polypeptide is being expressed in the cell. However, if such verification is not desired or required, a non-fused polypeptide portion of APC can be used. Such portion must be functional in binding to β-catenin. Preferably the portion will not contain at least one of the following domains: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain. These domains are apparently not required to mediate growth inhibition and apoptosis.

Polynucleotides which encode the APC-derived portion, whether in fusion protein form or as a polypeptide, can be regulated using any of the promoters, transcription terminators, enhancers, and transregulators known in the art. Expression can be regulated or constitutive as is desired. The regulation may be by an exogenous agent, such as a drug which can be administered, or by endogenous agents, such as developmentally or environmentally regulated factors. The polynucleotide may be isolated and purified away from other molecules, and in particular isolated and purified from full-length APC-encoding polynucleotides. Methods fof isolating and purifying polynucleotides of known sequence and/or size are known in the art, and any such method can be used. Any vector known in the art can be used, including viral vectors, plasmids, mini-chromosomes, and yeast artificial chromosomes. One particularly preferred vector is an adenovirus vector.

Any method known in the art can be used for administering polynucleotides to tumor cells or subjects. These include without limitation, transfection, liposomes, viral infection, particle bombardment, nanoparticles, microparticles, compacted nucleic acid complexes, intravenous, intradermal, subcutaneous, intramuscular, direct injection into a tumor. Combinations of these methods may also be used.

The portion of the APC gene which encodes the β-catenin binding site can be used in a gene delivery format. This portion comprises approximately amino acid residues 958 to 2075, although fewer residues may be sufficient to bind to β-catenin. For example, residues 1000-2000, 1100-1900, 1200-1800, or 1300-1700, may be sufficient to bind to β-catenin. Suitable techniques are known in the art for administering genes to tumors and tumor cells, and any such technique can be used. Suitable expression vectors are also known in the art and it is within the skill of the artisan to select an appropriate one. Upon expression in a tumor cell of the β-catenin binding portion of APC, β-catenin will be bound and titrated away from binding to

Tcf-4, thus preventing unregulated expression of the CRT target genes. Similarly, a polypeptide portion of APC containing the β-catenin binding site can be administered to cells to perform a titration of β-catenin. Techniques for such administration to cells is well known in the art. Cells which are treated with either the polynucleotide or the polypeptide can be used to study the interaction between APC and β-catenin, and for developing drugs which interfere with such binding. Such treatment can also induce apoptosis and inhibit cell growth.

Polynucleotides may be administered to cells, subjects, or patients according to the present invention for the purpose of screening for agents which enhance polynucleotide transfer to cells or enhance subsequent biological effects of the polynucleotides or their encoded proteins. Such biological effects include expression of the polynucleotide to form mRNA and/or protein, replication of the polynucleotide, hybridization to complementary messenger RNA and inhibition of its translation, and integration of the polynucleotide. According to the present invention, expression of a polypeptide or fusion protein encoded by the administered polynucleotide is preferred so that the polypeptide or fusion protein can bind to β-catenin. The result of such binding is inhibition of tumor cell growth and/or induction of apoptosis. Agents can also be screened for the ability to enhance the binding of the encoded polypeptide or fusion protein to β-catenin. Agents which can be screened include any test compounds or substances, whether natural products or synthetic, which can be administered to the cells, subject, or patient. Libraries or mixtures of compounds can be tested. The compounds or substances may be those for which a pharmaceutical effect is previously known or unknown. The compounds or substances may be delivered before, after, or concomitantly with the polynucleotides. They may be administered separately or in admixture with the polynucleotides. Integration of delivered DNA can be monitored by any means known in the

art. For example, Southern blotting of the delivered DNA can be performed. A change in the size of the fragments of the delivered polynucleotide indicates integration. Replication of the delivered polynucleotide can be monitored *inter alia* by monitoring incorporation of labeled nucleotides combined with hybridization to a probe for the delivered polynucleotides. Expression of the polynucleotide can be monitored by detecting production of RNA which hybridizes to the delivered polynucleotide or by detecting protein encoded by the delivered polynucleotide. The protein can be detected immunologically or by activity, for example. The expression of the protein can also be monitored by detecting the effects of β-catenin binding. Thus the administration of the polynucleotides according to the present invention provides an excellent system for screening agents for their ability to promote delivery, integration, hybridization, expression, replication or integration in cells or in an animal, preferably a mammal, more preferably a human.

Tumor cell inhibition can be measured or assessed according to any method known in the art. Tumor mass may be observed and measured. Time until palpable tumor development after administration of tumor cells to a nude mouse can be assessed. Survival time of animals with tumors can be measured. Incorporation of tritiated thymidine can be measured. Focus formation can be measured. Any method known in the art can be used, without limitation. Monitoring of apoptosis can also be done as is known in the art. DNA fragmentation, a hallmark of apoptosis, can be assayed by any means known in the art, including but not limited to electrophoresis on a gel, staining nuclei with TUNEL, and photometric enzyme immunoassay for the determination of cytoplasmic histone-associated DNA fragments. The assays can be performed *in vitro* or *in vivo*.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific

examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

This example identifies Tcf-4 as the expressed family member in colorectal epithelial cells and provides the complete sequence of the cloned cDNA.

There are four known members of the Tcf/Lef family in mammals: the lymphoid-specific factors Tcf- I and Lef- 1 (7,8), and the less well characterized Tcf-3 and 4(9). We performed **a** qualitative Reverse Transcriptase-Polymerase Chain Reaction assay for expression of the four *Tcf/Lef* genes on 43 colon tumor cell lines. While most colon cell lines expressed more than one of the genes, only hTcf-4 mRNA was expressed in essentially all lines.

We then screened a human fetal CDNA library and retrieved clones encoding full-length hTcf-4 (Fig. 1). A genomic fragment encoding, the HMG box region of hTcf-4 (7) was used to probe a human 12 week-fetal cDNA library in Lambda GT-11. Positive clones were subcloned into pBluescriptSK and sequenced. See SEQ ID NOs: 1 and 3. The predicted sequence of hTcf-4 was most similar to that of hTcf-1. Alternative splicing yielded two COOH-termini that were conserved between hTcf- I and hTcf-4. The NH_2-terminus, which in hTcf- 1, mLef-1 and *Xenopus* TCF-3 mediates binding to β-catenin (6), was also conserved in hTcf-4. Northern blot analysis of selected colon carcinoma cell lines revealed high-level expression of *hTcf-4* (Fig. 2A). Northern blot hybridizations (7) were performed with full-length *hTcf-1, hLef-I and hTcf-4* cDNA. Colon epithelial cells were freshly prepared from a mucosal preparation dissected from a healthy surgical colon sample. The sample was minced, and incubated with 1 mM dithiothreitol (DTT) in Hanks' medium to remove mucus. Single-cell suspensions were prepared by

incubation at RT in 0.75 mM EDTA in Hanks' medium. Epithelial cells were separated from lymphocytes by Percoll gradient centrifugation.

As evidenced by in situ hybridization (Fig. 2, B and C) and Northern blotting (Fig. 2A), *hTcf-4* MRNA was readily detectable in normal colonic epithelium, whereas *hTcf-I and hLef*-I were not detectable. In situ hybridization of 6 μ frozen sections of healthy colon biopsy samples were performed as described(10). *hTcf-4 cDNA* encoding amino acids 200 to 310 was amplified and labeled with Dig-11-dUTP (Boehringer Mannheim, Germany) by PCR. After hybridization and washing, the sections were sequentially incubated with mouse anti-Dig antibody (Boehringer) and a horseradish peroxidase conjugated rabbit antibody to mouse immunoglobulin (Dako, Glostrup, Denmark). The signal was visualized with diaminobenzidine, which produces a reddish-brown precipitate. Blue counterstaining was performed with hematoxylin.

EXAMPLE 2

This example demonstrates the interaction of Tcf-4 and β-catenin and their function as a transcriptional activating factor.

To investigate whether hTcf-4 functionally interacts with β-catenin, we used two sets of reporter constructs in a β -catenin-Tcf reporter gene assay (7). One contained three copies of the optimal Tcf motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal *c-Fos* promoter driven-luciferase expression (PTOPFLASH and PFOPFLASH). The second set contained three copies of the optimal motif, or three copies of the mutant motif, upstream of a minimal Herpes virus thymidine kinase promoter driven-Chloramphenicol Acetyl Transferase (CAT) expression (PTOPCAT and PFOPCAT, respectively). Reporter gene assays were performed as in (7). In brief, 2 x 10^6 cells were transfected with plasmids by electroporation. After 24 hours, cells were harvested and lysed in 1 mM DTT, 1 % Triton X-

100, 15 % glycerol, 25 mM Tris pH 7.8 and 8 mM $MgCl_2$. cDNAs encoding Myc-tagged versions of β-catenin and hTcf-4 were inserted into the mammalian expression vector pCDNA (Invitrogen). PCATCONTROL, encoding the CAT enzyme under the control of the SV40 promoter, was purchased from Promega.

Epitope-tagged hTcf-4 and a deletion mutant lacking, the NH_2-terminal 30 amino acids (ΔNhTcf-4) were cloned into the expression vector pCDNA. Transient transfections were performed in a murine B cell line (IIA1.6), that does not express any of the Tcf genes (6).

The TOPFLASH reporter was strongly transcribed upon cotransfection with the combination of β-catenin and hTcf-4 plasmids, but not with the individual plasmids or with the combination of β-catenin and ΔNhTcf-4 plasmids. No enhanced transcription was detected in cells transfected with the negative control PFOPFLASH (Fig. 3A). These results show that interaction of the NH_2-terminus of hTcf-4 with β-catenin results in transcriptional activation.

EXAMPLE 3

This example demonstrates the functional regulation of CRT transcriptional activation by wild-type APC.

In three $APC^{-/-}$ carcinoma cell lines, SW480, SW620 and DLD-1 (Fig. 3B), the PTOPFLASH reporter was 5-20 fold more actively transcribed than PFOPFLASH. Importantly, transfection of SW480 cells with the reporter gene and an APC-expression vector abrogated the transcriptional activity in a dose-dependent manner (Fig. 3B). In contrast APC had no effect on a cotransfected internal control (pCATCONTROL), or on the basal transcription of PFOPFLASH (Fig. 3B). The use of PTOPCAT and PFOPCAT instead of PTOPFLASH and PFOPFLASH led to comparable observations. The constitutive transcriptional activity of Tcf reporter genes in $APC^{-/-}$ colon carcinoma cells was in stark contrast to the inactivity of these genes in non-

colonic cell lines, including IIA1.6 B cells (Fig. 3A), the C57MG breast carcinoma cell line; the Jurkat and BW5147 T cell lines; the Daudi and NS1 B cell lines; the K562 erythromyeloid cell line; the HeLa cervical carcinoma line; the HepG2 hepatoma cell line; 3T3, 3T6, and Rat-I fibroblasts; and the kidney derived SV40-transformed COS cell line (7,16).

EXAMPLE 4

This example demonstrates that a functional β-catenin-hTcf-4 complex exists constitutively in $APC^{-/-}$ cells.

We used HT29-$APC^{-/-}$ colon carcinoma cells (12), in which APC is controlled by a metallothionein promoter. Induction by Zn^{++} restores wild-type levels of APC, and leads to apoptosis (12). HT29-Gal cells which carry a Zn^{++}-inducible LacZ gene were used as a control. The only Tcf family member expressed in HT29 is hTcf-4 (Fig. 2C). In nuclear extracts from uninduced HT29 derived transfectants, we readily detected hTcf-4 by gel retardation (Fig. 4). An additional band of slightly slower mobility was also observed. The addition of a β-catenin antibody resulted in the specific retardation of the latter band, indicating that it represented a β-catenin-hTcf-4 complex (Fig. 4) (12). After Zn^{++} induction for 20 hours, the β-catenin-hTcf-4 complex was diminished sixfold relative to uncomplexed hTcf-4 in HT29-APC1, while no significant change was observed in HT29-Gal cells (Fig. 4). Importantly, the overall levels of cellular β-catenin do not change during the induction period in HT29-APC1 cells (12).

Gel retardation assays were performed as described elsewhere (7). Extracts were prepared from intact nuclei that were washed four times to avoid contamination with cytoplasmic β-catenin. As the optimal Tcf/Lef probe, we used a double-stranded 15-mer CCCTTTGATCTTACC (SEQ ID NO: 3); the control probe was CCCTTTGGCCTTACC (SEQ ID NO: 4). (All oligonucleotides were from Isogene, Holland). The β-catenin antibody was purchased from Transduction Laboratories (Lexington, KY). A typical

binding reaction contained 3 μg nuclear protein, 0. 1 ng radiolabeled probe, 100 ng of dIdC, in 25 μl of binding buffer (60 mm KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol). Samples were incubated for 20 min at room temperature, antibody was added, and the samples incubated 20 min further.

On the basis of these data, we propose the following model. In normal colonic epithelium hTcf-4 is the only expressed member of the Tcf family. The interaction of β-catenin with hTcf-4 is regulated by APC. When appropriate extracellular signals are delivered to an epithelial cell, β-catenin accumulates in a form that is not complexed with GSK3β-APC, and that enables its nuclear transport and association with hTcf-4. The HMG domain of hTcf-4 binds in a sequence-specific fashion to the regulatory sequences of specific target genes; β-catenin supplies a transactivation domain. Thus, transcriptional activation of target genes occurs only when hTcf-4 is associated with β-catenin. The hTcf-4 target genes remain to be identified. However, the link with APC and catenin suggests that these genes may participate in the generation and turnover of epithelial cells. Upon loss of wild-type APC, monomeric β-catenin accumulates in the absence of extracellular stimuli, leading to uncontrolled transcription of the hTcf-4 target genes. The apparent *de novo* expression of other members of the *Tcf* family in some colon carcinoma cell lines might lead to a further deregulation of Tcf target gene expression by the same mechanism. The control of β-catenin -Tcf signaling is likely to be an important part of the gatekeeper function of APC (19), and its disruption an early step in malignant transformation.

EXAMPLE 5

This example demonstrates that mutant APC protein does not regulate CRT and that a complete set of 20-AA repeats in APC is required to mediate inhibition of CRT.

We tested four APC mutants (Fig. 5A) for their ability to inhibit β-catenin/Tcf-regulated transcription (CRT) in transfection assays. The first mutant, APC331Δ represents a type of mutation found in the germline of Familial Adenomatous Polyposis (FAP) patients as well as in sporadic tumors (15). The APC331Δ protein is truncated at codon 331, amino-terminal to the three 15-amino-acid (AA) β-catenin-binding repeats between codons 1020 and 1169. The second mutant, APC1309Δ, is the most common germline *APC* mutation (15), a 5-bp deletion that produces a frameshift at codon 1309 and truncation of the protein. The APC1309Δ protein retains the 15-AA β-catenin repeats but lacks the seven 20-AA repeats between codons 1323 and 2075 that have been implicated in binding and phosphorylation of β-catenin (*18*). The third mutant, APC1941Δ, represents one of the most distal somatic mutations observed in colorectal tumors (*25*). The APC1941Δ protein is truncated at codon 1941 and therefore contains the 15-AA repeats and all but the last two 20-AA repeats. Finally, APC2644Δ represents a germline mutation resulting from a 4-bp deletion in codon 2644. Patients with this type of unusual carboxyl-terminal mutation develop few polyps (attenuated polyposis) but have pronounced extracolonic disease, particularly desmoid tumors (*26*).

Each of the *APC* mutants was cotransfected with a CRT reporter into the SW480 colorectal cancer cell line. SW480 cells have truncated APC and constitutively active CRT which can be suppressed by exogenous WT APC. Although all four mutants produced comparable levels of APC protein after transfection, they varied in their CRT inhibitory activity. The three mutants found in patients with typical polyposis or cancer were markedly deficient in inhibition of CRT (Fig. 5B). The reduced activity of APC1309Δ and APC1941Δ suggests that β-catenin binding is not sufficient for APC-mediated inhibition of CRT and that the complete set of 20-AA repeats is required. Interestingly, the inhibitory activity of the APC2644Δ mutant associated with attenuated polyposis was comparable to that of WT APC (Fig. 5B), suggesting

that the DLG-binding domain at the carboxyl-terminus of APC is not required for down-regulation of CRT.

WT and mutant *APC* constructs (2 μg) were transfected into 293, SW480, and HCT116 cells using Lipofectamine (GIBCO/BRL, Gaithersburg). Protein was harvested 24 hours later and subjected to immunoblot analysis with APC monoclonal antibody FE9 (*23*). In HCT116 and 293 cells, exogenous WT APC comigrated with the endogenous APC. In SW480 cells, APC1309Δ comigrated with the endogenous mutant APC. In all other cases, the nonfunctional APC constructs (APC331Δ, APC 1309Δ, and APC1941Δ) produced as much or more protein than the CRT-functional forms of APC (APC WT and APC 2644Δ).

EXAMPLE 6

This example demonstrates that other components of the APC-regulatory pathway are affected in some cancer cells.

We evaluated CRT in two colorectal tumor cell lines (HCT116 and SW48) that express full-length APC (Fig. 6A). Both HCT116 and SW48 displayed constitutively active CRT and, in contrast to cell lines with truncated APC (DLD1 and SW480), this activity was not inhibited by exogenous WT APC (Fig. 5B, 6B). Other (noncolorectal cancer) cell lines expressing WT APC do not display constitutive CRT activity. These transfection results suggested that the constitutive CRT in HCT116 and SW48 might be due to an altered downstream component of the APC tumor suppressor pathway.

EXAMPLE 7

This example demonstrates a defect in the gene encoding β-catenin in some cancer cells, which affects CRT.

We evaluated the status of a likely candidate for a downstream component of the APC tumor suppressor pathway, β-catenin, in the same four lines. All four lines expressed similar amounts of apparently intact β-catenin, as assessed by immunoblots (Fig. 7A). However, sequence analysis revealed that both HCT116 and SW48 harbored mutations in the β-catenin gene (*CTNNB1*) (Fig. 7B). HCT116 had a 3-bp deletion that removed one AA (Ser-45), and SW48 had a C to A missense mutation that changed Ser-33 to Tyr. Analysis of paraffin-embedded archival tissue from the HCT116 patient confirmed the somatic nature of this mutation and its presence in the primary tumor prior to culture. Interestingly, both mutations affected serines that have been implicated in the down regulation of β-catenin through phosphorylation by the ZW3/GSK3β kinase in *Xenopus* embryos (Fig. 7C) (*27,28*).

Genomic DNA was isolated from paraffin-embedded normal and tumor tissue from the patient from whom the HCT116 cell line was derived. A 95 bp PCR product encompassing the mutation was then amplified by PCR and directly sequenced using THERMOSEQUENASE (Amersham). The 3 bp deletion was observed in tumor but not in normal tissue.

To test the generality of this mutational mechanism, we evaluated five primary colorectal cancers in which sequencing of the entire coding region of *APC* revealed no mutations (*25*). Three of these five tumors were found to contain *CTNNB1* mutations (S45F, S45F, and T44A) that altered potential ZW3/GSK3β phosphorylation sites (Fig. 7C). Each mutation appeared to affect only one of the two *CTNNB1* alleles and to be somatic.

Genomic DNA was isolated from frozen-sectioned colorectal cancers and a 1001 bp PCR product containing exon 3 of *CTNNB1* was then amplified by PCR and directly sequenced using ThermoSequenase

(Amersham). An ACC to GCC change at codon 41 (T41A) and a TCT to TTT at codon 45 (S45F) was observed in one and two tumors, respectively.

EXAMPLE 8

This example demonstrates dominant mutations of *CTNNB1* that render CRT insensitive to the effects of WT APC.

Because the β-catenin mutations were heterozygous, we hypothesized that the mutations might exert a dominant effect, rendering a fraction of cellular β-catenin insensitive to APC-mediated down regulation. To test this notion, we performed gel shift analyses with nuclear extracts from untransfected HCT116 cells. In contrast to noncolorectal cancer cell lines with intact *APC*, HCT116 cells contained a β-catenin/Tcf complex that gel-shifted an optimized Tcf-binding oligonucleotide, and this complex supershifted with anti-β-catenin (Fig. 8A). We also constructed β-catenin expression vectors and compared the biologic activity of the mutant β-catenin from HCT116 (β-Cat Δ45) and SW48 (β-Cat S33Y) with that of their WT counterpart. For these experiments, we used the 293 kidney epithelial cell line as it is highly transfectable, exhibits low endogenous CRT, and contains a high level of endogenous APC (Fig. 6A). In the presence of endogenous APC, both mutant β-catenins were at least 6-fold more active than the WT protein and this activity was inhibited by dominant-negative hTcf-4 (Fig. 8B).

