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(54) Titre : UTILISATION D'OLIGONUCLEOTIDES ANTISENS DE SIARN POUR SUPPRIMER L'EXPRESSION DE EIF-5A1

(54) Title: USE OF ANTISENSE OLIGONUCLEOTIDES OR SIRNA TO SUPPRESS EXPRESSION OF EIF-5A1

(57) **Abrégé/Abstract:**

The present invention relates to apoptosis specific eucaryotic initiation factor 5A (eIF-5A), referred to as apoptosis factor 5A1 or simply factor 5A1, apoptosis factor 5A1 nucleic acids and polypeptides and methods for inhibiting or suppressing apoptosis in cells using antisense nucleotides or siRNAs to inhibit expression of factor 5A1. The invention also relates to suppressing or inhibiting expression of pro-inflammatory cytokines by inhibiting expression of apoptosis factor 5A.



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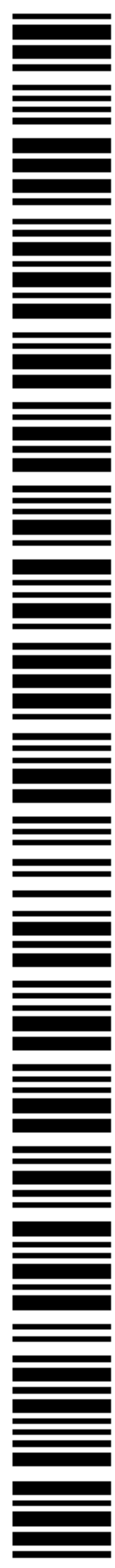
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(54) Title: USE OF ANTISENSE OLIGONUCLEOTIDES OR siRNA TO SUPPRESS EXPRESSION OF eIF-5A1

(57) Abstract: The present invention relates to apoptosis specific eucaryotic initiation factor 5A (eIF-5A), referred to as apoptosis factor 5A1 or simply factor 5A1, apoptosis factor 5A1 nucleic acids and polypeptides and methods for inhibiting or suppressing apoptosis in cells using antisense nucleotides or siRNAs to inhibit expression of factor 5A1. The invention also relates to suppressing or inhibiting expression of pro-inflammatory cytokines by inhibiting expression of apoptosis factor 5A.



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Use of Antisense Oligonucleotides or siRNA to Suppress Expression of eIF-5A1**RELATED APPLICATIONS**

This application is a continuation-in-part of U.S. application serial number 10/383,614, filed on March 10, 2003, which is a continuation-in-part of 10/277,969, filed October 23, 2002, which is a continuation-in-part of 10/200,148, filed on July 23, 2002, which is a continuation-in-part of U.S. application serial number 10/141,647, filed May 7, 2002, which is a continuation-in part of U.S. application serial number, 9/909,796, filed July 23, 2001, all of which are herein incorporated in their entirety. This application also claims priority to U.S. provisional 60/451,677, filed on March 5, 2003; U.S. provisional 60/476,194, filed on June 6, 2003; and U.S. provisional 60/504,731, filed on September 22, 2003, all of which are herein incorporated in their entirety.

FIELD OF THE INVENTION

The present invention relates to apoptosis-specific eucaryotic initiation Factor-5A (eIF-5A) or referred to as apoptosis Factor 5A or Factor 5A1 or apoptosis-specific eIF-5A and deoxyhypusine synthase (DHS). The present invention relates to apoptosis Factor 5A and DHS nucleic acids and polypeptides and methods for inhibiting expression of apoptosis Factor 5A and DHS.

BACKGROUND OF THE INVENTION

Apoptosis is a genetically programmed cellular event that is characterized by well-defined morphological features, such as cell shrinkage, chromatin condensation, nuclear fragmentation, and membrane blebbing. Kerr et al. (1972) Br. J. Cancer, 26, 239-257; Wyllie et al. (1980) Int. Rev. Cytol., 68, 251-306. It plays an important role in normal tissue development and homeostasis, and defects in the apoptotic program are thought to contribute to a wide range of human disorders ranging from neurodegenerative and autoimmunity disorders to neoplasms. Thompson (1995) Science, 267, 1456-1462; Mullauer et al. (2001) Mutat. Res, 488, 211-231. Although the morphological characteristics of apoptotic cells are well characterized, the molecular pathways that regulate this process have only begun to be elucidated.

One group of proteins that is thought to play a key role in apoptosis is a family of cysteine proteases, termed caspases, which appear to be required for most pathways of

apoptosis. Creagh & Martin (2001) *Biochem. Soc. Trans.*, 29, 696-701; Dales et al. (2001) *Leuk. Lymphoma*, 41, 247-253. Caspases trigger apoptosis in response to apoptotic stimuli by cleaving various cellular proteins, which results in classic manifestations of apoptosis, including cell shrinkage, membrane blebbing and DNA fragmentation. Chang & Yang (2000) *Microbiol. Mol. Biol. Rev.*, 64, 821-846.

Pro-apoptotic proteins, such as Bax or Bak, also play a key role in the apoptotic pathway by releasing caspase-activating molecules, such as mitochondrial cytochrome c, thereby promoting cell death through apoptosis. Martinou & Green (2001) *Nat. Rev. Mol. Cell. Biol.*, 2, 63-67; Zou et al. (1997) *Cell*, 90, 405-413. Anti-apoptotic proteins, such as Bcl-2, promote cell survival by antagonizing the activity of the pro-apoptotic proteins, Bax and Bak. Tsujimoto (1998) *Genes Cells*, 3, 697-707; Kroemer (1997) *Nature Med.*, 3, 614-620. The ratio of Bax:Bcl-2 is thought to be one way in which cell fate is determined; an excess of Bax promotes apoptosis and an excess of Bcl-2 promotes cell survival. Salomons et al. (1997) *Int. J. Cancer*, 71, 959-965; Wallace-Brodeur & Lowe (1999) *Cell Mol. Life Sci.*, 55, 64-75.

Another key protein involved in apoptosis is that encoded by the tumor suppressor gene p53. This protein is a transcription factor that regulates cell growth and induces apoptosis in cells that are damaged and genetically unstable, presumably through up-regulation of Bax. Bold et al. (1997) *Surgical Oncology*, 6, 133-142; Ronen et al., 1996; Schuler & Green (2001) *Biochem. Soc. Trans.*, 29, 684-688; Ryan et al. (2001) *Curr. Opin. Cell Biol.*, 13, 332-337; Zörnig et al. (2001) *Biochem. Biophys. Acta*, 1551, F1-F37.

The distinct morphological features that characterize cells undergoing apoptosis have given rise to a number of methods for assessing the onset and progress of apoptosis. One such feature of apoptotic cells that can be exploited for their detection is activation of a flippase, which results in externalization of phosphatidylserine, a phospholipid normally localized to the inner leaflet of the plasma membrane. Fadok *et al.* (1992) *J. Immunol.*, 149, 4029-4035. Apoptotic cells bearing externalized phosphatidylserine can be detected by staining with a phosphatidylserine-binding protein, Annexin V, conjugated to a fluorescent dye. The characteristic DNA fragmentation that occurs during the apoptotic process can be detected by labeling the exposed 3'-OH ends of the DNA fragments with fluorescein-labeled deoxynucleotides. Fluorescent dyes that bind nucleic acids, such as Hoescht 33258, can be used to detect chromatin condensation and nuclear fragmentation

in apoptotic cells. The degree of apoptosis in a cell population can also be inferred from the extent of caspase proteolytic activity present in cellular extracts.

As a genetically defined process, apoptosis, like any other developmental program, can be disrupted by mutation. Alterations in the apoptotic pathways are believed to play a key role in a number of disease processes, including cancer. Wyllie *et al.* (1980) *Int. Rev. Cytol.*, **68**, 251-306; Thompson (1995) *Science*, **267**, 1456-1462; Sen & D'Incalci (1992) *FEBS Letters*, **307**, 122-127; McDonnell *et al.* (1995) *Seminars in Cancer and Biology*, **6**, 53-60. Investigations into cancer development and progression have traditionally been focused on cellular proliferation. However, the important role that apoptosis plays in tumorigenesis has recently become apparent. In fact, much of what is now known about apoptosis has been learned using tumor models, since the control of apoptosis is invariably altered in some way in tumor cells. Bold *et al.* (1997) *Surgical Oncology*, **6**, 133-142.

Apoptosis can be triggered during tumor development by a variety of signals. Extracellular signals include growth or survival factor depletion, hypoxia and ionizing radiation. Internal signals that can trigger apoptosis include DNA damage, shortening telomeres, and oncogenic mutations that produce inappropriate proliferative signals. Lowe & Lin (2000) *Carcinogenesis*, **21**, 485-495. Ionizing radiation and nearly all cytotoxic chemotherapy agents used to treat malignancies are thought to act by triggering endogenous apoptotic mechanisms to induce cell death. Rowan & Fisher (1997) *Leukemia*, **11**, 457-465; Kerr *et al.* (1994) *Cancer*, **73**, 2013-2026; Martin & Schwartz (1997) *Oncology Research*, **9**, 1-5.

Evidence would suggest that early in the progression of cancer, tumor cells are sensitive to agents (such as ionizing radiation or chemotherapeutic drugs) that induce apoptosis. However, as the tumor progresses, the cells develop resistance to apoptotic stimuli. Naik *et al.* (1996) *Genes and Development*, **10**, 2105-2116. This may explain why early cancers respond better to treatment than more advanced lesions. The ability of late-stage cancers to develop resistance to chemotherapy and radiation therapy appears to be linked to alterations in the apoptotic pathway that limit the ability of tumor cells to respond to apoptotic stimuli. Reed *et al.* (1996) *Journal of Cellular Biology*, **60**, 23-32; Meyn *et al.* (1996) *Cancer Metastasis Reviews*, **15**, 119-131; Hannun (1997) *Blood*, **89**, 1845-1853; Reed (1995) *Toxicology Letters*, **82-83**, 155-158; Hickman (1996) *European Journal of Cancer*, **32A**, 921-926. Resistance to chemotherapy has been correlated to

overexpression of the anti-apoptotic gene bcl-2 and deletion or mutation of the pro-apoptotic bax gene in chronic lymphocytic leukemia and colon cancer, respectively.

The ability of tumor cells to successfully establish disseminated metastases also appears to involve alterations in the apoptotic pathway. Bold *et al.* (1997) *Surgical Oncology*, **6**, 133-142. For example, mutations in the tumor suppressor gene p53 are thought to occur in 70 % of tumors. Evan *et al.* (1995) *Curr. Opin. Cell Biol.*, **7**, 825-834. Mutations that inactivate p53 limit the ability of cells to induce apoptosis in response to DNA damage, leaving the cell vulnerable to further mutations. Ko & Prives (1996) *Genes and Development*, **10**, 1054-1072.

Therefore, apoptosis is intimately involved in the development and progression of neoplastic transformation and metastases, and a better understanding of the apoptotic pathways involved may lead to new potential targets for the treatment of cancer by the modulation of apoptotic pathways through gene therapy approaches. Bold *et al.* (1997) *Surgical Oncology*, **6**, 133-142.

The present invention relates to cloning of an eIF-5A cDNA that is up regulated immediately before the induction of apoptosis. This apoptosis-specific eIF-5A is likely to be a suitable target for intervention in apoptosis-causing disease states since it appears to act at the level of post-transcriptional regulation of downstream effectors and transcription factors involved in the apoptotic pathway. Specifically, the apoptosis-specific eIF-5A appears to selectively facilitate the translocation of mRNAs encoding downstream effectors and transcription factors of apoptosis from the nucleus to the cytoplasm, where they are subsequently translated. The ultimate decision to initiate apoptosis appears to stem from a complex interaction between internal and external pro- and anti-apoptotic signals. Lowe & Lin (2000) *Carcinogenesis*, **21**, 485-495. Through its ability to facilitate the translation of downstream apoptosis effectors and transcription factors, the apoptosis-related eIF-5A appears to tip the balance between these signals in favor of apoptosis.

As described previously, it is well established that anticancer agents induce apoptosis and that alterations in the apoptotic pathways can attenuate drug-induced cell death. Schmitt & Lowe (1999) *J. Pathol.*, **187**, 127-137. For example, many anticancer drugs upregulate p53, and tumor cells that have lost p53 develop resistance to these drugs. However, nearly all chemotherapy agents can induce apoptosis independently of p53 if the dose is sufficient, indicating that even in drug-resistant tumors, the pathways to apoptosis are not completely blocked. Wallace-Brodeur & Lowe (1999) *Cell Mol. Life Sci.*, **55**, 64-

75. This suggests that induction of apoptosis eIF-5A, even though it may not correct the mutated gene, may be able to circumvent the p53-dependent pathway and induce apoptosis by promoting alternative pathways.

Induction of apoptosis-related eIF-5A has the potential to selectively target cancer
5 cells while having little or no effect on normal neighboring cells. This arises because mitogenic oncogenes expressed in tumor cells provide an apoptotic signal in the form of specific species of mRNA that are not present in normal cells. Lowe *et al.* (1993) *Cell*, 74, 954-967; Lowe & Lin (2000) *Carcinogenesis*, 21, 485-495. For example, restoration of wild-type p53 in p53-mutant tumor cells can directly induce apoptosis as well as increase
10 drug sensitivity in tumor cell lines and xenographs. (Spitz *et al.*, 1996; Badie *et al.* 1998).

The selectivity of apoptosis-eIF-5A arises from the fact that it selectively facilitates translation of mRNAs for downstream apoptosis effectors and transcription factors by mediating their translocation from the nucleus into the cytoplasm. Thus, for apoptosis eIF-5A to have an effect, mRNAs for these effectors and transcription factors have to be
15 transcribed. Inasmuch as these mRNAs would be transcribed in cancer cells, but not in neighboring normal cells, it is to be expected that apoptosis eIF-5A would promote apoptosis in cancer cells but have minimal, if any, effect on normal cells. Thus, restoration of apoptotic potential in tumor cells with apoptosis-related eIF-5A may decrease the toxicity and side effects experienced by cancer patients due to selective
20 targeting of tumor cells. Induction of apoptotic eIF-5A also has the potential to potentiate the response of tumor cells to anti-cancer drugs and thereby improve the effectiveness of these agents against drug-resistant tumors. This in turn could result in lower doses of anti-cancer drugs for efficacy and reduced toxicity to the patient.

Alternations in the apoptotic pathways are also believed to play a role in
25 degeneration of retinal ganglion cells causing blindness due to glaucoma. Glaucoma describes a group of eye conditions in which increased intra-ocular pressure (IOP) leads to damage of the optic nerve and progressive blindness. Although glaucoma is currently managed by drugs or surgery to control IOP to reduce damage to the optic nerve or the use of neuro-protectors, there remains a need to protect retinal ganglion cells from
30 degeneration by apoptosis in the glaucomatous eye.

Cytokines have also been implicated in the apoptotic pathway. Biological systems require cellular interactions for their regulation, and cross-talk between cells generally involves a large variety of cytokines. Cytokines are mediators that are produced in

response to a wide variety of stimuli by many different cell types. Cytokines are pleiotropic molecules that can exert many different effects on many different cell types, but are especially important in regulation of the immune response and hematopoietic cell proliferation and differentiation. The actions of cytokines on target cells can promote cell survival, proliferation, activation, differentiation, or apoptosis depending on the particular cytokine, relative concentration, and presence of other mediators.

The use of anti-cytokines to treat autoimmune disorders (psoriasis, rheumatoid arthritis, Crohn's disease) is gaining popularity. The pro-inflammatory cytokines IL-1 and TNF play a large role in the pathology of these chronic disorders and anti-cytokine therapies that reduce the biological activities of these two cytokines can provide therapeutic benefits (Dinarello and Abraham, 2002).

Interleukin 1 (IL-1) is an important cytokine that mediates local and systemic inflammatory reactions and which can synergize with TNF in the pathogenesis of many disorders, including vasculitis, osteoporosis, neurodegenerative disorders, diabetes, lupus nephritis, and autoimmune disorders such as rheumatoid arthritis. The importance of IL-1 β in tumour angiogenesis and invasiveness was also recently demonstrated by the resistance of IL-1 β knockout mice to metastases and angiogenesis when injected with melanoma cells (Voronov *et al.*, 2003).

Interleukin 18 (IL-18) is a recently discovered member of the IL-1 family and is related by structure, receptors, and function to IL-1. IL-18 is a central cytokine involved in inflammatory and autoimmune disorders as a result of its ability to induce interferon-gamma (IFN- λ), TNF- α , and IL-1. IL-1 β and IL-18 are both capable of inducing production of TNF- α , a cytokine known to contribute to cardiac dysfunction during myocardial ischemia (Maekawa *et al.*, 2002). Inhibition of IL-18 by neutralization with an IL-18 binding protein was found to reduce ischemia-induced myocardial dysfunction in an ischemia/reperfusion model of suprafused human atrial myocardium (Dinarello, 2001). Neutralization of IL-18 using a mouse IL-18 binding protein was also able to decrease IFN- λ , TNF- α , and IL-1 β transcript levels and reduce joint damage in a collagen-induced arthritis mouse model (Banda *et al.*, 2003). A reduction of IL-18 production or availability may also prove beneficial to control metastatic cancer as injection of IL-18 binding protein in a mouse melanoma model successfully inhibited metastases (Carrascal *et al.*, 2003). As a further indication of its importance as a pro-inflammatory cytokine, plasma levels of IL-18 were elevated in patients with chronic liver disease and increased

levels were correlated with the severity of the disease (Ludwiczek *et al.*, 2002). Similarly, IL-18 and TNF- α were elevated in the serum of diabetes mellitus patients with nephropathy (Moriwaki *et al.*, 2003). Neuroinflammation following traumatic brain injury is also mediated by pro-inflammatory cytokines and inhibition of IL-18 by the IL-18 binding protein improved neurological recovery in mice following brain trauma (Yatsiv *et al.*, 2002).

TNF- α , a member of the TNF family of cytokines, is a pro-inflammatory cytokine with pleiotropic effects ranging from co-mitogenic effects on hematopoietic cells, induction of inflammatory responses, and induction of cell death in many cell types. TNF- α is normally induced by bacterial lipopolysaccharides, parasites, viruses, malignant cells and cytokines and usually acts beneficially to protect cells from infection and cancer. However, inappropriate induction of TNF- α is a major contributor to disorders resulting from acute and chronic inflammation such as autoimmune disorders and can also contribute to cancer, AIDS, heart disease, and sepsis (reviewed by Aggarwal and Natarajan, 1996; Sharma and Anker, 2002). Experimental animal models of disease (i.e. septic shock and rheumatoid arthritis) as well as human disorders (i.e. inflammatory bowel diseases and acute graft-versus-host disease) have demonstrated the beneficial effects of blocking TNF- α (Wallach *et al.*, 1999). Inhibition of TNF- α has also been effective in providing relief to patients suffering autoimmune disorders such as Crohn's disease (van Deventer, 1999) and rheumatoid arthritis (Richard-Miceli and Dougados, 2001). The ability of TNF- α to promote the survival and growth of B lymphocytes is also thought to play a role in the pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL) and the levels of TNF- α being expressed by T cells in B-CLL was positively correlated with tumour mass and stage of the disease (Bojarska-Junak *et al.*, 2002). Interleukin-1 β (IL-1 β) is a cytokine known to induce TNF- α production.

Deoxyhypusine synthase (DHS) and hypusine-containing eucaryotic translation initiation Factor-5A (eIF-5A) are known to play important roles in many cellular processes including cell growth and differentiation. Hypusine, a unique amino acid, is found in all examined eucaryotes and archaeobacteria, but not in eubacteria, and eIF-5A is the only known hypusine-containing protein. Park (1988) *J. Biol. Chem.*, 263, 7447-7449; Schümann & Klink (1989) *System. Appl. Microbiol.*, 11, 103-107; Bartig *et al.* (1990) *System. Appl. Microbiol.*, 13, 112-116; Gordon *et al.* (1987a) *J. Biol. Chem.*, 262, 16585-

16589. Active eIF-5A is formed in two post-translational steps: the first step is the formation of a deoxyhypusine residue by the transfer of the 4-aminobutyl moiety of spermidine to the α -amino group of a specific lysine of the precursor eIF-5A catalyzed by deoxyhypusine synthase; the second step involves the hydroxylation of this 4-aminobutyl moiety by deoxyhypusine hydroxylase to form hypusine.

The amino acid sequence of eIF-5A is well conserved between species, and there is strict conservation of the amino acid sequence surrounding the hypusine residue in eIF-5A, which suggests that this modification may be important for survival. Park et al. (1993) *Biofactors*, 4, 95-104. This assumption is further supported by the observation that inactivation of both isoforms of eIF-5A found to date in yeast, or inactivation of the DHS gene, which catalyzes the first step in their activation, blocks cell division. Schnier et al. (1991) *Mol. Cell. Biol.*, 11, 3105-3114; Sasaki et al. (1996) *FEBS Lett.*, 384, 151-154; Park et al. (1998) *J. Biol. Chem.*, 273, 1677-1683. However, depletion of eIF-5A protein in yeast resulted in only a small decrease in total protein synthesis suggesting that eIF-5A may be required for the translation of specific subsets of mRNA's rather than for protein global synthesis. Kang et al. (1993), "Effect of initiation factor eIF-5A depletion on cell proliferation and protein synthesis," in Tuite, M. (ed.), *Protein Synthesis and Targeting in Yeast*, NATO Series H. The recent finding that ligands that bind eIF-5A share highly conserved motifs also supports the importance of eIF-5A. Xu & Chen (2001) *J. Biol. Chem.*, 276, 2555-2561. In addition, the hypusine residue of modified eIF-5A was found to be essential for sequence-specific binding to RNA, and binding did not provide protection from ribonucleases.

In addition, intracellular depletion of eIF-5A results in a significant accumulation of specific mRNAs in the nucleus, indicating that eIF-5A may be responsible for shuttling specific classes of mRNAs from the nucleus to the cytoplasm. Liu & Tartakoff (1997) *Supplement to Molecular Biology of the Cell*, 8, 426a. Abstract No. 2476, 37th American Society for Cell Biology Annual Meeting. The accumulation of eIF-5A at nuclear pore-associated intranuclear filaments and its interaction with a general nuclear export receptor further suggest that eIF-5A is a nucleocytoplasmic shuttle protein, rather than a component of polysomes. Rosorius et al. (1999) *J. Cell Science*, 112, 2369-2380.

The first cDNA for eIF-5A was cloned from human in 1989 by Smit-McBride et al., and since then cDNAs or genes for eIF-5A have been cloned from various eukaryotes including yeast, rat, chick embryo, alfalfa, and tomato. Smit-McBride et al. (1989a) *J.*

Biol. Chem., 264, 1578-1583; Schnier et al. (1991) (yeast); Sano, A. (1995) in Imahori, M. et al. (eds), Polyamines, Basic and Clinical Aspects, VNU Science Press, The Netherlands, 81-88 (rat); Rinaudo & Park (1992) FASEB J., 6, A453 (chick embryo); Pay et al. (1991) Plant Mol. Biol., 17, 927-929 (alfalfa); Wang et al. (2001) J. Biol. Chem., 276, 17541-17549 (tomato).

Expression of eIF-5A mRNA has been explored in various human tissues and mammalian cell lines. For example, changes in eIF-5A expression have been observed in human fibroblast cells after addition of serum following serum deprivation. Pang & Chen (1994) J. Cell Physiol., 160, 531-538. Age-related decreases in deoxyhypusine synthase activity and abundance of precursor eIF-5A have also been observed in senescing fibroblast cells, although the possibility that this reflects averaging of differential changes in isoforms was not determined. Chen & Chen (1997b) J. Cell Physiol., 170, 248-254.

Studies have shown that eIF-5A may be the cellular target of viral proteins such as the human immunodeficiency virus type 1 Rev protein and human T cell leukemia virus type 1 Rex protein. Ruhl et al. (1993) J. Cell Biol., 123, 1309-1320; Katahira et al. (1995) J. Virol., 69, 3125-3133. Preliminary studies indicate that eIF-5A may target RNA by interacting with other RNA-binding proteins such as Rev, suggesting that these viral proteins may recruit eIF-5A for viral RNA processing. Liu et al. (1997) Biol. Signals, 6, 166-174.

Thus, although eIF5A and DHS are known, there remains a need in understanding how these proteins are involved in apoptotic pathways as well as cytokine stimulation to be able to modulate apoptosis and cytokine expression. The present invention fulfills this need.

SUMMARY OF INVENTION

The present invention relates to apoptosis specific eucaryotic initiation factor 5A (eIF-5A), referred to as apoptosis factor 5A1 or simply factor 5A1. The present invention also relates to apoptosis factor 5A1 nucleic acids and polypeptides and methods for inhibiting or suppressing apoptosis in cells using antisense nucleotides or siRNAs to inhibit expression of factor 5A1. The invention also relates to suppressing or inhibiting expression of pro-inflammatory cytokines by inhibiting expression of apoptosis factor 5A1. Further, the present invention relates to inhibiting or suppressing expression of p53 by inhibiting expression of apoptosis factor 5A1. The present invention also relates to a

method of increasing Bcl-2 expression by inhibiting or suppression expression of apoptosis factor 5A1 using antisense nucleotides or siRNAs. The present invention also provides a method of inhibiting production of cytokines, especially TNF- α in human epithelial cells. In another embodiment of the present invention, suppressing expression of apoptosis-specific eIF5A1 by the use of antisense oligonucleotides targeted at apoptosis-specific eIF5A1 provides methods of preventing retinal ganglion cell death in a glaucomatous eye. The present invention also provides methods of controlling the rate of dendritic cell maturation and PBMC activation by controlling the expression of eIF-5A with antisense oligonucleotides or siRNAs.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence and derived amino acid sequence of the 3' end of rat apoptosis-specific eIF-5A.

Figure 2 depicts the nucleotide sequence and derived amino acid sequence of the 5' end of rat apoptosis-specific eIF-5A cDNA.

Figure 3 depicts the nucleotide sequence of rat corpus luteum apoptosis-specific eIF-5A full-length cDNA.

Figure 4 depicts the nucleotide sequence and derived amino acid sequence of the 3' end of rat apoptosis-specific DHS cDNA.

Figure 5 is an alignment of the full-length nucleotide sequence of rat corpus luteum apoptosis-specific eIF-5A cDNA with the nucleotide sequence of human eIF-5A (Accession number BC000751 or NM_001970, SEQ ID NO:3).

Figure 6 is an alignment of the full-length nucleotide sequence of rat corpus luteum apoptosis-specific eIF-5A cDNA with the nucleotide sequence of human eIF-5A (Accession number NM-020390, SEQ ID NO:4).

Figure 7 is an alignment of the full-length nucleotide sequence of rat corpus luteum apoptosis-specific eIF-5A cDNA with the nucleotide sequence of mouse eIF-5A

(Accession number BC003889). Mouse nucleotide sequence (Accession number BC003889) is SEQ ID NO:5.

Figure 8 is an alignment of the derived full-length amino acid sequence of rat corpus luteum apoptosis-specific eIF-5A with the derived amino acid sequence of human eIF-5A (Accession number BC000751 or NM_001970).

Figure 9 is an alignment of the derived full-length amino acid sequence of rat corpus luteum apoptosis-specific eIF-5A with the derived amino acid sequence of human eIF-5A (Accession number NM_020390).

Figure 10 is an alignment of the derived full-length amino acid sequence of rat corpus luteum apoptosis-specific eIF-5A with the derived amino acid sequence of mouse eIF-5A (Accession number BC003889).

Figure 11 is an alignment of the partial-length nucleotide sequence of rat corpus luteum apoptosis-specific DHS cDNA with the nucleotide sequence of human DHS (Accession number BC000333, SEQ ID NO:8).

Figure 12 is a restriction map of rat corpus luteum apoptosis-specific eIF-5A cDNA.

Figure 13 is a restriction map of the partial-length rat apoptosis-specific DHS cDNA.

Figure 14 is a Northern blot (Figure 14A) and an ethidium bromide stained gel (Figure 14B) of total RNA probed with the ³²P-dCTP-labeled 3'-end of rat corpus luteum apoptosis-specific eIF-5A cDNA.

Figure 15 is a Northern blot (Figure 15A) and an ethidium bromide stained gel (Figure 15B) of total RNA probed with the ³²P-dCTP-labeled 3'-end of rat corpus luteum apoptosis-specific DHS cDNA.

Figure 16 depicts a DNA laddering experiment in which the degree of apoptosis in superovulated rat corpus lutea was examined after injection with PGF-2 α .

Figure 17 is an agarose gel of genomic DNA isolated from apoptosing rat corpus luteum showing DNA laddering after treatment of rats with PGF-2 α .

5 Figure 18 depicts a DNA laddering experiment in which the degree of apoptosis in dispersed cells of superovulated rat corpora lutea was examined in rats treated with spermidine prior to exposure to PGF-2 α (PGF-2 α).

10 Figure 19 depicts a DNA laddering experiment in which the degree of apoptosis in superovulated rat corpus lutea was examined in rats treated with spermidine and/or PGF-2 α .

Figure 20 is a Southern blot of rat genomic DNA probed with ³²P-dCTP-labeled partial-length rat corpus luteum apoptosis-specific eIF-5A cDNA.

15

Figure 21 depicts pHM6, a mammalian epitope tag expression vector (Roche Molecular Biochemicals).

20 Figure 22 is a Northern blot (Figure 22A) and ethidium bromide stained gel (Figure 22B) of total RNA isolated from COS-7 cells after induction of apoptosis by withdrawal of serum probed with the ³²P-dCTP-labeled 3'-untranslated region of rat corpus luteum apoptosis-specific DHS cDNA.

25 Figure 23 is a flow chart illustrating the procedure for transient transfection of COS-7 cells.

Figure 24 is a Western blot of transient expression of foreign proteins in COS-7 cells following transfection with pHM6.

30 Figure 25 illustrates enhanced apoptosis as reflected by increased caspase activity when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

Figure 26 illustrates enhanced apoptosis as reflected by increased DNA fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

5 Figure 27 illustrates detection of apoptosis as reflected by increased nuclear fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

Figure 28 illustrates enhanced apoptosis as reflected by increased nuclear fragmentation
10 when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

Figure 29 illustrates detection of apoptosis as reflected by phosphatidylserine exposure
15 when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

Figure 30 illustrates enhanced apoptosis as reflected by increased phosphatidylserine exposure when COS-7 cells were transiently transfected with pHM6 containing full-length
20 rat apoptosis-specific eIF-5A in the sense orientation.

Figure 31 illustrates enhanced apoptosis as reflected by increased nuclear fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

25 Figure 32 illustrates enhanced apoptosis when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation.

Figure 33 illustrates down-regulation of Bcl-2 when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense
30 orientation. Figure 33A is the Coomassie-blue-stained protein blot; Figure 33B is the corresponding Western blot.

Figure 34 is a Coomassie-blue-stained protein blot and the corresponding Western blot of COS-7 cells transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the antisense orientation using Bcl-2 as a probe.

5 Figure 35 is a Coomassie-blue-stained protein blot and the corresponding Western blot of COS-7 cells transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation using c-Myc as a probe.

Figure 36 is a Coomassie-blue-stained protein blot and the corresponding Western blot of
10 COS-7 cells transiently transfected with pHM6 containing full-length rat apoptosis-specific eIF-5A in the sense orientation when p53 is used as a probe.

Figure 37 is a Coomassie-blue-stained protein blot and the corresponding Western blot of expression of pHM6-full-length rat apoptosis-specific eIF-5A in COS-7 cells using an
15 anti-[HA]-peroxidase probe and a Coomassie-blue-stained protein blot and the corresponding Western blot of expression of pHM6-full-length rat apoptosis-specific eIF-5A in COS-7 cells when a p53 probe is used.

Figure 38 is an alignment of human eIF5A2 isolated from RKO cells with the sequence of
20 human eIF5A2 (Genbank accession number XM_113401).

Figure 39 is a graph depicting the percentage of apoptosis occurring in RKO and RKO-E6 cells following transient transfection. RKO and RKO-E6 cells were transiently transfected with pHM6-LacZ or pHM6-eIF5A1. RKO cells treated with Actinomycin D and
25 transfected with pHM6-eIF5A1 showed a 240% increase in apoptosis relative to cells transfected with pHM6-LacZ that were not treated with Actinomycin D. RKO-E6 cells treated with Actinomycin D and transfected with pHM6-eIF5A1 showed a 105% increase in apoptosis relative to cells transfected with pHM6-LacZ that were not treated with Actinomycin D.

30

Figure 40 is a graph depicting the percentage of apoptosis occurring in RKO cells following transient transfection. RKO cells were transiently transfected with pHM6-LacZ, pHM6-eIF5A1, pHM6-eIF5A2, or pHM6-truncated eIF5A1. Cells transfected with

pHM6-eIF5A1 showed a 25% increase in apoptosis relative to control cells transfected with pHM6-LacZ. This increase was not apparent for cells transfected with pHM6-eIF5A2 or pHM6-truncated eIF5A1.

5 Figure 41 is a graph depicting the percentage of apoptosis occurring in RKO cells following transient transfection. RKO cells were either left untransfected or were transiently transfected with pHM6-LacZ or pHM6-eIF5A1. After correction for transfection efficiency, 60 % of the cells transfected with pHM6-eIF5A1 were apoptotic.

10 Figure 42 provides the results of a flow cytometry analysis of RKO cell apoptosis following transient transfection. RKO cells were either left untransfected or were transiently transfected with pHM6-LacZ, pHM6-eIF5A1, pHM6-eIF5A2, or pHM6-truncated eIF5A1. The table depicts the percentage of cells undergoing apoptosis calculated based on the area under the peak of each gate. After correction for background
15 apoptosis in untransfected cells and for transfection efficiency, 80% of cells transfected with pHM6-eIF5A1 exhibited apoptosis. Cells transfected with pHM6-LacZ, pHM6-eIF5A2 or pHM6-truncated eIF5A1 exhibited only background levels of apoptosis.

Figure 43 provides Western blots of protein extracted from RKO cells treated with 0.25
20 µg/ml Actinomycin D for 0, 3, 7, 24, and 48 hours. The top panel depicts a Western blot using anti-p53 as the primary antibody. The middle panel depicts a Western blot using anti-eIF5A1 as the primary antibody. The bottom panel depicts the membrane used for the anti-eIF5A1 blot stained with Coomassie blue following chemiluminescent detection to demonstrate equal loading. p53 and eIF5A1 are both upregulated by treatment with
25 Actinomycin D.

Figure 44 is a bar graph showing that both apoptosis-specific eIF-5A (eIF5a) and proliferation eIF-5A (eIF5b) are expressed in heart tissue. The heart tissue was taken from patients receiving coronary artery bypass grafts (CABG). Gene expression levels of eIF5a
30 (light gray bar) are compared to eIF5b (dark gray bar). The X-axis are patient identifier numbers. The Y-axis is pg/ng of 18s (picograms of message RNA over nanograms of ribosomal RNA 18S).

Figure 45 is a bar graph showing that both apoptosis-specific eIF-5A (eIF5a) and proliferation eIF-5A (eIF5b) are expressed in heart tissue. The heart tissue was taken from patients receiving valve replacements. Gene expression levels of eIF5a (light gray bar) are compared to eIF5b (dark gray bar). The X-axis are patient identifier numbers. The Y-axis is pg/ng of 18s (picograms of message RNA over nanograms of ribosomal RNA 18S).

Figure 46 is a bar graph showing the gene expression levels measured by real-time PCR of apoptosis-specific eIF-5A (eIF5a) versus proliferation eIF-5A (eIF5b) in pre-ischemia heart tissue and post ischemia heart tissue. The Y-axis is pg/ng of 18s (picograms of message RNA over nanograms of ribosomal RNA 18S).

Figure 47 depicts schematically an experiment performed on heart tissue.

Figure 48 shows EKGs of heart tissue before and after ischemia was induced.

Figure 49 shows the lab bench with the set up of the experiment depicted in Figure 47.

Figures 50A-F report patient data where the levels of Apoptosis Factor eIF-5a (also denoted as eIF-5a1 or on the chart as IF5a1) are correlated with levels of IL-1 β and IL-18. Figure 50A is a chart of data obtained from coronary artery bypass graft (CABG) patients. Figure 50B is a chart of data obtained from valve replacement patients. Figure 50C is a graph depicting the correlation of apoptosis factor eIF-5a (Factor 5a1) to IL-18 in CABG patients. Figure 50D is a graph depicting the correlation of proliferating eIF-5a (Factor a2) to IL-18 in CABG patients. Figure 50E is a graph depicting the correlation of apoptosis factor eIF-5a (Factor 5a1) to IL-18 in valve replacement patients. Figure 50F is a graph depicting the correlation of proliferating eIF-5a (Factor a2) to IL-18 in valve replacement patients.

Figure 51 is a chart of the patient's data from which patients data used in figures 50A-F was obtained.

Figure 52 shows the levels of protein produced by RKO cells after being treated with antisense oligo 1, 2 and 3 (to apoptosis factor 5A). The RKO cells produced less

apoptosis factor 5A as well as less p53 after having been transfected with the antisense apoptosis factor 5A nucleotides.

Figure 53 shows uptake of the fluorescently labeled antisense oligonucleotide.

5

Figures 54 -58 show a decrease in the percentage of cells undergoing apoptosis in the cells having being treated with antisense apoptosis factor 5A oligonucleotides as compared to cells not having been transfected with the antisense apoptosis factor 5A oligonucleotides.

10 Figure 59 shows that treating lamina cribrosa cells with TNF- α and/or camptothecin caused an increase in the number of cells undergoing apoptosis.

Figure 60 and 61 show a decrease in the percentage of cells undergoing apoptosis in the cells having being treated with antisense apoptosis factor 5A oligonucleotides as compared
15 to cells not having been transfected with the antisense apoptosis factor 5A oligonucleotides.

Figure 62 shows that the lamina cribrosa cells uptake the labeled siRNA either in the presence of serum or without serum.

20

Figure 63 shows that cells transfected with apoptosis factor 5a siRNA produced less apoptosis factor 5a protein and in addition, produced more Bcl-2 protein. A decrease in apoptosis factor 5Aexpression correlates with an increase in BCL-2 expression.

25 Figure 64 shows that cells transfected with apoptosis factor 5a siRNA produced less apoptosis factor 5a protein.

Figures 65 - 67 shows that cells transfected with apoptosis factor 5a siRNA had a lower percentage of cells undergoing apoptosis after exposure to amptothecin and TNF- α .

30

Figure 68 are photographs of Hoescht-stained lamina cribrosa cell line # 506 transfected with siRNA and treated with camptothecin and TNF- α from the experiment described in figure 67 and Example 13. The apoptosing cells are seen as more brightly stained cells.

They have smaller nucleic because of chromatin condensing and are smaller and irregular in shape.

Figure 69 shows that IL-1 exposed HepG2 cells transfected with apoptosis factor 5A cells
5 secreted less TNF- α than non transfected cells.

Figure 70 shows the sequence of human apoptosis factor 5a (SEQ ID NO:*AA) and the sequences of 5 siRNAs of the present invention (SEQ ID NO:*1, *2, *3, *4 and *5).

10 Figure 71 shows the sequence of human apoptosis factor 5a (SEQ ID NO:*AA) and the sequences of 5 siRNAs of the present invention (SEQ ID NO:*6, *7 and *8).

Figure 72 shows the binding position of three antisense oligonucleotides targeted against human eIF5A1.

15

Figure 73a and b shows the nucleotide alignment and amino acid alignment of human eIF5A1 (apoptosis factor 5A) against human eIF5A2 (proliferating eIF5A).

Figure 74A provides a picture of a Western blot where siRNAs against eIF5A1 have
20 reduced, if not inhibited, the production of TNF- α in transfected HT-29 cells. Figure 74B provides the results of an ELISA.

Figure 75 provides the results of an ELISA. TNF- α production was reduced in cells treated with siRNAs against eIF5A1 as compared to control cells.

25

Figure 76 shows the time course of the U-937 differentiation experiment. See Example 16.

Figure 77 shows the results of a Western blot showing that eIF-5A1 is up-regulated during
30 monocyte differentiation and subsequence TNF- α secretion.

Figure 78 depicts stem cell differentiation and the use of siRNAs against eIF-5A1 to inhibit cytokine production.

Figure 79 is a bar graph showing that IL-8 is produced in response to TNF-alpha as well as in response to interferon. This graph shows that siRNA against eIF5A blocked almost all IL-8 produced in response to interferon as well as a significant amount of the IL-8

5 produced as a result of the combined treatment of interferon and TNF.

Figure 80 is another bar graph showing that IL-8 is produced in response to TNF-alpha as well as in response to interferon. This graph shows that siRNA against eIF5A blocked almost all IL-8 produced in response to interferon as well as a significant amount of the

10 IL-8 produced as a result of the combined treatment of interferon and TNF.

Figure 81 is a western blot of HT-29 cells treated with IFN gamma for 8 and 24 hours.

This blot shows upregulation (4 fold at 8 hours) of apoptosis eIF5A in response to interferon gamma in HT-29 cells.

15 Figure 82 is a characterization of lamina cribrosa cells by immunofluorescence. Lamina cribrosa cells (# 506) isolated from the optic nerve head of an 83-year old male were characterized by immunofluorescence. Primary antibodies were a) actin; b) fibronectin; c) laminin; and d) GFAP. All pictures were taken at 400 times magnification.

20 Figure 83: Apoptosis of lamina cribrosa cell line # 506 in response to treatment with camptothecin and TNF- α . Lamina cribrosa cell line # 506 cells were seeded at 40,000 cells per well onto an 8-well culture slide. Three days later the confluent LC cells were treated with either 10 ng/ml TNF- α , 50 μ M camptothecin, or 10 ng/ml TNF- α plus 50 μ M camptothecin. An equivalent volume of DMSO, a vehicle control for camptothecin, was added to the untreated control cells. The cells were stained with Hoescht 33258 48 hours after treatment and viewed by fluorescence microscopy using a UV filter. Cells with brightly stained condensed or fragmented nuclei were counted as apoptotic.

30 Figure 84: Expression of eIF5A during camptothecin or TNF-a plus camptothecin treatment. Lamina cribrosa cell # 506 cells were seeded at 40,000 cells per well onto a 24-well plate. Three days later the LC cells were treated with either 50 μ M camptothecin or 10 ng/ml TNF- α plus 50 μ M camptothecin and protein lysate was harvested 1, 4, 8, and 24

hours later. An equivalent volume of DMSO was added to control cells as a vehicle control and cell lysate was harvested 1 and 24 hours later. 5 µg of protein from each sample was separated by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with anti-eIF5A antibody. The bound antibody was detected by
5 chemiluminescence and exposed to x-ray film. The membrane was then stripped and re-blotted with anti-β-actin as an internal loading control.

Figure 85: Expression of eIF5A in lamina cribrosa cell lines # 506 and # 517 following transfection with siRNAs. Lamina cribrosa cell # 506 and # 517 cells were seeded at
10 10,000 cells per well onto a 24-well plate. Three days later the LC cells were transfected with either GAPDH siRNA, eIF5A siRNAs #1-4, or control siRNA # 5. Three days after transfection the protein lysate was harvested and 5 µg of protein from each sample was separated by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with anti-eIF5A antibody. The bound antibody was detected by chemiluminescence and
15 exposed to x-ray film. The membrane was then stripped and re-blotted with anti-β-actin as an internal loading control.

Figure 86: Apoptosis of lamina cribrosa cell line # 506 cells transfected with eIF5A siRNAs and treated with TNF-α and camptothecin. Lamina cribrosa cell line # 506 cells
20 were seeded at 7500 cells per well onto an 8-well culture slide. Three days later the LC cells were transfected with either GAPDH siRNA, eIF5A siRNAs #1-4, or control siRNA # 5. 72 hours after transfection, the transfected cells were treated with 10 ng/ml TNF-α plus 50 µM camptothecin. Twenty-four hours later the cells were stained with Hoescht 33258 and viewed by fluorescence microscopy using a UV filter. Cells with brightly
25 stained condensed or fragmented nuclei were counted as apoptotic. This graph represents the average of n=4 independent experiments.

Figure 87: Apoptosis of lamina cribrosa cell line # 517 cells transfected with eIF5A siRNA # 1 and treated with TNF-α and camptothecin. Lamina cribrosa cell line # 517 cells were
30 seeded at 7500 cells per well onto an 8-well culture slide. Three days later the LC cells were transfected with either eIF5A siRNA #1 or control siRNA # 5. 72 hours after transfection, the transfected cells were treated with 10 ng/ml TNF-α plus 50 µM camptothecin. Twenty-four hours later the cells were stained with Hoescht 33258 and

viewed by fluorescence microscopy using a UV filter. Cells with brightly stained condensed or fragmented nuclei were counted as apoptotic. The results of two independent experiments are represented here.

5 Figure 88: TUNEL-labeling of lamina cribosa cell line # 506 cells transfected with eIF5A siRNA # 1 and treated with TNF- α and camptothecin. Lamina cribrosa cell line # 506 cells were seeded at 7500 cells per well onto an 8-well culture slide. Three days later the LC cells were transfected with either eIF5A siRNA #1 or control siRNA # 5. 72 hours after transfection, the transfected cells were treated with 10 ng/ml TNF- α plus 50 μ M
10 camptothecin. Twenty-four hours later the cells were stained with Hoescht 33258 and DNA fragmentation was evaluated in situ using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. Panel A represents the slide observed by fluorescence microscopy using a fluorescein filter to visualize TUNEL-labeling of the fragmented DNA of apoptotic cells. Panel B represents the same slide
15 observed by through a UV filter to visualize the Hoescht-stained nuclei. The results are representative of two independent experiments. All pictures were taken at 400 times magnification.

Figure 89 depicts the design of siRNAs against eIF5A1

20

DETAILED DESCRIPTION OF THE INVENTION

Several isoforms of eukaryotic initiation factor 5a (eIF-5A) have been isolated and present in published databanks. It was thought that these isoforms were functionally redundant. The present inventors have discovered that one isoform is upregulated
25 immediately before the induction of apoptosis, which they have designated apoptosis factor 5A, or apoptosis-specific eIF-5A, or factor 5A1, or eIF5A1. The subject of the present invention is apoptosis factor 5A as well as DHS, which is involved in the activation of eIF-5A.

Apoptosis factor 5A is likely to be a suitable target for intervention in apoptosis-
30 causing disease states since it appears to act at the level of post-transcriptional regulation of downstream effectors and transcription factors involved in the apoptotic pathway. Specifically, apoptosis factor 5A appears to selectively facilitate the translocation of mRNAs encoding downstream effectors and transcription factors of apoptosis from the

nucleus to the cytoplasm, where they are subsequently translated. The ultimate decision to initiate apoptosis appears to stem from a complex interaction between internal and external pro- and anti-apoptotic signals. Lowe & Lin (2000) *Carcinogenesis*, 21, 485-495. Through its ability to facilitate the translation of downstream apoptosis effectors and transcription factors, the apoptosis factor 5A appears to tip the balance between these signals in favor of apoptosis.

Accordingly, the present invention provides a method of suppressing or reducing apoptosis in a cell by administering an agent that inhibits or reduces expression of either apoptosis factor 5A or DHS. One agent that can inhibit or reduce expression of apoptosis factor 5A or DHS is an antisense oligonucleotide.

Antisense oligonucleotides have been successfully used to accomplish both *in vitro* as well as *in vivo* gene-specific suppression. Antisense oligonucleotides are short, synthetic strands of DNA (or DNA analogs) that are antisense (or complimentary) to a specific DNA or RNA target. Antisense oligonucleotides are designed to block expression of the protein encoded by the DNA or RNA target by binding to the target and halting expression at the level of transcription, translation, or splicing. By using modified backbones that resist degradation (Blake *et al.*, 1985), such as replacement of the phosphodiester bonds in the oligonucleotides with phosphorothioate linkages to retard nuclease degradation (Matzura and Eckstein, 1968), antisense oligonucleotides have been used successfully both in cell cultures and animal models of disease (Hogrefe, 1999).

Preferably, the antisense oligonucleotides of the present invention have a nucleotide sequence encoding a portion of an apoptosis factor 5A polypeptide or an apoptosis-specific DHS polypeptide. The inventors have transfected various cell lines with antisense nucleotides encoding a portion of an apoptosis factor 5A polypeptide as described below and measured the number of cells undergoing apoptosis. The cells that were transfected with the antisense oligonucleotides showed a decrease in the number of cells undergoing apoptosis as compared to like cells not having been transfected with the antisense oligos. Figures 54 -58 show a decrease in the percentage of cells undergoing apoptosis in the cells having being treated with antisense apoptosis factor 5A oligonucleotides as compared to cells not having been transfected with the antisense apoptosis factor 5A oligonucleotides.

The present invention contemplates the use of many suitable nucleic acid sequences encoding an apoptosis factor 5A polypeptide or DHS polypeptide. For

example, SEQ ID NOS:1, 3, 4, 5, 11, 15, 19, 20, and 21 (apoptosis-factor 5A nucleic acid sequences), SEQ ID NOS:6 and 8 (apoptosis-specific DHS nucleic acid sequences), SEQ ID NOS:12 and 16 (apoptosis factor 5A sequences), and SEQ ID NO:7 (apoptosis-specific DHS polypeptide sequences), or portions thereof, provide suitable sequences. Other
5 preferred apoptosis factor 5A sequences include SEQ ID NO: *6, *7, and *8. Additional antisense nucleotides include those that have substantial sequence identity to those enumerated above (i.e. 90% homology) or those having sequences that hybridize under highly stringent conditions to the enumerated SEQ ID NOs. Additionally, other suitable sequences can be found using the known sequences as probes according to methods
10 known in the art.

The antisense oligonucleotides of the present invention may be single stranded, double stranded, DNA, RNA or a hybrid. The oligonucleotides may be modified by methods known in the art to increase stability, increase resistance to nuclease degradation or the like. These modifications are known in the art and include, but are not limited to
15 modifying the backbone of the oligonucleotide, modifying the sugar moieties, or modifying the base. Also inclusive in these modifications are various DNA-RNA hybrids or constructs commonly referred to as "gapped" oligonucleotides.

The present invention provides other agents that can inhibit or reduce expression of apoptosis factor 5A or DHS. One such agent is siRNAs. Small Inhibitory RNAs (siRNA)
20 have been emerging as a viable alternative to antisense oligonucleotides since lower concentrations are required to achieve levels of suppression that are equivalent or superior to those achieved with antisense oligonucleotides (Thompson, 2002). Long double-stranded RNAs have been used to silence the expression of specific genes in a variety of organisms such as plants, nematodes, and fruit flies. An RNase-III family enzyme called
25 Dicer processes these long double stranded RNAs into 21-23 nucleotide small interfering RNAs which are then incorporated into an RNA-induced silencing complex (RISC). Unwinding of the siRNA activates RISC and allows the single-stranded siRNA to guide the complex to the endogenous mRNA by base pairing. Recognition of the endogenous mRNA by RISC results in its cleavage and consequently makes it unavailable for
30 translation. Introduction of long double stranded RNA into mammalian cells results in a potent antiviral response which can be bypassed by use of siRNAs. (Elbashir *et al.*, 2001). SiRNA has been widely used in cell cultures and routinely achieves a reduction in specific gene expression of 90 % or more.

The use of siRNAs has also been gaining popularity in inhibiting gene expression in animal models of disease. A recent study that demonstrated that an siRNA against luciferase was able to block luciferase expression from a co-transfected plasmid in a wide variety of organs in post-natal mice using a hydrodynamic injection delivery technique (Lewis *et al.*, 2002). An siRNA against Fas, a receptor in the TNF family, injected hydrodynamically into the tail vein of mice was able to transfect greater than 80 % of hepatocytes and decrease Fas expression in the liver by 90 % for up to 10 days after the last injection (Song *et al.*, 2003). The Fas siRNA was also able to protect mice from liver fibrosis and fulminant hepatitis. The development of sepsis in mice treated with a lethal dose of lipopolysaccharide was inhibited by the use of an siRNA directed against TNF α (Sørensen *et al.*, 2003). SiRNA has the potential to be a very potent drug for the inhibition of specific gene expression *in vivo* in light of their long-lasting effectiveness in cell cultures and *in vivo*, their ability to transfect cells *in vivo*, and their resistance to degradation in serum (Bertrand *et al.*, 2002).

The present inventors have transfected cells with apoptosis factor 5A siRNAs and studied the effects on expression of apoptosis factor 5A. Figure 64 shows that cells transfected with apoptosis factor 5a siRNA produced less apoptosis factor 5a protein. Figures 64 - 67 show that cells transfected with apoptosis factor 5A siRNAs have a lower percentage of cells undergoing apoptosis after exposure to amptothecin and TNF- α as compared to cells not having been transfected with apoptosis factor 5A siRNAs.

Preferred siRNAs include those that have SEQ ID NO: *1, *2, *3, *4, and *5. Additional siRNAs include those that have substantial sequence identity to those enumerated (i.e. 90% homology) or those having sequences that hybridize under highly stringent conditions to the enumerated SEQ ID NOs.

Many important human diseases are caused by abnormalities in the control of apoptosis. These abnormalities can result in either a pathological increase in cell number (e.g. cancer) or a damaging loss of cells (e.g. degenerative diseases). As non-limiting examples, the methods and compositions of the present invention can be used to prevent or treat the following apoptosis-associated diseases and disorders: neurological/neurodegenerative disorders (e.g., Alzheimer's, Parkinson's, Huntington's, Amyotrophic Lateral Sclerosis (Lou Gehrig's Disease), autoimmune disorders (e.g., rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis), Duchenne Muscular Dystrophy (DMD), motor neuron disorders, ischemia, heart ischemia, chronic heart

failure, stroke, infantile spinal muscular atrophy, cardiac arrest, renal failure, atopic dermatitis, sepsis and septic shock, AIDS, hepatitis, glaucoma, diabetes (type 1 and type 2), asthma, retinitis pigmentosa, osteoporosis, xenograft rejection, and burn injury.

One such disease caused by abnormalities in the control of apoptosis is glaucoma.

5 Apoptosis is a critical factor leading to blindness in glaucoma patients. Glaucoma is a group of eye conditions arising from damage to the optic nerve that results in progressive blindness. Apoptosis has been shown to be a direct cause of this optic nerve damage.

Early work in the field of glaucoma research has indicated that elevated IOP leads to interference in axonal transport at the level of the lamina cribosa (a perforated,
10 collagenous connective tissue) that is followed by the death of retinal ganglion cells. Quigley and Anderson (1976) *Invest. Ophthalmol. Vis. Sci.*, **15**, 606-16; Minckler, Bunt, and Klock, (1978) *Invest. Ophthalmol. Vis. Sci.*, **17**, 33-50; Anderson and Hendrickson, (1974) *Invest. Ophthalmol. Vis. Sci.*, **13**, 771-83; Quigley *et al.*, (1980) *Invest. Ophthalmol. Vis. Sci.*, **19**, 505-17. Studies of animal models of glaucoma and post-
15 mortem human tissues indicate that the death of retinal ganglion cells in glaucoma occurs by apoptosis. Garcia-Valenzuela *e. al.*, (1995) *Exp. Eye Res.*, **61**, 33-44; Quigley *et al.*, (1995) *Invest. Ophthalmol. Vis. Sci.*, **36**, 774-786; Monard, (1998) In: Haefliger IO, Flammer J (eds) *Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma*, New York, NY, Lippincott-Raven, 213-220. The interruption of axonal transport as a result of
20 increased IOP may contribute to retinal ganglion cell death by deprivation of trophic factors. Quigley, (1995) *Aust N Z J Ophthalmol*, **23**(2), 85-91. Optic nerve head astrocytes in glaucomatous eyes have also been found to produce increased levels of some neurotoxic substances. For example, increased production of tumor necrosis factor- α (TNF- α) (Yan *et al.*, (2000) *Arch. Ophthalmol.*, **118**, 666-673), and nitric oxide
25 synthase (Neufeld *et al.*, (1997) *Arch. Ophthalmol.*, **115**, 497-503), the enzyme which gives rise to nitric oxide, has been found in the optic nerve head of glaucomatous eyes. Furthermore, increased expression of the inducible form of nitric oxide synthase (iNOS) and TNF- α by activated retinal glial cells have been observed in rat models of hereditary retinal diseases. Cotinet *et al.*, (1997) *Glia*, **20**, 59-69; de Kozak *et al.*, (1997) *Ocul. Immunol. Inflamm.*, **5**, 85-94; Goureau *et al.*, (1999) *J. Neurochem*, **72**, 2506-2515. In the
30 glaucomatous optic nerve head, excessive nitric oxide has been linked to the degeneration of axons of retinal ganglion cells. Arthur and Neufeld, (1999) *Surv Ophthalmol*, **43** (Suppl 1), S129-S135. Finally, increased production of TNF- α by retinal glial cells in response to

simulated ischemia or elevated hydrostatic pressure has been shown to induce apoptosis in cocultured retinal ganglion cells. Tezel and Wax, (2000) *J. Neurosci.*, **20(23)**, 8693-8700.

Protecting retinal ganglion cells from degeneration by apoptosis is under study as a potential new treatment for blindness due to glaucoma. Antisense oligonucleotides have been used by several groups to target key proteins in the apoptotic process in order to protect retinal ganglion cells from apoptotic cell death. Antisense oligonucleotides are short, synthetic strands of DNA (or DNA analogs) that are antisense (or complimentary) to a specific DNA or RNA target. Antisense oligonucleotides are designed to block expression of the protein encoded by the DNA or RNA target by binding to the target and halting expression at the level of transcription, translation, or splicing. One of the hurdles to using antisense oligonucleotides as a drug is the rapid degradation of oligonucleotides in blood and in cells by nucleases. This problem has been addressed by using modified backbones that resist degradation (Blake *et al.*, (1985) *Biochemistry*, **24**, 6139-6145) such as replacement of the phosphodiester bonds in the oligonucleotides with phosphorothioate linkages to retard nuclease degradation. Matzura and Eckstein, (1968) *Eur. J. Biochem.*, **3**, 448-452.

Antisense oligonucleotides have been used successfully in animal models of eye disease. In a model of transient global retinal ischemia, expression of caspase 2 was increased during ischemia, primarily in the inner nuclear and ganglion cell layers of the retina. Suppression of caspase using an antisense oligonucleotide led to significant histopathologic and functional improvement as determined by electroretinogram. Singh *et al.*, (2001) *J. Neurochem.*, **77(2)**, 466-75. Another study demonstrated that, upon transection of the optic nerve, retinal ganglion cells upregulate the pro-apoptotic protein Bax and undergo apoptosis. Repeated injections of a Bax antisense oligonucleotide into the temporal superior retina of rats inhibited the local expression of Bax and increased the number of surviving retinal ganglion cells following transaction of the optic nerve. Isenmann *et al.*, (1999) *Cell Death Differ.*, **6(7)**, 673-82.

Delivery of antisense oligonucleotides to retinal ganglion cells has been improved by encapsulating the oligonucleotides in liposomes, which were then coated with the envelope of inactivated hemagglutinating virus of Japan (HVJ; Sendai virus) by fusion (HVJ liposomes). Intravitreal injection into mice of FITC-labeled antisense oligonucleotides encapsulated in HVJ liposomes resulted in high fluorescence within 44 % of the cells in the ganglion layer which lasted three days while fluorescence with naked

FITC-labeled antisense oligonucleotide disappeared after one day. Hangai *et al.*, (1998) *Arch Ophthalmol*, 116(7), 976.