Together, these results indicate that disruption of APC-mediated regulation of CRT is critical for colorectal tumorigenesis. This is most commonly achieved by recessive inactivating mutations of both *APC* alleles but, as shown here, can also be achieved by dominant mutations of *CTNNB1* that render CRT insensitive to the effects of WT APC. Our results suggest that APC inhibition of CRT requires phosphorylation of β-catenin at multiple sites. These potential phosphorylation sites are consistent with the known specificity of ZW3/GSK3β (*29*) a serine kinase that negatively regulates

β-catenin in *Xenopus* and *Drosophila* cells (*27*) and that interacts with APC and β-catenin in mammalian cells (*23*). These results also suggest a functional basis for the occasional *CTNNB1* mutations observed in other tumor types (*30*) and illustrate how a critical pathway in human disease can be illuminated by the discovery of mutations in different components of the pathway. The next step in understanding *APC* function will be the identification of the genes that are activated by hTcf-4/β-catenin complexes and inhibited by WT APC. These genes are likely to be related to APC's ability to induce apoptosis in colorectal cancer cells (*31*).

EXAMPLE 9

Characterization of the Ad-Mini-ME recombinant adenovirus.

Understanding the structure-function relationships of APC has been hindered by the inability to readily restore APC tumor suppressor activity to human cells. To address this problem, we developed an adenovirus system that would allow the relatively facile evaluation of APC effects in a variety of cell lines. Three features of the adenovirus construction are of particular interest. First, to facilitate the actual construction of the adenoviral vectors, we used the AdEasy adenovirus system, which employs recombination in bacteria rather than in mammalian cells to generate recombinant adenovirus. Second, we included a GFP marker to allow easy identification of APC expressing cells. This eliminates problems related to differences in infectivity and allows the use of viral titers well below levels that result in virus-induced cytopathic effect (CPE). Avoiding such nonspecific CPE is especially important for assessing tumor suppressive effects. Finally, we generated a virus that expressed a fusion protein (GFP/cAPC) containing GFP fused to the central third of APC (Fig. 9A). The employment of a fusion protein ensured that expression of GFP was coupled with APC and allowed positive verification of cAPC expression. The growth suppressive effects of tumor suppressor genes impose a powerful negative selection force that can result in loss of expression of the tumor suppressor gene even in the presence of a positive selection marker.

The central portion of APC was chosen for the following experiments because it contains all of the known β-catenin and Axin/Conductin binding domains (**Fig. 9A**) and is sufficient for promoting β-catenin degradation. In keeping with precedent, the clone expressing this smaller version of APC Minus its amino- and carboxyl-terminal Ends was designated Ad-Mini-ME. (*37*) Infection of 293 and SW480 cells with Ad-Mini-ME resulted in the

production of a fusion protein of the expected size (150 kD fusion) as judged by Western blot analysis with an anti-GFP antibody (**Fig. 9B**). Fluorescence microscopy revealed that the GFP/cAPC fusion protein was diffusely present in the cytoplasm (**Fig. 9C**).

Ad-Mini-ME inhibits Tcf4/β-catenin mediated transactivation.

One of the best-characterized functions of APC is its ability to inhibit CRT. To investigate the effects of GFP/cAPC on this function, we generated stable DLD1 cell lines with either an integrated Tcf-4 responsive luciferase reporter (DOT cells) or a reporter driven by the SV40 promoter (Dluc cells). Infection of DOT cells with Ad-Mini-ME markedly suppressed luciferase activity, whereas a virus expressing GFP alone (Ad-GFP) had no effect (**Fig 10A**). This inhibition appeared to be specific as infection of Dluc cells with Ad-Mini-ME had no effect on luciferase activity (**Fig. 10B**). To determine whether the Ad-Mini-ME suppression could be extended to endogenous targets of CRT, we evaluated the expression of c-MYC and cyclin D1, two recently described targets of the APC/β-catenin/Tcf-4 pathway. Expression of c-MYC and cyclin D1 was examined in three human colorectal cancer cell lines. In DLD1 and SW480 CRT was constitutively activated because of APC mutations, while in HCT116 CRT activation was due to a β-catenin mutation.

Infection of SW480 and DLD1 cells with Ad-Mini-ME resulted in a marked reduction of c-MYC protein levels, and a minor reduction of cyclin D1 levels (**Fig. 11**). Ad-GFP infection had no inhibitory effect on either c-MYC or cyclin D1 protein levels in these cells. As expected, HCT116 cells, which possess a stabilizing β-catenin mutation, were relatively resistant to the effects of Ad-Mini-ME (**Fig. 11**).

The subcellular localization of β-catenin was dramatically altered by Ad-Mini-ME infection. Whereas mock-infected or Ad-GFP-infected DLD1 cells displayed predominantly nuclear β-catenin staining, cells infected with

Ad-Mini-ME showed cytoplasmic and membrane β-catenin staining with minimal nuclear staining (**Fig. 12A**). A similar alteration in the subcellular distribution of β-catenin was observed in SW480 cells (**Fig. 12B**). The selective depletion of nuclear β-catenin by Ad-Mini-ME is consistent with the model for APC action proposed previously.

Ad-Mini-ME suppresses cell growth and colony formation in colorectal cell lines.

In both DLD1 and SW480, Ad-Mini-ME infection resulted in a clear growth inhibition by day 2 (**Fig. 13A**). This growth inhibition was persistent, with Ad-Mini-ME infected DLD1 and SW480 cells failing to reach confluence even after 2 weeks of culture, long after mock- and Ad-GFP infected cells became superconfluent. Consistent with the effects of Ad-Mini-ME on CRT in HCT116 cells, the growth of Ad-Mini-ME infected HCT116 cells was only partially inhibited (**Fig. 13A**).

We next examined the effects of Ad-Mini-ME mediated CRT inhibition on colony formation in collagen gels using a series of eight colorectal cancer cell lines. The cells were flow sorted to select virally-infected cells, as described in Materials and Methods. Infection with Ad-Mini-ME resulted in a marked suppression of colony formation in SW480, DLD1, LoVo, HT29, SW837, and SW1417 cells, all of which possess mutated APC (**Fig. 13B**). Colony numbers were reduced by at least 96% in each of these lines compared with cells mock-infected or infected with Ad-GFP virus. Examination under phase-contrast microscopy revealed that most of the Ad-Mini-ME infected cells were pyknotic. A small number of growth-arrested single cells remained in the gels, and these continued to express APC as judged by fluorescence. In the few colonies that did form after Ad-Mini-ME infection, APC did not appear to be expressed. In contrast, cells with intact APC but mutated

β-catenin were able to form a significant number of colonies following Ad-Mini-ME infection (HCT116 and SW48 in Fig. **13B**).

Ad-Mini-ME induces apoptosis in colorectal cell lines.

Ad-Mini-ME-expressing cells revealed a gradual loss of the G1 peak and an accumulation of cells in the S and G_2 phases of the cell cycle. Five days after infection, all six colorectal cell lines with *APC* mutations demonstrated significantly increased apoptosis following Ad-Mini-ME infection (**Fig. 14**). In line with the effects of Ad-Mini-ME on CRT and growth, the mutant β-catenin containing cell lines, HCT116 and SW48, exhibited little increase in apoptosis in response to Ad-Mini-ME infection. Time course studies revealed that the first morphological signs of apoptosis were not evident in DLD1 and SW480 cells until 72 hours after plating. The induction of apoptosis was confirmed by Annexin V, which has been shown to bind to phosphatidylserine exposed on the outer leaflet of apoptotic cell membranes. The proportion of DLD1 and SW480 cells staining with Alexa568-labeled annexin V was in good agreement with the fraction of cells displaying morphological signs of apoptosis (>90% of annexin V-labeled cells displayed apoptotic nuclei).

Tumor growth in nude mice.

To investigate the effects of Ad-Mini-ME on colorectal cancer cells in vivo, Ad-GFP and Ad-Mini-ME-infected DLD1 cells were selected by flow sorting and injected subcutaneously into nude mice. Mice sacrificed three days after injection showed a large number of apoptotic tumor cells at the injection site (**Fig. 15**, H&E). Such changes were not evident after injection of mock-infected or Ad-GFP-infected cells. Apoptosis was further evaluated by immunoreactivity with the M30 antibody, which recognizes a caspase-cleaved epitope of cytokeratin 18. Ad-Mini-ME infected cells were diffusely positive for the M30 antibody, while only a small fraction of

Ad-GFP-infected cells stained with this antibody (Fig. 15, M30). Immunoperoxidase staining with an antibody specific for the proliferation marker Ki-67 showed a significant decrease in nuclear labeling of Ad-Mini-ME infected tumors, with only 10% of cells positive (Fig. 15, Ki-67). In contrast, over 90% of Ad-GFP-infected cells were Ki-67 positive. Tumors derived from Ad-GFP infected cells continued to grow in the nude mouse, eventually forming tumors larger than 1 cm in diameter, while there was no visible tumor formation after injection of Ad-Mini-ME infected cells after three weeks.

Materials and Methods

Cell Culture, Medium, and Reagents. 293 cells were purchased from Microbix Biosystems (Toronto, Canada) and 911 cells were kindly provided by Alex J. Van der Eb of the University of Leiden. Both lines were maintained in DMEM growth medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml of penicillin and 100 μg/ml of streptomycin. The human colon carcinoma cell lines, SW480, DLD1, HCT116, LoVo, SW48, HT29, SW837, and SW1417 were obtained from the American Type Culture Collection and cultivated in McCoy's 5A media (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml of penicillin and 100 μg/ml of streptomycin. The DOT and Dluc cell lines were derived from DLD1 cells as described below. Both cell lines were maintained in McCoy's 5A media (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone) and 0.4 mg/ml Hygromycin B (Calbiochem).

Generation of Recombinant Adenovirus Expressing Central Third of APC (Ad-Mini-ME). The recombinant adenovirus, Ad-Mini-ME, which

carried the central third of APC gene, was generated using a modified system as previously described. The central third of APC containing amino acids 958-2075 (nucleotides 2890-6240) was isolated from pCMV-APC by Bgl II digestion. This fragment was subcloned into the pEGFP- C1 (Clontech, Palo Alto, CA). The cassette containing the EGFP-tagged central third of APC gene (cAPC) was further subcloned into the shuttle vector (pShuttle) using Apal I and Mlu I digestion. Recombinant adenoviral plasmid was generated by homologous recombination in E. coli (BJ5183). BJ5183 cells were transformed using electroporation with pAdEasy-1 and pShuttle/EGFP-cAPC linearized with PmeI. Successful recombinants were identified by restriction endonuclease mapping. The recombinant EGFP-cAPC virus (Ad-Mini-ME) was produced in the 911 and 293 adenovirus packaging lines and the viral particles were purified by CsCl banding. The control virus (Ad-EGFP) with EGFP alone was also prepared and purified side by side. Viral titer was determined by a modified CPE[3] endpoint assay. A series of Ad-GFP infections was performed on HCT116 cells to determine the optimal multiplicity of infection (MOI) to avoid adenovirus-associated CPE. Typically, viral CPE could be observed at an MOI of above 100, which resulted in more than 80% of cells becoming fluorescent 18 hours after Ad-GFP infection. To avoid any cytopathic effects of viruses, we infected cells with a minimal MOI, generating fluorescence in 20-30% of the cells (MOI=5-11), then flow-sorted infected cells to obtain homogeneous populations.

Viral Infection and Cell Sorting. Viral stocks were predialyzed using 1% agarose in microcentrifuge tube. Three million cells were infected with either Ad-Mini-ME or Ad-GFP in a 75 cm^2 flask. After 18 hours of incubation at 37°C, cells were washed, trypsinized and subjected to fluorescence activated cell sorting. Cells with green fluorescence were

collected for experiments or were replated in culture flasks immediately after sorting.

Reporter Assay. DOT and Dluc cells were generated from DLD1 cells by cotransfection of pTK-hygro (Clontech) and a Tcf-4 responsive luciferase plasmid (pGL3-OT, Yu, J. unpublished data) or a constitutive luciferase plasmid (pGL3-control, Promega, Madison, WI), respectively. Clones were isolated and the sensitivity to CRT determined using a dominant negative Tcf-4 adenovirus (Yu, J. unpublished data). Luciferase reporter activity in the DOT clone was constitutively high as expected for a CRT responsive reporter in a colorectal cancer cell line with mutated APC. This constitutive activity was inhibited by dominant negative Tcf-4. In contrast, the luciferase activity in the Dluc clone was unaffected by dominant negative Tcf-4 as expected for expression driven by the SV40 promoter. To assess the effects of cAPC on CRT, DOT and Dluc cells were infected with Ad-GFP and Ad-Mini-ME. Eighteen hours after viral infection, equal numbers of GFP positive cells were pelleted, lysed, and collected for luciferase assays using Luciferase Assay Reagents (Promega).

Western Blot Analysis. Whole cell lysates were prepared in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 0.5% β-mecaptoethanol. Equal amounts of total protein from each lysate were loaded and separated on 4-12% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes. Western blots were developed by chemiluminescence (NEN Life Science, Boston, MA), detected by Kodak Image Station 440CF, and analyzed by 1D Image Analysis software (NEN Life Science). Primary antibodies included anti-GFP polyclonal antibody from Clontech (Palo Alto, CA), anti-c-MYC monoclonal antibody (9E10)

from Santa Cruz (Santa Cruz, CA), anti-cyclin D1 monoclonal antibody (A-12) from Santa Cruz, and anti-α-tubulin monoclonal antibody (TU-02) from Santa Cruz. Secondary peroxidase-conjugated antibodies were goat anti-mouse IgG and goat anti-rabbit IgG from Pierce (Rockford, IL).

Immunofluorescence Staining. Cells were infected with Ad-GFP or Ad-Mini-ME for 18 hours and sorted. Fluorescent cells were cultured on 8-well chamber CultureSlides (Becton Dickinson, Bedford, MA). After 8 hours, cells were fixed in 3% paraformaldehyde in PBS at room temperature for 8 min, then permeabilized with 0.3% NP-40 in PBS for another 8 min. After washing in PBS, the cells were incubated with primary mouse anti-β-catenin monoclonal antibody (1 μg/ml; Transduction Laboratories, Lexington, KY) at 4°C overnight. After washing, cells were incubated with biotinylated goat anti-mouse IgG (Pierce, Rockford, IL) at room temperature for 1 hour. The immunoreactivity was revealed using Alexa568-conjugated streptavidin (Molecular Probes, Eugene, OR) and cells were counterstained with 10 μg/ml Hoechst 33258. The cells were examined under a Nikon fluorescence microscope (Image Systems, Columbia, MD).

Cell Growth and Colony Formation Assay. 10^5 cells were plated in one well of a 24 well-plate. Cells were counted using a hemocytometer after trypsinization on days 1, 2, 3 and 5. For colony formation assays, each well of 24-well plates was precoated with 100 μl of collagen gel containing 50% type I collagen (Collaborative Biomedical Science, Bedford, MA), 40% culture medium, and 0.75% $NaHCO_3$ (Halttunen). 160 μl of collagen gel-cell suspension containing 10,000 cells, 45% type I collagen, 40% culture medium, and 0.075% $NaHCO_3$ was added to the wells. After solidification, each well was covered with 1 ml of culture medium and the

plates were incubated at 37°C. Twelve days after seeding, cells were stained with 0.05% crystal violet (Sigma, St. Louis, MO) containing 10% buffered formalin (Sigma).

Hoechst Staining and Annexin V Staining for Apoptosis Detection. Both attached and floating cells were harvested for staining. For Hoechst staining, $3x10^5$ cells were resuspended in 50 µl PBS and 350 µl of staining solution containing 0.6% Nonidet P-40, 3% paraformaldehyde, and 10 µg/ml Hoechst 33258. For annexin V staining, 10^5 cells were suspended in 100 ul annexin-binding buffer containing 10mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$. 5 µl of Alexa568-conjugated annexin V (Molecular Probes) was added and incubated at room temperature for 15 min, at which point an additional 400 µl of annexin-binding buffer was added to each sample. Apoptotic cells were defined as those cells containing condensed and/or fragmented nuclei after Hoechst staining or were fluorescent after annexin V staining. At least 500 cells were counted and the results were expressed as the percentage of apoptotic cells in each sample.

Experimental Animals and Immunoperoxidase. Eight pathogen-free female athymic nude mice (Harlan, Indianapolis, IN), 6-8 weeks old were injected with $3x10^6$ DLD1 cells infected either with Ad-GFP or Ad-Mini-ME in the subcutaneous tissues adjacent to the lower spine. Before injection, these cells were sorted and green fluorescent cells were collected and cultured for 24 hours. Three days after injection, two of the mice from each group were sacrificed and the tumors were formalin-fixed, paraffin-embedded, and processed for routine histological examination. Tissue sections of 5 micron thickness were deparaffinized and incubated with a mouse anti-Ki-67 antibody (AMAC Inc., Westbrook, ME) or with a mouse antibody specific for a caspase-cleaved epitope of cytokeratin 18

(M30, Boehringer Mannheim, Mannheim, Germany). Cells were incubated with antibodies at 4°C overnight, then washed and developed with an avidin-biotin complex peroxidase method (Biogenex, San Ramon, CA). Immunoreactivity was detected using the 3,3'-diaminobenzidine chromagen and counterstained with 0.1% hematoxylin (Sigma, St Louis, MO).

DISCUSSION

While the ability of APC to suppress intestinal tumorigenesis has been known for several years, the sequences required for this inhibition have not been well defined. In this study, we define a minimal portion of APC that is sufficient for its growth suppressive effects. Our results suggest that expression of the central third of APC is sufficient to inhibit cellular proliferation and induce apoptosis in colorectal cancer cells.

The biological effects of the central third of APC are likely related to abrogation of the APC/β-catenin/Tcf-4 signaling pathway. This conclusion is based on the fact that Ad-Mini-ME inhibits β-catenin nuclear translocation (Fig. 12), suppresses β-catenin/Tcf-4 mediated transcription in reporter assays (Fig. 10), and downregulates the expression of targets of the APC/β-catenin/Tcf-4 pathway (Fig. 11). Cellular proliferation and colony formation are dramatically suppressed by Ad-Mini-ME in cell lines which contain mutations in the *APC* gene, but are only partially inhibited in lines containing mutations of β-catenin that render it resistant to APC degradation.

At the cellular level, expression of the central third of APC eventually results in apoptosis of colorectal cancer cells containing APC mutations. This observation is consistent with those in a previous report, demonstrating apoptosis 60 hours after induction of full length APC expression. In both cases, the delay in appearance of apoptotic cells

suggests that APC-induced apoptosis may not be a direct result of suppression of CRT.

While the β-catenin binding domain in the central third of APC is sufficient for growth suppression by APC, it may not recapitulate all of the functions of this gene. For example, the carboxyl-terminal third of APC can associate with the human homolog of the Drosophila tumor suppressor gene discs large (hDLG) and EB-1. The latter has recently been implicated in the spindle checkpoint. In addition, a carboxyl-terminal fragment of APC has been shown to induce assembly and bundling of microtubules in vitro and has a role in directed cell migration. Like other canonical tumor suppressor genes, it is likely that APC functions at several levels to regulate cell growth and suppress neoplastic transformation. However, the finding that the middle third of APC is sufficient to inhibit tumor cell growth focuses further attention on the APC/β-catenin interaction. Future experiments to understand the upstream regulators and downstream transducers of this interaction should shed further light on tumorigenesis associated with defects in the APC pathway.