A method of preventing or modulating apoptosis of the present invention is directed to modulating apoptosis in the cells of the eye, such as but not limited to, astrocytes, retinal ganglion, retinal glial cells and lamina cribosa. Death of retinal ganglion cells in glaucoma occurs by apoptosis. Thus, providing a method of inhibiting apoptosis in retinal ganglion cells or by protecting retinal ganglion cells from degeneration by apoptosis provides a novel treatment for prevention of blindness due to glaucoma.

The present invention provides a method for preventing retinal ganglion cell death in a glaucomatous eye, by suppressing expression of apoptosis-specific eIF5A1. Inhibiting the expression of apoptosis-specific eIF5A1 reduces apoptosis. Apoptosis-specific eIF5A1 is a powerful gene that appears to regulate the entire apoptic process. Thus, controlling apoptosis in the optic nerve head indicates that blocking expression of apoptosis-specific eIF5A1 provides a treatment for glaucoma.

Suppression of expression of apoptosis-specific eIF5A1 is accomplished by administering antisense oligonucleotides or siRNAs targeted against human apoptosis-specific eIF5A1 to cells of the eye such as, but not limited to lamina cribosa, astrocytes, retinal ganglion, or retinal glial cells. Antisense oligonucleotides are as defined above, i.e. have a nucleotide sequence encoding at least a portion of an apoptosis-specific eIF5A1 polypeptide. Antisense oligonucleotides targeted against human apoptosis-specific eIF5A1 have a nucleotide sequence encoding at least a portion of human apoptosis-specific eIF5A1 polypeptide. Preferred antisense oligonucleotides comprise SEQ ID NO:26 or 27 or oligonucleotides that bind to a sequence complementary to SEQ ID NO:26 or 27 under high stringency conditions.

Another embodiment of the invention provides a method of suppressing expression of apoptosis-specific eIF5A1 in lamina cribosa cells, astrocyte cells, retinal ganglion cells or retinal glial cells. Antisense oligonucleotides, such as but not limited to, SEQ ID NO:26 and 27, targeted against human apoptosis-specific eIF5A1 are administered to lamina cribosa cells, astrocyte cells, retinal ganglion cells or retinal glial cells. The cells may be human.

In addition to having a role in apoptosis, eIF5A may also have a role in the immune response. The present inventors have discovered that apoptosis factor 5A levels

correlate with elevated levels of two cytokines (Interleukin 1-beta "IL-1 β " and interleukin 18 "IL-18") in ischemic heart tissue, thus further proving that apoptosis factor 5A is involved in cell death as it is present in ischemic heart tissue. Further, this apoptosis factor 5A/interleukin correlation has not been seen in non-ischemic heart tissue. See
5 Figures 50A-F and 51. Using PCR measurements, levels of apoptosis factor 5A, proliferating eIF-5a (eIF-5A2 - the other known isoform or referenced in some figures as eIF5b), IL-1 β , and IL-18 were measured and compared in various ischemic heart tissue (from coronary bypass graft and valve (mitral and atrial valve) replacement patients).

The correlation of apoptosis eIF-5a to these potent interleukins further suggests
10 that the inflammation and apoptosis pathways in ischemia may be controlled via controlling levels of apoptosis factor 5A. Further evidence that apoptosis factor 5A is involved in the immune response is suggested by the fact that human peripheral blood mononuclear cells (PBMCs) normally express very low levels of eIF5A, but upon stimulation with T-lymphocyte-specific stimuli expression of eIF5A increases
15 dramatically (Bevec *et al.*, 1994). This suggests a role for apoptosis factor 5A in T-cell proliferation and/or activation. Since activated T cells are capable of producing a wide variety of cytokines, it is also possible that apoptosis factor 5A may be required as a nucleocytoplasmic shuttle for cytokine mRNAs.

Another study looked at eIF-5A mRNA and cell surface marker expression in
20 human peripheral blood mononuclear cells (PBMCs) and blood cell lines treated with various maturation stimulating agents (Bevec *et al.*, Proc. Natl. Acad. Sci. USA, 91:10829-10833.(1994)). eIF-5A mRNA expression was induced in the PBMCs by numerous stimuli that also induced T-cell activation (Bevec *et al.*, 1994). Higher levels of PBMC eIF-5A mRNA expression were observed in HIV-1 patients than in healthy donors. The
25 authors of this study interpreted their results by suggesting that the eIF-5A mRNA was induced so that it could act as a nucleocytoplasmic shuttle for the important mRNAs necessary for T-cell activation and also for HIV-1 replication (Bevec *et al.*, 1994). EiF5A has been demonstrated to be a cellular binding factor for the HIV Rev protein and required for HIV replication (Ruhl *et al.* 1993).

30 The present inventors have studied the ability of eIF5A1 to promote translation of cytokines by acting as a nucleocytoplasmic shuttle for cytokine mRNAs *in vitro* using a cell line known to predictably produce cytokine(s) in response to a specific stimulus. Some recent studies have found that human liver cell lines can respond to cytokine

stimulation by inducing production of other cytokines. HepG2 is a well characterized human hepatocellular carcinoma cell line found to be sensitive to cytokines. In response to IL-1 β , HepG2 cells rapidly produce TNF- α mRNA and protein in a dose-dependent manner (Frede et al., 1996; Rowell et al., 1997; Wordemann et al., 1998). Thus, HepG2
5 cells were used as a model system to study the regulation of TNF- α production. The present inventors have shown that inhibition of eIF5A1 expression in HepG2 cells caused the cells to produce less TNF- α after having been transfected with antisense nucleotides directed toward apoptosis factor 5A.

Thus one embodiment of the present invention provides a method for reducing
10 levels of a cytokine in a cell. The method involves administering an agent to the cell capable of reducing expression of apoptosis factor 5A1. Reducing expression of apoptosis factor 5A1 causes a reduction in the expression of the cytokine, and thus leads to a decreased amount of the cytokine produced by cell. The cytokine is preferably a pro-inflammatory cytokine, including, but not limited to IL-1, IL-18, IL-6 and TNF- α .

15 An agent capable of reducing expression of apoptosis factor 5A may be an antisense nucleotide having a sequence complementary to apoptosis factor 5A. Preferably the antisense nucleotide has a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8 or is an antisense nucleotide that hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *6, *7, and
20 *8.

An agent may also comprise a siRNA having a sequence complementary to apoptosis factor 5A. Preferably the siRNA has a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4, and *5, or is a siRNA that hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID
25 NO: *1, *2, *3, *4, and *5. Figures 65 - 67 show that cells transfected with apoptosis factor 5A siRNAs have a lower percentage of cells undergoing apoptosis after exposure to amphotecin and TNF- α . An agent may also comprise antisense DHS nucleotides.

The present invention is also directed to a polynucleotide having a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8 or is an antisense
30 nucleotide that hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8.

The present invention is also directed to a siRNA having a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4, and *5 or is a siRNA that hybridizes

under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4, and *5.

The present invention is also directed to a method for reducing the expression of p53. This method involves administering an agent capable of reducing expression of apoptosis factor 5A, such as the antisense polynucleotides or the siRNAs described above. Reducing expression of apoptosis factor 5A1 reduces expression of p53 as shown in figure 52 and example 11.

The present invention is also directed to a method for increasing the expression of Bcl-2. This method entails administering an agent capable of reducing expression of apoptosis factor 5A. Preferred agents include the antisense oligonucleotides and siRNAs described above. Reducing of expression of apoptosis factor 5A1 increases expression of Bcl-2 as shown in figure 63 and example 13. Figure 63 shows that cells transfected with apoptosis factor 5a siRNA produced less apoptosis factor 5A1 protein and in addition, produced more Bcl-2 protein. Decreased in apoptosis factor 5A1 expression correlates with an increase in BCL-2 expression.

The present invention also provides a method for reducing levels of TNF-alpha in a patient in need thereof comprising administering to said patient either the antisense polynucleotide or siRNAs described above. As demonstrated in figure 69 and example 14, cells transfected with antisense factor 5A oligonucleotides of the present invention produced less TNF- α after induction with IL-1 than cells not transfected with such antisense oligonucleotides.

The present invention provides for a method of reducing levels of TNF-alpha in human epithelial cells. As demonstrated in Figures 74A and B and Figure 75 and example 15, reducing or inhibiting the expression of eIF5A1 causes a decrease, if not complete inhibition of the production of TNF-alpha in a human epithelial cell line. siRNAs against eIF5A1 were used to inhibit expression of eIF5A1. This inhibition of expression not only reduced or inhibited the production of TNF-alpha, but it also protected the cells from cytokine-induced apoptosis. By reducing expression of eIF5A1, the production of TNF- α was reduced. This dual effect provides a method of treating patients suffering from inflammatory bowel disorders such as Crohn's disease and ulcerative colitis, which are associated with an increased inflammation caused by TNF- α .

Thus, the present invention provides a method of treating pathological conditions characterized by an increased IL-1, TNF-alpha, IL-6 or IL-18 level comprising

administering to a mammal having said pathological condition, an agent to reduce expression of apoptosis Factor 5A.

Known pathological conditions characterized by an increase in IL-1, TNF-alpha, or IL-6 levels include, but are not limited to arthritis-rheumatoid and osteo arthritis, asthma, allergies, arterial inflammation, crohn's disease, inflammatory bowel disease, (ibd), ulcerative colitis, coronary heart disease, cystic fibrosis, diabetes, lupus, multiple sclerosis, graves disease, periodontitis, glaucoma & macular degeneration, ocular surface diseases including keratoconus, organ ischemia- heart, kidney, repurfusion injury, sepsis, multiple myeloma, organ transplant rejection, psoriasis and eczema.

Recent studies have suggested an important role for eIF-5A in the differentiation and activation of cells. When immature dendritic cells were induced to differentiate and mature, an induction of eIF-5A mRNA levels coincided with an elevation of CD83 protein expression (Kruse et al., J. Exp. Med. 191(9): 1581-1589 (2000)). Dendritic cells are antigen-presenting cells that sensitize helper and killer T cells to induce T cell-mediated immunity (Steinman, 1991). Immature dendritic cells lack the ability to stimulate T cells and require appropriate stimuli (i.e. inflammatory cytokines and/or microbial products) to mature into cells capable of activating T cells. The synthesis and surface expression of CD83 on mature dendritic cells is importantly involved in sensitizing helper and killer T cells and in inducing T cell-mediated immunity. When the immature dendritic cells were pre-treated with an inhibitor (GC7) of hypusination and thus an inhibitor of eIF-5A activation, the surface expression of CD83 was prevented (Kruse et al., 2000). The authors of this study interpreted their results that the eIF-5A was essential for the nucleocytoplasmic translocation of the CD83 mRNA and that by blocking hypusination and thus eIF-5A, CD83 expression and dendritic cell maturation were blocked (Kruse et al., 2000).

In both of these studies (Bevec *et al.*, 1994; Kruse *et al.*, 2000) implicating a role for eIF5A in the immune system, the authors did not specify nor identify which isoform of eIF5A they were examining, nor did they have a reason to. As briefly discussed above, humans are known to have two isoforms of eIF5A, eIF5A1 (apoptosis factor 5A1) and eIF5A2, both encoded on separate chromosomes. Prior to the present inventors discoveries it was believed that both of these isoforms were functionally redundant. The oligonucleotide described by Bevec *et al.* that was used to detect eIF5A mRNA in stimulated PBMCs had 100 % homology to human eIF5A1 and the study pre-dates the

cloning of eIF5A2. Similarly, the primers described by Kruse *et al.* that were used to detect eIF5A by reverse transcription polymerase chain reaction during dendritic cell maturation had 100 % homology to human eIF5A1.

The present invention relates to controlling the expression of eIF-5A1 to control the rate of dendritic cell maturation and PBMC activation, which in turn may control the rate of T cell-mediated immunity. The present inventors studied the role of eIF-5A1 in the differentiation of monocytes into adherent macrophages using the U-937 cell line, as U-937 is known to express eIF-5A mRNA (Bevec *et al.*, 1994). U-937 is a human monocyte cell line that grows in suspension and will become adherent and differentiate into macrophages upon stimulation with PMA. When PMA is removed by changing the media, the cells become quiescent and are then capable of producing cytokines (Barrios-Rodiles *et al.*, J. Immunol. 163:963-969 (1999)). In response to lipopolysaccharide (LPS), a factor found on the outer membrane of many bacteria known to induce a general inflammatory response, the macrophages produce both TNF- α and IL-1 β (Barrios-Rodiles *et al.*, 1999). See Figure 78 showing a chart of stem cell differentiation and the resultant production of cytokines. The U-937 cells also produce IL-6 and IL-10 following LPS-stimulation (Izeboud *et al.*, J. Receptor & Signal Transduction Research, 19(1-4):191-202. (1999)).

Using U-937 cells, it was shown that eIF-5A1 is upregulated during monocyte differentiation and TNF- α secretion. See Figure 77. Accordingly, one aspect of the invention provides for a method of inhibiting or delaying maturation of macrophages to inhibit or reduce the production of cytokines. This method involves providing an agent that is capable of reducing the expression of either DHS or eIF-5A1. By reducing or eliminating expression of DHS, eIF-5A1 activation will be reduced or eliminated. Since, eIF-5A1 is upregulated during monocyte differentiation and TNF- α secretion, it is believed that it is necessary for these events to occur. Thus, by reducing or eliminating activation of eIF-5A1 or by directly reducing or eliminating eIF-5A1 expression, monocyte differentiation and TNF- α secretion can be reduced or eliminated. Any agent capable of reducing the expression of DHS or eIF-5A1 may be used and includes, but is not limited to antisense oligonucleotides or siRNAs as described herein.

As used herein, the term "substantial sequence identity" or "substantial homology" is used to indicate that a sequence exhibits substantial structural or functional equivalence with another sequence. Any structural or functional differences between sequences having

substantial sequence identity or substantial homology will be *de minimus*; that is, they will not affect the ability of the sequence to function as indicated in the desired application.

Differences may be due to inherent variations in codon usage among different species, for example. Structural differences are considered *de minimus* if there is a significant amount

5 of sequence overlap or similarity between two or more different sequences or if the different sequences exhibit similar physical characteristics even if the sequences differ in length or structure. Such characteristics include, for example, the ability to hybridize under defined conditions, or in the case of proteins, immunological crossreactivity, similar enzymatic activity, etc. The skilled practitioner can readily determine each of these

10 characteristics by art known methods.

Additionally, two nucleotide sequences are "substantially complementary" if the sequences have at least about 70 percent or greater, more preferably 80 percent or greater, even more preferably about 90 percent or greater, and most preferably about 95 percent or greater sequence similarity between them. Two amino acid sequences are substantially

15 homologous if they have at least 50%, preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95% similarity between the active, or functionally relevant, portions of the polypeptides.

To determine the percent identity of two sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a

20 second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% or more of the length of a reference sequence is aligned for comparison purposes. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a

25 position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account

30 the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity and similarity between two sequences can be accomplished using a mathematical algorithm.

(Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular
5 Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against sequence databases to, for example, identify other family members or related sequences. Such searches can be performed
10 using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program. BLAST protein searches can be performed with the XBLAST program to obtain amino acid sequences homologous to the proteins of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in
15 Altschul et al. (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

The term "functional derivative" of a nucleic acid is used herein to mean a homolog or analog of the gene or nucleotide sequence. A functional derivative may retain
20 at least a portion of the function of the given gene, which permits its utility in accordance with the invention. "Functional derivatives" of the apoptosis factor 5A polypeptide as described herein are fragments, variants, analogs, or chemical derivatives of apoptosis factor 5A that retain at least a portion of the apoptosis factor 5A activity or immunological cross reactivity with an antibody specific for apoptosis factor 5A. A fragment of the
25 apoptosis factor 5A polypeptide refers to any subset of the molecule.

Functional variants can also contain substitutions of similar amino acids that result in no change or an insignificant change in function. Amino acids that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham et al. (1989) *Science* 244:1081-1085). The
30 latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as kinase activity or in assays such as an in vitro proliferative activity. Sites that are critical for binding partner/substrate binding can also be determined by structural analysis such as

crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith et al. (1992) J. Mol. Biol. 224:899-904; de Vos et al. (1992) Science 255:306-312).

A "variant" refers to a molecule substantially similar to either the entire gene or a fragment thereof, such as a nucleotide substitution variant having one or more substituted
5 nucleotides, but which maintains the ability to hybridize with the particular gene or to encode mRNA transcript which hybridizes with the native DNA. A "homolog" refers to a fragment or variant sequence from a different animal genus or species. An "analog" refers to a non-natural molecule substantially similar to or functioning in relation to the entire molecule, a variant or a fragment thereof.

10 Variant peptides include naturally occurring variants as well as those manufactured by methods well known in the art. Such variants can readily be identified/made using molecular techniques and the sequence information disclosed herein. Further, such variants can readily be distinguished from other proteins based on sequence and/or structural homology to the eIF-5A or DHS proteins of the present invention. The degree
15 of homology/identity present will be based primarily on whether the protein is a functional variant or non-functional variant, the amount of divergence present in the paralog family and the evolutionary distance between the orthologs.

Non-naturally occurring variants of the eIF-5A or DHS proteins of the present invention can readily be generated using recombinant techniques. Such variants include,
20 but are not limited to deletions, additions and substitutions in the amino acid sequence of the proteins. For example, one class of substitutions are conserved amino acid substitution. Such substitutions are those that substitute a given amino acid in a protein by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, and Ile;
25 interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between the amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe and Tyr. Guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie et al., Science 247:1306-1310 (1990).

30 The term "hybridization" as used herein is generally used to mean hybridization of nucleic acids at appropriate conditions of stringency as would be readily evident to those skilled in the art depending upon the nature of the probe sequence and target sequences. Conditions of hybridization and washing are well known in the art, and the adjustment of

conditions depending upon the desired stringency by varying incubation time, temperature and/or ionic strength of the solution are readily accomplished. *See, e.g.* Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbour Press, Cold Spring Harbor, New York, 1989.

5 The choice of conditions is dictated by the length of the sequences being hybridized, in particular, the length of the probe sequence, the relative G-C content of the nucleic acids and the amount of mismatches to be permitted. Low stringency conditions are preferred when partial hybridization between strands that have lesser degrees of complementarity is desired. When perfect or near perfect complementarity is desired,
10 high stringency conditions are preferred. For typical high stringency conditions, the hybridization solution contains 6X S.S.C., 0.01 M EDTA, 1X Denhardt's solution and 0.5% SDS. Hybridization is carried out at about 68°C for about 3 to 4 hours for fragments of cloned DNA and for about 12 to 16 hours for total eucaryotic DNA. For lower stringencies, the temperature of hybridization is reduced to about 42° C below the melting
15 temperature (T_m) of the duplex. The T_m is known to be a function of the G-C content and duplex length as well as the ionic strength of the solution.

 As used herein, the phrase "hybridizes to a corresponding portion" of a DNA or RNA molecule means that the molecule that hybridizes, e.g., oligonucleotide, polynucleotide, or any nucleotide sequence (in sense or antisense orientation) recognizes
20 and hybridizes to a sequence in another nucleic acid molecule that is of approximately the same size and has enough sequence similarity thereto to effect hybridization under appropriate conditions. For example, a 100 nucleotide long sense molecule will recognize and hybridize to an approximately 100 nucleotide portion of a nucleotide sequence, so long as there is about 70% or more sequence similarity between the two sequences. It is to
25 be understood that the size of the "corresponding portion" will allow for some mismatches in hybridization such that the "corresponding portion" may be smaller or larger than the molecule which hybridizes to it, for example 20-30% larger or smaller, preferably no more than about 12-15% larger or smaller.

 In addition, functional variants of polypeptides can also contain substitution of
30 similar amino acids that result in no change or an insignificant change in function. Amino acids that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham et al., *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every

residue in the molecule. The resulting mutant molecules are then tested for biological activity or in assays.

For example, an analog of apoptosis factor 5A refers to a non-natural protein or peptidomimetic substantially similar to either the entire protein or a fragment thereof.

5 Chemical derivatives of apoptosis factor 5A contain additional chemical moieties not normally a part of the peptide or peptide fragment. Modifications can be introduced into peptide or fragment thereof by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

10 It is understood that the nucleic acids and polypeptides of the present invention, where used in an animal for the purpose of prophylaxis or treatment, will be administered in the form of a composition additionally comprising a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable carriers include, for example, one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as
15 combinations thereof. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the binding proteins. The compositions of the injection can, as is well known in the art, be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to
20 the mammal.

The compositions of this invention can be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions, dispersions or suspensions, liposomes, suppositories, injectable and infusible solutions. The preferred form depends on the intended mode of administration and
25 therapeutic application.

Such compositions can be prepared in a manner well known in the pharmaceutical art. In making the composition the active ingredient will usually be mixed with a carrier, or diluted by a carrier, and/or enclosed within a carrier which can, for example, be in the form of a capsule, sachet, paper or other container. When the carrier serves as a diluent, it
30 can be a solid, semi-solid, or liquid material, which acts as a vehicle, excipient or medium for the active ingredient. Thus, the composition can be in the form of tablets, lozenges, sachets, cachets, elixirs, suspensions, aerosols (as a solid or in a liquid medium), ointments containing for example up to 10% by weight of the active compound, soft and hard gelatin

capsules, suppositories, injection solutions, suspensions, sterile packaged powders and as a topical patch.

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration. The Examples are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way. The examples do not include detailed descriptions of conventional methods. Such methods are well known to those of ordinary skill in the art and are described in numerous publications. Detailed descriptions of conventional methods, such as those employed in the construction of vectors and plasmids, the insertion of nucleic acids encoding polypeptides into such vectors and plasmids, the introduction of plasmids into host cells, and the expression and determination thereof of genes and gene products can be obtained from numerous publication, including Sambrook, J. et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press. All references mentioned herein are incorporated in their entirety.

EXAMPLES

EXAMPLE 1

Visualization of Apoptosis in Rat Corpus Luteum by DNA Laddering

The degree of apoptosis was determined by DNA laddering. Genomic DNA was isolated from dispersed corpus luteal cells or from excised corpus luteum tissue using the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer's instructions. Corpus luteum tissue was excised before the induction of apoptosis by treatment with PGF-2 α , 1 and 24 hours after induction of apoptosis. The isolated DNA was end-labeled by incubating 500 ng of DNA with 0.2 μ Ci [α -³²P]dCTP, 1 mM Tris, 0.5 mM EDTA, 3 units of Klenow enzyme, and 0.2 pM each of dATP, dGTP, and dTTP at room temperature for 30 minutes. Unincorporated nucleotides were removed by passing the sample through a 1 ml Sepadex G-50 column according to Sambrook et al. The samples were then resolved by Tris-acetate-EDTA (1.8 %) gel electrophoresis. The gel was dried for 30 minutes at room temperature under vacuum and exposed to x-ray film at - 80° C for 24 hours.

In one experiment, the degree of apoptosis in superovulated rat corpus lutea was examined either 0, 1, or 24 hours after injection with PGF-2 α . In the 0 hour control, the ovaries were removed without PGF-2 α injection. Laddering of low molecular weight

DNA fragments reflecting nuclease activity associated with apoptosis is not evident in control corpus luteum tissue excised before treatment with PGF-2 α , but is discernible within 1 hour after induction of apoptosis and is pronounced by 24 hours after induction of apoptosis, which is shown in FIG. 16. In this figure, the top panel is an autoradiograph of the Northern blot probed with the ³²P-dCTP-labeled 3'-untranslated region of rat corpus luteum apoptosis-specific DHS cDNA. The lower panel is the ethidium bromide stained gel of total RNA. Each lane contains 10 μ g RNA. The data indicates that there is down-regulation of eIF-5A transcript following serum withdrawal.

In another experiment, the corresponding control animals were treated with saline instead of PGF-2 α . Fifteen minutes after treatment with saline or PGF-2 α , corpora lutea were removed from the animals. Genomic DNA was isolated from the corpora lutea at 3 hours and 6 hours after removal of the tissue from the animals. DNA laddering and increased end labeling of genomic DNA are evident 6 hours after removal of the tissue from the PGF-2 α -treated animals, but not at 3 hours after removal of the tissue. See FIG. 17. DNA laddering reflecting apoptosis is also evident when corpora lutea are excised 15 minutes after treatment with PGF-2 α and maintained for 6 hours under *in vitro* conditions in EBSS (Gibco). Nuclease activity associated with apoptosis is also evident from more extensive end labeling of genomic DNA.

In another experiment, superovulation was induced by subcutaneous injection with 500 μ g of PGF-2 α . Control rats were treated with an equivalent volume of saline solution. Fifteen to thirty minutes later, the ovaries were removed and minced with collagenase. The dispersed cells from rats treated with PGF-2 α were incubated in 10 mm glutamine + 10 mm spermidine for 1 hour and for a further 5 hours in 10 mm glutamine without spermidine (lane 2) or in 10 mm glutamine + 10 mm spermidine for 1 hour and for a further 5 hours in 10 mm glutamine + 1 mm spermidine (lane 3). Control cells from rats treated with saline were dispersed with collagenase and incubated for 1 hour and a further 5 hours in glutamine only (lane 1). Five hundred nanograms of DNA from each sample was labeled with [α -³²P]-dCTP using klenow enzyme, separated on a 1.8 % agarose gel, and exposed to film for 24 hours. Results are shown in FIG. 18.

In yet another experiment, superovulated rats were injected subcutaneously with 1 mg/100 g body weight of spermidine, delivered in three equal doses of 0.333 mg/100 g body weight, 24, 12, and 2 hours prior to a subcutaneous injection with 500 μ g PGF-2 α . Control rats were divided into three sets: no injections, three injections of spermidine but

no PGF-2 α ; and three injections with an equivalent volume of saline prior to PGF-2 α treatment. Ovaries were removed from the rats either 1 hour and 35 minutes or 3 hours and 45 minutes after prostaglandin treatment and used for the isolation of DNA. Five hundred nanograms of DNA from each sample was labeled with [α -³²P]-dCTP using
5 Klenow enzyme, separated on a 1.8 % agarose gel, and exposed to film for 24 hours: lane 1, no injections (animals were sacrificed at the same time as for lanes 3-5); lane 2, three injections with spermidine (animals were sacrificed at the same time as for lanes 3-5); lane 3, three injections with saline followed by injection with PGF-2 α (animals were sacrificed 1 h and 35 min after treatment with PGF-2 α); lane 4, three injections with spermidine
10 followed by injection with PGF-2 α (animals were sacrificed 1 h and 35 min after treatment with PGF-2 α); lane 5, three injections with spermidine followed by injection with PGF-2 α (animals were sacrificed 1 h and 35 min after treatment with PGF-2 α); lane 6, three injections with spermidine followed by injection with PGF-2 α (animals were sacrificed 3 h and 45 min after treatment with PGF-2 α); lane 7, three injections with spermidine
15 followed by injection with PGF-2 α (animals were sacrificed 3 h and 45 min after treatment with PGF-2 α). Results are shown in FIG. 19.

RNA Isolation

Total RNA was isolated from corpus luteum tissue removed from rats at various
20 times after PGF-2 α induction of apoptosis. Briefly, the tissue (5 g) was ground in liquid nitrogen. The ground powder was mixed with 30 ml guanidinium buffer (4 M guanidinium isothiocyanate, 2.5 mM NaOAc pH 8.5, 0.8% β -mercaptoethanol). The mixture was filtered through four layers of Miracloth and centrifuged at 10,000g at 4° C for 30 minutes. The supernatant was then subjected to cesium chloride density gradient
25 centrifugation at 11,200g for 20 hours. The pelleted RNA was rinsed with 75% ethanol, resuspended in 600 ml DEPC-treated water and the RNA precipitated at -70° C with 1.5 ml 95% ethanol and 60 ml of 3M NaOAc.

Genomic DNA Isolation and Laddering

30 Genomic DNA was isolated from extracted corpus luteum tissue or dispersed corpus luteal cells using the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer's instructions. The DNA was end-labeled by incubating 500 ng of DNA with 0.2 μ Ci [α -³²P]dCTP, 1 mM Tris, 0.5 mM EDTA, 3 units of Klenow enzyme, and 0.2

pM each of dATP, dGTP, and dTTP, at room temperature for 30 minutes. Unincorporated nucleotides were removed by passing the sample through a 1-ml Sephadex G-50 column according to the method described by Maniatis et al. The samples were then resolved by Tris-acetate-EDTA (2 %) gel electrophoresis. The gel was dried for 30 minutes at room temperature under vacuum and exposed to x-ray film at - 80° C for 24 hours.

Plasmid DNA Isolation, DNA Sequencing

The alkaline lysis method described by Sambrook et al., *supra*, was used to isolate plasmid DNA. The full-length positive cDNA clone was sequenced using the dideoxy sequencing method. Sanger et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467. The open reading frame was compiled and analyzed using BLAST search (GenBank, Bethesda, MD) and sequence alignment was achieved using a BCM Search Launcher: Multiple Sequence Alignments Pattern-Induced Multiple Alignment Method (see F. Corpet, Nuc. Acids Res., 16:10881-10890, (1987). Sequences and sequence alignments are shown in FIGS. 5-11.

Northern Blot Hybridization of Rat Corpus Luteum RNA

Twenty milligrams of total RNA isolated from rat corpus luteum at various stages of apoptosis were separated on 1% denatured formaldehyde agarose gels and immobilized on nylon membranes. The full-length rat apoptosis-specific eIF-5A cDNA (SEQ ID NO:1) labeled with ³²P-dCTP using a random primer kit (Boehringer) was used to probe the membranes 7 x 10⁷. Alternatively, full length rat apoptosis-specific DHS cDNA (SEQ ID NO:6) labeled with ³²P-dCTP using a random primer kit (Boehringer) was used to probe the membranes (7 x 10⁷ cpm). The membranes were washed once with 1x SSC, 0.1% SDS at room temperature and three times with 0.2x SSC, 0.1% SDS at 65° C. The membranes were dried and exposed to X-ray film overnight at -70° C.