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CLAIMS

1. A fusion protein which comprises an enzyme covalently linked to a portion of APC which comprises its β-catenin binding domain.
2. The fusion protein of claim 1 wherein the β-catenin binding domain consists of at least amino acids 958 to 2075.
3. The fusion protein of claim 1 wherein the enzyme is GFP.
4. An isolated and purified polynucleotide which encodes the fusion protein of claim 1.
5. An isolated and purified polynucleotide which encodes the fusion protein of claim 2.
6. An isolated and purified polypeptide comprising a portion of APC which comprises its β-catenin binding domain.
7. The isolated and purified polypeptide of claim 6 wherein the binding domain consists of at least amino acids 958 to 2075.
8. An isolated and purified polynucleotide which encodes the polypeptide of claim 6.
9. An isolated and purified polynucleotide which encodes the polypeptide of claim 7.
10. A method for inhibiting the growth of a tumor cell comprising: administering to a tumor cell a polynucleotide according to claim 8, whereby growth of the tumor cell is inhibited.
11. The method of claim 10 wherein the tumor cell is in vitro.
12. The method of claim 10 wherein the tumor cell is in a human body.
13. The method of claim 10 wherein the polynucleotide is part of an adenovirus.
14. A method for inducing apoptosis of a tumor cell comprising: administering to a tumor cell a polynucleotide according to claim 8, whereby apoptosis of tumor cells is induced.
15. The method of claim 14 wherein the tumor cell is in vitro.

16. The method of claim 14 wherein the tumor cell is in a human body.

17. The method of claim 14 wherein the polynucleotide is part of an adenovirus.

18. An adenovirus vector which comprises the polynucleotide of claim 8.

19. An adenovirus vector which comprises the polynucleotide of claim 9.

20. The adenovirus vector of claim 19 which is Ad-mini-ME.

21. The fusion protein of claim 1 which lacks at last one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.

22. The polynucleotide of claim 4 wherein the fusion protein lacks at least one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.

23. The isolated and purified polypeptide of claim 6 which lacks at least one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.

24. The isolated and purified polynucleotide of claim 8 wherein the polypeptide lacks at lest one APC domain selected from the group consisting of: oligomerization domain, armadillo repeat domain, human homolog of Drosophila tumor suppressor gene discs large domain, and EB1 binding domain.

25. A method for treating a patient with colorectal cancer or other cancer associated with FAP, the method comprising the step of:

administering to the patient a nucleotide sequence comprising a portion of the APC coding sequence, said portion consisting of the β-catenin binding site.

26. A method for treating a patient with colorectal cancer or other cancer associated with FAP, the method comprising the step of:

administering to the patient a polypeptide comprising a portion of the APC coding sequence, said portion consisting of the β-catenin binding site.

FIG. IA

FIG. 1B

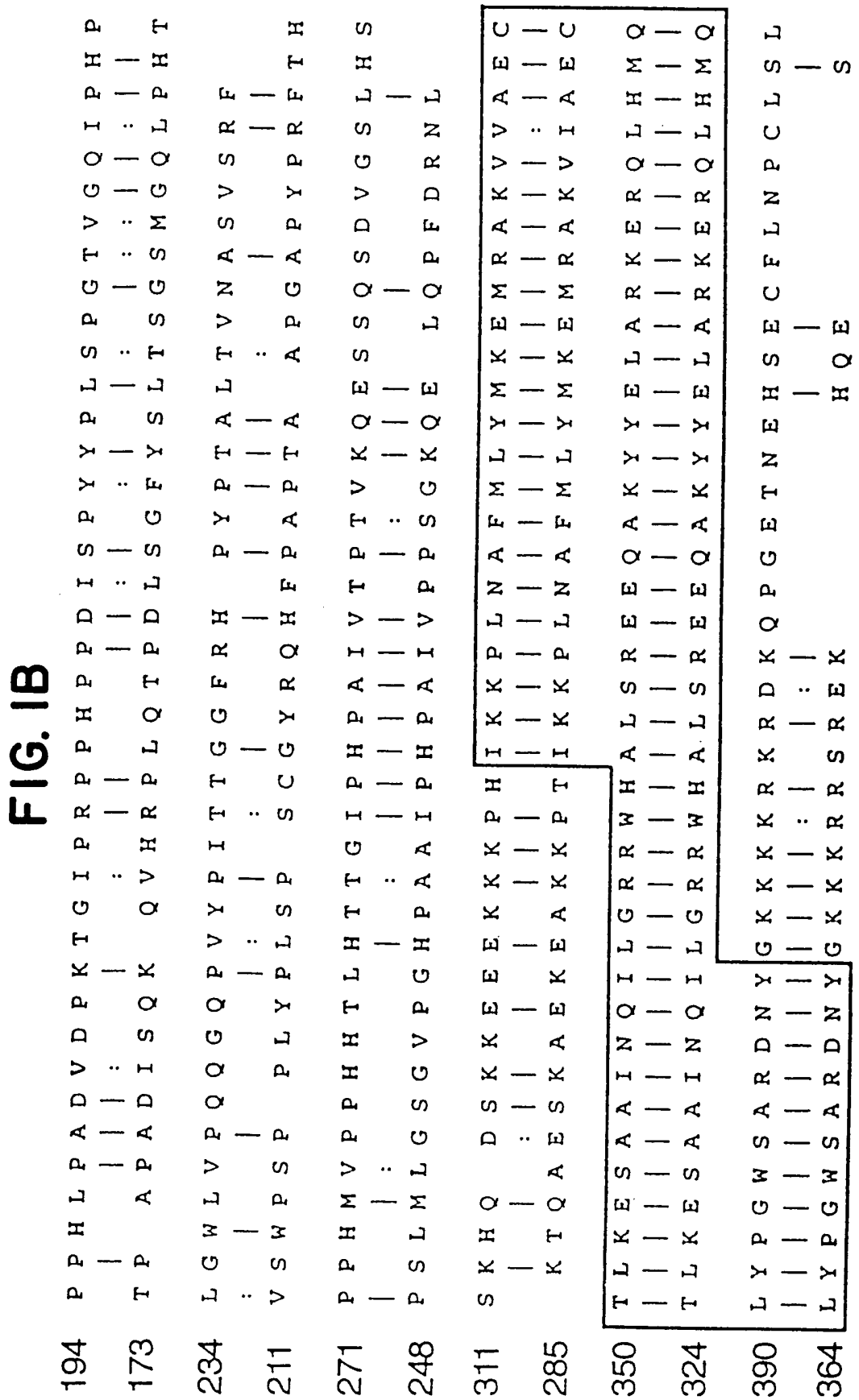


FIG. IC

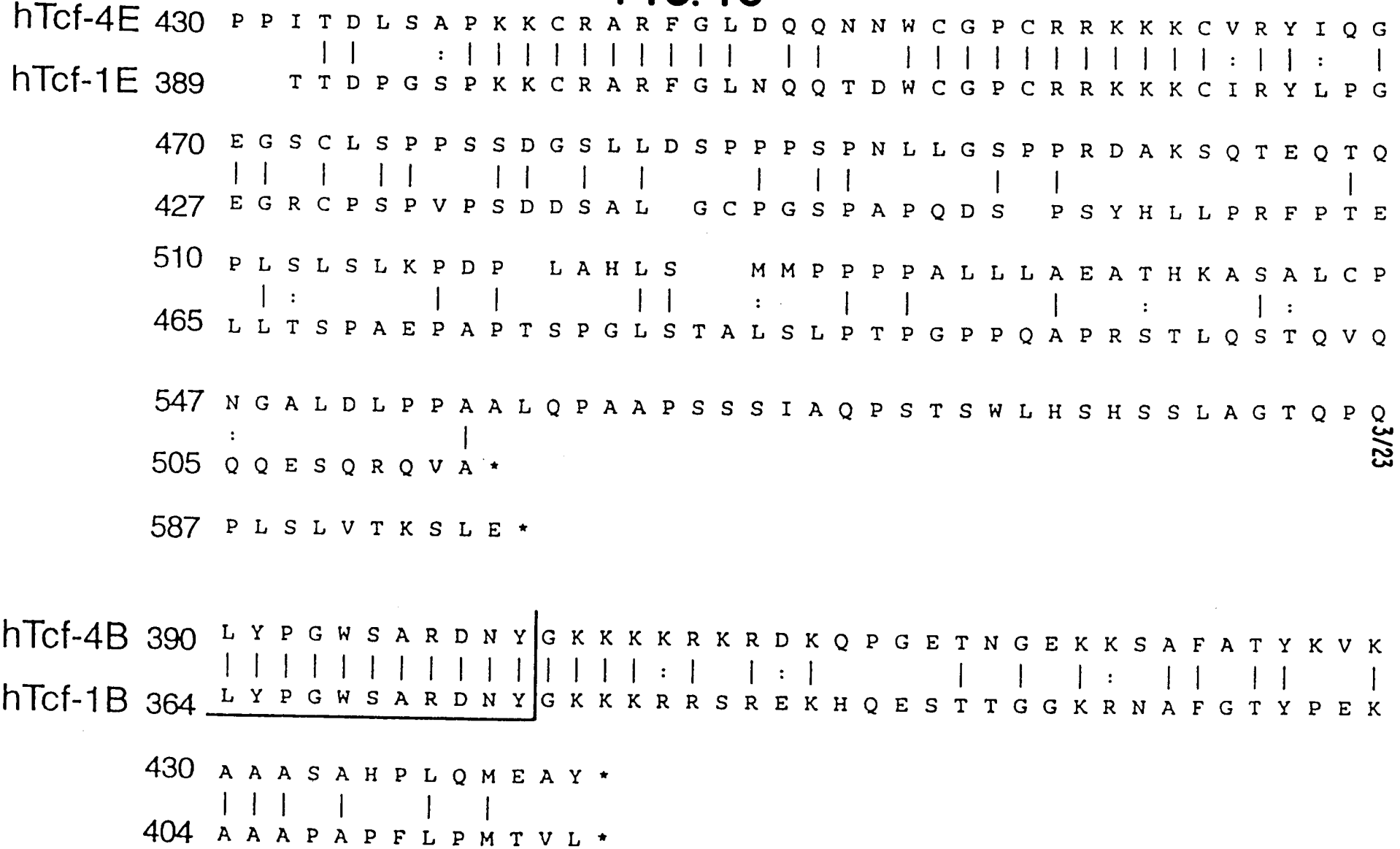


FIG. 2A

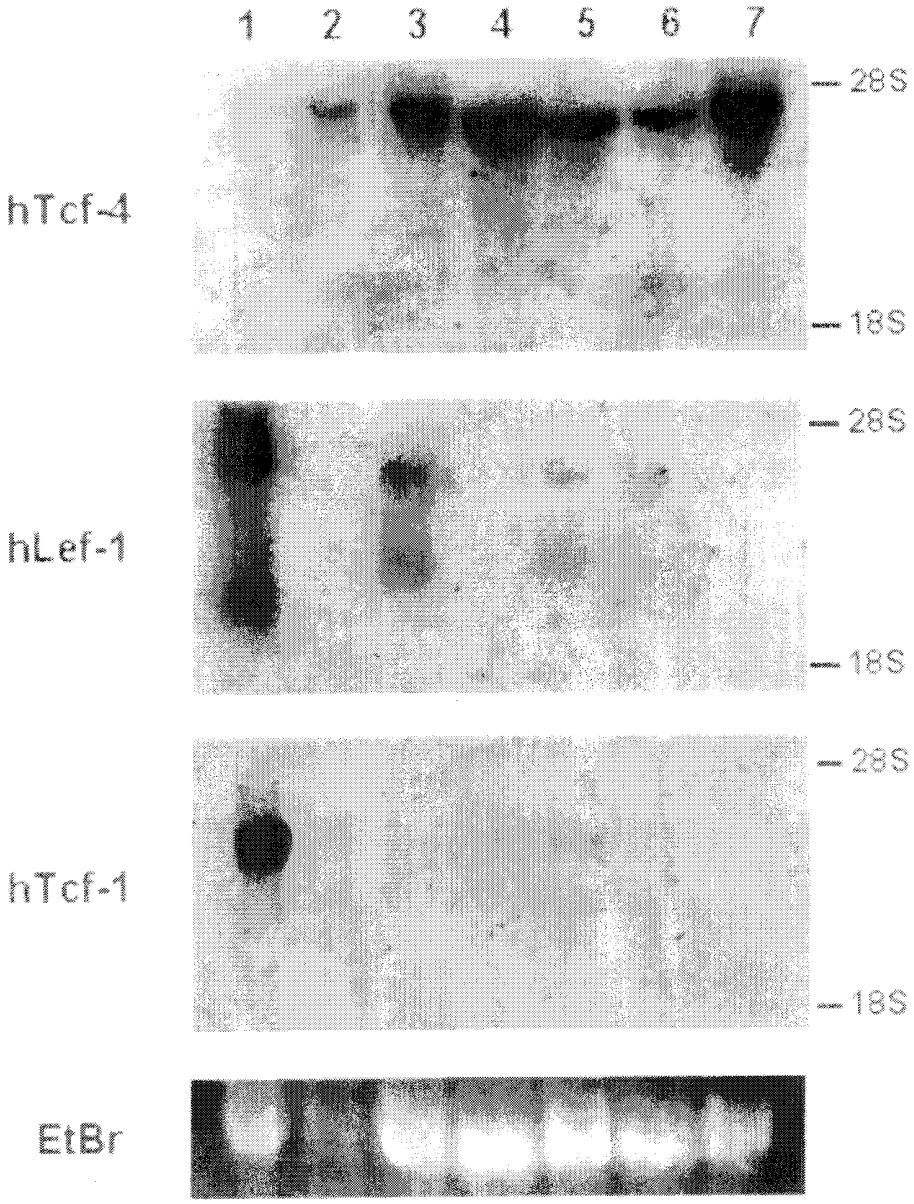


FIG. 2B

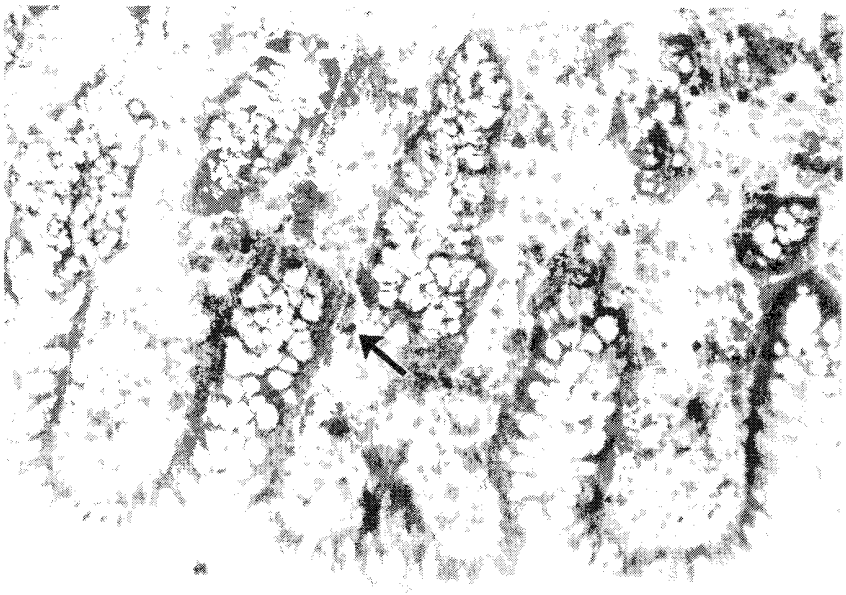


FIG. 2C

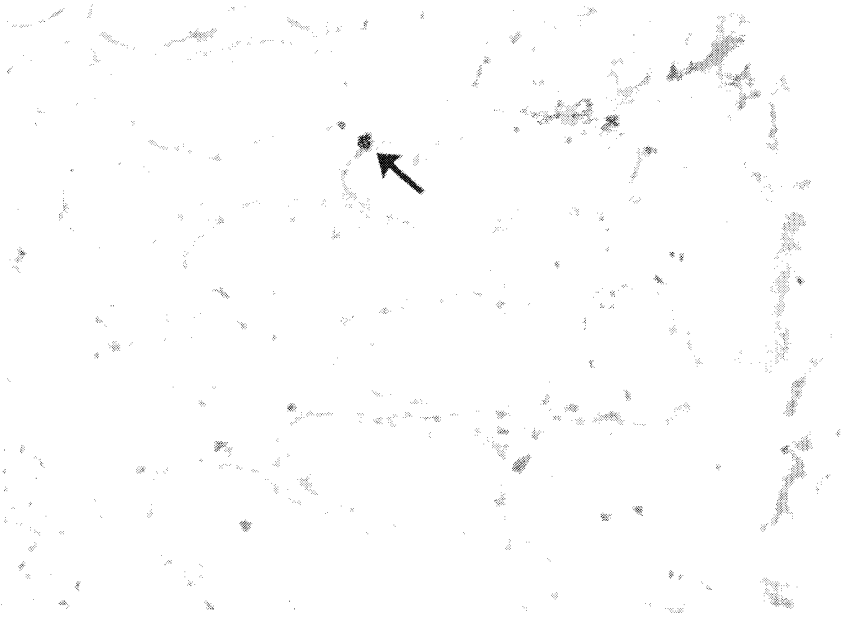


FIG. 3A

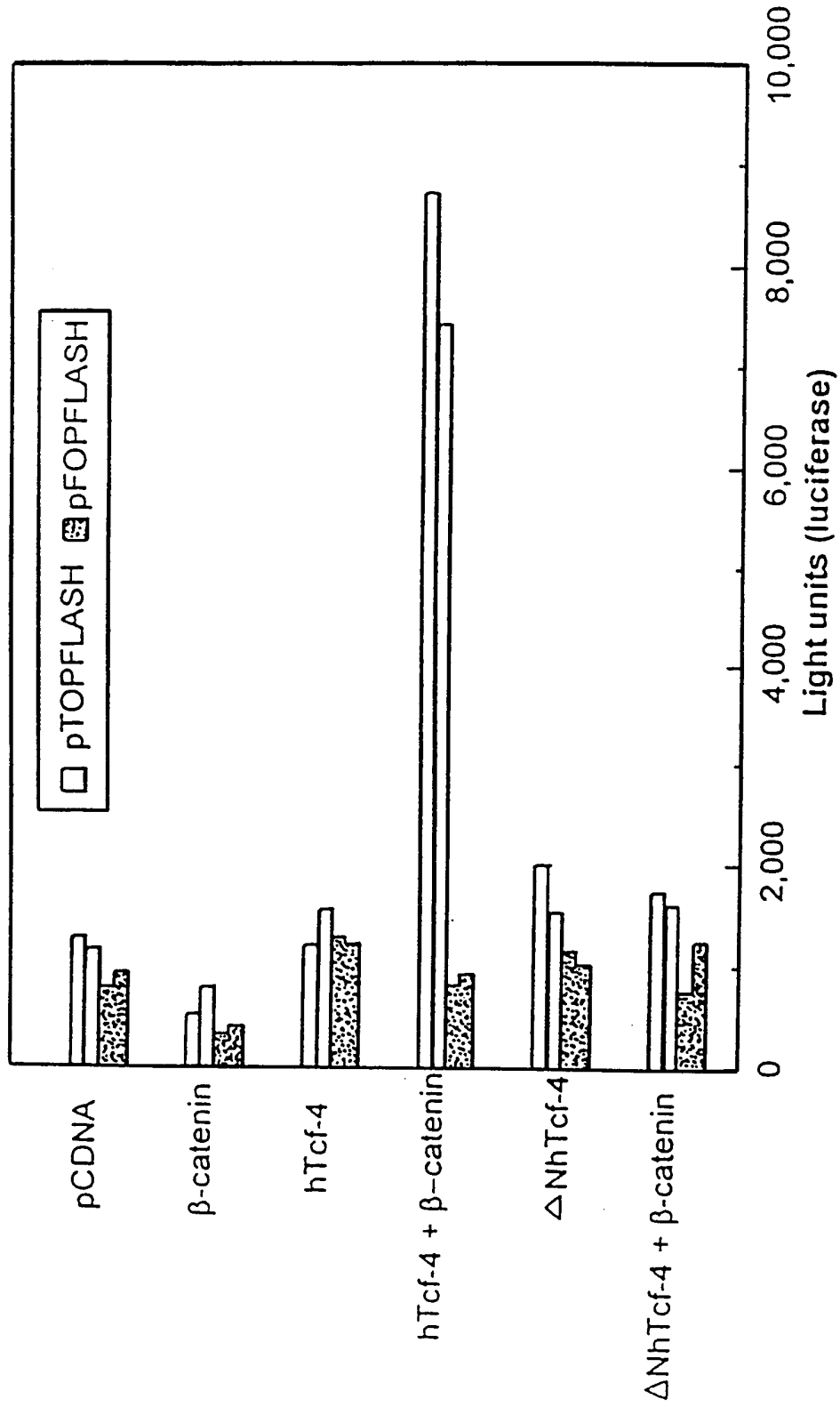

pTOPFLASH
pFOPFLASH
0
2,000
4,000
6,000
8,000
10,000
Light units (luciferase)
pCDNA
β-catenin
hTcf-4
hTcf-4 + β-catenin
ΔNhTcf-4
ΔNhTcf-4 + β-catenin

FIG. 3C

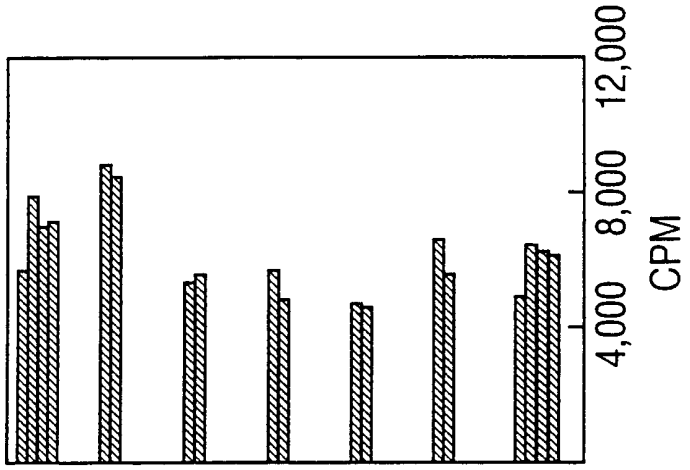

4,000
8,000
12,000
CPM

FIG. 3B

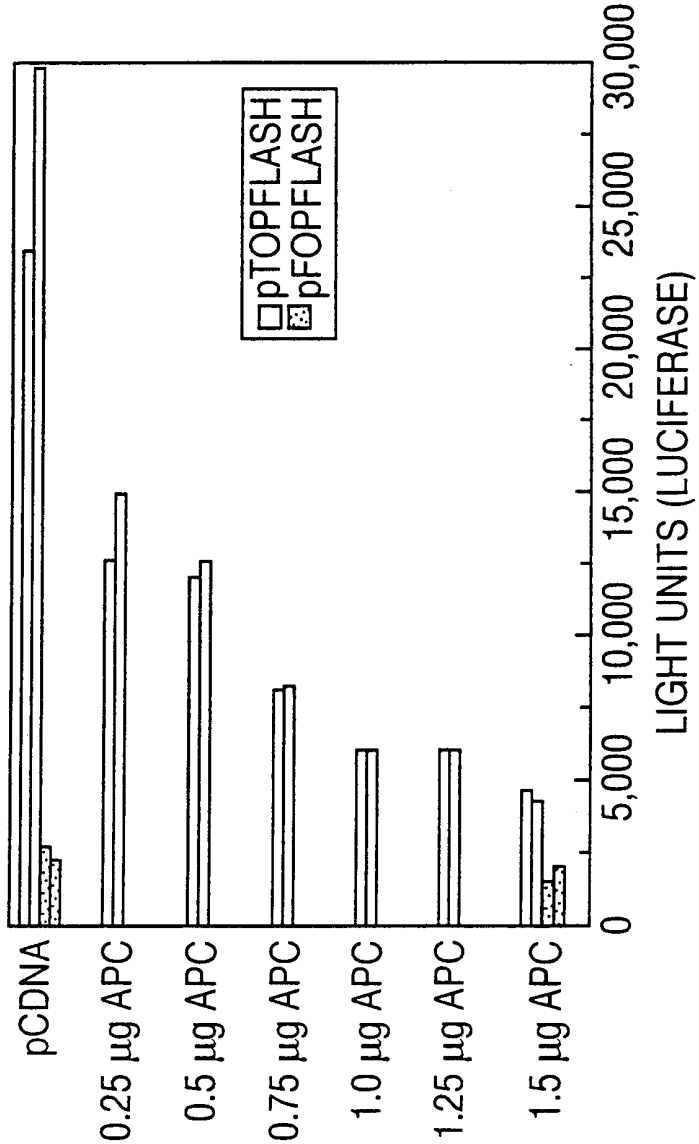

pTOPFLASH
pFOPFLASH
0
5,000
10,000
15,000
20,000
25,000
30,000
LIGHT UNITS (LUCIFERASE)
pCDNA
0.25 µg APC
0.5 µg APC
0.75 µg APC
1.0 µg APC
1.25 µg APC
1.5 µg APC

FIG. 4

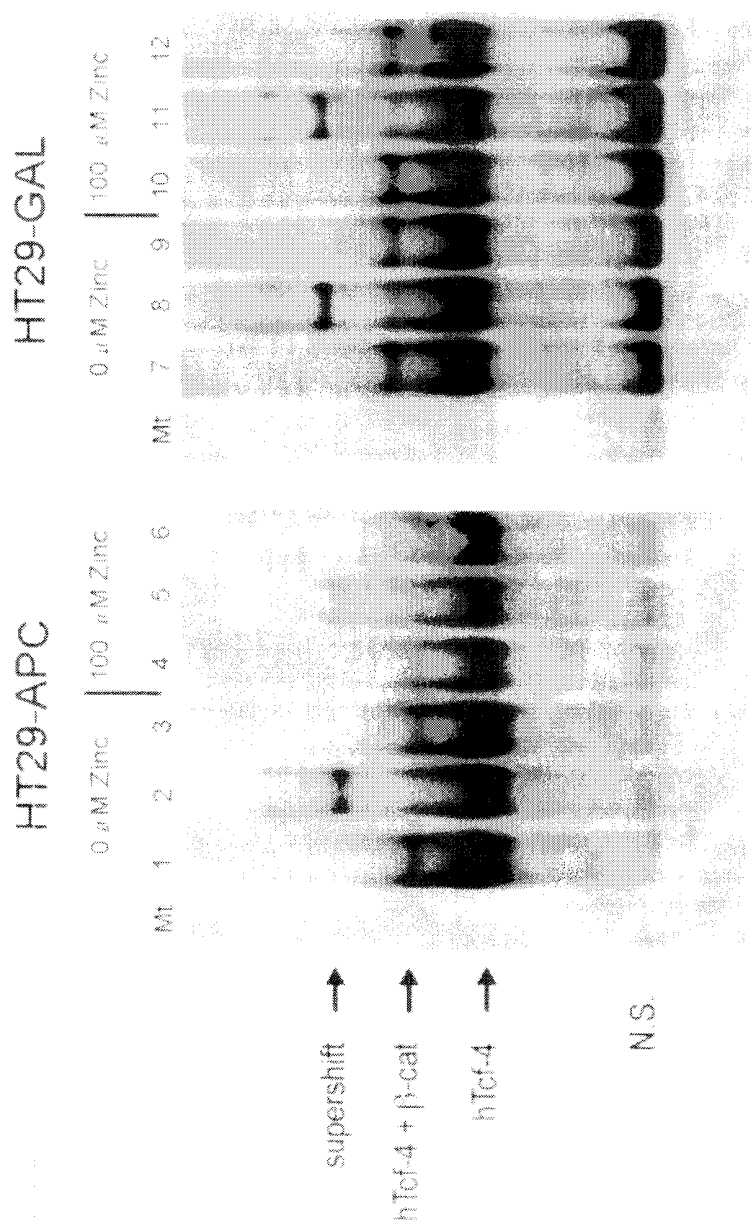

HT29-GAL
0 μM Zinc
100 μM Zinc
Mt 7 8 9 10 11 12
HT29-APC
0 μM Zinc
100 μM Zinc
Mt 1 2 3 4 5 6
supershift
hTcf-4 + β-cat
hTcf-4
N.S.

FIG. 5A

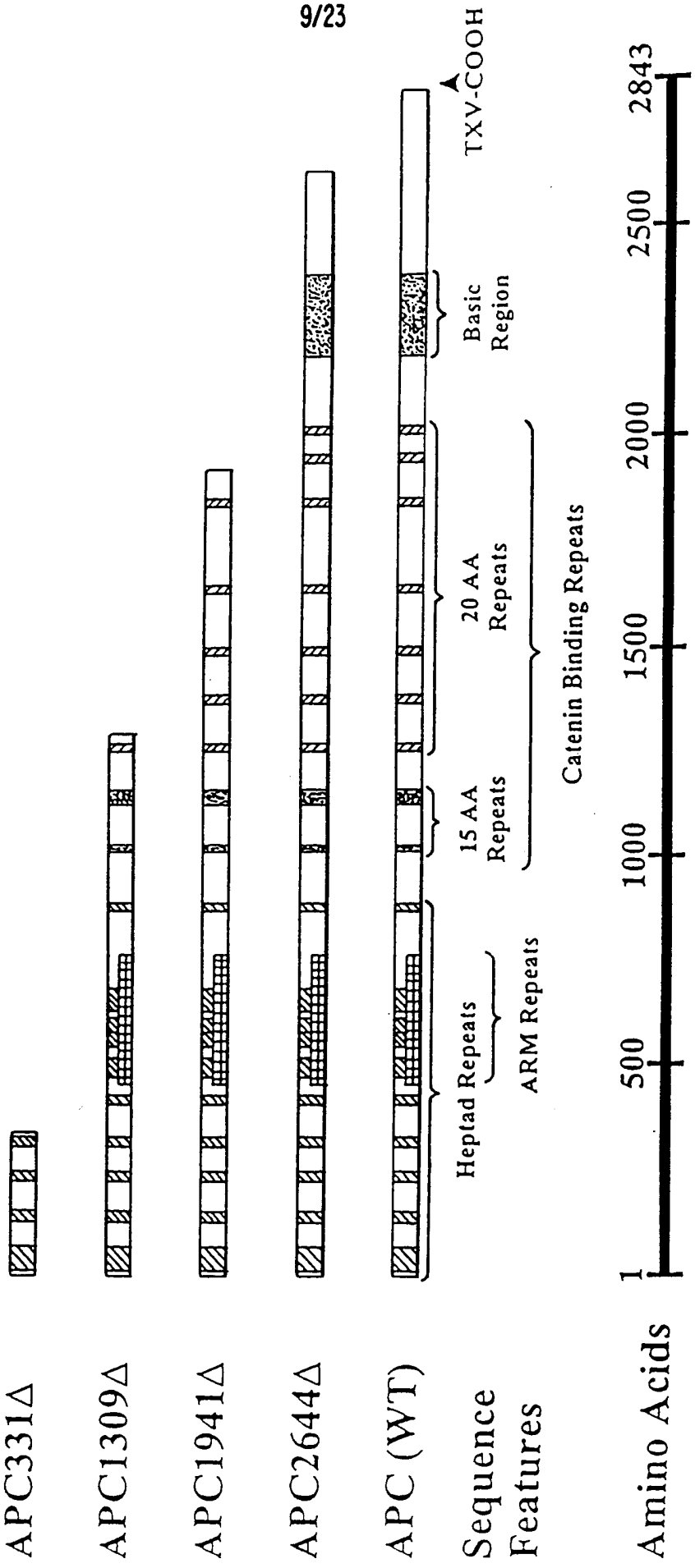

APC331Δ
APC1309Δ
APC1941Δ
APC2644Δ
APC (WT)
Sequence Features
Amino Acids
Heptad Repeats
ARM Repeats
15 AA Repeats
20 AA Repeats
Catenin Binding Repeats
Basic Region
TXV-COOH
1
500
1000
1500
2000
2500
2843

FIG. 5B

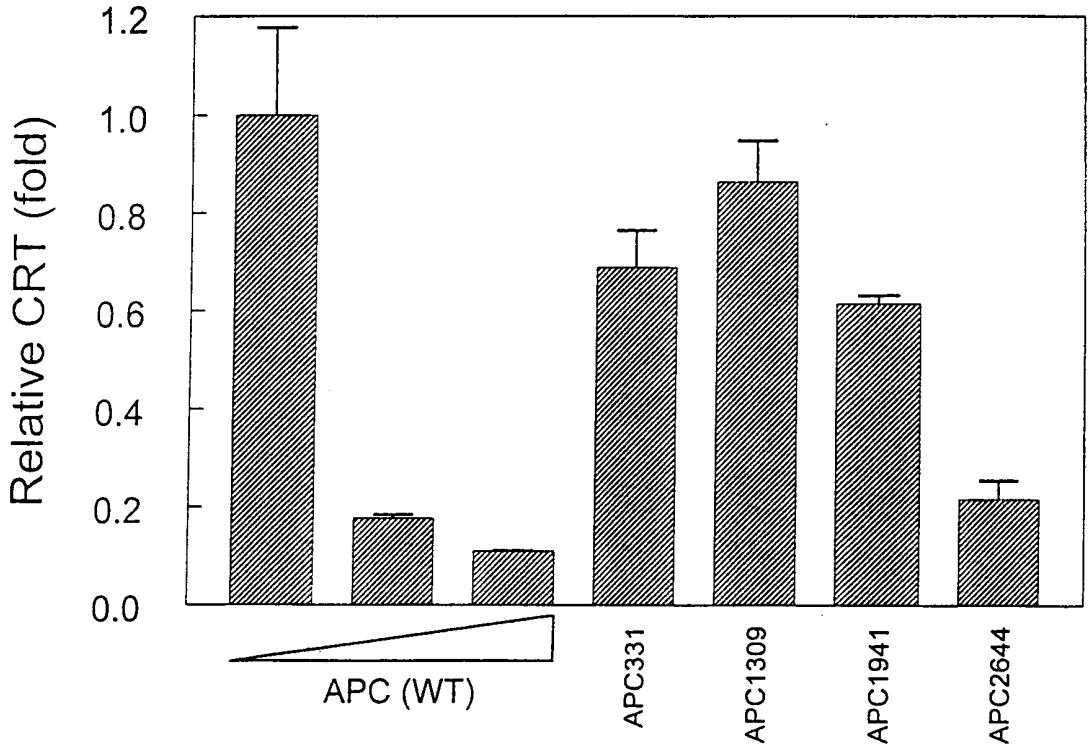

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative CRT (fold)
APC (WT)
APC331
APC1309
APC1941
APC2644

FIG. 6B

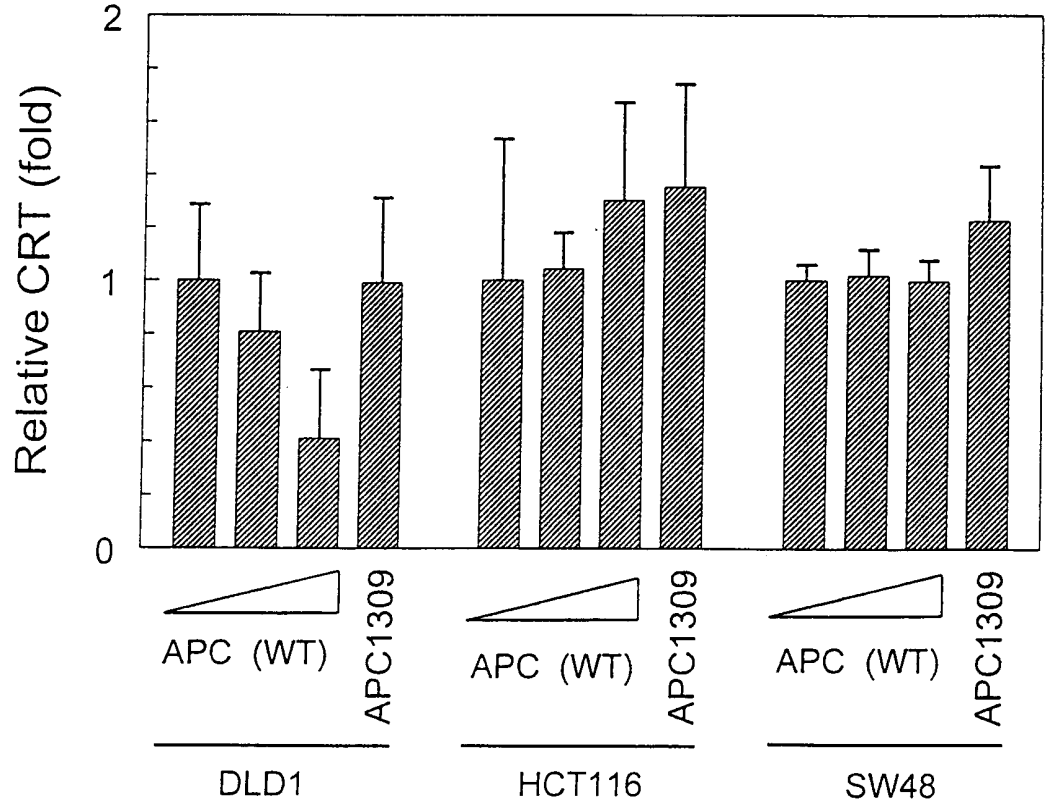

2
1
0
Relative CRT (fold)
APC (WT)
APC1309
APC (WT)
APC1309
APC (WT)
APC1309
DLD1
HCT116
SW48

FIG. 6A

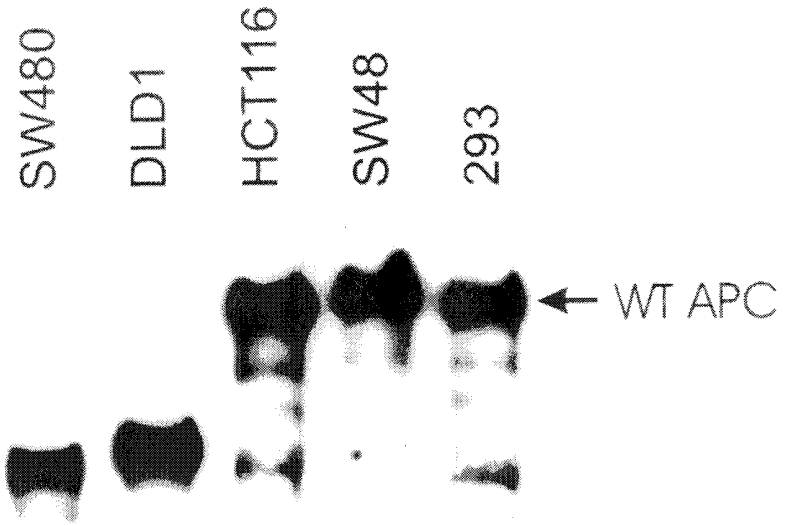
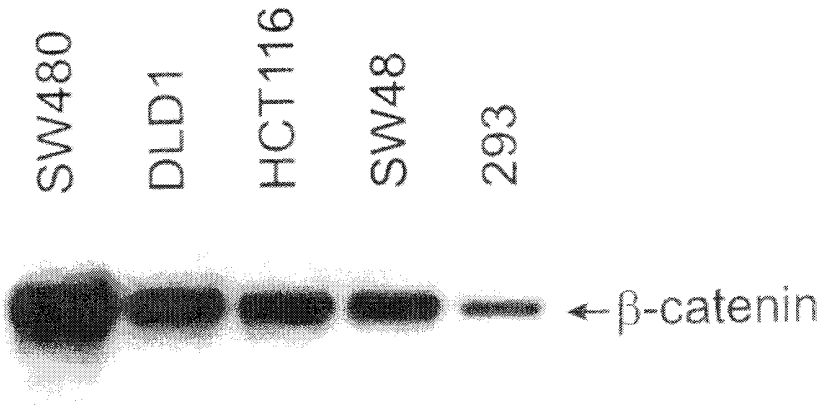

SW480
DLD1
HCT116
SW48
293
WT APC

FIG. 7A


SW480
DLD1
HCT116
SW48
293
β-catenin

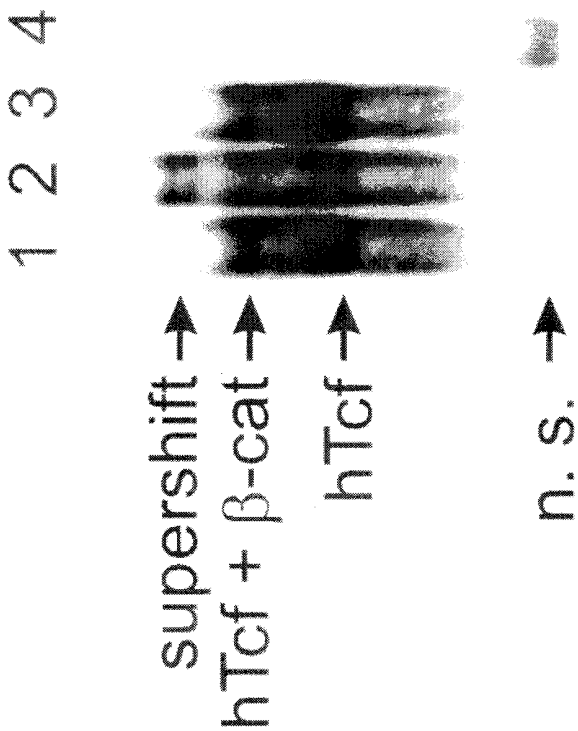

1 2 3 4
supershift
hTcf + β-cat
hTcf
n. s.