As can be seen, eIF-5A and DHS are both upregulated in apoptosing corpus luteum tissue. Expression of apoptosis-specific eIF-5A is significantly enhanced after induction of apoptosis by treatment with PGF-2α – low at time zero, increased substantially within 1 hour of treatment, increased still more within 8 hours of treatment and increased slightly within 24 hours of treatment (FIG. 14). Expression of DHS was low at time zero, increased substantially within 1 hour of treatment, increased still more within 8 hours of treatment and increased again slightly within 24 hours of treatment (FIG. 15).

Generation of an Apoptosing Rat Corpus Luteum RT-PCR Product Using Primers Based on Yeast, Fungal and Human eIF-5A Sequences

A partial-length apoptosis-specific eIF-5A sequence (SEQ ID NO:11) corresponding to the 3' end of the gene was generated from apoptosing rat corpus luteum RNA template by RT-PCR using a pair of oligonucleotide primers designed from yeast, fungal and human eIF-5A sequences. The upstream primer used to isolate the 3' end of the rat eIF-5A gene is a 20 nucleotide degenerate primer: 5'

TCSAARACHGGNAAGCAYGG 3' (SEQ ID NO:9), wherein S is selected from C and G; R is selected from A and G; H is selected from A, T, and C; Y is selected from C and T; and N is any nucleic acid. The downstream primer used to isolate the 3' end of the rat eIF-5A gene contains 42 nucleotides: 5' GCGAAGCTTCCATGG

CTCGAGTTTTTTTTTTTTTTTTTTTTTTT 3' (SEQ ID NO:10). A reverse transcriptase polymerase chain reaction (RT-PCR) was carried out. Briefly, using 5 mg of the downstream primer, a first strand of cDNA was synthesized. The first strand was then used as a template in a RT-PCR using both the upstream and downstream primers.

Separation of the RT-PCR products on an agarose gel revealed the presence a 900 bp fragment, which was subcloned into pBluescriptTM (Stratagene Cloning Systems, LaJolla, CA) using blunt end ligation and sequenced (SEQ ID NO:11). The cDNA sequence of the 3' end is SEQ ID NO:11 and the amino acid sequence of the 3' end is SEQ ID NO:12. See FIGS. 1-2.

A partial-length apoptosis-specific eIF-5A sequence (SEQ ID NO:15) corresponding to the 5' end of the gene and overlapping with the 3' end was generated from apoptosing rat corpus luteum RNA template by RT-PCR. The 5' primer is a 24-mer having the sequence, 5' CAGGTCTAGAGTTGGAATCGAAGC 3' (SEQ ID NO:13), that was designed from human eIF-5A sequences. The 3' primer is a 30-mer having the sequence, 5' ATATCTCGAGCCTT GATTGCAACAGCTGCC 3' (SEQ ID NO:14) that was designed according to the 3' end RT-PCR fragment. A reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out. Briefly, using 5 mg of the downstream primer, a first strand of cDNA was synthesized. The first strand was then used as a template in a RT-PCR using both the upstream and downstream primers.

Separation of the RT-PCR products on an agarose gel revealed the presence a 500 bp fragment, which was subcloned into pBluescriptTM (Stratagene Cloning Systems,

LaJolla, CA) using XbaI and XhoI cloning sites present in the upstream and downstream primers, respectively, and sequenced (SEQ ID NO:15). The cDNA sequence of the 5' end is SEQ ID NO:15, and the amino acid sequence of the 5' end is SEQ ID NO:16. *See* FIG. 2.

5 The sequences of the 3' and 5' ends of the rat apoptosis-specific eIF-5A (SEQ ID NO:11 and SEQ ID NO:15, respectively) overlapped and gave rise to the full-length cDNA sequence (SEQ ID NO:1). This full-length sequence was aligned and compared with sequences in the GeneBank data base. *See* FIGS. 1-2. The cDNA clone encodes a 154 amino acid polypeptide (SEQ ID NO:2) having a calculated molecular mass of 16.8 KDa. The nucleotide sequence, SEQ ID NO:1, for the full length cDNA of the rat apoptosis-specific corpus luteum eIF-5A gene obtained by RT-PCR is depicted in FIG. 3 and the corresponding derived amino acid sequence is SEQ ID NO:9. The derived full-length amino acid sequence of eIF-5A was aligned with human and mouse eIF-5a sequences. *See* FIG. 7-9.

15 *Generation of an Apoptosing Rat Corpus Luteum RT-PCR Product Using Primers Based on a Human DHS Sequence*

A partial-length apoptosis-specific DHS sequence (SEQ ID NO:6) corresponding to the 3' end of the gene was generated from apoptosing rat corpus luteum RNA template by RT-PCR using a pair of oligonucleotide primers designed from a human DHS sequence. The 5' primer is a 20-mer having the sequence, 5' GTCTGTGTATTATTGGGCCC 3' (SEQ ID NO. 17); the 3' primer is a 42-mer having the sequence, 5' GCGAAGCTTCCATGGC TCGAGTTTTTTTTTTTTTTTTTTTTTTT 3' (SEQ ID NO:18). A reverse transcriptase polymerase chain reaction (RT-PCR) was carried out. Briefly, using 5 mg of the downstream primer, a first strand of cDNA was synthesized. The first strand was then used as a template in a RT-PCR using both the upstream and downstream primers.

Separation of the RT-PCR products on an agarose gel revealed the presence a 606 bp fragment, which was subcloned into pBluescriptTM (Stratagene Cloning Systems, LaJolla, CA) using blunt end ligation and sequenced (SEQ ID NO:6). The nucleotide sequence (SEQ ID NO:6) for the partial length cDNA of the rat apoptosis-specific corpus luteum DHS gene obtained by RT-PCR is depicted in FIG. 4 and the corresponding derived amino acid sequence is SEQ ID NO.7.

Isolation of Genomic DNA and Southern Analysis

Genomic DNA for southern blotting was isolated from excised rat ovaries. Approximately 100 mg of ovary tissue was divided into small pieces and placed into a 15 ml tube. The tissue was washed twice with 1 ml of PBS by gently shaking the tissue suspension and then removing the PBS using a pipette. The tissue was resuspended in 2.06 ml of DNA-buffer (0.2 M Tris-HCl pH 8.0 and 0.1 mM EDTA) and 240 μ l of 10 % SDS and 100 μ l of proteinase K (Boehringer Mannheim; 10 mg/ml) was added. The tissue was placed in a shaking water bath at 45° C overnight. The following day another 100 μ l of proteinase K (10 mg/ml) was added and the tissue suspension was incubated in a water-bath at 45 °C for an additional 4 hours. After the incubation the tissue suspension was extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform:iso-amyl alcohol (24:1). Following the extractions 1/10th volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol were added. A glass pipette sealed and formed into a hook using a Bunsen burner was used to pull the DNA threads out of solution and to transfer the DNA into a clean microcentrifuge tube. The DNA was washed once in 70 % ethanol and air-dried for 10 minutes. The DNA pellet was dissolved in 500 μ l of 10 mM Tris-HCl (pH 8.0), 10 μ l of RNase A (10 mg/ml) was added, and the DNA was incubated for 1 hour at 37 °C. The DNA was extracted once with phenol:chloroform:iso-amyl alcohol (25:24:1) and the DNA was precipitated by adding 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The DNA was pelleted by centrifugation for 10 minutes at 13,000 x g at 4° C. The DNA pellet was washed once in 70 % ethanol and dissolved in 200 μ l 10 mM Tris-HCl (pH 8.0) by rotating the DNA at 4 °C overnight.

For Southern blot analysis, genomic DNA isolated from rat ovaries was digested with various restriction enzymes that either do not cut in the endogenous gene or cut only once. To achieve this, 10 μ g genomic DNA, 20 μ l 10X reaction buffer and 100 U restriction enzyme were reacted for five to six hours in a total reaction volume of 200 μ l. Digested DNA was loaded onto a 0.7 % agarose gel and subjected to electrophoresis for 6 hours at 40 volts or overnight at 15 volts. After electrophoresis, the gel was depurinated for 10 minutes in 0.2 N HCl followed by two 15-minute washes in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and two 15 minute washes in neutralizing buffer (1.5 M NaCl, 0.5

M Tris-HCl pH 7.4). The DNA was transferred to a nylon membrane, and the membrane was prehybridized in hybridization solution (40 % formamide, 6 X SSC, 5 X Denhart's, solution (1 X Denhart's solution is 0.02 % Ficoll, 0.02 % PVP, and 0.02 % BSA), 0.5 % SDS, and 1.5 mg of denatured salmon sperm DNA). A 700 bp PCR fragment of the 3' UTR of rat eIF-5A cDNA (650 bp of 3' UTR and 50 bp of coding) was labeled with [α - ^{32} P]-dCTP by random priming and added to the membrane at 1×10^6 cpm/ml.

Similarly, a 606 bp PCR fragment of the rat DHS cDNA (450 bp coding and 156 bp 3' UTR) was random prime labeled with [α - ^{32} P]-dCTP and added at 1×10^6 cpm/ml to a second identical membrane. The blots were hybridized overnight at 42° C and then washed twice with 2 X SSC and 0.1 % SDS at 42° C and twice with 1 X SSC and 0.1 % SDS at 42° C. The blots were then exposed to film for 3-10 days.

Rat corpus genomic DNA was cut with restriction enzymes as indicated on FIG. 20 and probed with ^{32} P-dCTP-labeled full-length eIF-5A cDNA. Hybridization under high stringency conditions revealed hybridization of the full-length cDNA probe to several restriction fragments for each restriction enzyme digested DNA sample, indicating the presence of several isoforms of eIF-5A. Of particular note, when rat genomic DNA was digested with EcoRV, which has a restriction site within the open reading frame of apoptosis-specific eIF-5A, two restriction fragments of the apoptosis-specific isoform of eIF-5A were detectable in the Southern blot. The two fragments are indicated with double arrows in FIG. 20. The restriction fragment corresponding to the apoptosis-specific isoform of eIF-5A is indicated by a single arrow in the lanes labeled EcoR1 and BamH1, restriction enzymes for which there are no cut sites within the open reading frame. These results suggest that the apoptosis-specific eIF-5A is a single copy gene in rat. As shown in FIGS. 5 through 13, the eIF-5A gene is highly conserved across species, and so it would be expected that there is a significant amount of conservation between isoforms within any species.

Figure 21 shows a Southern blot of rat genomic DNA probed with ^{32} P-dCTP-labeled partial-length rat corpus luteum apoptosis-specific DHS cDNA. The genomic DNA was cut with EcoRV, a restriction enzyme that does not cut the partial-length cDNA used as a probe. Two restriction fragments are evident indicating that there are two copies of the gene or that the gene contains an intron with an EcoRV site.

EXAMPLE 2

The present example demonstrates modulation of apoptosis with apoptosis factor 5A and DHS.

Culturing of COS-7 Cells and Isolation of RNA

5 COS-7, an African green monkey kidney fibroblast-like cell line transformed with a mutant of SV40 that codes for wild-type T antigen, was used for all transfection-based experiments. COS-7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 0.584 grams per liter of L-glutamine, 4.5 g of glucose per liter, and 0.37 % sodium bicarbonate. The culture media was supplemented with 10 % fetal bovine serum
10 (FBS) and 100 units of penicillin/streptomycin. The cells were grown at 37° C in a humidified environment of 5 % CO₂ and 95 % air. The cells were subcultured every 3 to 4 days by detaching the adherent cells with a solution of 0.25 % trypsin and 1 mM EDTA. The detached cells were dispensed at a split ratio of 1:10 in a new culture dish with fresh media.

15 COS-7 cells to be used for isolation of RNA were grown in 150-mm tissue culture treated dishes (Corning). The cells were harvested by detaching them with a solution of trypsin-EDTA. The detached cells were collected in a centrifuge tube, and the cells were pelleted by centrifugation at 3000 rpm for 5 minutes. The supernatant was removed, and the cell pellet was flash-frozen in liquid nitrogen. RNA was isolated from the frozen cells
20 using the GenElute Mammalian Total RNA Miniprep kit (Sigma) according to the manufacturer's instructions.

Construction of Recombinant Plasmids and Transfection of COS-7 Cells

Recombinant plasmids carrying the full-length coding sequence of rat apoptosis
25 eIF-5A in the sense orientation and the 3' untranslated region (UTR) of rat apoptosis eIF-5A in the antisense orientation were constructed using the mammalian epitope tag expression vector, pHM6 (Roche Molecular Biochemicals), which is illustrated in FIG. 21. The vector contains the following: CMV promoter - human cytomegalovirus immediate-early promoter/enhancer; HA - nonapeptide epitope tag from influenza hemagglutinin;
30 BGH pA - Bovine growth hormone polyadenylation signal; f1 ori - f1 origin; SV40 ori - SV40 early promoter and origin; Neomycin - Neomycin resistance (G418) gene; SV40 pA - SV40 polyadenylation signal; Col E1 - ColE1 origin; Ampicillin- Ampicillin resistance gene. The full-length coding sequence of rat apoptosis eIF-5A and the 3' UTR of rat

apoptosis eIF-5A were amplified by PCR from the original rat eIF-5A RT-PCR fragment in pBluescript (SEQ ID NO:1). To amplify the full-length eIF-5A the primers used were as follows: Forward 5' GCCAAGCTTAATGGCAGATGATTT GG 3' (Hind3) and Reverse 5' CTGAATTCCAGT TATTTTGCCATGG 3' (EcoR1). To amplify the 3' UTR rat eIF-5A the primers used were as follows: forward 5' AATGAATTCCGCCATGACAGAGGAGGC 3' (EcoR1) and reverse 5' GCGAAGCTTCCATGGCTCGAGTTTTTTTTTTTTTTTTTTTTTTT 3' (Hind3).

The full-length rat eIF-5A PCR product isolated after agarose gel electrophoresis was 430 bp in length while the 3' UTR rat eIF-5A PCR product was 697 bp in length.

Both PCR products were subcloned into the Hind 3 and EcoR1 sites of pHM6 to create pHM6-full-length eIF-5A and pHM6-antisense 3'UTReIF-5A. The full-length rat eIF-5A PCR product was subcloned in frame with the nonapeptide epitope tag from influenza hemagglutinin (HA) present upstream of the multiple cloning site to allow for detection of the recombinant protein using an anti-[HA]-peroxidase antibody. Expression is driven by the human cytomegalovirus immediate-early promoter/enhancer to ensure high level expression in mammalian cell lines. The plasmid also features a neomycin-resistance (G418) gene, which allows for selection of stable transfectants, and a SV40 early promoter and origin, which allows episomal replication in cells expressing SV40 large T antigen, such as COS-7.

COS-7 cells to be used in transfection experiments were cultured in either 24 well cell culture plates (Corning) for cells to be used for protein extraction, or 4 chamber culture slides (Falcon) for cells to be used for staining. The cells were grown in DMEM media supplemented with 10 % FBS, but lacking penicillin/streptomycin, to 50 to 70 % confluency. Transfection medium sufficient for one well of a 24-well plate or culture slide was prepared by diluting 0.32 µg of plasmid DNA in 42.5 µl of serum-free DMEM and incubating the mixture at room temperature for 15 minutes. 1.6 µl of the transfection reagent, LipofectAMINE (Gibco, BRL), was diluted in 42.5 µl of serum-free DMEM and incubated for 5 minutes at room temperature. After 5 minutes the LipofectAMINE mixture was added to the DNA mixture and incubated together at room temperature for 30 to 60 minutes. The cells to be transfected were washed once with serum-free DMEM before overlaying the transfection medium and the cells were placed back in the growth chamber for 4 hours.

After the incubation, 0.17 ml of DMEM + 20 % FBS was added to the cells. The cells were the cultured for a further 40 hours before either being induced to undergo apoptosis prior to staining or harvested for Western blot analysis. As a control, mock transfections were also performed in which the plasmid DNA was omitted from the transfection medium.

Protein Extraction and Western Blotting

Protein was isolated for Western blotting from transfected cells by washing the cells twice in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, and 0.24 g/L KH₂PO₄) and then adding 150 µl of hot SDS gel-loading buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2 % SDS, 0.1 % bromophenol blue, and 10 % glycerol). The cell lysate was collected in a microcentrifuge tube, heated at 95° C for 10 minutes, and then centrifuged at 13,000 x g for 10 minutes. The supernatant was transferred to a fresh microcentrifuge tube and stored at -20° C until ready for use.

For Western blotting, 2.5 or 5 µg of total protein was separated on a 12 % SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was then incubated for one hour in blocking solution (5 % skim milk powder, 0.02 % sodium azide in PBS) and washed three times for 15 minutes in PBS-T (PBS + 0.05 % Tween-20). The membrane was stored overnight in PBS-T at 4 °C. After being warmed to room temperature the next day, the membrane was blocked for 30 seconds in 1 µg/ml polyvinyl alcohol. The membrane was rinsed 5 times in deionized water and then blocked for 30 minutes in a solution of 5 % milk in PBS. The primary antibody was preincubated for 30 minutes in a solution of 5 % milk in PBS prior to incubation with the membrane.

Several primary antibodies were used. An anti-[HA]-peroxidase antibody (Roche Molecular Biochemicals) was used at a dilution of 1:5000 to detect expression of the recombinant proteins. Since this antibody is conjugated to peroxidase, no secondary antibody was necessary, and the blot was washed and developed by chemiluminescence. The other primary antibodies that were used are monoclonal antibodies from Oncogene that recognize p53 (Ab-6), Bcl-2 (Ab-1), and c-Myc (Ab-2). The monoclonal antibody to p53 was used at a dilution of 0.1 µg/ml, and the monoclonal antibodies to Bcl-2 and c-Myc were both used at a dilution of 0.83 µg/ml. After incubation with primary antibody for 60 to 90 minutes, the membrane was washed 3 times for 15 minutes in PBS-T.

Secondary antibody was then diluted in 1 % milk in PBS and incubated with the membrane for 60 to 90 minutes. When p53 (Ab-6) was used as the primary antibody, the secondary antibody used was a goat anti-mouse IgG conjugated to alkaline phosphatase (Rockland) at a dilution of 1:1000. When Bcl-2 (Ab-1) and c-Myc (Ab-2) were used as the primary antibody, a rabbit anti-mouse IgG conjugated to peroxidase (Sigma) was used at a dilution of 1:5000. After incubation with the secondary antibody, the membrane was washed 3 times in PBS-T.

Two detection methods were used to develop the blots, a colorimetric method and a chemiluminescent method. The colorimetric method was used only when p53 (Ab-6) was used as the primary antibody in conjunction with the alkaline phosphatase-conjugated secondary antibody. Bound antibody was visualized by incubating the blot in the dark in a solution of 0.33 mg/mL nitro blue tetrazolium, 0.165 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, 100 mM NaCl, 5 mM MgCl₂, and 100 mM Tris-HCl (pH 9.5). The color reaction was stopped by incubating the blot in 2 mM EDTA in PBS. A chemiluminescent detection method was used for all other primary antibodies, including anti-[HA]-peroxidase, Bcl-2 (Ab-1), and c-Myc (Ab-2). The ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech) was used to detect peroxidase-conjugated bound antibodies. In brief, the membrane was lightly blotted dry and then incubated in the dark with a 40:1 mix of reagent A and reagent B for 5 minutes. The membrane was blotted dry, placed between sheets of acetate, and exposed to X-ray film for time periods varying from 10 seconds to 10 minutes.

Induction of Apoptosis in COS 7 Cells

Two methods were used to induce apoptosis in transfected COS-7 cells, serum deprivation and treatment with Actinomycin D, *streptomyces* sp (Calbiochem). For both treatments, the medium was removed 40 hours post-transfection. For serum starvation experiments, the media was replaced with serum- and antibiotic-free DMEM. Cells grown in antibiotic-free DMEM supplemented with 10 % FBS were used as a control. For Actinomycin D induction of apoptosis, the media was replaced with antibiotic-free DMEM supplemented with 10 % FBS and 1 µg/ml Actinomycin D dissolved in methanol. Control cells were grown in antibiotic-free DMEM supplemented with 10 % FBS and an equivalent volume of methanol. For both methods, the percentage of apoptotic cells was

determined 48 hours later by staining with either Hoescht or Annexin V-Cy3. Induction of apoptosis was also confirmed by Northern blot analyses, as shown in FIG. 22.

Hoescht Staining

5 The nuclear stain, Hoescht, was used to label the nuclei of transfected COS-7 cells in order to identify apoptotic cells based on morphological features such as nuclear fragmentation and condensation. A fixative, consisting of a 3:1 mixture of absolute methanol and glacial acetic acid, was prepared immediately before use. An equal volume of fixative was added to the media of COS-7 cells growing on a culture slide and
10 incubated for 2 minutes. The media/fixative mixture was removed from the cells and discarded, and 1 ml of fixative was added to the cells. After 5 minutes the fixative was discarded, and 1 ml of fresh fixative was added to the cells and incubated for 5 minutes. The fixative was discarded, and the cells were air-dried for 4 minutes before adding 1 ml of Hoescht stain (0.5 µg/ml Hoescht 33258 in PBS). After a 10-minute incubation in the
15 dark, the staining solution was discarded and the slide was washed 3 times for 1 minute with deionized water. After washing, 1 ml of McIlvaine's buffer (0.021 M citric acid, 0.058 M Na₂HPO₄·7H₂O; pH 5.6) was added to the cells, and they were incubated in the dark for 20 minutes. The buffer was discarded, the cells were air-dried for 5 minutes in the dark and the chambers separating the wells of the culture slide were removed. A few
20 drops of Vectashield mounting media for fluorescence (Vector Laboratories) was added to the slide and overlaid with a coverslip. The stained cells were viewed under a fluorescence microscope using a UV filter. Cells with brightly stained or fragmented nuclei were scored as apoptotic.

25 *Annexin V-Cy3 Staining*

 An Annexin V-Cy3 apoptosis detection kit (Sigma) was used to fluorescently label externalized phosphatidylserine on apoptotic cells. The kit was used according to the manufacturer's protocol with the following modifications. In brief, transfected COS-7 cells growing on four chamber culture slides were washed twice with PBS and three times
30 with 1 X Binding Buffer. 150 µl of staining solution (1 µg/ml AnnCy3 in 1 X Binding Buffer) was added, and the cells were incubated in the dark for 10 minutes. The staining solution was then removed, and the cells were washed 5 times with 1 X Binding Buffer. The chamber walls were removed from the culture slide, and several drops of 1 X Binding

Buffer were placed on the cells and overlaid with a coverslip. The stained cells were analyzed by fluorescence microscopy using a green filter to visualize the red fluorescence of positively stained (apoptotic) cells. The total cell population was determined by counting the cell number under visible light.

5

EXAMPLE 3

The present example demonstrates modulation of apoptosis with apoptosis factor 5A and DHS.

Using the general procedures and methods described in the previous examples, FIG. 23 is a flow chart illustrating the procedure for transient transfection of COS-7 cells, in which cells in serum-free medium were incubated in plasmid DNA in lipofectAMINE for 4 hours, serum was added, and the cells were incubated for a further 40 hours. The cells were then either incubated in regular medium containing serum for a further 48 hours before analysis (i.e. no further treatment), deprived of serum for 48 hours to induce apoptosis before analysis, or treated with actinomycin D for 48 hours to induce apoptosis before analysis.

FIG. 22 is a Western blot illustrating transient expression of foreign proteins in COS-7 cells following transfection with pHM6. Protein was isolated from COS-7 cells 48 hours after either mock transfection, or transfection with pHM6-LacZ, pHM6-Antisense 3' rF5A (pHM6-Antisense 3' UTR rat apoptosis eIF-5A), or pHM6-Sense rF5A (pHM6-Full length rat apoptosis eIF-5A). Five μ g of protein from each sample was fractionated by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with anti-[HA]-peroxidase. The bound antibody was detected by chemiluminescence and exposed to x-ray film for 30 seconds. Expression of LacZ (lane 2) and of sense rat apoptosis eIF-5A (lane 4) is clearly visible.

As described above, COS-7 cells were either mock transfected or transfected with pHM6-Sense rF5A (pHM6-Full length rat eIF-5A). Forty hours after transfection, the cells were induced to undergo apoptosis by withdrawal of serum for 48 hours. The caspase proteolytic activity in the transfected cell extract was measured using a fluorometric homogenous caspase assay kit (Roche Diagnostics). DNA fragmentation was also measured using the FragEL DNA Fragmentation Apoptosis Detection kit (Oncogene) which labels the exposed 3'-OH ends of DNA fragments with fluorescein-labeled deoxynucleotides.

Additional COS-7 cells were either mock transfected or transfected with pHM6-Sense rF5A (pHM6-Full length rat eIF-5A). Forty hours after transfection, the cells were either grown for an additional 48 hours in regular medium containing serum (no further treatment), induced to undergo apoptosis by withdrawal of serum for 48 hours or induced to undergo apoptosis by treatment with 0.5 µg/ml of Actinomycin D for 48 hours. The cells were either stained with Hoescht 33258, which depicts nuclear fragmentation accompanying apoptosis, or stained with Annexin V-Cy3, which depicts phosphatidylserine exposure accompanying apoptosis. Stained cells were also viewed by fluorescence microscopy using a green filter and counted to determine the percentage of cells undergoing apoptosis. The total cell population was counted under visible light.

FIG. 25 illustrates enhanced apoptosis as reflected by increased caspase activity when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. Expression of rat apoptosis-induced eIF-5A resulted in a 60% increase in caspase activity.

FIG. 26 illustrates enhanced apoptosis as reflected by increased DNA fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. Expression of rat apoptosis-induced eIF-5A resulted in a 273% increase in DNA fragmentation. FIG. 27 illustrates detection of apoptosis as reflected by increased nuclear fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. There is a greater incidence of fragmented nuclei in cells expressing rat apoptosis-induced eIF-5A. FIG. 28 illustrates enhanced apoptosis as reflected by increased nuclear fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. Expression of rat apoptosis-induced eIF-5A resulted in a 27 % and 63 % increase in nuclear fragmentation over control in non-serum starved and serum starved samples, respectively.

FIG. 29 illustrates detection of apoptosis as reflected by phosphatidylserine exposure when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. FIG. 30 illustrates enhanced apoptosis as reflected by increased phosphatidylserine exposure when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. Expression of rat apoptosis-induced eIF-5A resulted in a 140 % and

198 % increase in phosphatidylserine exposure over control, in non-serum starved and serum starved samples, respectively.

FIG. 31 illustrates enhanced apoptosis as reflected by increased nuclear fragmentation when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. Expression of rat apoptosis-induced eIF-5A resulted in a 115 % and 62 % increase in nuclear fragmentation over control in untreated and treated samples, respectively. FIG. 32 illustrates a comparison of enhanced apoptosis under conditions in which COS-7 cells transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation were either given no further treatment or treatment to induce apoptosis.

EXAMPLE 4

The present example demonstrates modulation of apoptotic activity following administration of apoptosis factor 5A and DHS.

COS-7 cells were either mock transfected, transfected with pHM6-LacZ or transfected with pHM6-Sense rF5A (pHM6-Full length rat eIF-5A) and incubated for 40 hours. Five μ g samples of protein extract from each sample were fractionated by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with a monoclonal antibody that recognizes Bcl-2. Rabbit anti-mouse IgG conjugated to peroxidase was used as a secondary antibody, and bound antibody was detected by chemiluminescence and exposure to x-ray film. Results are shown in FIG. 32. Less Bcl-2 is detectable in cells transfected with pHM6-Sense rF5A than in those transfected with pHM6-LacZ; therefore, Bcl-2 is down-regulated.

Additional COS-7 cells were either mock transfected, transfected with pHM6-antisense 3' rF5A (pHM6-antisense 3' UTR of rat apoptosis-specific eIF-5A) or transfected with pHM6-Sense rF5A (pHM6-Full length rat apoptosis-specific eIF-5A). Forty hours after transfection, the cells were induced to undergo apoptosis by withdrawal of serum for 48 hours. Five μ g samples of protein extract from each sample were fractionated by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with a monoclonal antibody that recognizes Bcl-2. Rabbit anti-mouse IgG conjugated to peroxidase was used as a secondary antibody, and bound antibody was detected by chemiluminescence and exposure to x-ray film.

Also additionally, COS-7 cells were either mock transfected, transfected with pHM6-LacZ or transfected with pHM6-Sense rF5A (pHM6-Full length rat eIF-5A) and incubated for 40 hours. Five μ g samples of protein extract from each sample were fractionated by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with a monoclonal antibody that recognizes p53. Goat anti-mouse IgG conjugated to alkaline phosphatase was used as a secondary antibody, and bound antibody was detected colorimetrically.

Finally, COS-7 cells were either mock transfected, transfected with pHM6-LacZ or transfected with pHM6-Sense rF5A (pHM6-Full length rat eIF-5A) and incubated for 40 hours. Five μ g samples of protein extract from each sample were fractionated by SDS-PAGE, transferred to a PVDF membrane, and probed with a monoclonal antibody that recognizes p53. Corresponding protein blots were probed with anti-[HA]-peroxidase to determine the level of rat apoptosis-specific eIF-5A expression. Goat anti-mouse IgG conjugated to alkaline phosphatase was used as a secondary antibody, and bound antibody was detected by chemiluminescence.

FIG. 33 illustrates downregulation of Bcl-2 when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. The upper panel illustrates the Coomassie-blue-stained protein blot; the lower panel illustrates the corresponding Western blot. Less Bcl-2 is detectable in cells transfected with pHM6-Sense rF5A than in those transfected with pHM6-LacZ.

FIG. 34 illustrates upregulation of Bcl-2 when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the antisense orientation. The upper panel illustrates the Coomassie-blue-stained protein blot; the lower panel illustrates the corresponding Western blot. More Bcl-2 is detectable in cells transfected with pHM6-antisense 3' rF5A than in those mock transfected or transfected with pHM6-Sense rF5A.