FIG. 8A

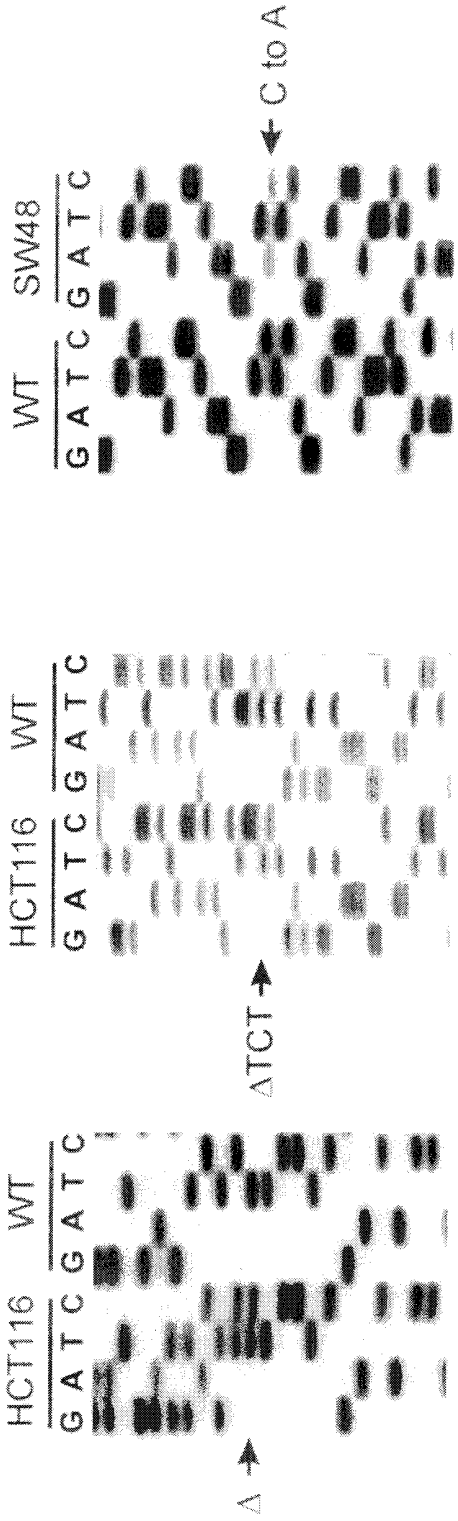

C to A
SW48
WT
G A T C G A T C
ΔTCT
HCT116
WT
G A T C G A T C
Δ
HCT116
WT
G A T C G A T C

FIG. 7B

FIG. 7C

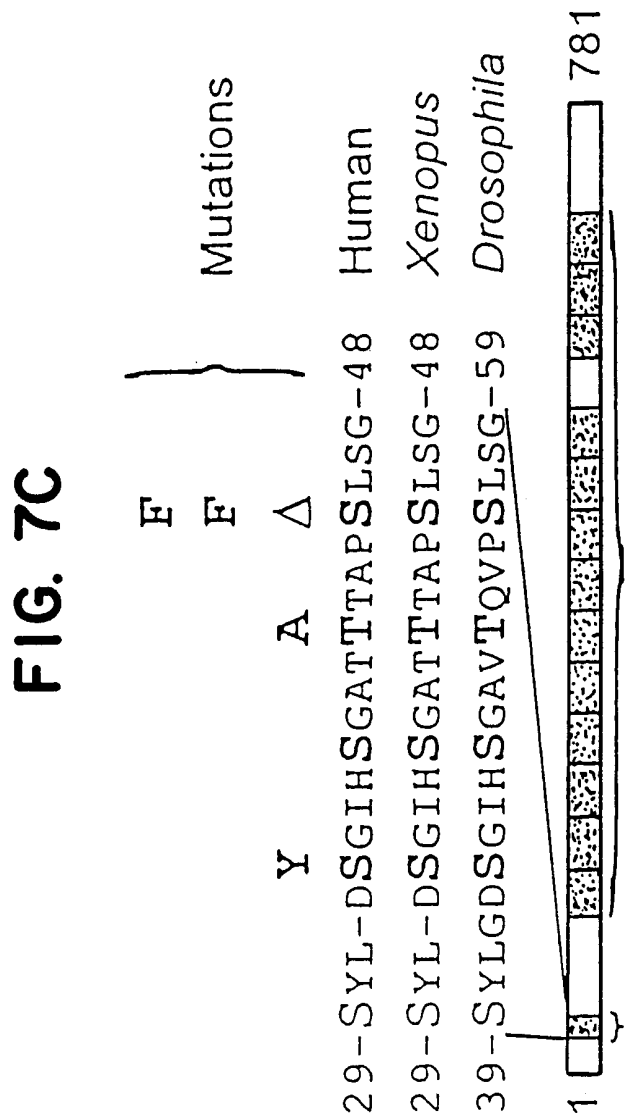

Mutations
F
F
Δ
A
Y
29-SYL-DSGIHSGATTTAPSLSG-48 Human
29-SYL-DSGIHSGATTTAPSLSG-48 Xenopus
39-SYLGDSGIHSGAVTQVPSLSG-59 Drosophila
1
781
GSK3β Sites
Armadillo Repeats

FIG. 8B

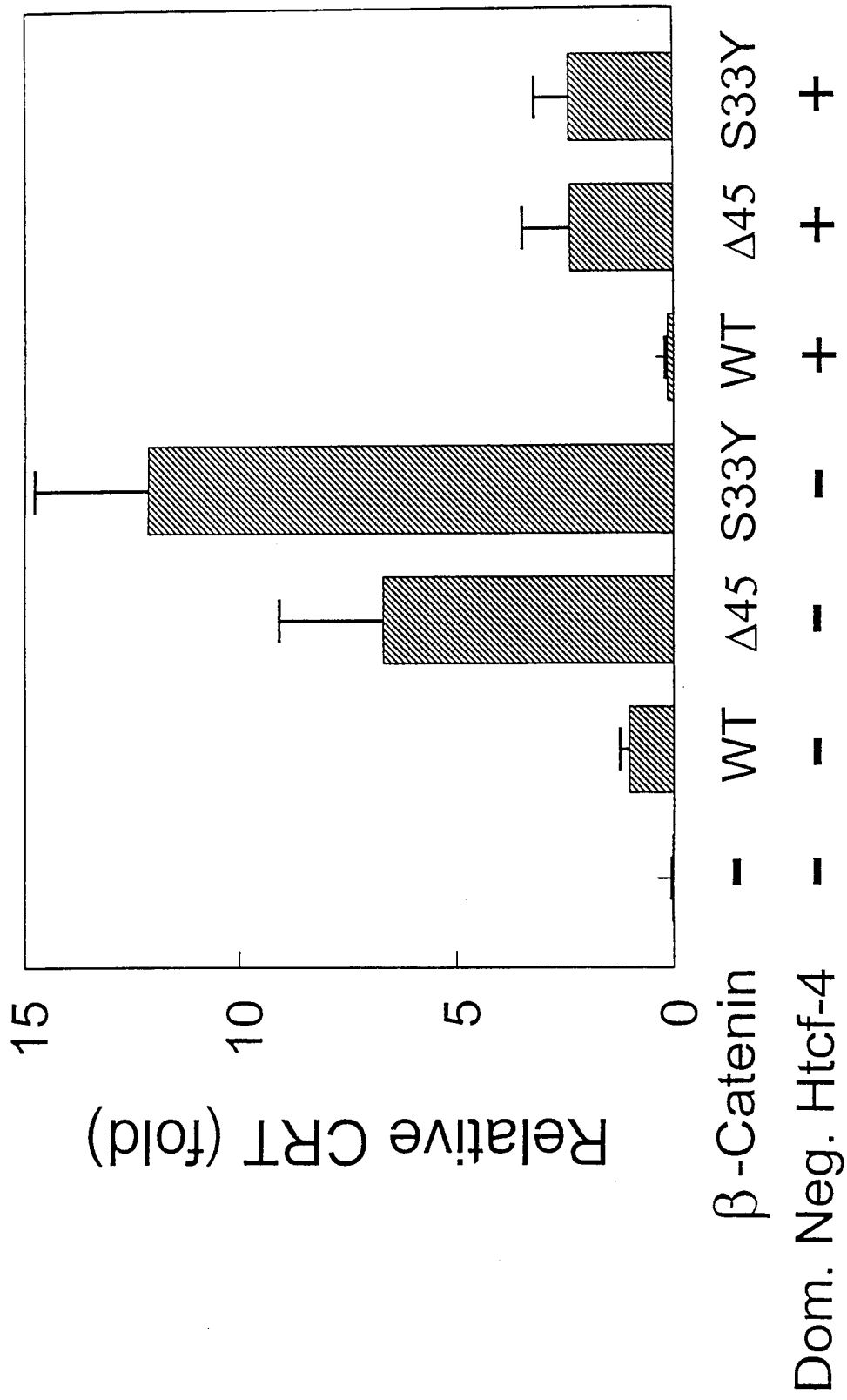

Relative CRT (fold)
0
5
10
15
β-Catenin - WT Δ45 S33Y WT Δ45 S33Y
Dom. Neg. Htcf-4 - - - - + + +

FIG. 9A

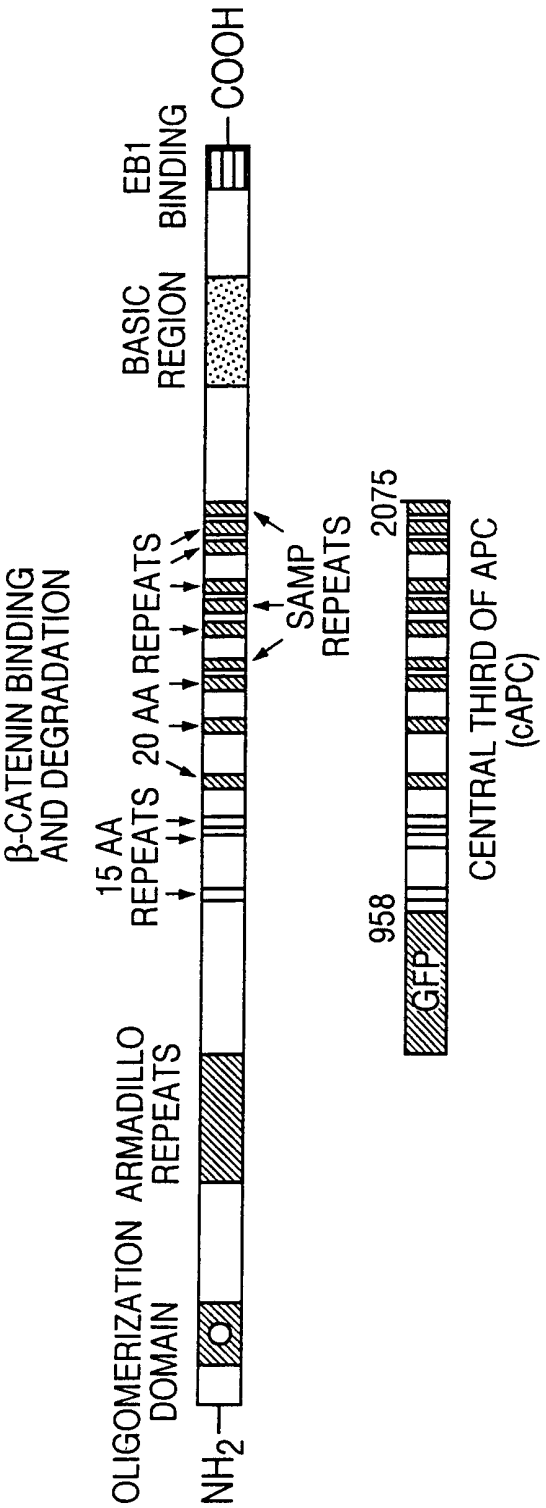

β-CATENIN BINDING AND DEGRADATION
15 AA REPEATS
20 AA REPEATS
BASIC REGION
EB1 BINDING
COOH
SAMP REPEATS
2075
958
GFP
CENTRAL THIRD OF APC (cAPC)
OLIGOMERIZATION DOMAIN
ARMADILLO REPEATS
NH2

FIG. 9B

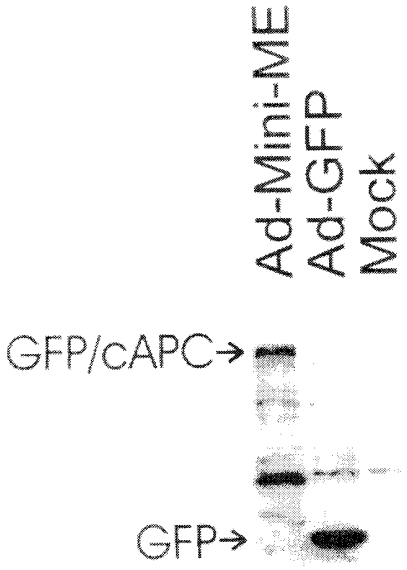


FIG. 9C

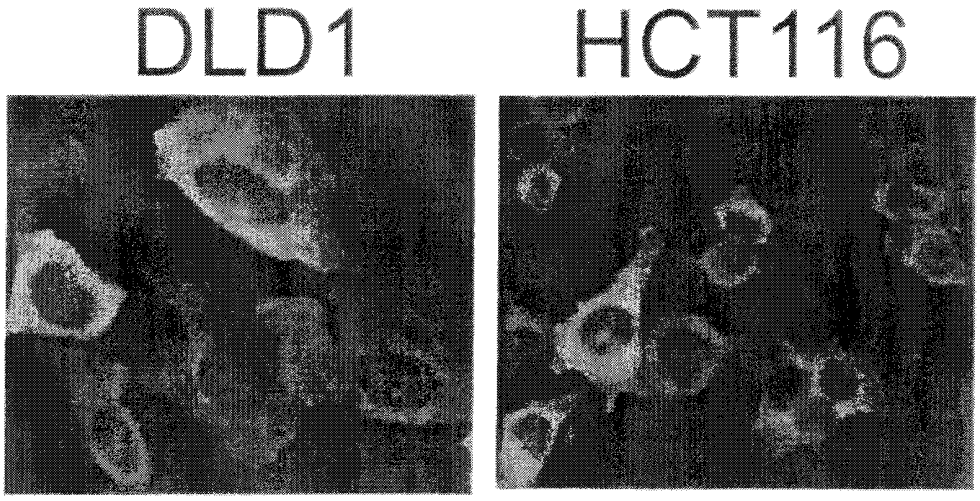


FIG. 10A

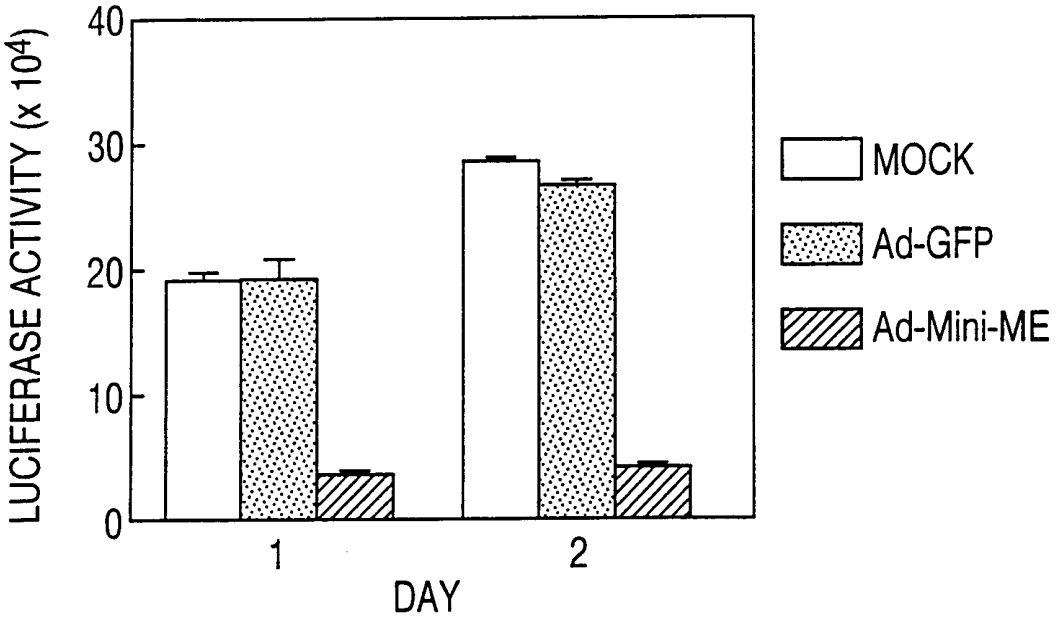


FIG. 10B

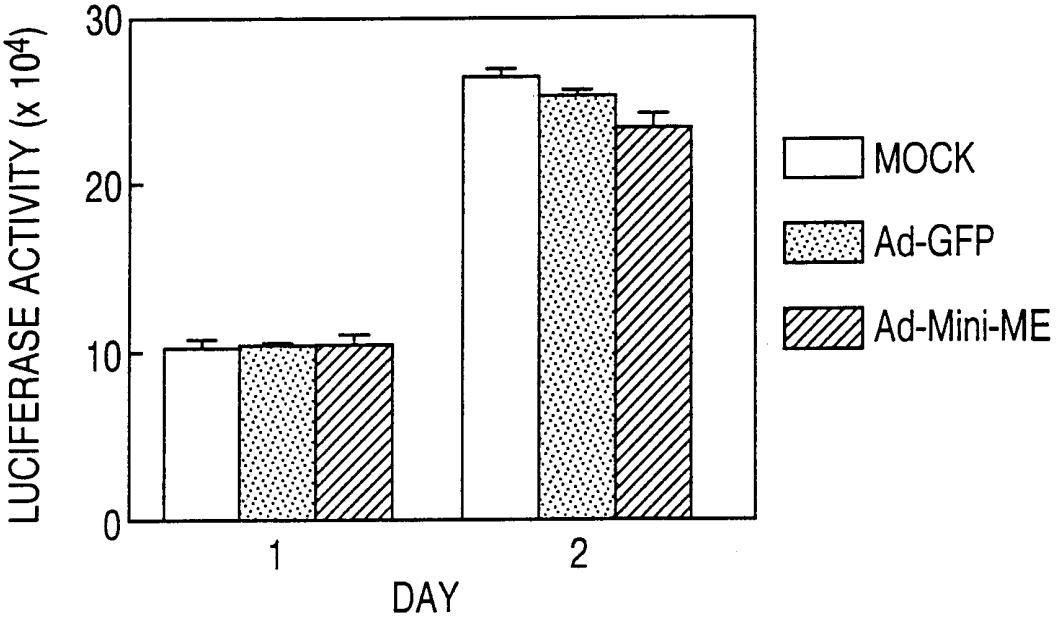


FIG. II

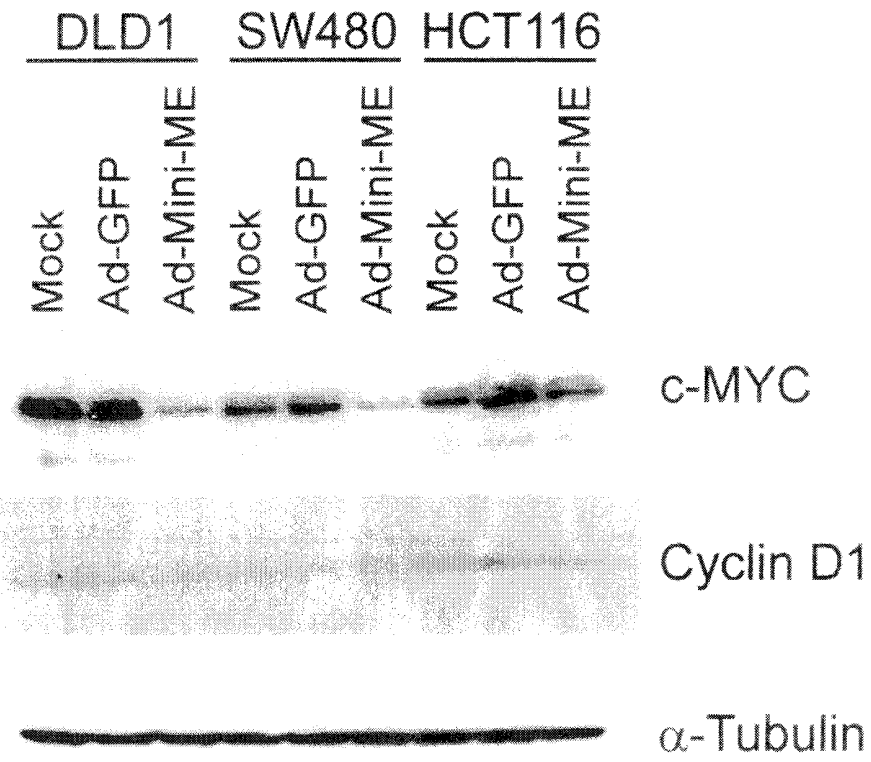

DLD1
SW480
HCT116
Mock
Ad-GFP
Ad-Mini-ME
Mock
Ad-GFP
Ad-Mini-ME
Mock
Ad-GFP
Ad-Mini-ME
c-MYC
Cyclin D1
α-Tubulin

FIG. 12A

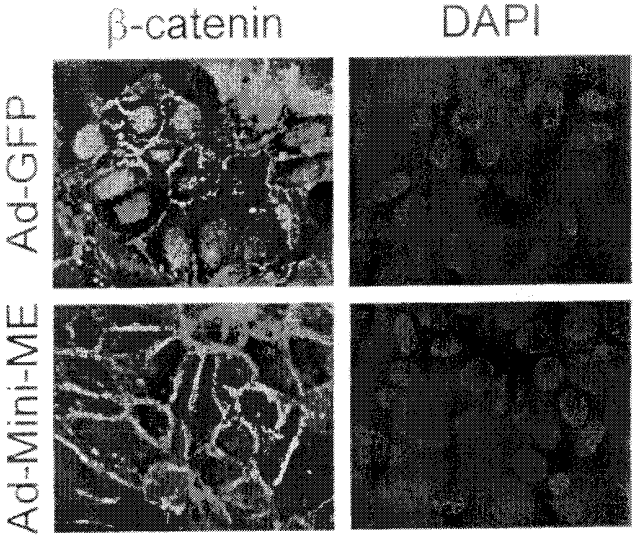


FIG. 12B

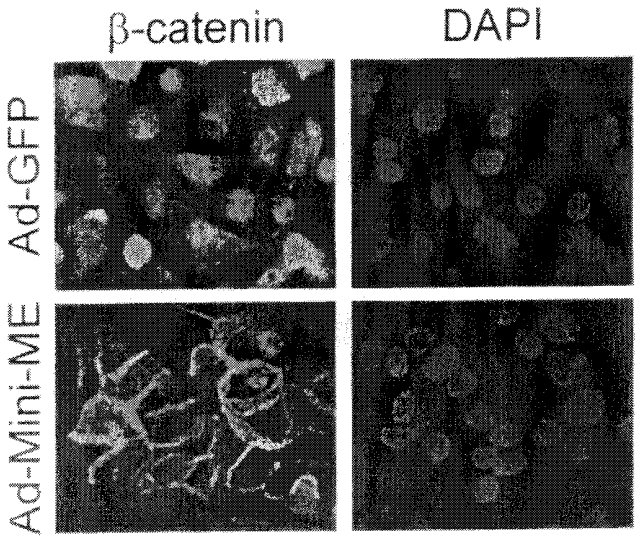
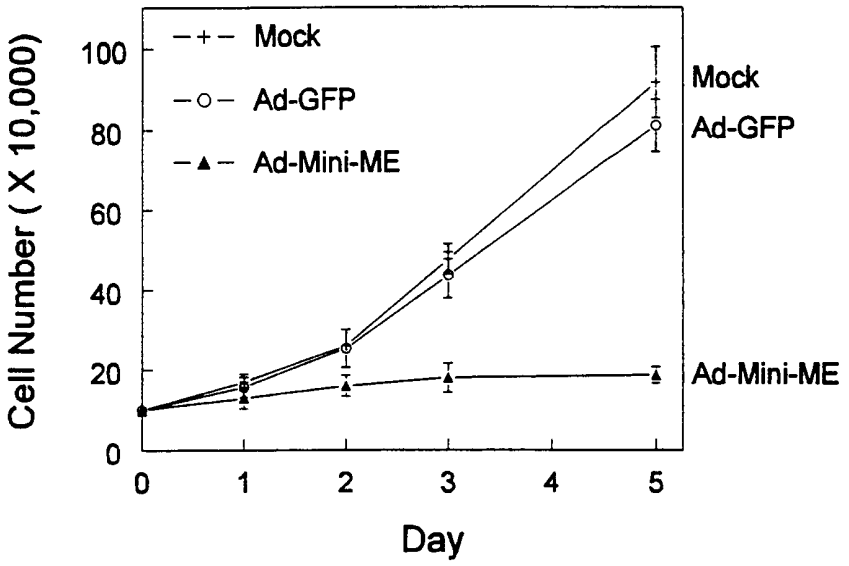
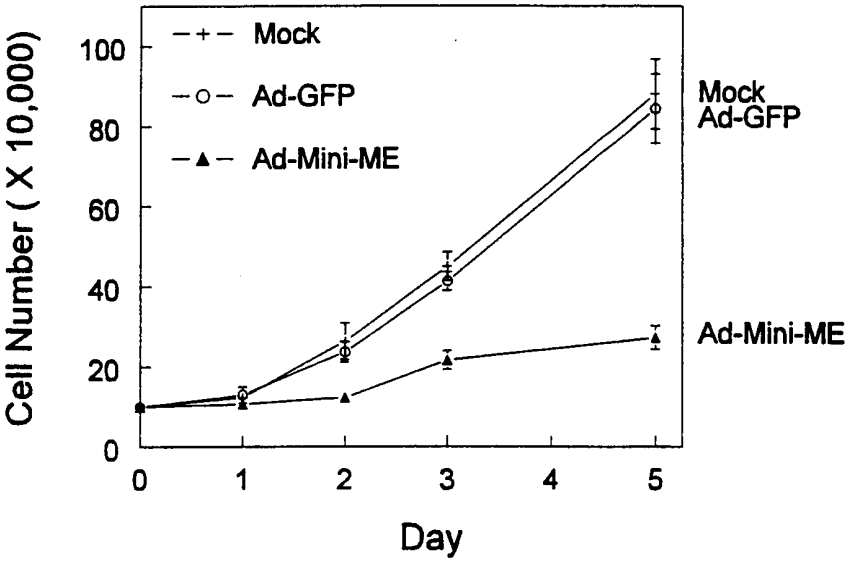


FIG. 13A

DLD1



SW480



HCT116

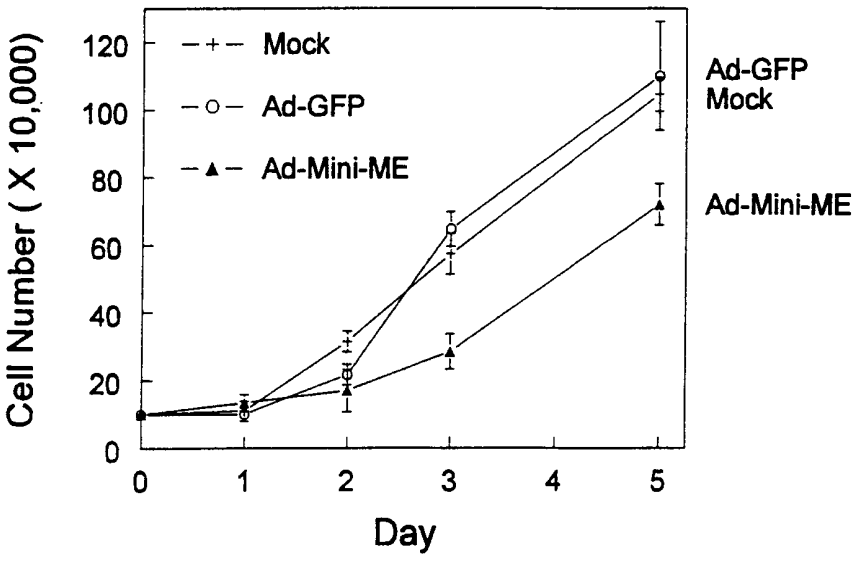
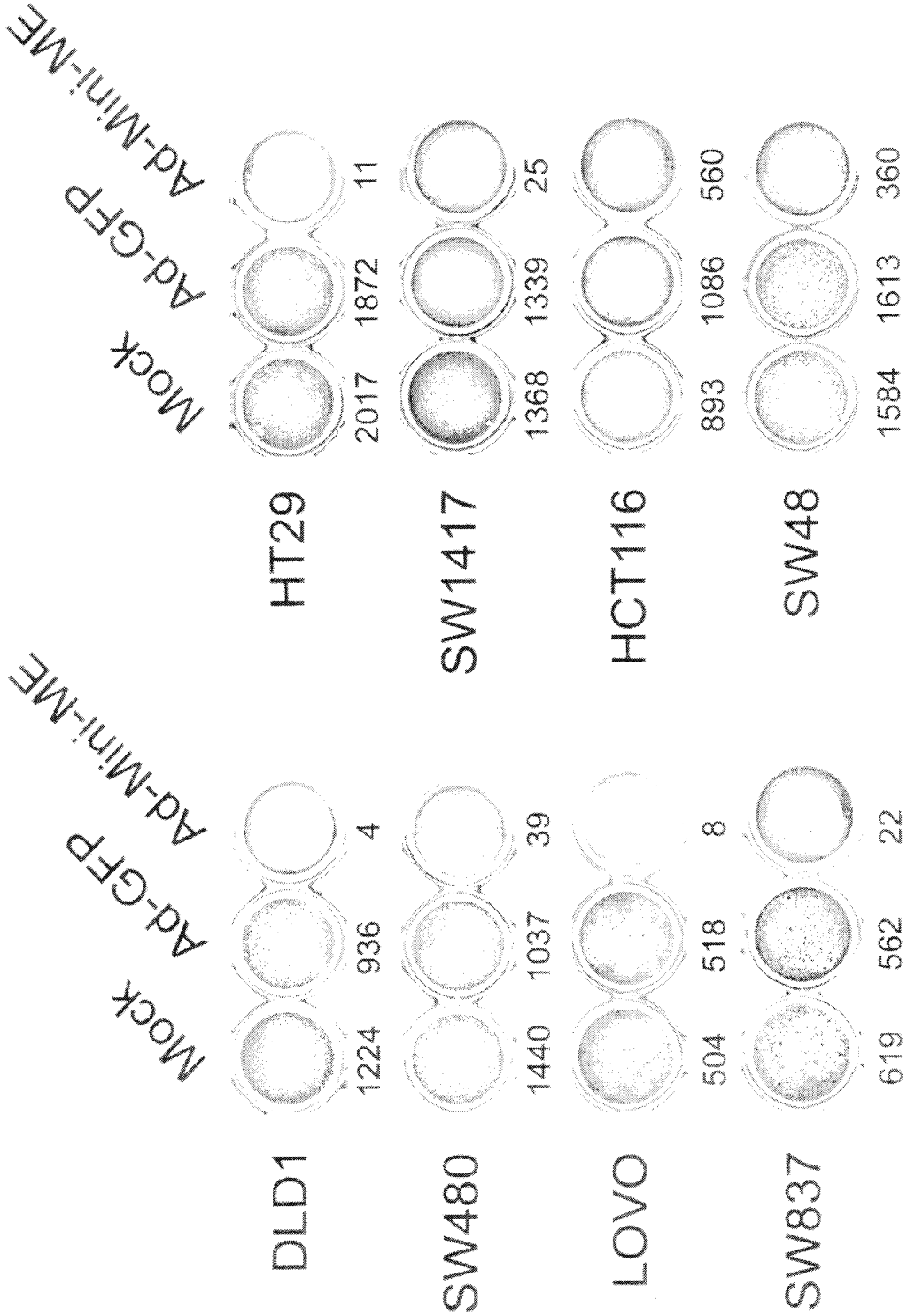


FIG. 13B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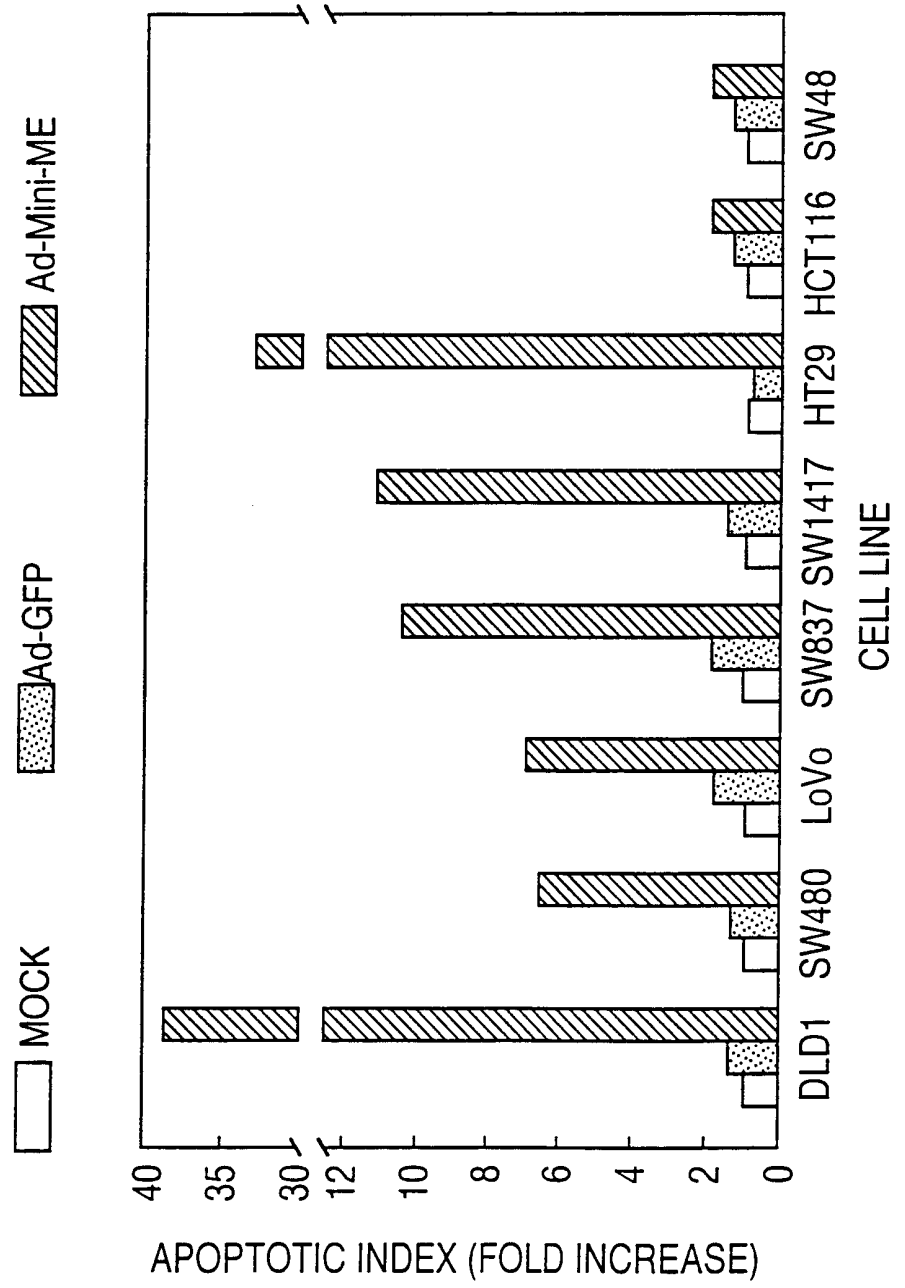
Ad-Mini-ME
HT29
2017 1872 11
SW1417
1368 1339 25
HCT116
893 1086 560
SW48
1584 1613 360
DLD1
1224 936 4
SW480
1440 1037 39
LOVO
504 518 8
SW837
619 562 22

FIG. 14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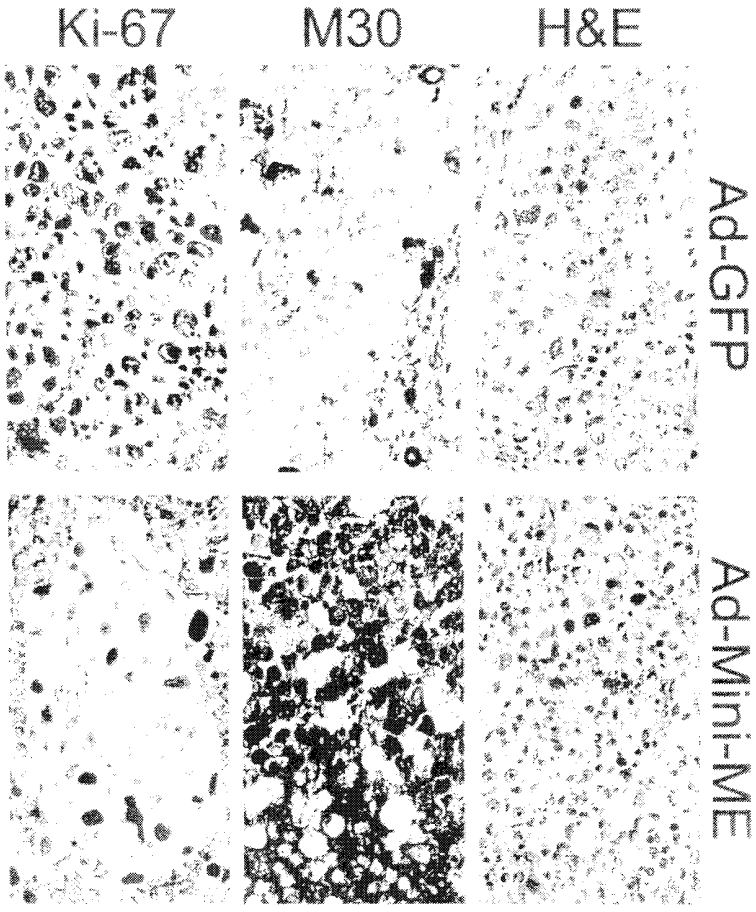
Ad-GFP
Ad-Mini-ME
40
35
30
12
10
8
6
4
2
0
APOPTOTIC INDEX (FOLD INCREASE)
DLD1
SW480
LoVo
SW837
SW1417
HT29
HCT116
SW48
CELL LINE

FIG. 15



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20 25 30
Glu Asn Ser Ser Ala Glu Arg Asp Leu Ala Asp Val Lys Ser Ser Leu
35 40 45
Val Asn Glu Ser Glu Thr Asn Gln Asn Ser Ser Ser Asp Ser Glu Ala
50 55 60
Glu Arg Arg Pro Pro Pro Arg Ser Glu Ser Phe Arg Asp Lys Ser Arg
65 70 75 80
Glu Ser Leu Glu Glu Ala Ala Lys Arg Gln Asp Gly Gly Leu Phe Lys
85 90 95
Gly Pro Pro Tyr Pro Gly Tyr Pro Phe Ile Met Ile Pro Asp Leu Thr
100 105 110
Ser Pro Tyr Leu Pro Lys Arg Ser Val Ser Pro Thr Ala Arg Thr Tyr
115 120 125
Leu Gln Met Lys Trp Pro Leu Leu Asp Val Gln Ala Gly Ser Leu Gln
130 135 140
Ser Arg Gln Ala Leu Lys Asp Ala Arg Ser Pro Ser Pro Ala His Ile
145 150 155 160
Val Ser Asn Lys Val Pro Val Val Gln His Pro His His Val His Pro
165 170 175
Leu Thr Pro Leu Ile Thr Tyr Ser Asn Glu His Phe Thr Pro Gly Asn
180 185 190
Pro Pro Pro His Leu Pro Ala Asp Val Asp Pro Lys Thr Gly Ile Pro
195 200 205
Arg Pro Pro His Pro Pro Asp Ile Ser Pro Tyr Tyr Pro Leu Ser Pro
210 215 220
Gly Thr Val Gly Gln Ile Pro His Pro Leu Gly Trp Leu Val Pro Gln
225 230 235 240
Gln Gly Gln Pro Val Tyr Pro Ile Thr Thr Gly Gly Phe Arg His Pro
245 250 255
Tyr Pro Thr Ala Leu Thr Val Asn Ala Ser Val Ser Arg Phe Pro Pro
260 265 270
His Met Val Pro Pro His His Thr Leu His Thr Thr Gly Ile Pro His
275 280 285
Pro Ala Ile Val Thr Pro Thr Val Lys Gln Glu Ser Ser Gln Ser Asp