FIG. 35 illustrates upregulation of c-Myc when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. The upper panel illustrates the Coomassie-blue-stained protein blot; the lower panel illustrates the corresponding Western blot. More c-Myc is detectable in cells transfected with pHM6-Sense rF5A than in those transfected with pHM6-LacZ or the mock control.

FIG. 36 illustrates upregulation of p53 when COS-7 cells were transiently transfected with pHM6 containing full-length rat apoptosis-induced eIF-5A in the sense orientation. The upper panel illustrates the Coomassie-blue-stained protein blot; the lower panel illustrates the corresponding Western blot. More p53 is detectable in cells transfected with pHM6-Sense rF5A than in those transfected with pHM6-LacZ or the mock control.

FIG. 37 illustrates the dependence of p53 upregulation upon the expression of pHM6-full length rat apoptosis-induced eIF-5A in COS-7 cells. In the Western blot probed with anti-[HA]-peroxidase, the upper panel illustrates the Coomassie-blue-stained protein blot and the lower panel illustrates the corresponding Western blot. More rat apoptosis-induced eIF-5A is detectable in the first transfection than in the second transfection. In the Western blot probed with anti-p53, the upper panel in A illustrates a corresponding Coomassie-blue-stained protein blot and the lower panel illustrates the Western blot with p53. For the first transfection, more p53 is detectable in cells transfected with pHM6-Sense rF5A than in those transfected with pHM6-LacZ or the mock control. For the second transfection in which there was less expression of rat apoptosis-induced eIF-5A, there was no detectable difference in levels of p53 between cells transfected with pHM6-Sense rF5A, pHM6-LacZ or the mock control.

EXAMPLE 5

Figure 47 depicts an experiment run on heart tissue to mimic the beating of a human heart and the subsequent induced heart attack. Figure 49 shows the laboratory bench set up. A slice of human heart tissue removed during valve replacement surgery was hooked up to electrodes. A small weight was attached to the heart tissue to ease in measuring the strength of the heart beats. The electrodes provided an electrical stimulus to get the tissue to start beating. The levels of gene expression for both apoptosis-specific eIF-5A (eIF-5a) and proliferating eIF-5A (eIF5b) were measured in the heart tissue before ischemia was induced. See Figure 46. In the pre-ischemic heart tissue low levels of both eIF-5a and 5eIFb were produced and their levels were in relative balance. During this time, oxygen and carbon dioxide were delivered in a buffer to the heart at 92.5% and 7.5 %, respectively. The heart tissue was thus exposed to normal oxygen levels and the expression levels of apoptosis-specific eIF-5A (eIF5a) and proliferating eIF-5A (eIF5b) measured. Later, the oxygen levels was reduced and the nitrogen levels was increased, to

induce hypoxia and ischemia and finally a "heart attack." The heart tissue stopped beating. The oxygen levels were then returned to normal, the heart tissue was pulsed again with an electrical stimulus to start the heart beating again. After the "heart attack" the expression levels of apoptosis-specific eIF-5a and proliferating eIF-5A (eIF5b) were again measured. This time, there was a significant increase in the level of expression of the apoptosis-specific eIF-5A levels, whereas the increase in the level of expression of proliferating eIF-5A (eIF5b) was noticeably less. See Figure 46.

After the "heart attack" the heart did not beat as strong, as indicated by less compression/movement of the attached weight, thus indicating that the heart tissue cells were being killed rapidly due to the presence of apoptosis-specific eIF-5A.

The EKG results are depicted in Figure 48. On the left side of the panels a normal heart beat is shown (the pre-ischemic heart tissue). After the "heart attack" (straight line), and the re-initiation of the heart beat, the EKG shows decreased activity due to muscle cell death. The EKG shows relative loss in strength of heart beat.

EXAMPLE 6: Human cell line culture conditions

Human Lamina Cribrosa and Astrocyte Culture

Paired human eyes were obtained within 48 hours *post mortem* from the Eye Bank of Canada, Ontario Division. Optic nerve heads (with attached pole) were removed and placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic/antimycotic, glutamine, and 10% FBS for 3 hours. The optic nerve head (ONH) button was retrieved from each tissue sample and minced with fine dissecting scissors into four small pieces. Explants were cultured in 12.5 cm² plastic culture flasks in DMEM medium. Growth was observed within one month in viable explants. Once the cells reached 90% confluence, they were trypsinized and subjected to differential subculturing to produce lamina cribrosa (LC) and astrocyte cell populations. Specifically, LC cells were subcultured in 25 cm² flasks in DMEM supplemented with gentamycin, glutamine, and 10% FBS, whereas astrocytes were expanded in 25cm² flasks containing EBM complete medium (Clonetics) with no FBS. FBS was added to astrocyte cultures following 10 days of subculture. Cells were maintained and subcultured as per this protocol.

Cell populations obtained by differential subculturing were characterized for identity and population purity using differential fluorescent antibody staining on 8 well culture slides. Cells were fixed in 10 % formalin solution and washed three times with

Dulbecco's Phosphate Buffered Saline (DPBS). Following blocking with 2 % nonfat milk in DPBS, antibodies were diluted in 1% BSA in DPBS and applied to the cells in 6 of the wells. The remaining two wells were treated with only 1 % bovine serum albumin (BSA) solution and no primary antibody as controls. Cells were incubated with the primary
5 antibodies for one hour at room temperature and then washed three times with DPBS. Appropriate secondary antibodies were diluted in 1 % BSA in DPBS, added to each well and incubated for 1 hour. Following washing with DPBS, the chambers separating the wells of the culture slide were removed from the slide, and the slide was immersed in double distilled water and then allowed to air-dry. Fluoromount (Vector Laboratories)
10 was applied to each slide and overlayed by 22x60 mm coverglass slips.

Immunofluorescent staining was viewed under a fluorescent microscope with appropriate filters and compared to the control wells that were not treated with primary antibody. All primary antibodies were obtained from Sigma unless otherwise stated. All secondary antibodies were purchased from Molecular Probes. Primary antibodies used to
15 identify LC cells were: anti-collagen I, anti-collagen IV, anti-laminin, anti-cellular fibronectin. Primary antibodies used to identify astrocytes were: anti-galactocerebroside (Chemicon International), anti-A2B5 (Chemicon International), anti-NCAM, anti-human Von willebrand Factor. Additional antibodies used for both cell populations included anti-glial fibrillary (GFAP) and anti-alpha-smooth muscle actin. Cell populations were
20 determined to be comprised of LC cells if they stained positively for collagen I, collagen IV, laminin, cellular fibronectin, alpha smooth muscle actin and negatively for glial fibrillary (GFAP). Cell populations were determined to be comprised of astrocytes if they stained positively for NCAM, glial fibrillary (GFAP), and negatively for galactocerebroside, A2B5, human Von willebrand Factor, and alpha smooth muscle actin.

25 In this preliminary study, three sets of human eyes were used to initiate cultures. LC cell lines # 506, # 517, and # 524 were established from the optic nerve heads of and 83-year old male, a 17-year old male, and a 26-year old female, respectively. All LC cell lines have been fully characterized and found to contain greater than 90 % LC cells.

30 *RKO Cell Culture*

RKO (American Type Culture Collection CRL-2577), a human colon carcinoma cell line expressing wild-type p53, was used to test the antisense oligonucleotides for the ability to suppress eIF5A1 protein expression. RKO were cultured in Minimum Essential

Medium Eagle (MEM) with non-essential amino acids, Earle's salts, and L-glutamine. The culture media was supplemented with 10 % fetal bovine serum (FBS) and 100 units of penicillin/streptomycin. The cells were grown at 37 °C in a humidified environment of 5 % CO₂ and 95 % air. The cells were subcultured every 3 to 4 days by detaching the
5 adherent cells with a solution of 0.25 % trypsin and 1 mM EDTA. The detached cells were dispensed at a split ratio of 1:10 to 1:12 into a new culture dish with fresh media.

HepG2 Cell Culture

HepG2, a human hepatocellular carcinoma cell line, was used to test the ability of
10 an antisense oligo directed against human eIF5A1 to block production of TNF- α in response to treatment with IL-1 β . HepG2 cells were cultured in DMEM supplemented with gentamycin, glutamine, and 10% FBS and grown at 37 °C in a humidified environment of 5 % CO₂ and 95 % air.

15 EXAMPLE 7

Induction of Apoptosis

Apoptosis was induced in RKO and lamina cribrosa cells using Actinomycin D, an RNA polymerase inhibitor, and camptothecin, a topoisomerase inhibitor, respectively. Actinomycin D was used at a concentration of 0.25 μ g/ml and camptothecin was used at a
20 concentration of 20, 40, or 50 μ M. Apoptosis was also induced in lamina cribrosa cells using a combination of camptothecin (50 μ M) and TNF- α (10 ng/ml). The combination of camptothecin and TNF- α was found to be more effective at inducing apoptosis than either camptothecin or TNF- α alone.

25 *Antisense Oligonucleotides*

A set of three antisense oligonucleotides targeted against human eIF5A1 were designed by, and purchased from, Molecula Research Labs. The sequence of the first antisense oligonucleotide targeted against human eIF5A1 (#1) was 5' CCT GTC TCG AAG TCC AAG TC 3'. The sequence of the second antisense oligonucleotide targeted
30 against human eIF5A1 (#2) was 5' GGA CCT TGG CGT GGC CGT GC 3'. The sequence of the third antisense oligonucleotide targeted against human eIF5A1 (#3) was 5' CTC GTA CCT CCC CGC TCT CC 3'. The control oligonucleotide had the sequence 5' CGT

ACC GGT ACG GTT CCA GG 3'. A fluorescein isothiocyanate (FITC)-labeled antisense oligonucleotide (Molecular Research Labs) was used to monitor transfection efficiency and had the sequence 5' GGA CCT TGG CGT GGC CGT GCX 3', where X is the FITC label. All antisense oligonucleotides were fully phosphorothioated.

Transfection of Antisense Oligonucleotides

The ability of the eIF5A1 antisense oligonucleotides to block eIF5A1 protein expression was tested in RKO cells. RKO cells were transfected with antisense oligonucleotides using the transfection reagent, Oligofectamine (Invitrogen). Twenty four hours prior to transfection, the cells were split onto a 24 well plate at 157,000 per well in MEM media supplemented with 10 % FBS but lacking penicillin/streptomycin. Twenty four hours later the cells had generally reached a confluency of approximately 50%. RKO cells were either mock transfected, or transfected with 100 nM or 200 nM of antisense oligonucleotide. Transfection medium sufficient for one well of an 24 well plate was prepared by diluting 0, 1.25, or 2.5 μ l of a 20 μ M stock of antisense oligonucleotide with serum-free MEM to a final volume of 42.5 μ l and incubating the mixture at room temperature for 15 minutes. 1.5 μ l of Oligofectamine was diluted in 6 μ l of serum-free MEM and incubated for 7.5 minutes at room temperature. After 5 minutes the diluted Oligofectamine mixture was added to the DNA mixture and incubated together at room temperature for 20 minutes. The cells were washed once with serum-free MEM before adding 200 μ l of MEM to the cells and overlaying 50 μ l of transfection medium. The cells were placed back in the growth chamber for 4 hours. After the incubation, 125 μ l of MEM + 30 % FBS was added to the cells. The cells were then cultured for a further 48 hours, treated with 0.25 μ g/ml Actinomycin D for 24 hours, and then cell extract was harvested for Western blot analysis.

Transfection of lamina cribrosa cells was also tested using 100 and 200 nM antisense oligonucleotide and Oligofectamine using the same procedure described for RKO cells. However, effective transfection of lamina cribrosa cells was achieved by simply adding antisense oligonucleotide, diluted from 1 μ M to 10 μ M in serum-free media, to the cells for 24 hours and thereafter replacing the media with fresh antisense oligonucleotides diluted in serum-containing media every 24 hours for a total of two to five days.

The efficiency of antisense oligonucleotide transfection was optimized and monitored by performing transfections with an FITC-labeled antisense oligonucleotide having the same sequence as eIF5A1 antisense oligonucleotide # 2 but conjugated to FITC at the 3' end. RKO and lamina cribrosa cells were transfected with the FITC-labeled
5 antisense oligonucleotide on an 8-well culture slide. Forty-eight hours later the cells were washed with PBS and fixed for 10 minutes in 3.7 % formaldehyde in PBS. The wells were removed and mounting media (Vectashield) was added, followed by a coverslip. The cells were then visualized under UV light on a fluorescent microscope nucleus using a fluorescein filter (Green H546, filter set 48915) and cells fluorescing bright green were
10 determined to have taken up the oligonucleotide.

Detection of Apoptosis

Following transfection of lamina cribrosa cells with antisense oligonucleotides and induction of apoptosis with camptothecin, the percentage of cells undergoing apoptosis in
15 cells treated with either control antisense oligonucleotide or antisense oligonucleotide eIF5A1 SEQ ID NO:26 was determined. Two methods were used to detect apoptotic lamina cribrosa cells – Hoescht staining and DeadEnd™ Fluorometric TUNEL. The nuclear stain, Hoescht, was used to label the nuclei of lamina cribrosa cells in order to identify apoptotic cells based on morphological features such as nuclear fragmentation and
20 condensation. A fixative, consisting of a 3:1 mixture of absolute methanol and glacial acetic acid, was prepared immediately before use. An equal volume of fixative was added to the media of cells growing on a culture slide and incubated for 2 minutes. The media/fixative mixture was removed from the cells and discarded and 1 ml of fixative was added to the cells. After 5 minutes the fixative was discarded and 1 ml of fresh fixative
25 was added to the cells and incubated for 5 minutes. The fixative was discarded and the cells were air-dried for 4 minutes before adding 1 ml of Hoescht stain (0.5 µg/ml Hoescht 33258 in PBS). After a 10 minute incubation in the dark, the staining solution was discarded, the chambers separating the wells of the culture slide were removed, and the slide was washed 3 times for 1 minute with deionized water. After washing, a few drops
30 of McIlvaine's buffer (0.021 M citric acid, 0.058 M Na₂HPO₄·7H₂O; pH 5.6) was added to the cells and overlaid with a coverslip. The stained cells were viewed under a fluorescent microscope using a UV filter. Cells with brightly stained or fragmented nuclei were scored as apoptotic. A minimum of 200 cells were counted per well.

The DeadEnd™ Fluorometric TUNEL (Promega) was used to detect the DNA fragmentation that is a characteristic feature of apoptotic cells. Following Hoescht staining, the culture slide was washed briefly with distilled water, and further washed by immersing the slide twice for 5 minutes in PBS (137mM NaCl, 2.68mM KCl, 1.47mM KH₂PO₄, 8.1mM Na₂HPO₄), blotting the slide on paper towel between washes. The cells were permeabilized by immersing them in 0.2 % Triton X-100 in PBS for 5 minutes. The cells were then washed again by immersing the slide twice for 5 minutes in PBS and blotting the slide on paper towel between washes. 25 µl of equilibration buffer [200 mM potassium cacodylate (pH 6.6), 25 mM Tris-HCl (pH 6.6), 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride] was added per well and incubated for 5 to 10 minutes. During equilibration, 30 µl of reaction mixture was prepared for each well by mixing in a ratio of 45:5:1, respectively, equilibration buffer, nucleotide mix [50 µM fluorescein-12-dUTP, 100 µM dATP, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA], and terminal deoxynucleotidyl transferase enzyme (Tdt, 25 U/µl). After the incubation in equilibration buffer, 30 µl of reaction mixture was added per well and overlaid with a coverslip. The reaction was allowed to proceed in the dark at 37°C for 1 hour. The reaction was terminated by immersing the slide in 2 X SSC [0.3 M NaCl, and 30mM sodium citrate (pH 7.0)] and incubating for 15 minutes. The slide was then washed by immersion in PBS three times for 5 minutes. The PBS was removed by sponging around the wells with a Kim wipe, a drop of mounting media (Oncogene research project, JA1750-4ML) was added to each well, and the slide was overlaid with a coverslip. The cells were viewed under a fluorescent microscope using a UV filter (UV-G 365, filter set 487902) in order to count the Hoescht-stained nuclei. Any cells with brightly stained or fragmented nuclei were scored as apoptotic. Using the same field of view, the cells were then viewed using a fluorescein filter (Green H546, filter set 48915) and any nuclei fluorescing bright green were scored as apoptotic. The percentage of apoptotic cells in the field of view was calculated by dividing the number of bright green nuclei counted using the fluorescein filter by the total number of nuclei counted under the UV filter. A minimum of 200 cells were counted per well.

Figures 54-57 depict the results of these studies. The percentage of apoptotic cells in samples having been transfected with apoptosis-specific eIF5A1 is clearly much less than seen in cells having been transfected with the control oligonucleotide.

Protein Extraction and Western Blotting

Protein from transfected RKO cells was harvested for Western blot analysis by washing the cells with PBS, adding 40 µl of hot lysis buffer [0.5% SDS, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0)] per well. The cells were scraped and the
5 resulting extract was transferred to a microfuge tube, boiled for 5 minutes, and stored at -20°C. The protein was quantitated using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's instructions.

For Western blotting 5 µg of total protein was separated on a 12 % SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene
10 difluoride membrane. The membrane was then incubated for one hour in blocking solution (5 % skim milk powder in PBS) and washed three times for 15 minutes in 0.05 % Tween-20/PBS. The membrane was stored overnight in PBS-T at 4 °C. After being warmed to room temperature the next day, the membrane was blocked for 30 seconds in 1 µg/ml polyvinyl alcohol. The membrane was rinsed 5 times in deionized water and then
15 blocked for 30 minutes in a solution of 5 % milk in 0.025 % Tween-20/PBS. The primary antibody was preincubated for 30 minutes in a solution of 5 % milk in 0.025% Tween-20/PBS prior to incubation with the membrane.

Several primary antibodies were used. A monoclonal antibody from Oncogene which recognizes p53 (Ab-6) and a polyclonal antibody directed against a synthetic
20 peptide (amino-CRLPEGDLGKEIEQKYD-carboxy) (SEQ ID NO:33) homologous to the c-terminal end of human eIF5A1 that was raised in chickens (Gallus Immunotech). An anti-β-actin antibody (Oncogene) was also used to demonstrate equal loading of protein. The monoclonal antibody to p53 was used at a dilution of 0.05 µg/ml, the antibody against eIF5A1 was used at a dilution of 1:1000, and the antibody against actin was used at a
25 dilution of 1:20,000. After incubation with primary antibody for 60 to 90 minutes, the membrane was washed 3 times for 15 minutes in 0.05% Tween-20/PBS. Secondary antibody was then diluted in 1 % milk in 0.025 % Tween-20/PBS and incubated with the membrane for 60 to 90 minutes. When p53 (Ab-6) was used as the primary antibody, the secondary antibody used was a rabbit anti-mouse IgG conjugated to peroxidase (Sigma) at
30 a dilution of 1:5000. When anti-eIF5A1 was used as the primary antibody, a rabbit anti-chicken IgY conjugated to peroxidase (Gallus Immunotech) was used at a dilution of 1:5000. The secondary antibody used with actin was a goat anti-mouse IgM conjugated to

peroxidase (Calbiochem) used at a dilution of 1:5000. After incubation with the secondary antibody, the membrane was washed 3 times in PBS-T.

The ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech) was used to detect peroxidase-conjugated bound antibodies. In brief, the membrane was
 5 lightly blotted dry and then incubated in the dark with a 40:1 mix of reagent A and reagent B for 5 minutes. The membrane was blotted dry, placed between sheets of acetate, and exposed to X-ray film for time periods varying from 10 seconds to 30 minutes. The membrane was stripped by submerging the membrane in stripping buffer [100 mM 2-Mercaptoethanol, 2 % SDS, and 62.5 mM Tris-HCl (pH 6.7)], and incubating at 50°C for
 10 30 minutes. The membrane was then rinsed in deionized water and washed twice for 10 minutes in large volumes of 0.05 % Tween-20/PBS. Membranes were stripped and re-blotted up to three times.

EXAMPLE 8

15 Construction of siRNA

Small inhibitory RNAs (siRNAs) directed against human eIF5A1 were used to specifically suppress expression of eIF5A1 in RKO and lamina cribrosa cells. Six siRNAs were generated by *in vitro* transcription using the *Silencer*TM siRNA Construction Kit (Ambion Inc.). Four siRNAs were generated against human eIF5A1 (siRNAs # 1 to # 4).
 20 Two siRNAs were used as controls; an siRNA directed against GAPDH provided in the kit, and an siRNA (siRNA # 5) which had the reverse sequence of the eIF5A1-specific siRNA # 1 but does not itself target eIF5A1. The siRNAs were generated according to the manufacturer's protocol. In brief, DNA oligonucleotides encoding the desired siRNA strands were used as templates for T7 RNA polymerase to generate individual strands of
 25 the siRNA following annealing of a T7 promoter primer and a fill-in reaction with Klenow fragment. Following transcription reactions for both the sense and antisense strands, the reactions were combined and the two siRNA strands were annealed, treated with DNase and RNase, and then column purified. The sequence of the DNA oligonucleotides (T7 primer annealing site underlined) used to generate the siRNAs were: siRNA # 1 antisense
 30 5' AAAGGAATGACTTCCAGCTGACCTGTCTC 3' and siRNA # 1 sense 5' AATCAGCTGGAAGTCATTCCTCCTGTCTC 3'; siRNA # 2 antisense 5' AAGATCGTCGAGATGTCTACTCCTGTCTC 3' and siRNA # 2 sense 5' AAAGTAGACATCTCGACGATCCCTGTCTC 3'; siRNA # 3 antisense 5'

AAGGTCCATCTGGTTGGTATTCCTGTCTC 3' and siRNA # 3 sense 5'
 AAAATACCAACCAGATGGACCCCTGTCTC 3'; siRNA # 4 antisense 5'
 AAGCTGGACTCCTCCTACACACCTGTCTC 3' and siRNA # 4 sense 5'
 AATGTGTAGGAGGAGTCCAGCCCTGTCTC 3'; siRNA # 5 antisense 5'
 5 AAAGTCGACCTTCAGTAAGGACCTGTCTC 3' and siRNA # 5 sense 5'
 AATCCTTACTGAAGGTCGACTCCTGTCTC 3'.

The *Silencer*TM siRNA Labeling Kit – FAM (Ambion) was used to label GAPDH siRNA with FAM in order to monitor the uptake of siRNA into RKO and lamina cribrosa cells. After transfection on 8-well culture slides, cells were washed with PBS and fixed
 10 for 10 minutes in 3.7 % formaldehyde in PBS. The wells were removed and mounting media (Vectashield) was added, followed by a coverslip. Uptake of the FAM-labeled siRNA was visualized under a fluorescent microscope under UV light using a fluorescein filter. The GAPDH siRNA was labeled according to the manufacturer's protocol.

15 *Transfection of siRNA*

RKO cells and lamina cribrosa cells were transfected with siRNA using the same transfection protocol. RKO cells were seeded the day before transfection onto 8-well culture slides or 24-well plates at a density of 46,000 and 105,800 cells per well, respectively. Lamina cribrosa cells were transfected when cell confluence was at 40 to 70
 20 % and were generally seeded onto 8-well culture slides at 7500 to 10,000 cells per well three days prior to transfection. Transfection medium sufficient for one well of an 8-well culture slide was prepared by diluting 25.5 pmoles of siRNA stock to a final volume of 21.2 µl in Opti-Mem (Sigma). 0.425 µl of Lipofectamine 2000 was diluted to a final volume of 21.2 µl in Opti-Mem and incubated for 7 to 10 minutes at room temperature.
 25 The diluted Lipofectamine 2000 mixture was then added to the diluted siRNA mixture and incubated together at room temperature for 20 to 30 minutes. The cells were washed once with serum-free media before adding 135 µl of serum-free media to the cells and overlaying the 42.4 µl of transfection medium. The cells were placed back in the growth chamber for 4 hours. After the incubation, 65 µl of serum-free media + 30 % FBS was
 30 added to the cells. Transfection of siRNA into cells to be used for Western blot analysis were performed in 24-well plates using the same conditions as the transfections in 8-well slides except that the volumes were increased by 2.3 fold.

Following transfection, RKO and lamina cribrosa cells were incubated for 72 hours prior to collection of cellular extract for Western blot analysis. In order to determine the effectiveness of the siRNAs directed against eIF5A1 to block apoptosis, lamina cribrosa cells were treated with 50 μ M of camptothecin (Sigma) and 10 ng/ml of TNF- α (Leinco Technologies) to induce apoptosis either 48 or 72 hours after transfection. The cells were stained with Hoescht either 24 or 48 hours later in order to determine the percentage of cells undergoing apoptosis.

EXAMPLE 9

10 *Detection of Apoptosis*

Following transfection of lamina cribrosa cells with antisense oligonucleotides and induction of apoptosis with camptothecin, the percentage of cells undergoing apoptosis in cells treated with either control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 was determined. Two methods were used to detect apoptotic lamina cribrosa cells – Hoescht staining and DeadEnd™ Fluorometric TUNEL. The nuclear stain, Hoescht, was used to label the nuclei of lamina cribrosa cells in order to identify apoptotic cells based on morphological features such as nuclear fragmentation and condensation. A fixative, consisting of a 3:1 mixture of absolute methanol and glacial acetic acid, was prepared immediately before use. An equal volume of fixative was added to the media of cells growing on a culture slide and incubated for 2 minutes. The media/fixative mixture was removed from the cells and discarded and 1 ml of fixative was added to the cells. After 5 minutes the fixative was discarded and 1 ml of fresh fixative was added to the cells and incubated for 5 minutes. The fixative was discarded and the cells were air-dried for 4 minutes before adding 1 ml of Hoescht stain (0.5 μ g/ml Hoescht 33258 in PBS). After a 10 minute incubation in the dark, the staining solution was discarded, the chambers separating the wells of the culture slide were removed, and the slide was washed 3 times for 1 minute with deionized water. After washing, a few drops of McIlvaine's buffer (0.021 M citric acid, 0.058 M Na₂HPO₄·7H₂O; pH 5.6) was added to the cells and overlaid with a coverslip. The stained cells were viewed under a fluorescent microscope using a UV filter. Cells with brightly stained or fragmented nuclei were scored as apoptotic. A minimum of 200 cells were counted per well.

The DeadEnd™ Fluorometric TUNEL (Promega) was used to detect the DNA fragmentation that is a characteristic feature of apoptotic cells. Following Hoescht

staining, the culture slide was washed briefly with distilled water, and further washed by immersing the slide twice for 5 minutes in PBS (137mM NaCl, 2.68mM KCl, 1.47mM KH₂PO₄, 8.1mM Na₂HPO₄), blotting the slide on paper towel between washes. The cells were permeabilized by immersing them in 0.2 % Triton X-100 in PBS for 5 minutes. The cells were then washed again by immersing the slide twice for 5 minutes in PBS and blotting the slide on paper towel between washes. 25 µl of equilibration buffer [200 mM potassium cacodylate (pH 6.6), 25 mM Tris-HCl (pH 6.6), 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride] was added per well and incubated for 5 to 10 minutes. During equilibration, 30 µl of reaction mixture was prepared for each well by mixing in a ratio of 45:5:1, respectively, equilibration buffer, nucleotide mix [50 µM fluorescein-12-dUTP, 100 µM dATP, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA], and terminal deoxynucleotidyl transferase enzyme (Tdt, 25 U/µl). After the incubation in equilibration buffer, 30 µl of reaction mixture was added per well and overlayed with a coverslip. The reaction was allowed to proceed in the dark at 37°C for 1 hour. The reaction was terminated by immersing the slide in 2 X SSC [0.3 M NaCl, and 30mM sodium citrate (pH 7.0)] and incubating for 15 minutes. The slide was then washed by immersion in PBS three times for 5 minutes. The PBS was removed by sponging around the wells with a Kim wipe, a drop of mounting media (Oncogene research project, JA1750-4ML) was added to each well, and the slide was overlayed with a coverslip. The cells were viewed under a fluorescent microscope using a UV filter (UV-G 365, filter set 487902) in order to count the Hoescht-stained nuclei. Any cells with brightly stained or fragmented nuclei were scored as apoptotic. Using the same field of view, the cells were then viewed using a fluorescein filter (Green H546, filter set 48915) and any nuclei fluorescing bright green were scored as apoptotic. The percentage of apoptotic cells in the field of view was calculated by dividing the number of bright green nuclei counted using the fluorescein filter by the total number of nuclei counted under the UV filter. A minimum of 200 cells were counted per well.

Protein Extraction and Western Blotting

Protein from transfected RKO cells was harvested for Western blot analysis by washing the cells with PBS, adding 40 µl of hot lysis buffer [0.5% SDS, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0)] per well. The cells were scraped and the resulting extract was transferred to an eppendorf, boiled for 5 minutes, and stored at -

20°C. The protein was quantitated using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's instructions.

For Western blotting 5 µg of total protein was separated on a 12 % SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was then incubated for one hour in blocking solution (5 % skim milk powder in PBS) and washed three times for 15 minutes in 0.05 % Tween-20/PBS. The membrane was stored overnight in PBS-T at 4 °C. After being warmed to room temperature the next day, the membrane was blocked for 30 seconds in 1 µg/ml polyvinyl alcohol. The membrane was rinsed 5 times in deionized water and then blocked for 30 minutes in a solution of 5 % milk in 0.025 % Tween-20/PBS. The primary antibody was preincubated for 30 minutes in a solution of 5 % milk in 0.025% Tween-20/PBS prior to incubation with the membrane.