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    290                 295                 300
Val Gly Ser Leu His Ser Ser Lys His Gln Asp Ser Lys Lys Glu Glu
305                 310                 315                 320
Glu Lys Lys Lys Pro His Ile Lys Lys Pro Leu Asn Ala Phe Met Leu
                325                 330                 335
Tyr Met Lys Glu Met Arg Ala Lys Val Val Ala Glu Cys Thr Leu Lys
            340                 345                 350
Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg Trp His Ala Leu
        355                 360                 365
Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Glu Leu Ala Arg Lys Glu Arg
    370                 375                 380
Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala Arg Asp Asn Tyr
385                 390                 395                 400
Gly Lys Lys Lys Lys Arg Lys Arg Asp Lys Gln Pro Gly Glu Thr Asn
                405                 410                 415
Gly Glu Lys Lys Ser Ala Phe Ala Thr Tyr Lys Val Lys Ala Ala Ala
            420                 425                 430
Ser Ala His Pro Leu Gln Met Glu Ala Tyr
        435                 440
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<212> PRT
<213> Homo sapiens

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            20                  25                  30
Glu Asn Ser Ser Ala Glu Arg Asp Leu Ala Asp Val Lys Ser Ser Leu
        35                  40                  45
Val Asn Glu Ser Glu Thr Asn Gln Asn Ser Ser Ser Asp Ser Glu Ala
    50                  55                  60
Glu Arg Arg Pro Pro Pro Arg Ser Glu Ser Phe Arg Asp Lys Ser Arg
65                  70                  75                  80
Glu Ser Leu Glu Glu Ala Ala Lys Arg Gln Asp Gly Gly Leu Phe Lys
                85                  90                  95
Gly Pro Pro Tyr Pro Gly Tyr Pro Phe Ile Met Ile Pro Asp Leu Thr
            100                 105                 110
Ser Pro Tyr Leu Pro Asn Gly Ser Val Ser Pro Thr Ala Arg Thr Tyr
        115                 120                 125
Leu Gln Met Lys Trp Pro Leu Leu Asp Val Gln Ala Gly Ser Leu Gln
    130                 135                 140
Ser Arg Gln Ala Leu Lys Asp Ala Arg Ser Pro Ser Pro Ala His Ile
145                 150                 155                 160
Val Ser Asn Lys Val Pro Val Val Gln His Pro His His Val His Pro
                165                 170                 175
Leu Thr Pro Leu Ile Thr Tyr Ser Asn Glu His Phe Thr Pro Gly Asn
            180                 185                 190
Pro Pro Pro His Leu Pro Ala Asp Val Asp Pro Lys Thr Gly Ile Pro
        195                 200                 205
Arg Pro Pro His Pro Pro Asp Ile Ser Pro Tyr Tyr Pro Leu Ser Pro
    210                 215                 220
Gly Thr Val Gly Gln Ile Pro His Pro Leu Gly Trp Leu Val Pro Gln
225                 230                 235                 240
Gln Gly Gln Pro Val Tyr Pro Ile Thr Thr Gly Gly Phe Arg His Pro
                245                 250                 255
Tyr Pro Thr Ala Leu Thr Val Asn Ala Ser Val Ser Arg Phe Pro Pro
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```
            260                 265                 270
His Met Val Pro Pro His His Thr Leu His Thr Thr Gly Ile Pro His
        275                 280                 285
Pro Ala Ile Val Thr Pro Thr Val Lys Gln Glu Ser Ser Gln Ser Asp
    290                 295                 300
Val Gly Ser Leu His Ser Ser Lys His Gln Asp Ser Lys Lys Glu Glu
305                 310                 315                 320
Glu Lys Lys Lys Pro His Ile Lys Lys Pro Leu Asn Ala Phe Met Leu
                325                 330                 335
Tyr Met Lys Glu Met Arg Ala Lys Val Val Ala Glu Cys Thr Leu Lys
            340                 345                 350
Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg Trp His Ala Leu
        355                 360                 365
Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Glu Leu Ala Arg Lys Glu Arg
    370                 375                 380
Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala Arg Asp Asn Tyr
385                 390                 395                 400
Gly Lys Lys Lys Lys Arg Lys Arg Asp Lys Gln Pro Gly Glu Thr Asn
                405                 410                 415
Glu His Ser Glu Cys Phe Leu Asn Pro Cys Leu Ser Leu Pro Pro Ile
            420                 425                 430
Thr Asp Leu Ser Ala Pro Lys Lys Cys Arg Ala Arg Phe Gly Leu Asp
        435                 440                 445
Gln Gln Asn Asn Trp Cys Gly Pro Cys Arg Arg Lys Lys Lys Cys Val
    450                 455                 460
Arg Tyr Ile Gln Gly Glu Gly Ser Cys Leu Ser Pro Pro Ser Ser Asp
465                 470                 475                 480
Gly Ser Leu Leu Asp Ser Pro Pro Pro Ser Pro Asn Leu Leu Gly Ser
                485                 490                 495
Pro Pro Arg Asp Ala Lys Ser Gln Thr Glu Gln Thr Gln Pro Leu Ser
            500                 505                 510
Leu Ser Leu Lys Pro Asp Pro Leu Ala His Leu Ser Met Met Pro Pro
        515                 520                 525
Pro Pro Ala Leu Leu Leu Ala Glu Ala Thr His Lys Ala Ser Ala Leu
    530                 535                 540
Cys Pro Asn Gly Ala Leu Asp Leu Pro Pro Ala Ala Leu Gln Pro Ala
545                 550                 555                 560
Ala Pro Ser Ser Ser Ile Ala Gln Pro Ser Thr Ser Trp Leu His Ser
                565                 570                 575
His Ser Ser Leu Ala Gly Thr Gln Pro Gln Pro Leu Ser Leu Val Thr
            580                 585                 590
Lys Ser Leu Glu
        595
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<212> PRT
<213> Homo sapiens

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Lys Met Glu Asn Ser Asn Leu Arg Gln Glu Leu Glu Asp Asn Ser Asn
            20                  25                  30
His Leu Thr Lys Leu Glu Thr Glu Ala Ser Asn Met Lys Glu Val Leu
        35                  40                  45
Lys Gln Leu Gln Gly Ser Ile Glu Asp Glu Ala Met Ala Ser Ser Gly
    50                  55                  60
Gln Ile Asp Leu Leu Glu Arg Leu Lys Glu Leu Asn Leu Asp Ser Ser
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```
65                  70                  75                  80
Asn Phe Pro Gly Val Lys Leu Arg Ser Lys Met Ser Leu Arg Ser Tyr
                85                  90                  95
Gly Ser Arg Glu Gly Ser Val Ser Ser Arg Ser Gly Glu Cys Ser Pro
            100                 105                 110
Val Pro Met Gly Ser Phe Pro Arg Arg Gly Phe Val Asn Gly Ser Arg
        115                 120                 125
Glu Ser Thr Gly Tyr Leu Glu Glu Leu Glu Lys Glu Arg Ser Leu Leu
    130                 135                 140
Leu Ala Asp Leu Asp Lys Glu Glu Lys Glu Lys Asp Trp Tyr Tyr Ala
145                 150                 155                 160
Gln Leu Gln Asn Leu Thr Lys Arg Ile Asp Ser Leu Pro Leu Thr Glu
                165                 170                 175
Asn Phe Ser Leu Gln Thr Asp Met Thr Arg Arg Gln Leu Glu Tyr Glu
            180                 185                 190
Ala Arg Gln Ile Arg Val Ala Met Glu Glu Gln Leu Gly Thr Cys Gln
        195                 200                 205
Asp Met Glu Lys Arg Ala Gln Arg Arg Ile Ala Arg Ile Gln Gln Ile
    210                 215                 220
Glu Lys Asp Ile Leu Arg Ile Arg Gln Leu Leu Gln Ser Gln Ala Thr
225                 230                 235                 240
Glu Ala Glu Arg Ser Ser Gln Asn Lys His Glu Thr Gly Ser His Asp
                245                 250                 255
Ala Glu Arg Gln Asn Glu Gly Gln Gly Val Gly Glu Ile Asn Met Ala
            260                 265                 270
Thr Ser Gly Asn Gly Gln Gly Ser Thr Thr Arg Met Asp His Glu Thr
        275                 280                 285
Ala Ser Val Leu Ser Ser Ser Ser Thr His Ser Ala Pro Arg Arg Leu
    290                 295                 300
Thr Ser His Leu Gly Thr Lys Val Glu Met Val Tyr Ser Leu Leu Ser
305                 310                 315                 320
Met Leu Gly Thr His Asp Lys Asp Asp Met Ser Arg Thr Leu Leu Ala
                325                 330                 335
Met Ser Ser Ser Gln Asp Ser Cys Ile Ser Met Arg Gln Ser Gly Cys
            340                 345                 350
Leu Pro Leu Leu Ile Gln Leu Leu His Gly Asn Asp Lys Asp Ser Val
        355                 360                 365
Leu Leu Gly Asn Ser Arg Gly Ser Lys Glu Ala Arg Ala Arg Ala Ser
    370                 375                 380
Ala Ala Leu His Asn Ile Ile His Ser Gln Pro Asp Asp Lys Arg Gly
385                 390                 395                 400
Arg Arg Glu Ile Arg Val Leu His Leu Leu Glu Gln Ile Arg Ala Tyr
                405                 410                 415
Cys Glu Thr Cys Trp Glu Trp Gln Glu Ala His Glu Pro Gly Met Asp
            420                 425                 430
Gln Asp Lys Asn Pro Met Pro Ala Pro Val Glu His Gln Ile Cys Pro
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    450                 455                 460
Ala Met Asn Glu Leu Gly Gly Leu Gln Ala Ile Ala Glu Leu Leu Gln
465                 470                 475                 480
Val Asp Cys Glu Met Tyr Gly Leu Thr Asn Asp His Tyr Ser Ile Thr
                485                 490                 495
Leu Arg Arg Tyr Ala Gly Met Ala Leu Thr Asn Leu Thr Phe Gly Asp
            500                 505                 510
Val Ala Asn Lys Ala Thr Leu Cys Ser Met Lys Gly Cys Met Arg Ala
        515                 520                 525
Leu Val Ala Gln Leu Lys Ser Glu Ser Glu Asp Leu Gln Gln Val Ile
    530                 535                 540
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545                 550                 555                 560
Lys Thr Leu Arg Glu Val Gly Ser Val Lys Ala Leu Met Glu Cys Ala
                565                 570                 575
Leu Glu Val Lys Lys Glu Ser Thr Leu Lys Ser Val Leu Ser Ala Leu
            580                 585                 590
Trp Asn Leu Ser Ala His Cys Thr Glu Asn Lys Ala Asp Ile Cys Ala
        595                 600                 605
Val Asp Gly Ala Leu Ala Phe Leu Val Gly Thr Leu Thr Tyr Arg Ser
    610                 615                 620
Gln Thr Asn Thr Leu Ala Ile Ile Glu Ser Gly Gly Gly Ile Leu Arg
625                 630                 635                 640
Asn Val Ser Ser Leu Ile Ala Thr Asn Glu Asp His Arg Gln Ile Leu
                645                 650                 655
Arg Glu Asn Asn Cys Leu Gln Thr Leu Leu Gln His Leu Lys Ser His
            660                 665                 670
Ser Leu Thr Ile Val Ser Asn Ala Cys Gly Thr Leu Trp Asn Leu Ser
        675                 680                 685
Ala Arg Asn Pro Lys Asp Gln Glu Ala Leu Trp Asp Met Gly Ala Val
    690                 695                 700
Ser Met Leu Lys Asn Leu Ile His Ser Lys His Lys Met Ile Ala Met
705                 710                 715                 720
Gly Ser Ala Ala Ala Leu Arg Asn Leu Met Ala Asn Arg Pro Ala Lys
                725                 730                 735
Tyr Lys Asp Ala Asn Ile Met Ser Pro Gly Ser Ser Leu Pro Ser Leu
            740                 745                 750
His Val Arg Lys Gln Lys Ala Leu Glu Ala Glu Leu Asp Ala Gln His
        755                 760                 765
Leu Ser Glu Thr Phe Asp Asn Ile Asp Asn Leu Ser Pro Lys Ala Ser
    770                 775                 780
His Arg Ser Lys Gln Arg His Lys Gln Ser Leu Tyr Gly Asp Tyr Val
785                 790                 795                 800
Phe Asp Thr Asn Arg His Asp Asp Asn Arg Ser Asp Asn Phe Asn Thr
                805                 810                 815
Gly Asn Met Thr Val Leu Ser Pro Tyr Leu Asn Thr Thr Val Leu Pro
            820                 825                 830
Ser Ser Ser Ser Ser Arg Gly Ser Leu Asp Ser Ser Arg Ser Glu Lys
        835                 840                 845
Asp Arg Ser Leu Glu Arg Glu Arg Gly Ile Gly Leu Gly Asn Tyr His
    850                 855                 860
Pro Ala Thr Glu Asn Pro Gly Thr Ser Ser Lys Arg Gly Leu Gln Ile
865                 870                 875                 880
Ser Thr Thr Ala Ala Gln Ile Ala Lys Val Met Glu Glu Val Ser Ala
                885                 890                 895
Ile His Thr Ser Gln Glu Asp Arg Ser Ser Gly Ser Thr Thr Glu Leu
            900                 905                 910
His Cys Val Thr Asp Glu Arg Asn Ala Leu Arg Arg Ser Ser Ala Ala
        915                 920                 925
His Thr His Ser Asn Thr Tyr Asn Phe Thr Lys Ser Glu Asn Ser Asn
    930                 935                 940
Arg Thr Cys Ser Met Pro Tyr Ala Lys Leu Glu Tyr Lys Arg Ser Ser
945                 950                 955                 960
Asn Asp Ser Leu Asn Ser Val Ser Ser Ser Asp Gly Tyr Gly Lys Arg
                965                 970                 975
Gly Gln Met Lys Pro Ser Ile Glu Ser Tyr Ser Glu Asp Asp Glu Ser
            980                 985                 990
Lys Phe Cys Ser Tyr Gly Gln Tyr Pro Ala Asp Leu Ala His Lys Ile
        995                 1000                1005
His Ser Ala Asn His Met Asp Asp Asn Asp Gly Glu Leu Asp Thr Pro
```

```
    1010                1015                1020
Ile Asn Tyr Ser Leu Lys Tyr Ser Asp Glu Gln Leu Asn Ser Gly Arg
1025                1030                1035                1040
Gln Ser Pro Ser Gln Asn Glu Arg Trp Ala Arg Pro Lys His Ile Ile
                1045                1050                1055
Glu Asp Glu Ile Lys Gln Ser Glu Gln Arg Gln Ser Arg Asn Gln Ser
            1060                1065                1070
Thr Thr Tyr Pro Val Tyr Thr Glu Ser Thr Asp Asp Lys His Leu Lys
        1075                1080                1085
Phe Gln Pro His Phe Gly Gln Gln Glu Cys Val Ser Pro Tyr Arg Ser
    1090                1095                1100
Arg Gly Ala Asn Gly Ser Glu Thr Asn Arg Val Gly Ser Asn His Gly
1105                1110                1115                1120
Ile Asn Gln Asn Val Ser Gln Ser Leu Cys Gln Glu Asp Asp Tyr Glu
                1125                1130                1135
Asp Asp Lys Pro Thr Asn Tyr Ser Glu Arg Tyr Ser Glu Glu Glu Gln
            1140                1145                1150
His Glu Glu Glu Glu Arg Pro Thr Asn Tyr Ser Ile Lys Tyr Asn Glu
        1155                1160                1165
Glu Lys Arg His Val Asp Gln Pro Ile Asp Tyr Ser Leu Lys Tyr Ala
    1170                1175                1180
Thr Asp Ile Pro Ser Ser Gln Lys Gln Ser Phe Ser Phe Ser Lys Ser
1185                1190                1195                1200
Ser Ser Gly Gln Ser Ser Lys Thr Glu His Met Ser Ser Ser Ser Glu
                1205                1210                1215
Asn Thr Ser Thr Pro Ser Ser Asn Ala Lys Arg Gln Asn Gln Leu His
            1220                1225                1230
Pro Ser Ser Ala Gln Ser Arg Ser Gly Gln Pro Gln Lys Ala Ala Thr
        1235                1240                1245
Cys Lys Val Ser Ser Ile Asn Gln Glu Thr Ile Gln Thr Tyr Cys Val
    1250                1255                1260
Glu Asp Thr Pro Ile Cys Phe Ser Arg Cys Ser Ser Leu Ser Ser Leu
1265                1270                1275                1280
Ser Ser Ala Glu Asp Glu Ile Gly Cys Asn Gln Thr Thr Gln Glu Ala
                1285                1290                1295
Asp Ser Ala Asn Thr Leu Gln Ile Ala Glu Ile Lys Glu Lys Ile Gly
            1300                1305                1310
Thr Arg Ser Ala Glu Asp Pro Val Ser Glu Val Pro Ala Val Ser Gln
        1315                1320                1325
His Pro Arg Thr Lys Ser Ser Arg Leu Gln Gly Ser Ser Leu Ser Ser
    1330                1335                1340
Glu Ser Ala Arg His Lys Ala Val Glu Phe Ser Ser Gly Ala Lys Ser
1345                1350                1355                1360
Pro Ser Lys Ser Gly Ala Gln Thr Pro Lys Ser Pro Pro Glu His Tyr
                1365                1370                1375
Val Gln Glu Thr Pro Leu Met Phe Ser Arg Cys Thr Ser Val Ser Ser
            1380                1385                1390
Leu Asp Ser Phe Glu Ser Arg Ser Ile Ala Ser Ser Val Gln Ser Glu
        1395                1400                1405
Pro Cys Ser Gly Met Val Ser Gly Ile Ile Ser Pro Ser Asp Leu Pro
    1410                1415                1420
Asp Ser Pro Gly Gln Thr Met Pro Pro Ser Arg Ser Lys Thr Pro Pro
1425                1430                1435                1440
Pro Pro Pro Gln Thr Ala Gln Thr Lys Arg Glu Val Pro Lys Asn Lys
                1445                1450                1455
Ala Pro Thr Ala Glu Lys Arg Glu Ser Gly Pro Lys Gln Ala Ala Val
            1460                1465                1470
Asn Ala Ala Val Gln Arg Val Gln Val Leu Pro Asp Ala Asp Thr Leu
        1475                1480                1485
```

Leu His Phe Ala Thr Glu Ser Thr Pro Asp Gly Phe Ser Cys Ser Ser
1490 1495 1500
Ser Leu Ser Ala Leu Ser Leu Asp Glu Pro Phe Ile Gln Lys Asp Val
1505 1510 1515 1520
Glu Leu Arg Ile Met Pro Pro Val Gln Glu Asn Asp Asn Gly Asn Glu
1525 1530 1535
Thr Glu Ser Glu Gln Pro Lys Glu Ser Asn Glu Asn Gln Glu Lys Glu
1540 1545 1550
Ala Glu Lys Thr Ile Asp Ser Glu Lys Asp Leu Leu Asp Asp Ser Asp
1555 1560 1565
Asp Asp Asp Ile Glu Ile Leu Glu Glu Cys Ile Ile Ser Ala Met Pro
1570 1575 1580
Thr Lys Ser Ser Arg Lys Ala Lys Lys Pro Ala Gln Thr Ala Ser Lys
1585 1590 1595 1600
Leu Pro Pro Pro Val Ala Arg Lys Pro Ser Gln Leu Pro Val Tyr Lys
1605 1610 1615
Leu Leu Pro Ser Gln Asn Arg Leu Gln Pro Gln Lys His Val Ser Phe
1620 1625 1630
Thr Pro Gly Asp Asp Met Pro Arg Val Tyr Cys Val Glu Gly Thr Pro
1635 1640 1645
Ile Asn Phe Ser Thr Ala Thr Ser Leu Ser Asp Leu Thr Ile Glu Ser
1650 1655 1660
Pro Pro Asn Glu Leu Ala Ala Gly Glu Gly Val Arg Gly Gly Ala Gln
1665 1670 1675 1680
Ser Gly Glu Phe Glu Lys Arg Asp Thr Ile Pro Thr Glu Gly Arg Ser
1685 1690 1695
Thr Asp Glu Ala Gln Gly Gly Lys Thr Ser Ser Val Thr Ile Pro Glu
1700 1705 1710
Leu Asp Asp Asn Lys Ala Glu Glu Gly Asp Ile Leu Ala Glu Cys Ile
1715 1720 1725
Asn Ser Ala Met Pro Lys Gly Lys Ser His Lys Pro Phe Arg Val Lys
1730 1735 1740
Lys Ile Met Asp Gln Val Gln Gln Ala Ser Ala Ser Ser Ser Ala Pro
1745 1750 1755 1760
Asn Lys Asn Gln Leu Asp Gly Lys Lys Lys Lys Pro Thr Ser Pro Val
1765 1770 1775
Lys Pro Ile Pro Gln Asn Thr Glu Tyr Arg Thr Arg Val Arg Lys Asn
1780 1785 1790
Ala Asp Ser Lys Asn Asn Leu Asn Ala Glu Arg Val Phe Ser Asp Asn
1795 1800 1805
Lys Asp Ser Lys Lys Gln Asn Leu Lys Asn Asn Ser Lys Asp Phe Asn
1810 1815 1820
Asp Lys Leu Pro Asn Asn Glu Asp Arg Val Arg Gly Ser Phe Ala Phe
1825 1830 1835 1840
Asp Ser Pro His His Tyr Thr Pro Ile Glu Gly Thr Pro Tyr Cys Phe
1845 1850 1855
Ser Arg Asn Asp Ser Leu Ser Ser Leu Asp Phe Asp Asp Asp Asp Val
1860 1865 1870
Asp Leu Ser Arg Glu Lys Ala Glu Leu Arg Lys Ala Lys Glu Asn Lys
1875 1880 1885
Glu Ser Glu Ala Lys Val Thr Ser His Thr Glu Leu Thr Ser Asn Gln
1890 1895 1900
Gln Ser Ala Asn Lys Thr Gln Ala Ile Ala Lys Gln Pro Ile Asn Arg
1905 1910 1915 1920
Gly Gln Pro Lys Pro Ile Leu Gln Lys Gln Ser Thr Phe Pro Gln Ser
1925 1930 1935
Ser Lys Asp Ile Pro Asp Arg Gly Ala Ala Thr Asp Glu Lys Leu Gln
1940 1945 1950
Asn Phe Ala Ile Glu Asn Thr Pro Val Cys Phe Ser His Asn Ser Ser

1955 1960 1965
Leu Ser Ser Leu Ser Asp Ile Asp Gln Glu Asn Asn Asn Lys Glu Asn
1970 1975 1980
Glu Pro Ile Lys Glu Thr Glu Pro Pro Asp Ser Gln Gly Glu Pro Ser
1985 1990 1995 2000
Lys Pro Gln Ala Ser Gly Tyr Ala Pro Lys Ser Phe His Val Glu Asp
2005 2010 2015
Thr Pro Val Cys Phe Ser Arg Asn Ser Ser Leu Ser Ser Leu Ser Ile
2020 2025 2030
Asp Ser Glu Asp Asp Leu Leu Gln Glu Cys Ile Ser Ser Ala Met Pro
2035 2040 2045
Lys Lys Lys Lys Pro Ser Arg Leu Lys Gly Asp Asn Glu Lys His Ser
2050 2055 2060
Pro Arg Asn Met Gly Gly Ile Leu Gly Glu Asp Leu Thr Leu Asp Leu
2065 2070 2075 2080
Lys Asp Ile Gln Arg Pro Asp Ser Glu His Gly Leu Ser Pro Asp Ser
2085 2090 2095
Glu Asn Phe Asp Trp Lys Ala Ile Gln Glu Gly Ala Asn Ser Ile Val
2100 2105 2110
Ser Ser Leu His Gln Ala Ala Ala Ala Ala Cys Leu Ser Arg Gln Ala
2115 2120 2125
Ser Ser Asp Ser Asp Ser Ile Leu Ser Leu Lys Ser Gly Ile Ser Leu
2130 2135 2140
Gly Ser Pro Phe His Leu Thr Pro Asp Gln Glu Glu Lys Pro Phe Thr
2145 2150 2155 2160
Ser Asn Lys Gly Pro Arg Ile Leu Lys Pro Gly Glu Lys Ser Thr Leu
2165 2170 2175
Glu Thr Lys Lys Ile Glu Ser Glu Ser Lys Gly Ile Lys Gly Gly Lys
2180 2185 2190
Lys Val Tyr Lys Ser Leu Ile Thr Gly Lys Val Arg Ser Asn Ser Glu
2195 2200 2205
Ile Ser Gly Gln Met Lys Gln Pro Leu Gln Ala Asn Met Pro Ser Ile
2210 2215 2220
Ser Arg Gly Arg Thr Met Ile His Ile Pro Gly Val Arg Asn Ser Ser
2225 2230 2235 2240
Ser Ser Thr Ser Pro Val Ser Lys Lys Gly Pro Pro Leu Lys Thr Pro
2245 2250 2255
Ala Ser Lys Ser Pro Ser Glu Gly Gln Thr Ala Thr Thr Ser Pro Arg
2260 2265 2270
Gly Ala Lys Pro Ser Val Lys Ser Glu Leu Ser Pro Val Ala Arg Gln
2275 2280 2285
Thr Ser Gln Ile Gly Gly Ser Ser Lys Ala Pro Ser Arg Ser Gly Ser
2290 2295 2300
Arg Asp Ser Thr Pro Ser Arg Pro Ala Gln Gln Pro Leu Ser Arg Pro
2305 2310 2315 2320
Ile Gln Ser Pro Gly Arg Asn Ser Ile Ser Pro Gly Arg Asn Gly Ile
2325 2330 2335
Ser Pro Pro Asn Lys Leu Ser Gln Leu Pro Arg Thr Ser Ser Pro Ser
2340 2345 2350
Thr Ala Ser Thr Lys Ser Ser Gly Ser Gly Lys Met Ser Tyr Thr Ser
2355 2360 2365
Pro Gly Arg Gln Met Ser Gln Gln Asn Leu Thr Lys Gln Thr Gly Leu
2370 2375 2380
Ser Lys Asn Ala Ser Ser Ile Pro Arg Ser Glu Ser Ala Ser Lys Gly
2385 2390 2395 2400
Leu Asn Gln Met Asn Asn Gly Asn Gly Ala Asn Lys Lys Val Glu Leu
2405 2410 2415
Ser Arg Met Ser Ser Thr Lys Ser Ser Gly Ser Glu Ser Asp Arg Ser
2420 2425 2430

Glu Arg Pro Val Leu Val Arg Gln Ser Thr Phe Ile Lys Glu Ala Pro
2435 2440 2445
Ser Pro Thr Leu Arg Arg Lys Leu Glu Glu Ser Ala Ser Phe Glu Ser
2450 2455 2460
Leu Ser Pro Ser Ser Arg Pro Ala Ser Pro Thr Arg Ser Gln Ala Gln
2465 2470 2475 2480
Thr Pro Val Leu Ser Pro Ser Leu Pro Asp Met Ser Leu Ser Thr His
2485 2490 2495
Ser Ser Val Gln Ala Gly Gly Trp Arg Lys Leu Pro Pro Asn Leu Ser
2500 2505 2510
Pro Thr Ile Glu Tyr Asn Asp Gly Arg Pro Ala Lys Arg His Asp Ile
2515 2520 2525
Ala Arg Ser His Ser Glu Ser Pro Ser Arg Leu Pro Ile Asn Arg Ser
2530 2535 2540
Gly Thr Trp Lys Arg Glu His Ser Lys His Ser Ser Ser Leu Pro Arg
2545 2550 2555 2560
Val Ser Thr Trp Arg Arg Thr Gly Ser Ser Ser Ser Ile Leu Ser Ala
2565 2570 2575
Ser Ser Glu Ser Ser Glu Lys Ala Lys Ser Glu Asp Glu Lys His Val
2580 2585 2590
Asn Ser Ile Ser Gly Thr Lys Gln Ser Lys Glu Asn Gln Val Ser Ala
2595 2600 2605
Lys Gly Thr Trp Arg Lys Ile Lys Glu Asn Glu Phe Ser Pro Thr Asn
2610 2615 2620
Ser Thr Ser Gln Thr Val Ser Ser Gly Ala Thr Asn Gly Ala Glu Ser
2625 2630 2635 2640
Lys Thr Leu Ile Tyr Gln Met Ala Pro Ala Val Ser Lys Thr Glu Asp
2645 2650 2655
Val Trp Val Arg Ile Glu Asp Cys Pro Ile Asn Asn Pro Arg Ser Gly
2660 2665 2670
Arg Ser Pro Thr Gly Asn Thr Pro Pro Val Ile Asp Ser Val Ser Glu
2675 2680 2685
Lys Ala Asn Pro Asn Ile Lys Asp Ser Lys Asp Asn Gln Ala Lys Gln
2690 2695 2700
Asn Val Gly Asn Gly Ser Val Pro Met Arg Thr Val Gly Leu Glu Asn
2705 2710 2715 2720
Arg Leu Asn Ser Phe Ile Gln Val Asp Ala Pro Asp Gln Lys Gly Thr
2725 2730 2735
Glu Ile Lys Pro Gly Gln Asn Asn Pro Val Pro Val Ser Glu Thr Asn
2740 2745 2750
Glu Ser Ser Ile Val Glu Arg Thr Pro Phe Ser Ser Ser Ser Ser Ser
2755 2760 2765
Lys His Ser Ser Pro Ser Gly Thr Val Ala Ala Arg Val Thr Pro Phe
2770 2775 2780
Asn Tyr Asn Pro Ser Pro Arg Lys Ser Ser Ala Asp Ser Thr Ser Ala
2785 2790 2795 2800
Arg Pro Ser Gln Ile Pro Thr Pro Val Asn Asn Asn Thr Lys Lys Arg
2805 2810 2815
Asp Ser Lys Thr Asp Ser Thr Glu Ser Ser Gly Thr Gln Ser Pro Lys
2820 2825 2830
Arg His Ser Gly Ser Tyr Leu Val Thr Ser Val Lys Arg Gly Arg Met
2835 2840 2845
Lys Leu Arg Lys Phe Tyr Val Asn Tyr Asn Cys Tyr Ile Asp Ile Leu
2850 2855 2860
Phe Gln Met Lys Leu Lys Thr Glu Lys Phe Cys Lys Val Phe Leu Leu
2865 2870 2875 2880
Glu Gly Phe Cys Ser Gly Ser His Ile Tyr Thr Leu Ser Ser Leu Val
2885 2890 2895
Leu Phe Trp Glu Ala Leu Leu Met Val Arg Lys Lys Ile Val Lys Pro

```
            2900                2905                2910
Ser Met Phe Val Gln Tyr Val Leu His Val Phe Lys Val Ala Pro Ile
        2915                2920                2925
Pro Thr Ser Phe Asn Tyr Cys Leu Ser Asn Asn Glu His Tyr Arg Lys
    2930                2935                2940
Ile Tyr Ile Ala Val Ile Asn His Phe Ile Ile Asn Leu Asn Leu His
2945                2950                2955                2960
Gln Gly Lys Ile Gly Ile Tyr Ala Lys Lys Asn Val Phe
                2965                2970
```

<210> 8
<211> 486
<212> PRT
<213> Homo sapiens

<400> 8

```
Met Pro Gln Leu Asp Ser Gly Gly Gly Gly Ala Gly Gly Gly Asp Asp
 1               5                  10                  15
Leu Gly Ala Pro Asp Glu Leu Leu Ala Phe Gln Asp Glu Gly Glu Glu
            20                  25                  30
Gln Asp Asp Lys Ser Arg Asp Ser Ala Gly Pro Glu Arg Asp Leu Ala
        35                  40                  45
Glu Leu Lys Ser Ser Leu Val Asn Glu Ser Glu Gly Ala Ala Gly Ser
    50                  55                  60
Ala Gly Ile Pro Gly Val Pro Gly Ala Gly Ala Gly Ala Arg Gly Glu
65                  70                  75                  80
Ala Glu Ala Leu Gly Arg Glu His Arg Ala Gln Arg Leu Phe Pro Asp
                85                  90                  95
Lys Leu Pro Glu Pro Leu Glu Asp Gly Leu Lys Ala Pro Glu Cys Thr
            100                 105                 110
Ser Gly Met Tyr Lys Glu Thr Val Tyr Ser Ala Phe Asn Leu Leu Met
        115                 120                 125
His Tyr Pro Pro Pro Ser Gly Ala Gly Gln His Pro Gln Pro Gln Pro
    130                 135                 140
Pro Leu His Lys Ala Asn Gln Pro Pro His Gly Val Pro Gln Leu Ser
145                 150                 155                 160
Leu Tyr Glu His Phe Asn Ser Pro His Pro Thr Pro Ala Pro Ala Asp
                165                 170                 175
Ile Ser Gln Lys Gln Val His Arg Pro Leu Gln Thr Pro Asp Leu Ser
            180                 185                 190
Gly Phe Tyr Ser Leu Thr Ser Gly Ser Met Gly Gln Leu Pro His Thr
        195                 200                 205
Val Ser Trp Pro Ser Pro Pro Leu Tyr Pro Leu Ser Pro Ser Cys Gly
    210                 215                 220
Tyr Arg Gln His Phe Pro Ala Pro Thr Ala Ala Pro Gly Ala Pro Tyr
225                 230                 235                 240
Pro Arg Phe Thr His Pro Ser Leu Met Leu Gly Ser Gly Val Pro Gly
                245                 250                 255
His Pro Ala Ala Ile Pro His Pro Ala Ile Val Pro Pro Ser Gly Lys
            260                 265                 270
Gln Glu Leu Gln Pro Phe Asp Arg Asn Leu Lys Thr Gln Ala Glu Ser
        275                 280                 285
Lys Ala Glu Lys Glu Ala Lys Lys Pro Thr Ile Lys Lys Pro Leu Asn
    290                 295                 300
Ala Phe Met Leu Tyr Met Lys Glu Met Arg Ala Lys Val Ile Ala Glu
305                 310                 315                 320
Cys Thr Leu Lys Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg
                325                 330                 335
Trp His Ala Leu Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Glu Leu Ala
```

```
            340                 345                 350
Arg Lys Glu Arg Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala
        355                 360                 365
Arg Asp Asn Tyr Gly Lys Lys Lys Arg Arg Ser Arg Glu Lys His Gln
    370                 375                 380
Glu Ser Thr Thr Gly Gly Lys Arg Asn Ala Phe Gly Thr Tyr Pro Glu
385                 390                 395                 400
Lys Ala Ala Ala Pro Ala Pro Phe Leu Pro Met Thr Val Leu Ala Ala
                405                 410                 415
Pro Gly Pro Gln Leu Pro Arg Thr His Pro His Thr Ile Cys Cys Pro
            420                 425                 430
Ala Ser Pro Gln Asn Cys Leu Leu Ala Leu Arg Ser Arg His Leu His
        435                 440                 445
Pro Gln Val Ser Pro Leu Leu Ser Ala Ser Gln Pro Gln Gly Pro His
    450                 455                 460
Arg Pro Pro Ala Ala Pro Cys Arg Ala His Arg Tyr Ser Asn Arg Asn
465                 470                 475                 480
Leu Arg Asp Arg Trp Pro
                485
```

<210> 9
<211> 511
<212> PRT
<213> Homo sapiens

<400> 9

```
Met Pro Gln Leu Asp Ser Gly Gly Gly Gly Ala Gly Gly Gly Asp Asp
 1               5                  10                  15
Leu Gly Ala Pro Asp Glu Leu Leu Ala Phe Gln Asp Glu Gly Glu Glu
            20                  25                  30
Gln Asp Asp Lys Ser Arg Asp Ser Ala Gly Pro Glu Arg Asp Leu Ala
        35                  40                  45
Glu Leu Lys Ser Ser Leu Val Asn Glu Ser Glu Gly Ala Ala Gly Ser
    50                  55                  60
Ala Gly Ile Pro Gly Val Pro Gly Ala Gly Ala Gly Ala Arg Gly Glu
65                  70                  75                  80
Ala Glu Ala Leu Gly Arg Glu His Arg Ala Gln Arg Leu Phe Pro Asp
                85                  90                  95
Lys Leu Pro Glu Pro Leu Glu Asp Gly Leu Lys Ala Pro Glu Cys Thr
            100                 105                 110
Ser Gly Met Tyr Lys Glu Thr Val Tyr Ser Ala Phe Asn Leu Leu Met
        115                 120                 125
His Tyr Pro Pro Pro Ser Gly Ala Gly Gln His Pro Gln Pro Gln Pro
    130                 135                 140
Pro Leu His Lys Ala Asn Gln Pro Pro His Gly Val Pro Gln Leu Ser
145                 150                 155                 160
Leu Tyr Glu His Phe Asn Ser Pro His Pro Thr Pro Ala Pro Ala Asp
                165                 170                 175
Ile Ser Gln Lys Gln Val His Arg Pro Leu Gln Thr Pro Asp Leu Ser
            180                 185                 190
Gly Phe Tyr Ser Leu Thr Ser Gly Ser Met Gly Gln Leu Pro His Thr
        195                 200                 205
Val Ser Trp Pro Ser Pro Pro Leu Tyr Pro Leu Ser Pro Ser Cys Gly
    210                 215                 220
Tyr Arg Gln His Phe Pro Ala Pro Thr Ala Ala Pro Gly Ala Pro Tyr
225                 230                 235                 240
Pro Arg Phe Thr His Pro Ser Leu Met Leu Gly Ser Gly Val Pro Gly
                245                 250                 255
His Pro Ala Ala Ile Pro His Pro Ala Ile Val Pro Pro Ser Gly Lys
```

```
            260                 265                 270
Gln Glu Leu Gln Pro Phe Asp Arg Asn Leu Lys Thr Gln Ala Glu Ser
        275                 280                 285
Lys Ala Glu Lys Glu Ala Lys Lys Pro Thr Ile Lys Lys Pro Leu Asn
    290                 295                 300
Ala Phe Met Leu Tyr Met Lys Glu Met Arg Ala Lys Val Ile Ala Glu
305                 310                 315                 320
Cys Thr Leu Lys Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg
                325                 330                 335
Trp His Ala Leu Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Glu Leu Ala
            340                 345                 350
Arg Lys Glu Arg Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala
        355                 360                 365
Arg Asp Asn Tyr Gly Lys Lys Lys Arg Arg Ser Arg Glu Lys His Gln
    370                 375                 380
Glu Ser Thr Thr Asp Pro Gly Ser Pro Lys Lys Cys Arg Ala Arg Phe
385                 390                 395                 400
Gly Leu Asn Gln Gln Thr Asp Trp Cys Gly Pro Cys Arg Arg Lys Lys
                405                 410                 415
Lys Cys Ile Arg Tyr Leu Pro Gly Glu Gly Arg Cys Pro Ser Pro Val
            420                 425                 430
Pro Ser Asp Asp Ser Ala Leu Gly Cys Pro Gly Ser Pro Ala Pro Gln
        435                 440                 445
Asp Ser Pro Ser Tyr His Leu Leu Pro Arg Phe Pro Thr Glu Leu Leu
    450                 455                 460
Thr Ser Pro Ala Glu Pro Ala Pro Thr Ser Pro Gly Leu Ser Thr Ala
465                 470                 475                 480
Leu Ser Leu Pro Thr Pro Gly Pro Pro Gln Ala Pro Arg Ser Thr Leu
                485                 490                 495
Gln Ser Thr Gln Val Gln Gln Gln Glu Ser Gln Arg Gln Val Ala
            500                 505                 510
```

<210> 10
<211> 20
<212> PRT
<213> Homo sapiens

<400> 10
Ser Tyr Leu Asp Ser Gly Ile His Ser Gly Ala Thr Thr Thr Ala Pro
1 5 10 15
Ser Leu Ser Gly
20

<210> 11
<211> 21
<212> PRT
<213> Drosophila melanogaster

<400> 11
Ser Tyr Leu Gly Asp Ser Gly Ile His Ser Gly Ala Val Thr Gln Val
1 5 10 15
Pro Ser Leu Ser Gly
20