Several primary antibodies were used. A monoclonal antibody from Oncogene which recognizes p53 (Ab-6; Oncogene), a monoclonal which recognizes human bcl-2 (Oncogene), and a polyclonal antibody directed against a synthetic peptide (amino-CRLPEGDLGKEIEQKYD-carboxy) homologous to the c-terminal end of human eIF5A1 that was raised in chickens (Gallus Immunotech). An anti-β-actin antibody (Oncogene) was also used to demonstrate equal loading of protein. The monoclonal antibody to p53 was used at a dilution of 0.05 µg/ml, the antibody against bcl-2 was used at a dilution of 1:3500, the antibody against eIF5A1 was used at a dilution of 1:1000, and the antibody against actin was used at a dilution of 1:20,000. After incubation with primary antibody for 60 to 90 minutes, the membrane was washed 3 times for 15 minutes in 0.05% Tween-20/PBS. Secondary antibody was then diluted in 1 % milk in 0.025 % Tween-20/PBS and incubated with the membrane for 60 to 90 minutes. When p53 (Ab-6) was used as the primary antibody, the secondary antibody used was a rabbit anti-mouse IgG conjugated to peroxidase (Sigma) at a dilution of 1:5000. When anti-eIF5A1 was used as the primary antibody, a rabbit anti-chicken IgY conjugated to peroxidase (Gallus Immunotech) was used at a dilution of 1:5000. The secondary antibody used with actin was a goat anti-mouse IgM conjugated to peroxidase (Calbiochem) used at a dilution of 1:5000. After incubation with the secondary antibody, the membrane was washed 3 times in PBS-T.

The ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech) was used to detect peroxidase-conjugated bound antibodies. In brief, the membrane was lightly blotted dry and then incubated in the dark with a 40:1 mix of reagent A and reagent

B for 5 minutes. The membrane was blotted dry, placed between sheets of acetate, and exposed to X-ray film for time periods varying from 10 seconds to 30 minutes. The membrane was stripped by submerging the membrane in stripping buffer [100 mM 2-Mercaptoethanol, 2 % SDS, and 62.5 mM Tris-HCl (pH 6.7)], and incubating at 50°C for 5 30 minutes. The membrane was then rinsed in deionized water and washed twice for 10 minutes in large volumes of 0.05 % Tween-20/PBS. Membranes were stripped and re-probed up to three times.

EXAMPLE 10

10 *Quantification of HepG2 TNF- α Production*

HepG2 cells were plated at 20,000 cells per well onto 48-well plates. Seventy two hours later the media was removed and fresh media containing either 2.5 μ M control antisense oligonucleotide or 2.5 μ M antisense oligonucleotide eIF5A1 # 2 was added to the cells. Fresh media containing antisense oligonucleotides was added after twenty four 15 hours. After a total of 48 hours incubation with the oligonucleotides, the media was replaced with media containing interleukin 1 β (IL-1 β , 1000 pg/ml; Leinco Technologies) and incubated for 6 hours. The media was collected and frozen (-20°C) for TNF- α quantification. Additional parallel incubations with untreated cells (without antisense oligonucleotide and IL-1 β) and cells treated with only IL-1 β were used for controls. All 20 treatments were done in duplicate. TNF- α released into the media was measured by ELISA assays (Assay Designs Inc.) according to the manufacturer's protocol.

EXAMPLE 11

The following experiments show that antisense apoptosis factor 5A nucleotides 25 were able to inhibit expression of apoptosis factor 5A as well as p53.

RKO cells were either left untransfected, mock transfected, or transfected with 200 nM of antisense oligonucleotides eIF5A1 # 1, # 2, or # 3. RKO cells were also transfected with 100 nM of antisense oligonucleotide eIF5A1 # 2. Forty-eight hours after 30 transfection, the cells were treated with 0.25 μ g/ml Actinomycin D. Twenty-four hours later, the cell extract was harvested and 5 μ g of protein from each sample was separated on an SDS-PAGE gel, transferred to a PVDF membrane, and Western blotted with an

antibody against eIF5A1. After chemiluminescent detection, the membrane was stripped and reprobbed with an antibody against p53. After chemiluminescent detection, the membrane was stripped again and reprobbed with an antibody against actin. See figure 52 which shows the levels of protein produced by RKO cells after being treated with antisense oligo 1, 2 and 3 (to apoptosis factor 5A). The RKO cells produced less apoptosis factor 5A as well as less p53 after having been transfected with the antisense apoptosis factor 5A nucleotides.

EXAMPLE 12

The following experiments show that antisense apoptosis factor 5A nucleotides were able to reduce apoptois.

In one experiment, the lamina cribrosa cell line # 506 was either (A) transfected with 100 nM of FITC-labeled antisense oligonucleotide using Oligofectamine transfection reagent or (B) transfected with 10 μ M of naked FITC-labeled antisense oligonucleotide diluted directly in serum-free media. After 24 hours fresh media containing 10 % FBS and fresh antisense oligonucleotide diluted to 10 μ M was added to the cells. The cells, (A) and (B), were fixed after a total of 48 hours and visualized on a fluorescent microscope under UV light using a fluorescein filter. Figure 53 shows uptake of the flourescently labeled antisense oligonucleotide.

In another experiment, the lamina cribrosa cell line # 506 was transfected with 10 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of 4 days. Forty-eight hours after beginning antisense oligonucleotide treatment, the cells were treated with either 20 μ M or 40 μ M camptothecin for 48 hours. Antisense oligonucleotide and camptothecin-containing media was changed daily. The percentage of apoptotic cells was determined by labeling the cells with Hoescht and TUNEL. See figure 54.

In another experiment, the lamina cribrosa cell line # 506 was transfected with 10 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2. Twenty-four hours later the media was changed and fresh antisense oligonucleotides were added. Forty-eight hours after beginning antisense oligonucleotide treatment, the antisense-oligonucleotides were removed and the cells were treated with 20 μ M camptothecin for 3 days. The camptothecin-containing media was changed daily. The

percentage of apoptotic cells was determined by labeling the cells with Hoescht and TUNEL. See Figure 55.

In yet another experiment, the lamina cribrosa cell line # 517 was transfected with 1 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of five days. Forty-eight hours after beginning antisense oligonucleotide treatment, the cells were treated with 20 μ M camptothecin for either 3 or 4 days. Antisense oligonucleotide and camptothecin-containing media was changed daily. The percentage of apoptotic cells was determined by labeling the cells with Hoescht and TUNEL. See Figure 56.

In another experiment, the lamina cribrosa cell line # 517 was transfected with 2.5 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of five days. Forty-eight hours after beginning antisense oligonucleotide treatment, the cells were treated with 40 μ M camptothecin for 3 days. Antisense oligonucleotide and camptothecin-containing media was changed daily. The percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 57.

In another experiment, the lamina cribrosa cell line # 517 was transfected with either 1 μ M or 2.5 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of five days. Forty-eight hours after beginning antisense oligonucleotide treatment, the cells were treated with 40 μ M camptothecin for 3 days. Antisense oligonucleotide and camptothecin-containing media was changed daily. The percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 58.

In another experiment, the lamina cribrosa cell line # 517 was left either untreated, or was treated with 10 ng/ml TNF- α , 50 μ M camptothecin, or 10 ng/ml TNF- α and 50 μ M camptothecin. The percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 59.

In another experiment, the lamina cribrosa cell lines # 506 and # 517 were transfected with either 2.5 μ M or 5 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of two days. Fresh media containing antisense oligonucleotides was added after 24 hours. Forty-eight hours after beginning antisense oligonucleotide treatment, the cells were treated with 50 μ M camptothecin and 10 ng/ml TNF- α for 2 days. The percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 60.

In another experiment, the lamina cribrosa cell lines # 506, # 517, and # 524 were transfected with 2.5 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of two days. Fresh media containing antisense oligonucleotides was added after 24 hours. Forty-eight hours after beginning antisense oligonucleotide treatment, the cells were treated with 50 μ M camptothecin and 10 ng/ml TNF- α for 2 days. The percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 61.

EXAMPLE 13

The following experiments show that cells transfected with siRNAs targeted against apoptosis factor 5A expressed less apoptosis factor 5A. The experiments also show that siRNAs targeted against apoptosis factor 5A were able to reduce apoptosis.

In one experiment, the lamina cribrosa cell line # 517 was transfected with 100 nM of FAM-labeled siRNA using Lipofectamine 2000 transfection reagent either with serum (A) or without serum (B) during transfection. The cells, (A) and (B), were fixed after a total of 24 hours and visualized on a fluorescent microscope under UV light using a fluorescein filter. See figure 62.

In another experiment, RKO cells were transfected with 100 nM of siRNA either in the presence or absence of serum during the transfection. Six siRNAs were transfected, two control siRNAs (siRNA # 5 and one targeted against GAPDH) and four targeted against eIF5A1 (siRNA # 1 to # 4). Seventy-two hours after transfection, the cell extract was harvested and 5 μ g of protein from each sample was separated on an SDS-PAGE gel, transferred to a PVDF membrane, and Western blotted with an antibody against eIF5A1. After chemiluminescent detection, the membrane was stripped and re-probed with an antibody against bcl-2. After chemiluminescent detection, the membrane was stripped again and re-probed with an antibody against actin. See figure 63.

In another experiment, lamina Cribrosa cell lines # 506 and # 517 were transfected with 100 nM of siRNA. Six siRNAs were transfected, two control siRNAs (siRNA # 5 and one targeted against GAPDH) and four targeted against eIF5A1 (siRNA # 1 to # 4). Seventy-two hours after transfection, the cell extract was harvested and 5 μ g of protein from each sample was separated on an SDS-PAGE gel, transferred to a PVDF membrane, and Western blotted with an antibody against eIF5A1. After chemiluminescent detection, the membrane was stripped and re-probed with an antibody against actin. See figure 64.

In another experiment, the lamina cribrosa cell line # 506 was transfected with 100 nm of siRNA. Six siRNAs were transfected, two control siRNAs (siRNA # 5 and one targeted against GAPDH) and four targeted against eIF5A1 (siRNA # 1 to # 4). Forty-eight hours after transfection, the media was replaced with media containing 50 μ M camptothecin and 10 ng/ml TNF- α . Twenty-four hours later, the percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 65.

In another experiment, the lamina cribrosa cell line # 506 was transfected with 100 nm of siRNA. Six siRNAs were transfected, two control siRNAs (siRNA # 5 and one targeted against GAPDH) and four targeted against eIF5A1 (siRNA # 1 to # 4). Seventy-two hours after transfection, the media was replaced with media containing 50 μ M camptothecin and 10 ng/ml TNF- α . Twenty-four hours later, the percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 66.

In another experiment, the lamina cribrosa cell line # 506 was either left untransfected or was transfected with 100 nm of siRNA. Six siRNAs were transfected, two control siRNAs (siRNA # 5 and one targeted against GAPDH) and four targeted against eIF5A1 (siRNA # 1 to # 4). Seventy-two hours after transfection, the media was replaced with media containing 50 μ M camptothecin and 10 ng/ml TNF- α . Fresh media was also added to the untransfected, untreated control cells. Forty-eight hours later, the percentage of apoptotic cells was determined by labeling the cells with Hoescht. See figure 67.

Photographs of Hoescht-stained lamina cribrosa cell line # 506 transfected with siRNA and treated with camptothecin and TNF- α from the experiment described in figure 67 and example 13. See figure 68.

EXAMPLE 14

This example shows that treating a human cell line with antisense oligonucleotides directed against apoptosis factor 5A causes the cells to produce less TNF- α .

HepG2 cells were treated with 2.5 μ M of either the control antisense oligonucleotide or antisense oligonucleotide eIF5A1 # 2 for a total of two days. Fresh media containing antisense oligonucleotides was added after 24 hours. Additional cells were left untreated for two days. Forty-eight hours after the beginning of treatment, the cells were treated with IL-1 β (1000 pg/ml) in fresh media for 6 hours. At the end of the experiment, the media was collected and frozen (-20°C) for TNF- α quantification. TNF- α

released into the media was measured using ELISA assays purchased from Assay Designs Inc. See figure 69.

EXAMPLE 15

5 HT-29 cells (human colon adenocarcinoma) were transfected with either an siRNA against eIF5A1 or with a control siRNA with the reverse sequence. The siRNA used is as follows:

Position 690 (3'UTR) % G/C=48

5' AAGCUGGACUCCUCCUACACA 3'

10

The control siRNA used is as follows:

% G/C=39

5' AAACACAUCCUCCUCAGGUCG 3'

15 After 48 hours the cells were treated with interferon-gamma (IFN-gamma) for 16 hours. After 16 hours the cells were washed with fresh media and treated with lipopolysaccharide (LPS) for 8 or 24 hours. At each time point (8 or 24 hours) the cell culture media was removed from the cells, frozen, and the TNF-alpha present in the media was quantitated by ELISA. The cell lysate was also harvested, quantitated for protein, and used to adjust
20 the TNF-alpha values to pg/mg protein (to adjust for differences in cell number in different wells). The results of the Western blot and Elisa are provided in Figures 74 A and B. Figure 75 are the results of the same experiment except the cells were at a higher density.

25 EXAMPLE 16

Tissue culture conditions of U-937 cell line

U-937 is a human monocyte cell line that grows in suspension and will become adherent and differentiate into macrophages upon stimulation with PMA (ATCC Number CRL-1593.2)(cells not obtained directly from ATCC). Cells were maintained in RPMI
30 1640 media with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% fetal bovine serum in a 37°C CO₂ (5%) incubator. Cells were split into fresh media (1:4 or 1:5 split ratio) twice a week and the cell density was always kept between 10⁵ and 2 x 10⁶ cells/ml. Cells were cultured in

suspension in tissue culture-treated plastic T-25 flasks and experiments were conducted in 24-well plates.

Time course experiment

Two days before the start of an experiment, the cell density was adjusted to 3×10^5 cells/ml media. On the day of the experiment, the cells were harvested in log phase. The cell suspension was transferred to 15ml tubes and centrifuged at $400 \times g$ for 10 mins at room temperature. The supernatant was aspirated and the cell pellet was washed/resuspended with fresh media. The cells were again centrifuged at $400 \times g$ for 10 mins, the supernatant was aspirated, and the cell pellet was finally resuspended in fresh media. Equal volumes of cell suspension and trypan blue solution (0.4% trypan blue dye in PBS) were mixed and the live cells were counted using a haemocytometer and a microscope. The cells were diluted to 4×10^5 cells/ml.

A 24-well plate was prepared by adding either PMA or DMSO (vehicle control) to each well. 1ml of cell suspension was added to each well so that each well contained 400,000 cells, 0.1 % DMSO +/- 162 nM PMA. The cells were maintained in a 37°C CO_2 (5%) incubator. Separate wells of cells were harvested at times 0, 24, 48, 72, 96, 99 and 102 h. See Figure 76 for a summary of the experimental time points and additions.

The media was changed at 72 h. Since some cells were adherent and others were in suspension, care was taken to avoid disrupting the adherent cells. The media from each well was carefully transferred into corresponding microcentrifuge tubes and the tubes were centrifuged at $14,000 \times g$ for 3 min. The tubes were aspirated, the cell pellets were resuspended in fresh media (1 ml, (-) DMSO, (-) PMA), and returned to their original wells. The cells become quiescent in this fresh media without PMA. At 96 h, LPS (100 ng/ml) was added and cells were harvested at 3h (99h) and 6h (102h) later.

At the time points, the suspension cells and media were transferred from each well into microcentrifuge tubes. The cells were pelleted at $14,000 \times g$ for 3 min. The media (supernatant) was transferred to clean tubes and stored (-20°C) for ELISA/cytokine analysis. The cells remaining in the wells were washed with PBS (1ml, 37°C) and this PBS was also used to wash the cell pellets in the corresponding microcentrifuge tubes. The cells were pelleted again at $14,000 \times g$ for 3 min. The cells were lysed with boiling lysis buffer (50 mM Tris pH 7.4 and 2% SDS). The adherent cells and the suspension cells from each well were pooled. The samples were boiled and then stored at -20°C .

Western Blotting

The protein concentration in each cell sample was determined by the BCA (bicinchoninic acid) method using BSA (bovine serum albumin) as the standard protein. Protein samples (5 µg total protein) were separated by 12% SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked with polyvinyl alcohol (1 µg/ml, 30 sec) and with 5% skim milk in PBS-t (1 h). The membranes were probed with a mouse monoclonal antibody raised against human eIF-5A (BD Biosciences cat # 611976; 1:20,000 in 5% skim milk, 1 h). The membranes were washed 3x10 mins PBS-t. The secondary antibody was a horseradish peroxidase-conjugated antimouse antibody (Sigma, 1:5000 in 1% skim milk, 1 h). The membranes were washed 3x10 mins PBS-t. The protein bands were visualized by chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

To demonstrate that similar amounts of protein were loaded on each gel lane, the membranes were stripped and reprobed for actin. Membranes were stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7; 50°C for 30 mins), washed, and then blocked as above. The membranes were probed with actin primary antibody (actin monoclonal antibody made in mouse; Oncogene, Ab-1; 1:20,000 in 5% skim milk). The secondary antibody, washing, and detection were the same as above.

Figure 77 shows that eIF5A is upregulated during monocyte (U-397) differentiation and subsequent TNF-α secretion.

EXAMPLE 17: Suppression of Il-8 production in response to interferon gamma by eIF5A siRNA

HT-29 (human colon adenocarcinoma) cells were transfected with siRNA directed to apoptosis eIF5A. Approximately 48 hours after transfection the media was changed so that some of the test samples had media with interferon gamma and some of the samples had media without interferon gamma. 16 hours after interferon gamma addition, the cells were washed, and the media, with or without TNF-alpha, was placed on the cells. The media (used for ELISA detection of IL-8) and the cell lysate was harvested 8 or 24 hours later.

Figures 79 and 80 show that IL-8 is produced in response to TNF-alpha as well as in response to interferon. Priming the cells with interferon gamma prior to TNF treatment causes the cells to produce more IL-8 than either treatment alone. This may be due to the known upregulation of the TNF receptor 1 in response to interferon, so 'priming' the cells with interferon allows them to respond to TNF better since the cells have more receptors. siRNA against eIF5A had no effect on IL-8 production in response to TNF alone (previous experiment) however, the siRNA blocked almost all IL-8 produced in response to interferon as well as a significant amount of the IL-8 produced as a result of the combined treatment of interferon and TNF. These results show that by using siRNAs directed against apoptosis eIF-5A, the inventors have the interferon signalling pathway leading to IL-8, but not the TNF pathway. Figure 81 is a western showing upregulation (4 fold at 8 hours) of apoptosis eIF5A in response to interferon gamma in HT-29 cells.

EXAMPLE 18

Human Lamina Cribrosa Culture

Paired human eyes were obtained within 48 hours *post mortem* from the Eye Bank of Canada, Ontario Division. Optic nerve heads (with attached pole) were removed and placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic/antimycotic, glutamine, and 10% FBS for 3 hours. The optic nerve head (ONH) button was retrieved from each tissue sample and minced with fine dissecting scissors into four small pieces. Explants were cultured in 12.5 cm² plastic culture flasks in DMEM medium. Growth was observed within one month in viable explants. Once the cells reached 90% confluence, they were trypsinized and subjected to differential subculturing to produce lamina cribrosa (LC) and astrocyte cell populations. LC cells were enriched by subculture in 25 cm² flasks in DMEM supplemented with gentamycin, glutamine, and 10% FBS. Cells were maintained and subcultured as per this protocol.

The identity and population purity of cells populations obtained by differential subculturing was characterized using differential fluorescent antibody staining on 8 well culture slides. Cells were fixed in 10 % formalin solution and washed three times with Dulbecco's Phosphate Buffered Saline (DPBS). Following blocking with 2 % nonfat milk in DPBS, antibodies were diluted in 1% BSA in DPBS and applied to the cells in 6 of the

wells. The remaining two wells were treated with only 1 % bovine serum albumin (BSA) solution and only secondary antibody as controls. Cells were incubated with the primary antibodies for one hour at room temperature and then washed three times with DPBS.

Appropriate secondary antibodies were diluted in 1 % BSA in DPBS, added to each well and incubated for 1 hour. Following washing with DPBS, the slide was washed in water, air-dried, and overlayed with Fluoromount (Vector Laboratories). Immunofluorescent staining was viewed under a fluorescent microscope with appropriate filters and compared to the control wells that were not treated with primary antibody. All primary antibodies were obtained from Sigma unless otherwise stated. All secondary antibodies were purchased from Molecular Probes. Primary antibodies used to identify LC cells were: anti-collagen I, anti-collagen IV, anti-laminin, anti-cellular fibronectin, anti-glial fibrillary acidic protein (GFAP), and anti-alpha-smooth muscle actin. Cell populations were determined to be comprised of LC cells if they stained positively for collagen I, collagen IV, laminin, cellular fibronectin, alpha smooth muscle actin and negatively for glial fibrillary (GFAP). In this study, two sets of human eyes were used to initiate cultures. LC cell lines # 506 and # 517 were established from the optic nerve heads of an 83-year old male and a 17-year old male, respectively. All LC cell lines have been fully characterized and found to contain greater than 90 % LC cells.

Treatment of LC cells

Apoptosis was induced in lamina cribrosa cells using a combination of 50 μ M camptothecin (Sigma) and 10 ng/ml TNF- α (Leinco Technologies). The combination of camptothecin and TNF- α was found to be more effective at inducing apoptosis than either camptothecin or TNF- α alone.

Construction and transfection of siRNAs

Small inhibitory RNAs (siRNAs) directed against human eIF5A were used to specifically suppress expression of eIF5A in lamina cribrosa cells. Six siRNAs were generated by *in vitro* transcription using the *Silencer*TM siRNA Construction Kit (Ambion Inc.). Four siRNAs were generated against human eIF5A1 (siRNAs # 1 to # 4). Two siRNAs were used as controls; an siRNA directed against GAPDH provided in the kit, and an siRNA (siRNA # 5), which had the reverse sequence of the eIF5A-specific siRNA # 1, but does not itself target eIF5A. The siRNAs were generated according to the

manufacturer's protocol. The eIF5A and control siRNA targets had the following sequences: siRNA # 1 5' AAAGGAATGACTTCCAGCTGA 3'; siRNA # 2 5' AAGATCGTCGAGATGTCTACT 3'; siRNA # 3 5' AAGGTCCATCTGGTTGGTATT 3'; siRNA # 4 5' AAGCTGGACTCCTCCTACACA 3'; siRNA # 5'

5 AAAGTCGACCTTCAGTAAGGA 3'. Lamina cribrosa cells were transfected with siRNA using LipofectAMINE 2000. Lamina cribrosa cells were transfected when cell confluence was at 40 to 70 % and were generally seeded onto 8-well culture slides at 7500 cells per well three days prior to transfection. Transfection medium sufficient for one well of an 8-well culture slide was prepared by diluting 25.5 pmoles of siRNA to a final
10 volume of 21.2 μ l in Opti-Mem (Sigma). 0.425 μ l of Lipofectamine 2000 was diluted to a final volume of 21.2 μ l in Opti-Mem and incubated for 7 to 10 minutes at room temperature. The diluted Lipofectamine 2000 mixture was then added to the diluted siRNA mixture and incubated together at room temperature for 20 to 30 minutes. The cells were washed once with serum-free media before adding 135 μ l of serum-free media
15 to the cells and overlaying 42.4 μ l of transfection medium. The cells were placed back in the growth chamber for 4 hours. After the incubation, 65 μ l of serum-free media plus 30 % FBS was added to the cells. Transfection of siRNA into cells to be used for Western blot analysis were performed in 24-well plates using the same conditions as the transfections in 8-well slides except that the volumes were increased by 2.3 fold.
20 Following transfection, lamina cribrosa cells were incubated for 72 hours prior to treatment with 50 μ M of camptothecin (Sigma) and 10 ng/ml of TNF- α (Leinco Technologies) to induce apoptosis. Cell lysates were then harvested for Western blotting or the cells were examined for apoptosis

25 *Detection of apoptotic cells*

Transfected cells that had been treated with TNF- α and camptothecin for 24 hours were stained with Hoescht 33258 in order to determine the percentage of cells undergoing apoptosis. Briefly, cells were fixed with a 3:1 mixture of absolute methanol and glacial acetic acid and then incubated with Hoescht stain (0.5 μ g/ml Hoescht 33258 in PBS).
30 After a 10 minute incubation in the dark, the staining solution was discarded, the chambers separating the wells of the culture slide were removed, and the slide was washed 3 times for 1 minute with deionized water. After washing, a few drops of McIlvaine's buffer

(0.021 M citric acid, 0.058 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; pH 5.6) was added to the cells and overlaid with a coverslip. The stained cells were viewed under a fluorescent microscope using a UV filter. Cells with brightly stained or fragmented nuclei were scored as apoptotic. A minimum of 200 cells were counted per well. The DeadEnd™ Fluorometric TUNEL (Promega) was also used to detect the DNA fragmentation that is a characteristic feature of apoptotic cells. Following Hoescht staining, the culture slide was washed briefly with distilled water, and further washed by immersing the slide twice for 5 minutes in PBS (137mM NaCl, 2.68mM KCl, 1.47mM KH_2PO_4 , 8.1mM Na_2HPO_4), blotting the slide on paper towel between washes. The cells were permeabilized by immersing them in 0.2 % Triton X-100 in PBS for 5 minutes. The cells were then washed again by immersing the slide twice for 5 minutes in PBS and blotting the slide on paper towel between washes. 25 μl of equilibration buffer [200 mM potassium cacodylate (pH 6.6), 25 mM Tris-HCl (pH 6.6), 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride] was added per well and incubated for 5 to 10 minutes. During equilibration, 30 μl of reaction mixture was prepared for each well by mixing in a ratio of 45:5:1, respectively, equilibration buffer, nucleotide mix [50 μM fluorescein-12-dUTP, 100 μM dATP, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA], and terminal deoxynucleotidyl transferase enzyme (Tdt, 25 U/ μl). After the incubation in equilibration buffer, 30 μl of reaction mixture was added per well and overlaid with a coverslip. The reaction was allowed to proceed in the dark at 37°C for 1 hour. The reaction was terminated by immersing the slide in 2 X SSC [0.3 M NaCl, and 30mM sodium citrate (pH 7.0)] and incubating for 15 minutes. The slide was then washed by immersion in PBS three times for 5 minutes. The PBS was removed by sponging around the wells with a Kim wipe, a drop of mounting media (Oncogene research project, JA1750-4ML) was added to each well, and the slide was overlaid with a coverslip. The cells were viewed under a fluorescent microscope using a UV filter (UV-G 365, filter set 487902) in order to count the Hoescht-stained nuclei. Any cells with brightly stained or fragmented nuclei were scored as apoptotic. Using the same field of view, the cells were then viewed using a fluorescein filter (Green H546, filter set 48915) and any nuclei fluorescing bright green were scored as apoptotic. The percentage of apoptotic cells in the field of view was calculated by dividing the number of bright green nuclei counted using the fluorescein filter by the total number of nuclei counted under the UV filter. A minimum of 200 cells were counted per well.

Protein extraction and Western blot analysis

Protein was isolated for Western blotting from lamina cribrosa cells growing on 24-well plates by washing the cells twice in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, and 0.24 g/L KH₂PO₄) and then adding 50 µl of lysis buffer [2 % SDS, 50 mM Tris-HCl (pH 7.4)]. The cell lysate was collected in a microcentrifuge tube, boiled for 5 minutes and stored at – 20 °C until ready for use. Protein concentrations were determined using the Bicinchoninic Acid Kit (BCA; Sigma). For Western blotting, 5 µg of total protein was separated on a 12 % SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was then incubated for one hour in blocking solution (5 % skim milk powder, 0.02 % sodium azide in PBS) and washed three times for 15 minutes in PBS-T (PBS + 0.05 % Tween-20). The membrane was stored overnight in PBS-T at 4 °C. After being warmed to room temperature the next day, the membrane was blocked for 30 seconds in 1 µg/ml polyvinyl alcohol. The membrane was rinsed 5 times in deionized water and then blocked for 30 minutes in a solution of 5 % milk in PBS. The primary antibody was preincubated for 30 minutes in a solution of 5 % milk in PBS prior to incubation with the membrane. The primary antibodies used were anti-eIF5A (BD Transduction Laboratories) at 1:20,000 and anti-β-actin (Oncogene). The membranes were washed three times in PBS-T and incubated for 1 hour with the appropriate HRP-conjugated secondary antibodies diluted in 1 % milk in PBS. The blot was washed and the ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech) was used to detect the peroxidase-conjugated bound antibodies.

Results

Two lamina cribrosa (LC) cell lines were established from optic nerve heads obtained from male donors ranging in age from 83 years (#506) to 17 years (#517). The cells isolated from the human lamina cribrosa had the same broad, flat morphology with prominent nucleus observed in other studies (Lambert *et al.*, 2001). Consistent with the characterizations of other groups, the LC cells showed immunoreactivity to alpha smooth muscle actin (Fig. 82a) as well as to a number of extracellular matrix proteins including cellular fibronectin (Fig. 82b), laminin (Fig. 82c), collagen I, and collagen IV (data not shown) (Clark *et al.*, 1995; Hernandez *et al.*, 1998; Hernandez and Yang, 2000; Lambert

et al.; 2001). Negative immunoreactivity of the LC cells to glial fibrillary acidic protein (GFAP) was also observed consistent with previous findings (Fig 82d) (Lambert *et al.*, 2001). These findings support the identification of the isolated cells as being LC cells rather than optic nerve head astrocytes.

5 Since TNF- α is believed to play an important role during the glaucomatous process, the susceptibility of LC cells to the cytotoxic effects of TNF- α was examined. Confluent LC cells were exposed to either camptothecin, TNF- α , or a combination of camptothecin and TNF- α for 48 hours (Fig. 83). Hoescht staining revealed that TNF- α alone was not cytotoxic to LC cells. Treatment with camptothecin resulted in
10 approximately 30 % cell death of the LC cells. However, a synergistic increase in apoptosis was observed when LC cells were treated with both camptothecin and TNF- α , a treatment which resulted in the death of 45 % of LC cells by 48 hours. These results indicate that LC cells are capable of responding to the cytotoxic effects of TNF- α when primed for apoptosis by camptothecin.

15 EIF5A is a nucleocytoplasmic shuttle protein known to be necessary for cell division and recently suggested to also be involved during apoptosis. We examined the expression of eIF5A protein in LC cells being induced to undergo apoptosis by either camptothecin, or camptothecin plus TNF- α . The expression of eIF5A did not alter significantly upon treatment with camptothecin except perhaps to decrease slightly (Fig.
20 84A). However, a significant upregulation of eIF5A protein was observed after 8 and 24 hours of camptothecin plus TNF- α treatment (Fig. 84B). These results indicate that eIF5A expression is induced specifically by exposure TNF- α and expression correlates to the induction of apoptosis. This points to a role for eIF5A in the apoptotic pathway downstream of TNF- α receptor binding.

25 In order to examine the importance of eIF5A expression during TNF- α -induced apoptosis in LC cells, a series of four siRNAs (siRNAs # 1 to # 4) targeting eIF5A were designed and synthesized by *in vitro* transcription. To determine the effectiveness of the siRNAs in suppressing eIF5A protein expression, LC cell lines # 506 and # 517 were transfected with each of the siRNAs and expression of eIF5A protein in the cell lysate was
30 examined 72 hours later (Fig. 85). For comparison, cells were also transfected with either an siRNA against GAPDH and/or a control siRNA (siRNA # 5) having the same chemical composition as siRNA #1 but which does not recognize eIF5A. All siRNAs directed

against eIF5A were capable of significantly suppressing eIF5A expression in both LC cell lines (Fig. 85). The GAPDH siRNA was used as an additional control because, unlike the control siRNA # 5 which simply has the reverse sequence of siRNA # 1 and does not have a cellular target, it is an active siRNA capable of suppressing the expression of its target protein, GAPDH (data not shown). All four siRNAs against eIF5A were also capable of protecting transfected LC cells (# 506) from apoptosis induced by 24 hour treatment with TNF- α and camptothecin (Fig. 86). Using Hoescht staining to detect cell death, the siRNAs (siRNAs # 1 to # 4) were found to be able to reduce apoptosis of LC cells by 59 % (siRNA # 1), 35 % (siRNA # 2), 50 % (siRNA # 3), and 69 % (siRNA # 4).

Interestingly, the siRNA against GAPDH was also able to reduce apoptosis of LC cells by 42 % (Fig. 86). GAPDH is known to have cellular functions outside of its role as a glycolytic enzyme, including a proposed function during apoptosis of cerebellar neurons (Ishitani and Chuang, 1996; Ishitani *et al.*, 1996a; Ishitani *et al.*, 1996b). In a similar experiment we also demonstrated that siRNA # 1 was able to reduce apoptosis of the LC line # 517 by 53 % in response to TNF- α and camptothecin indicating that eIF5A siRNAs are protective for LC cells isolated from different optic nerve heads (Fig. 87). These results indicate that eIF5A does have a function during apoptosis and may be an important intermediate in the pathway leading to TNF- α -induced apoptosis in LC cells.

In order to confirm that LC cells exposed to TNF- α and camptothecin were dying by classical apoptosis, DNA fragmentation was evaluated in situ using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. LC cells (# 506) were treated with TNF- α and camptothecin for 24 hours, 3 days after transfection with either an eIF5A siRNA (siRNA # 1) or a control siRNA (siRNA # 5). The cells were also stained with Hoescht to facilitate visualization of the nuclei. 46 % of LC cells transfected with the control siRNA were positive for TUNEL staining while only 8 % of LC cells transfected with eIF5A siRNA # 1 were positively labeled indicating that the eIF5A siRNA provided greater than 80 % protection from apoptosis (Fig. 88). Similar results were obtained with eIF5A siRNA # 4 which provided greater than 60 % protection from apoptosis relative to the control siRNA (data not shown).

We Claim:

1. A method for reducing levels of a cytokine in a cell comprising administering an agent capable of reducing expression of apoptosis factor 5A1, wherein the reducing of expression of apoptosis factor 5A1 reduces expression of said cytokine thereby reducing
5 levels of the cytokine in the cell.
2. The method of claim 1 wherein the cytokine is a pro-inflammatory cytokine.
3. The method of claim 2 wherein the cytokine is IL-1, IL-18, IL-6 or TNF- α .
- 10 4. The method of claim 1 wherein the agent comprises an antisense nucleotide having a sequence complementary to apoptosis factor 5A1.
5. The method of claim 1 wherein the agent comprises a siRNA having a sequence
15 complementary to apoptosis factor 5A1.
6. The method of claim 4 wherein the antisense nucleotide has a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8.
- 20 7. The method of claim 4 wherein the antisense nucleotide hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8.
8. The method of claim 5 wherein the siRNA has a sequence selected from the group
25 consisting of SEQ ID NO: *1, *2, *3, *4 and *5.
9. The method of claim 5 wherein the siRNA hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4 and *5.
- 30 10. A polynucleotide having a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8.

11. A siRNA having a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4 and *5.

12. A method for reducing the expression of p53 comprising administering an agent
5 capable of reducing expression of apoptosis factor 5A, wherein the reducing of expression of apoptosis factor 5A1 reduces expression of p53.

13. A method for increasing expression of Bcl-2 comprising administering an agent
capable of reducing expression of apoptosis factor 5A, wherein the reducing of expression
10 of apoptosis factor 5A1 increases expression of p53.

14. A method for reducing levels of TNF-alpha in a patient in need thereof comprising administering to said patient the antisense polynucleotide of claim 6 or 7.

15. A method for reducing levels of TNF-alpha in a patient in need thereof comprising administering to said patient the siRNA of claim 8 or 9.

16. A method of treating pathological conditions characterized by an increased IL-1, TNF-alpha, or Il-6 level comprising administering to a mammal having said pathological
20 condition, an agent to reduce expression of Factor 5a1.

17. The method of claim 16 wherein said pathological conditions are selected from the group consisting of arthritis-rheumatoid and osteo arthritis, asthma, allergies, arterial inflammation, crohn's disease, ibd, ulcerative colitis, coronary heart disease, cystic
25 fibrosis, diabetes, lupus, multiple sclerosis, graves disease, periodontitis, glaucoma & macular degeneration, ocular surface diseases including keratoconus, organ ischemia-heart, kidney, repurfusion injury, sepsis, multiple myeloma, organ transplant rejection psoriasis and eczema.

18. The method of claim 1 wherein the cells epithelial cells
30

19. A method for inhibiting or eliminating monocyte differentiation comprising administering an agent capable of reducing expression of apoptosis factor 5A1, wherein

the reducing of expression of apoptosis factor 5A1 inhibits or eliminates monocyte differentiation.

20. The method of claim 19 wherein the agent comprises an antisense nucleotide having a
5 sequence complementary to apoptosis factor 5a1.

21. The method of claim 19 wherein the agent comprises a siRNA having a sequence complementary to apoptosis factor 5a1.

10 22. The method of claim 20 wherein the antisense nucleotide has a sequence selected from the group consisting of SEQ ID NO: *6, *7, and *8.

23. The method of claim 20 wherein the antisense nucleotide hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *6,
15 *7, and *8.

24. The method of claim 21 wherein the siRNA has a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4 and *5.

20 25. The method of claim 21 wherein the siRNA hybridizes under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: *1, *2, *3, *4 and *5.

26. A method for preventing retinal ganglion cell death in a glaucomatous eye, said
25 method comprising suppressing expression of apoptosis-specific eIF5A1 in retinal ganglion cells.

27. The method of claim 26 wherein the suppressing expression of apoptosis-specific eIF5A1 comprises administration of antisense oligonucleotides to said retinal ganglion
30 cells, wherein said antisense oligonucleotides are targeted against human apoptosis-specific eIF5A1.

28. The method of claim 27 wherein said antisense oligonucleotide is SEQ ID NO:26 or 27.

29. The method of claim 28 wherein said antisense oligonucleotide is SEQ ID NO:26 or
5 an oligonucleotide that hybridizes under stringent conditions to a sequence complementary to SEQ ID NO:26.

30. The method of claim 28 wherein said antisense oligonucleotide is SEQ ID NO:27 or
10 an oligonucleotide that hybridizes under stringent conditions to a sequence complementary to SEQ ID NO:27.

31. A method of suppressing expression of apoptosis-specific eIF5A1 in lamina cribosa
cells, the method comprising transfecting lamina cribosa cells with antisense
oligonucleotides targeted against human apoptosis-specific eIF5A1, wherein said antisense
15 oligonucleotides suppress expression of apoptosis-specific eIF5A1 in said cells.

32. The method of claim 31 wherein the lamina cribosa cells are human.

33. The method of claim 31 wherein the antisense oligonucleotide is SEQ ID NO:26 or an
20 oligonucleotide that hybridizes under stringent conditions to a sequence complementary to SEQ ID NO:26.

34. The method of claim 31 wherein the antisense oligonucleotide is SEQ ID NO:27 or an
oligonucleotide that hybridizes under stringent conditions to a sequence complementary to
25 SEQ ID NO:27.

35. A method of suppressing expression of apoptosis-specific eIF5A1 in astrocyte cells,
the method comprising transfecting astrocyte cells with antisense oligonucleotides targeted
against human apoptosis-specific eIF5A1, wherein said antisense oligonucleotides suppress
30 expression of apoptosis-specific eIF5A1 in said cells.

36. The method of claim 35 wherein said astrocyte cells are human.

TCGAAGACCGGTAAGCACGGCCATGCCAAGGTCCATCTGGTTGGTATTGATATTTTTACTGGGAAGAAATAT
S K T G K H G H A K V H L V G I D I F T G K K Y
GAAGATATCTGCCCGTCGACTCATAACATGGATGTCCCAACATCAAAAGGAATGATTTCCAGCTGATTGGC
E D I C P S T H N M D V P N I K R N D F Q L I G
ATCCAGGATGGGTACCTATCCCTGCTCCAGGACAGTGGGGAGGTACGAGAGGACCTTCGTCTGCCTGAGGGA
I Q D G Y L S L L Q D S G E V R E D L R L P E G
GACCTTGGCAAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCCGCCATG
D L G K E I E Q K Y D C G E E I L I T V L S A M
ACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAAATAACTGGCTTCCAGGGTGGCGGTGGTGGCAGCA
T E E A A V A I K A M A K
GTGATCCATGAGCCTACAGAGGCCCTCCCCAGCTCTGGCTGGGCCCTTGGCTGGACTCCTATCCAATTTA
TTTGACGTTTTATTTTGGTTTTCTCACCCTTCAAACCTGTGGGGAGACCCTGCCCTTACCTAGCTCCCT
TGGCCAGGCATGAGGGAGCCATGGCCTTGGTGAAGCTACCTGCCTCTTCTCTCGCAGCCCTGATGGGGGAAA
GGGAGTGGGTACTGCCTGTGGTTTAGGTTCCCCTCTCCCTTTTTCTTTTTAATTCAATTTGGAATCAGAAAG
CTGTGGATTCTGGCAAATGGTCTTGTGTCTTTATCCCACTCAAACCCATCTGGTCCCCTGTTCTCCATAGT
CCTTCACCCCCAAGCACCCTGACAGACTGGGGACCAGCCCCCTTCCCTGCCTGTGTCTTCCCAAACCCC
TCTATAGGGGTGACAAGAAGAGGAGGGGGGGAGGGGACACGATCCCTCCTCAGGCATCTGGGAAGGCCTTGC
CCCCATGGGCTTTACCCTTTCCTGTGGGCTTTCTCCCTGACACATTTGTTAAAAATCAAACCTGAATAAAAC
TACAAGTTTAATATGAAAAAAAAAAAAAAAAAAAAA
(972 NT, 109 aa)

FIG. 1

CAGGTCTAGAGTTGGAATCGAAGCCTCTTAAAATGGCAGATGATTTGGACTTCGAGACAGGAGATGCAGGGG
M A D D L D F E T G D A G
CCTCAGCCACCTTCCCAATGCAGTGCTCAGCATTACGTAAGAATGGTTTTGTGGTGCTCAAGGGCCGGCCAT
A S A T F P M Q C S A L R K N G F V V L K G R P
GTAAGATCGTCGAGATGTCTACTTCGAAGACTGGCAAGCATGGCCATGCCAAGGTCCATCTGGTTGGTATTG
C K I V E M S T S K T G K H G H A K V H L V G I
ATATTTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCGACTCATAACATGGATGTCCCCAACATCAAAA
D I F T G K K Y E D I C P S T H N M D V P N I K
GGAATGATTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCCCTGCTCCAGGACAGTGGGGAGGTACGAG
R N D F Q L I G I Q D G Y L S L L Q D S G E V R
AGGACCTTCGTCTGCCTGAGGGAGACCTTGGCAAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCC
E D L R L P E G D L G K E I E Q K Y D C G E E I
TGATCACAGTGCTGTCCGCCATGACAGAGGAGGCAGCTGTTGCAATCAAGGCTCGAG
L I T V L S A M T E E A A V A I K A

(488 NT, 151 aa)

FIG.2

CAGGTCTAGAGTTGGAATCGAAGCCTCTTAAAATGGCAGATGATTTGGACTTCGAGACAGGAGATGCAGGGG
M A D D L D F E T G D A G 13
CCTCAGCCACCTTCCCAATGCAGTGCTCAGCATTACGTAAGAATGGTTTTGTGGTGCTCAAGGGCCGGCCAT 144
A S A T F P M Q C S A L R K N G F V V L K G R P
GTAAGATCGTCGAGATGTCTACTTCGAAGACTGGCAAGCATGGCCATGCCAAGGTCCATCTGGTTGGTATTG
C K I V E M S T S K T G K H G H A K V H L V G I 61
ATATTTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCGACTCATAACATGGATGTCCCAACATCAAAA 288
D I F T G K K Y E D I C P S T H N M D V P N I K
GGAATGATTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCCCTGCTCCAGGACAGTGGGGAGGTACGAG
R N D F Q L I G I Q D G Y L S L L Q D S G E V R 109
AGGACCTTCGTCTGCCTGAGGGAGACCTTGGCAAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCC 432
E D L R L P E G D L G K E I E Q K Y D C G E E I
TGATCACAGTGCTGTCCGCCATGACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAATAACTGGCTT
L I T V L S A M T E E A A V A I K A M A K * 154
CCAGGGTGGCGGTGGTGGCAGCAGTGATCCATGAGCCTACAGAGGCCCTCCCCAGCTCTGGCTGGGCCCT 576
TGGCTGGACTCCTATCCAATTTATTTGACGTTTTATTTTGGTTTTCTCACCCCTTCAAAGTGTGGGGAGA
CCCTGCCCTTCACCTAGCTCCCTTGGCCAGGCATGAGGGAGCCATGGCCTTGGTGAAGCTACCTGCCTCTTC 720
TCTCGCAGCCCTGATGGGGGAAAGGGAGTGGGTACTGCCTGTGGTTTAGGTTCCCTCTCCCTTTTTCTTTT
TAATTCAATTTGGAATCAGAAAGCTGTGGATTCTGGCAAATGGTCTTGTGTCCTTTATCCCACTCAAACCCA 864
TCTGGTCCCCTGTTCTCCATAGTCCTTCACCCCCAAGCACCCTGACAGACTGGGGACCAGCCCCCTTCCCT
GCCTGTGTCTCTTCCCAAACCCCTCTATAGGGGTGACAAGAAGAGGAGGGGGGAGGGGACACGATCCCTCC 1008
TCAGGCATCTGGGAAGGCCTTGCCCCCATGGGCTTTACCCTTTCCTGTGGGCTTTCTCCCTGACACATTTGT
TAAAAATCAAACCTGAATAAAACTACAAGTTTAATATGAAAAAAAAAAAAAAAAAAAAA 1139

(1139 NT, 154 aa)

FIG.3

GCTGTGTATTATTGGGCCCATAAGAACCACATACCTGTGCTGAGTCCTGCACTCACAGACGGCTCACTGGGT
A V Y Y W A H K N H I P V L S P A L T D G S L G
GACATGATCTTTTTCCATTCCTATAAAAACCCAGGCTTGGTCCTGGACATCGTTGAAGACCTGCGGCTCATC
D M I F F H S Y K N P G L V L D I V E D L R L I
AACATGCAGGCCATTTTCGCCAAGCGCACTGGGATGATCATCCTGGGTGGAGGCGTGGTCAAGCACCACATC
N M Q A I F A K R T G M I I L G G G V V K H H I
GCCAATGCTAACCTCATGCGGAATGGAGCTGACTACGCTGTTTATATCAACACAGCCCAGGAGTTTGATGGC
A N A N L M R N G A D Y A V Y I N T A Q E F D G
TCAGACTCAGGAGCCCGGCCAGATGAGGCTGTCTCCTGGGGCAAGATCCGGATGGATGCACAGCCAGTAAAG
S D S G A R P D E A V S W G K I R M D A Q P V K
GTCTATGCTGATGCATCTCTGGTTTTCCCTTGCTGGTGGCTGAGACATTCGCCCAAAGGCAGATGCCTTC
V Y A D A S L V F P L L V A E T F A Q K A D A F
AGAGCTGAGAAGAATGAGGACTGAGCAGATGGGTAAAGACGGAGGCTTCTGCCACACCTTTATTTATTATT
R A E K N E D
GCATACCAACCCCTCCTGGGCCCTCTCCTTGGTCAGCAGCATCTTGAGAATAAATGGCCTTTTTGTTGGTTT
CTGTAAAAAAGGACTTTAAAAA

(606 NT, 151 aa)

FIG.4

rat vs. human(BC000751 or NM_001970) 96.5% identity (coding)

```

      10      20      30      40      50      60
rat   ATGGCAGATGATTTGGACTTCGAGACAGGAGATGCAGGGGCCTCAGCCACCTTCCCAATG
      .....
human ATGGCAGATGACTTGGACTTCGAGACAGGAGATGCAGGGGCCTCAGCCACCTTCCCAATG
      10      20      30      40      50      60

      70      80      90     100     110     120
rat   CAGTGCTCAGCATTACGTAAGAATGGTTTTGTGGTGCTCAAGGGCCGGCCATGTAAGATC
      .....
human CAGTGCTCAGCATTACGTAAGAATGGCTTTGTGGTGCTCAAAGGGCCGGCCATGTAAGATC
      70      80      90     100     110     120

      130     140     150     160     170     180
rat   GTCGAGATGTCTACTTCGAAGACTGGCAAGCATGGCCATGCCAAGGTCCATCTGGTTGGT
      .....
human GTCGAGATGTCTACTTCGAAGACTGGCAAGCACGGCCACGCCAAGGTCCATCTGGTTGGT
      130     140     150     160     170     180

      190     200     210     220     230     240
rat   ATTGATATTTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCGACTCATAACATGGAT
      .....
human ATTGACATCTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCAACTCATAATATGGAT
      190     200     210     220     230     240

      250     260     270     280     290     300
rat   GTCCCAACATCAAAGGAATGATTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCC
      .....
human GTCCCAACATCAAAGGAATGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCA
      250     260     270     280     290     300

      310     320     330     340     350     360
rat   CTGCTCCAGGACAGTGGGGAGGTACGAGAGGACCTTCGTCTGCCTGAGGGAGACCTTGGC
      .....
human CTGCTCCAGGACAGCGGGGAGGTACGAGAGGACCTTCGTCTCCCTGAGGGAGACCTTGGC
      310     320     330     340     350     360

      370     380     390     400     410     420
rat   AAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCCGCC
      .....
human AAGGAGATTGAGCAGAAGTACGACTGTGGAGAAGAGATCCTGATCACGGTGCTGTCTGCC
      370     380     390     400     410     420

      430     440     450     460
rat   ATGACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAAA
      .....
human ATGACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAAA
      430     440     450     460
```

FIG.5

rat vs. human(NM_020390) 72.5% identity (coding)

```

      10      20      30      40      50      60
rat  ATGGCAGATGATTTGGACTTCGAGACAGGAGATGCAGGGGCCCTCAGCCACCTTCCCAATG
      :::::  :: :  : :: ::  :: :::::  :::::  ::  ::::  :::  :::  :::
human ATGGCAGACGAAATTGATTTCACTACTGGAGATGCCGGGGCTTCCAGCACTTACCCTATG
      10      20      30      40      50      60

      70      80      90     100     110     120
rat  CAGTGCTCAGCATTACGTAAGAATGGTTTTGTGGTGCTCAAGGGCCGCCATGTAAGATC
      :::::  :: :  : :: :  : :: :  : :::::  :: :  : :::::  :: :  :
human CAGTGCTCGGCCTTGCGCAAAAACGGCTTCGTGGTGCTCAAAGGACGACCATGCAAAATA
      70      80      90     100     110     120

      130     140     150     160     170     180
rat  GTCGAGATGTCTACTTCTGAAGACTGGCAAGCATGGCCATGCCAAGGTCCATCTGGTTGGT
      :: :::::  :::::  :: :::::  :::::  :::::  :: :  : :::::
human GTGGAGATGTCAACTTCCAAAACGGAAAGCATGGTCATGCCAAGGTTACCTTGTTGGA
      130     140     150     160     170     180

      190     200     210     220     230     240
rat  ATTGATATTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCGACTCATAACATGGAT
      :::::  :: :  : :::::  :::::  :: :  : :::::  :::::  :::::
human ATTGATATTTTCACGGGCAAAAATATGAAGATATTTGTCCTTCTACTCACAACATGGAT
      190     200     210     220     230     240

      250     260     270     280     290     300
rat  GTCCCCAACATCAAAGGAATGATTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCC
      :: :  : :: :  : :::::  :: :::::  :::::  :: :  : :::::  :::::
human GTTCCAAATATTAAGAGAAATGATTATCAACTGATATGCATTCAAGATGGTTACCTTTCC
      250     260     270     280     290     300

      310     320     330     340     350     360
rat  CTGCTCCAGGACAGTGGGGAGGTACGAGAGGACCTTCGTCTGCCTGAGGGAGACCTTGGC
      :::::  :: :  : :::  : :: :  : :::::  ::  :::::  :: :  : :::
human CTGCTGACAGAACTGGTGAAGTTCGTGAGGATCTTAAACTGCCAGAAGGTGAACTAGGC
      310     320     330     340     350     360

      370     380     390     400     410     420
rat  AAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCCGCC
      :: :  : :: :  : :: :  : :::::  : :  : : : :  :: :  : :::
human AAAGAAATAGAGGGAAAATACAATGCAGGTGAAGATGTACAGGTGTCTGTCATGTGTGCA
      370     380     390     400     410     420

      430     440     450     460
rat  ATGACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAAA
      ::::  :: :  : :::::  :: :  : :: :  : :: :  : :::::
human ATGAGTGAAGAATATGCTGTAGCCATAAAACCCT- -GCAAAAT
      430     440     450     460

```

FIG.6

rat vs. mouse (BC003889) 98.3% identity (coding)

	10	20	30	40	50	60
rat	ATGGCAGATGATTTGGACTTCGAGACAGGAGATGCAGGGGCCTCAGCCACCTTCCCAATG					
	::					
mouse	ATGGCAGATGATTTGGACTTCGAGACAGGAGATGCAGGGGCCTCAGCCACCTTCCCAATG					
	10	20	30	40	50	60
	70	80	90	100	110	120
rat	CAGTGCTCAGCATTACGTAAGAATGGTTTTGTGGTGCTCAAGGGCCGGCCATGTAAGATC					
	::					
mouse	CAGTGCTCAGCATTACGTAAGAATGGTTTTGTGGTGCTCAAAGGGCCGGCCATGTAAGATC					
	70	80	90	100	110	120
	130	140	150	160	170	180
rat	GTCGAGATGTCTACTTCGAAGACTGGCAAGCATGGCCATGCCAAGGTCCATCTGGTTGGT					
	::					
mouse	GTCGAGATGTCTACTTCGAAGACTGGCAAGCATGGCCATGCCAAGGTCCATCTGGTTGGC					
	130	140	150	160	170	180
	190	200	210	220	230	240
rat	ATTGATATTTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCGACTCATAACATGGAT					
	::::: ::					
mouse	ATTGACATTTTTACTGGGAAGAAATATGAAGATATCTGCCCGTCGACTCATAATATGGAT					
	190	200	210	220	230	240
	250	260	270	280	290	300
rat	GTCCCCAACATCAAAAGGAATGATTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCC					
	:::::::::::::::::::::::: :::::::::: ::::::::::::::::::::::::::::::::::					
mouse	GTCCCCAACATCAAACGGAATGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCC					
	250	260	270	280	290	300
	310	320	330	340	350	360
rat	CTGCTCCAGGACAGTGGGGAGGTACGAGAGGACCTTCGTCTGCCTGAGGGAGACCTTGGC					
	::					
mouse	CTGCTCCAGGACAGTGGGGAGGTACGAGAGGACCTTCGTCTGCCTGAAGGAGACCTTGGC					
	310	320	330	340	350	360
	370	380	390	400	410	420
rat	AAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCCGCC					
	:: ::					
mouse	AAGGAGATTGAGCAGAAGTATGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCTGCC					
	370	380	390	400	410	420
	430	440	450	460		
rat	ATGACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAAA					
	::					
mouse	ATGACAGAGGAGGCAGCTGTTGCAATCAAGGCCATGGCAAAA					
	430	440	450	460		

FIG.7

rat vs. human(BC000751 or NM_001970) 100.0% identity

	10	20	30	40	50	60
rat	MADDLDFETGDAGASATFPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG					
						
human	MADDLDFETGDAGASATFPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG					
	10	20	30	40	50	60
	70	80	90	100	110	120
rat	IDIFTGKKYEDICPSTHNMDVPNIKRNDFQLIGIQDGYLSLLQDSGEVREDLRLPEGDLG					
						
human	IDIFTGKKYEDICPSTHNMDVPNIKRNDFQLIGIQDGYLSLLQDSGEVREDLRLPEGDLG					
	70	80	90	100	110	120
	130	140	150			
rat	KEIEQKYDCGEEILITVLSAMTEEA A VAIKAMAK					
						
human	KEIEQKYDCGEEILITVLSAMTEEA A VAIKAMAK					
	130	140	150			

FIG.8

rat vs. human(NM_020390) 82.5% identity

	10	20	30	40	50	60
rat	MADDLDFETGDAGASATFPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG					
						
human	MADEIDFTTGDAGASSTYPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG					
	10	20	30	40	50	60
	70	80	90	100	110	120
rat	IDIFTGKKYEDICPSTHNMDVPNIKRNDYQLIGIQDGYLSLLQDSGEVREDLRLPEGDLG					
						
human	IDIFTGKKYEDICPSTHNMDVPNIKRNDYQLICIQDGYLSLLTETGEVREDLKLPEGELG					
	70	80	90	100	110	120
	130	140	150			
rat	KEIEQKYDCGEEILITVLSAMTEEA AVAIKAMAK					
	 :. :. :. :					
human	KEIEGKYNAGEDVQVSVMCAMSEEYAVAIKP-CK					
	130	140	150			

FIG.9

rat vs. mouse (BC003889)100.0% identity

	10	20	30	40	50	60
rat	MADDLDFETGDAGASATFPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG					
						
mouse	MADDLDFETGDAGASATFPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG					
	10	20	30	40	50	60
	70	80	90	100	110	120
rat	IDIFTGKKYEDICPSTHNMDVPNIKRNDFQLIGIQDGYLSLLQDSGEVREDLRLPEGDLG					
						
mouse	IDIFTGKKYEDICPSTHNMDVPNIKRNDFQLIGIQDGYLSLLQDSGEVREDLRLPEGDLG					
	70	80	90	100	110	120
	130	140	150			
rat	KEIEQKYDCGEEILITVLSAMTEEA AVAIKAMAK					
						
mouse	KEIEQKYDCGEEILITVLSAMTEEA AVAIKAMAK					
	130	140	150			

FIG. 10

rat vs. human (BC000333) 87.4% identity (coding)

```

      10      20      30      40      50      60
rat  GCTGTGTATTATTGGGCCCATAAGAACCACATACCTGTGCTGAGTCCTGCACTCACAGAC
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human TCCGTGTATTACTGGGCCCAGAAGAACCACATCCCTGTGTTTAGTCCCGCACTTACAGAC
      10      20      30      40      50      60

      70      80      90     100     110     120
rat  GGCTCACTGGGTGACATGATCTTTTTCCATTCTATAAAAACCCAGGCTTGGTCCTGGAC
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human GGCTCGCTGGGCGACATGATCTTCTTCCATTCTACAAGAACCCGGGCCTGGTCCTGGAC
      70      80      90     100     110     120

      130     140     150     160     170     180
rat  ATCGTTGAAGACCTGCGGCTCATCAACATGCAGGCCATTTTCGCCAAGCGCACTGGGATG
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human ATCGTTGAGGACCTGAGGCTCATCAACACACAGGCCATCTTTGCCAAGTGCCTGGGATG
      130     140     150     160     170     180

      190     200     210     220     230     240
rat  ATCATCCTGGGTGGAGGCGTGGTCAAGCACCACATCGCCAATGCTAACCTCATGCGGAAT
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human ATCATTCTGGGCGGGGCGTGGTCAAGCACCACATTGCCAATGCCAACCTCATGCGGAAC
      190     200     210     220     230     240

      250     260     270     280     290     300
rat  GGAGCTGACTACGCTGTTTATATCAACACAGCCCAGGAGTTTGATGGCTCAGACTCAGGA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human GGGGCCGACTACGCTGTTTACATCAACACAGCCCAGGAGTTTGATGGCTCTGACTCAGGT
      250     260     270     280     290     300

      310     320     330     340     350     360
rat  GCCCGGCCAGATGAGGCTGTCTCCTGGGGCAAGATCCGGATGGATGCACAGCCAGTAAAG
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human GCCCGACCAGACGAGGCTGTCTCCTGGGGCAAGATCCGGGTGGATGCACAGCCCGTCAAG
      310     320     330     340     350     360

      370     380     390     400     410     420
rat  GTCTATGCTGATGCATCTCTGGTTTTCCCCTTGCTGGTGGCTGAGACATTCGCCCAAAG
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human GTCTATGCTGACGCTCCCTGGTCTTCCCCCTGCTTGTGGCTGAAACCTTTGCCCAGAAG
      370     380     390     400     410     420

      430     440     450
rat  GCAGATGCCTTCAGAGCTGAGAAGAATGAGGAC
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
human ATGGATGCCTTCATGCATGAGAAGAACGAGGAC
      430     440     450
```

FIG. 11

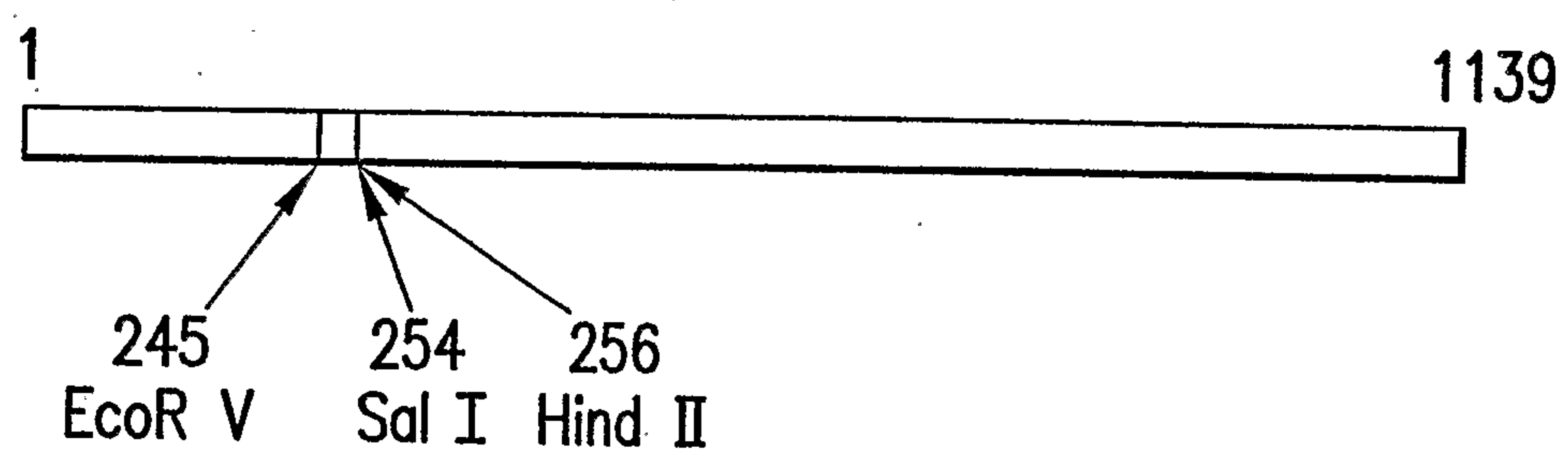


FIG.12

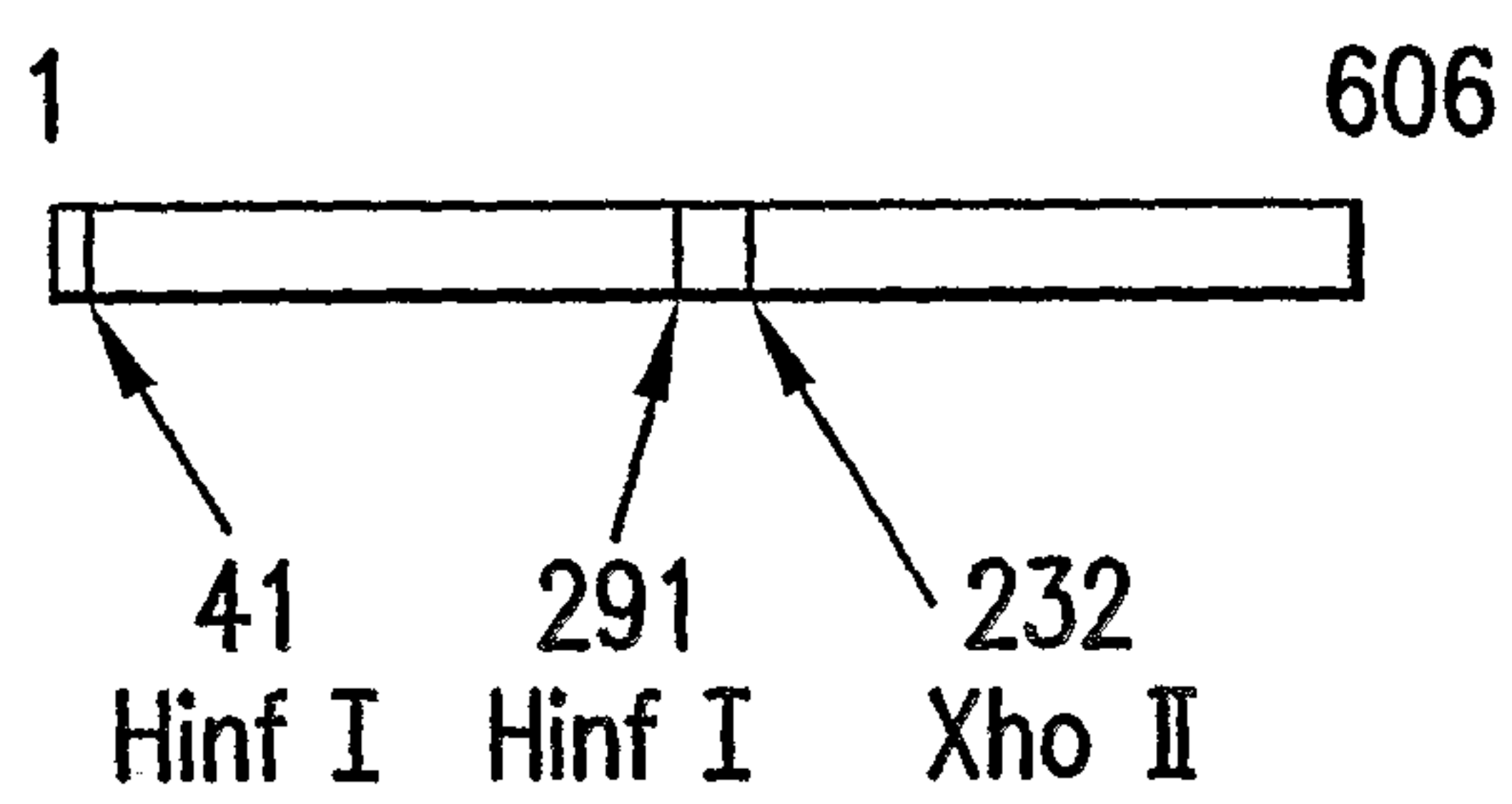


FIG.13

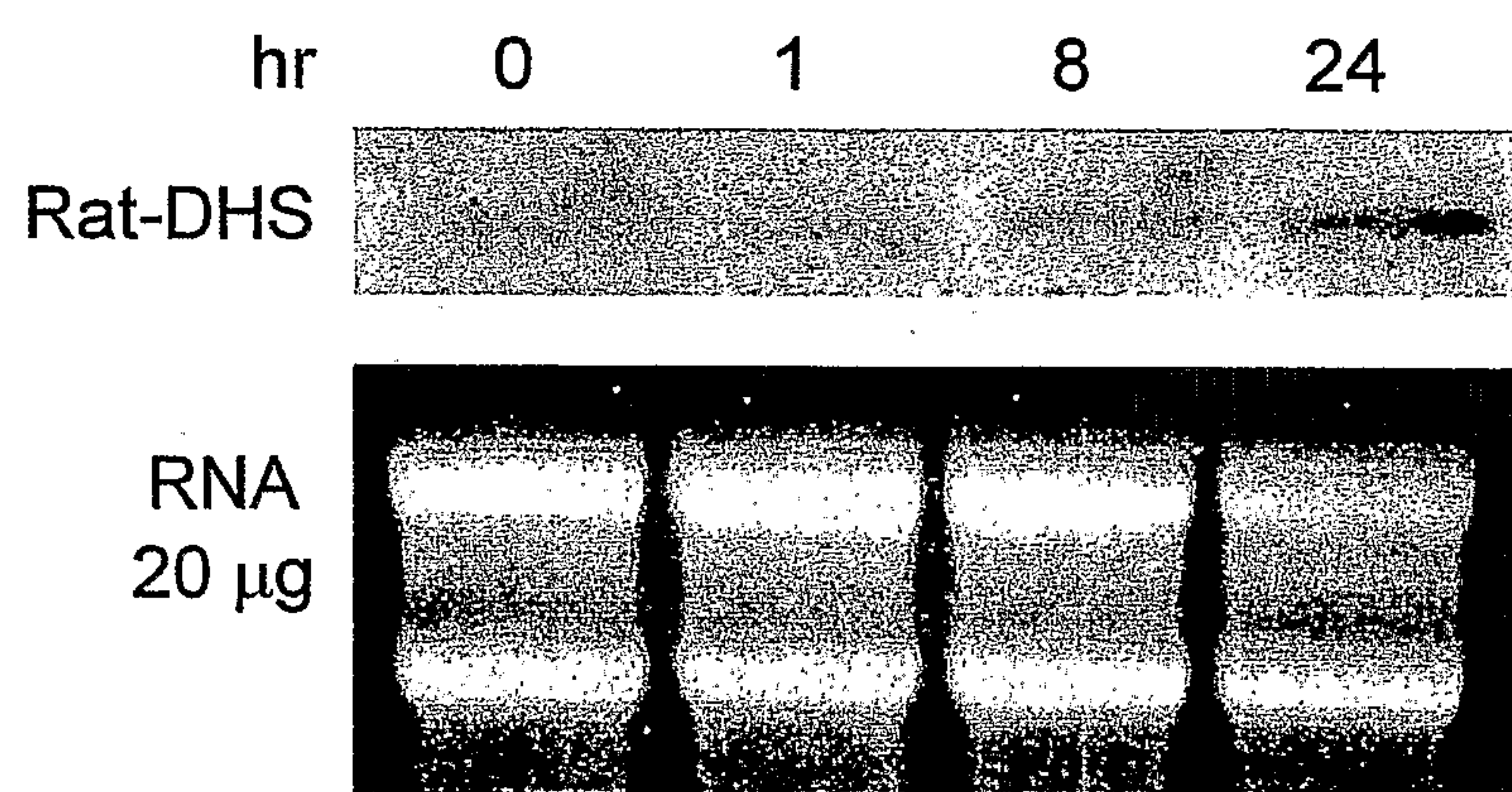


FIG.15

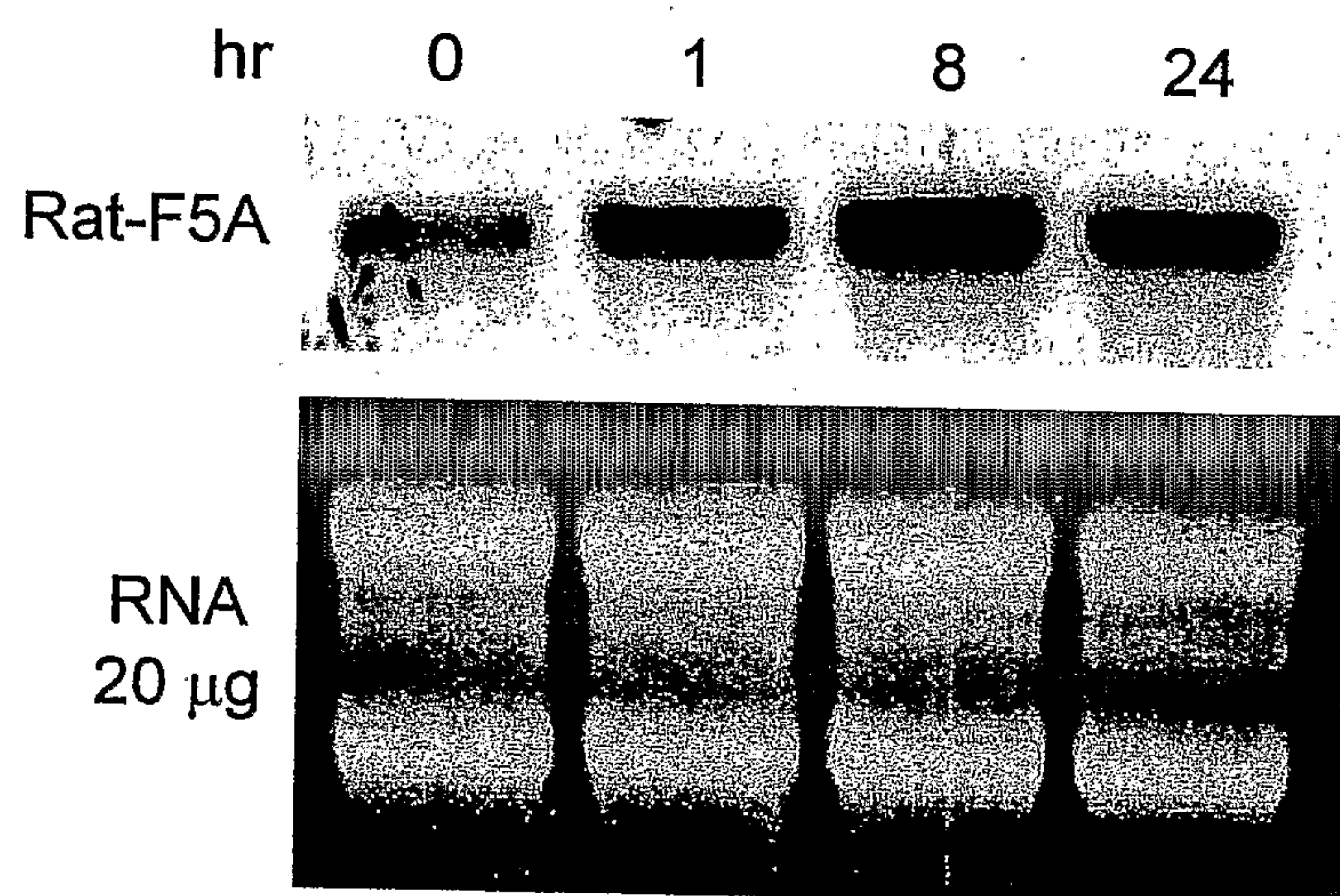


FIG.14



FIG.16

Saline – 3 hours *in vitro*
PGF-2 α – 3 hours *in vitro*
PGF-2 α – 6 hours *in vitro*

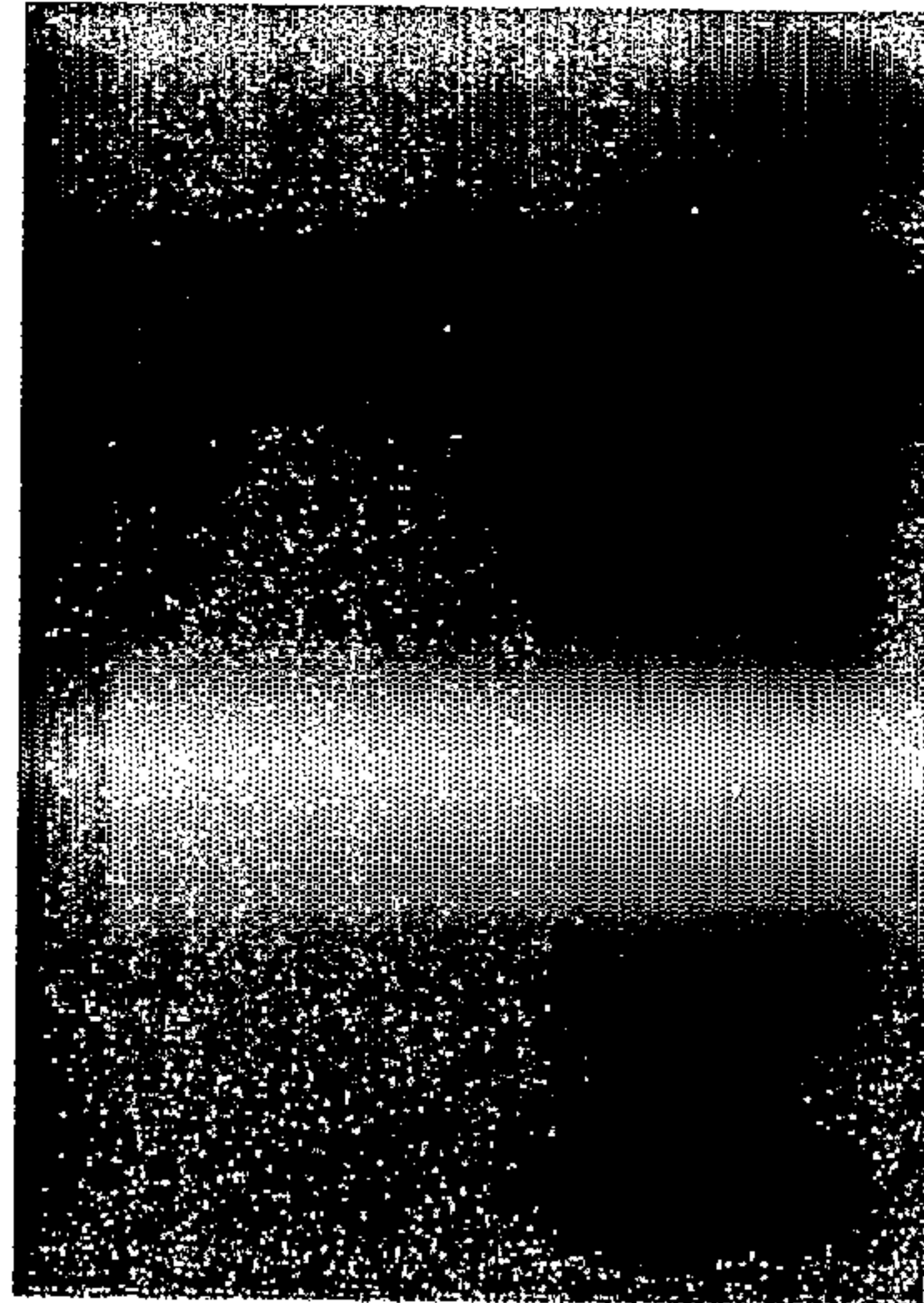


FIG.17

1 Saline Control

2 PGF-2 α – 1 hr 10 mM sperm.

3 PGF-2 α – 1 hr 10 mM sperm. + 5 hr 1 mM sperm.

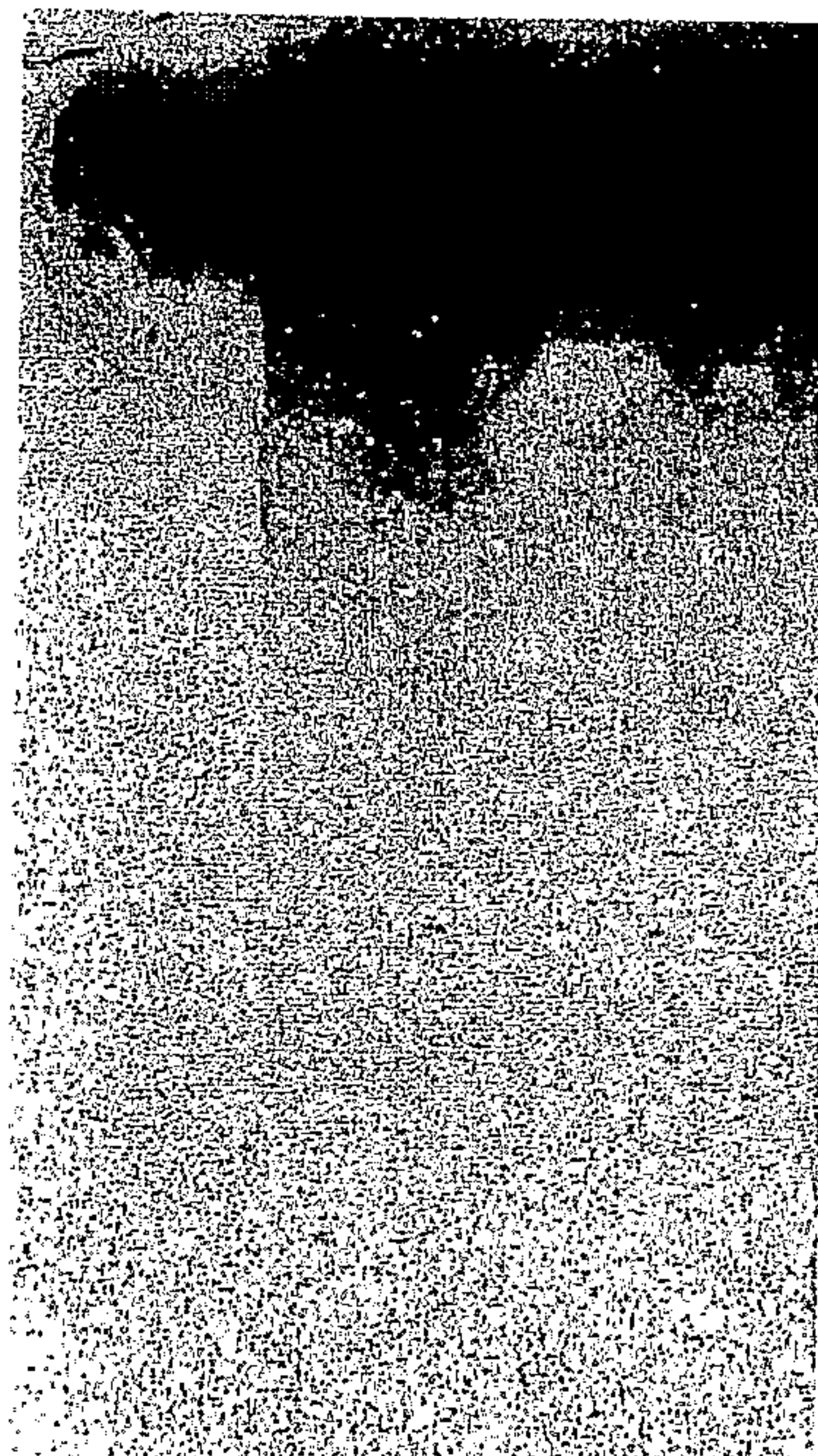


FIG.18

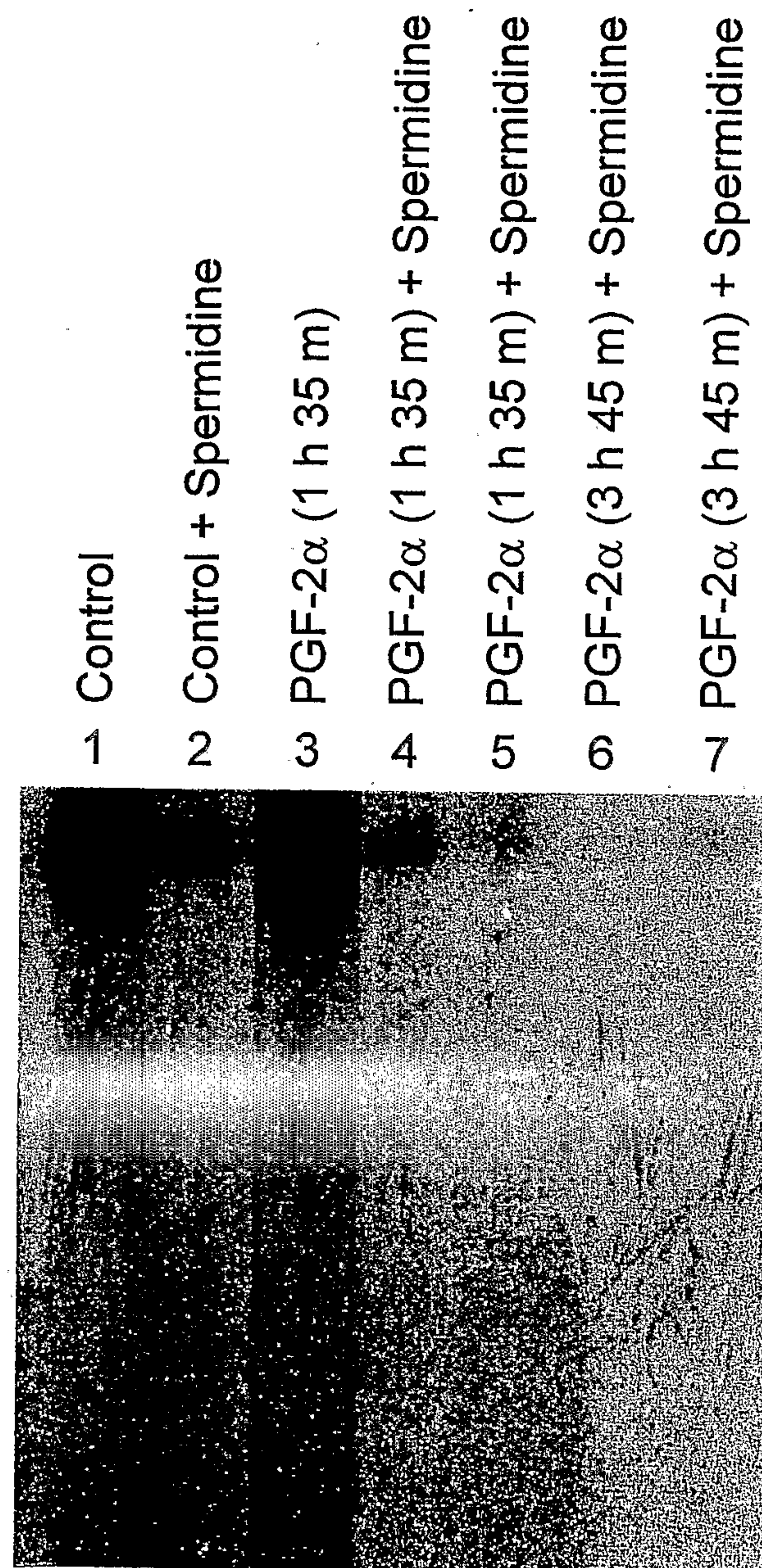


FIG. 19

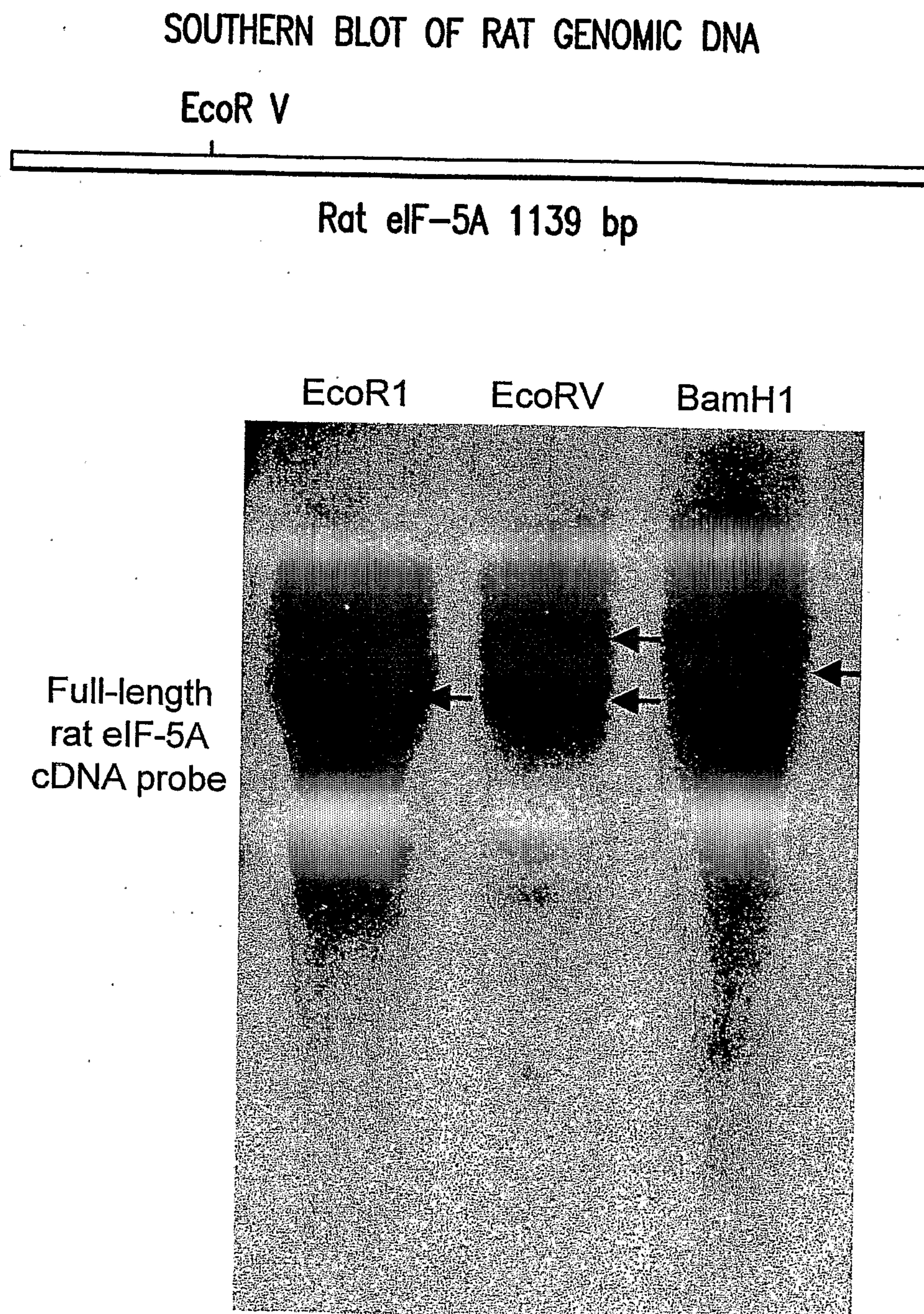


FIG.20

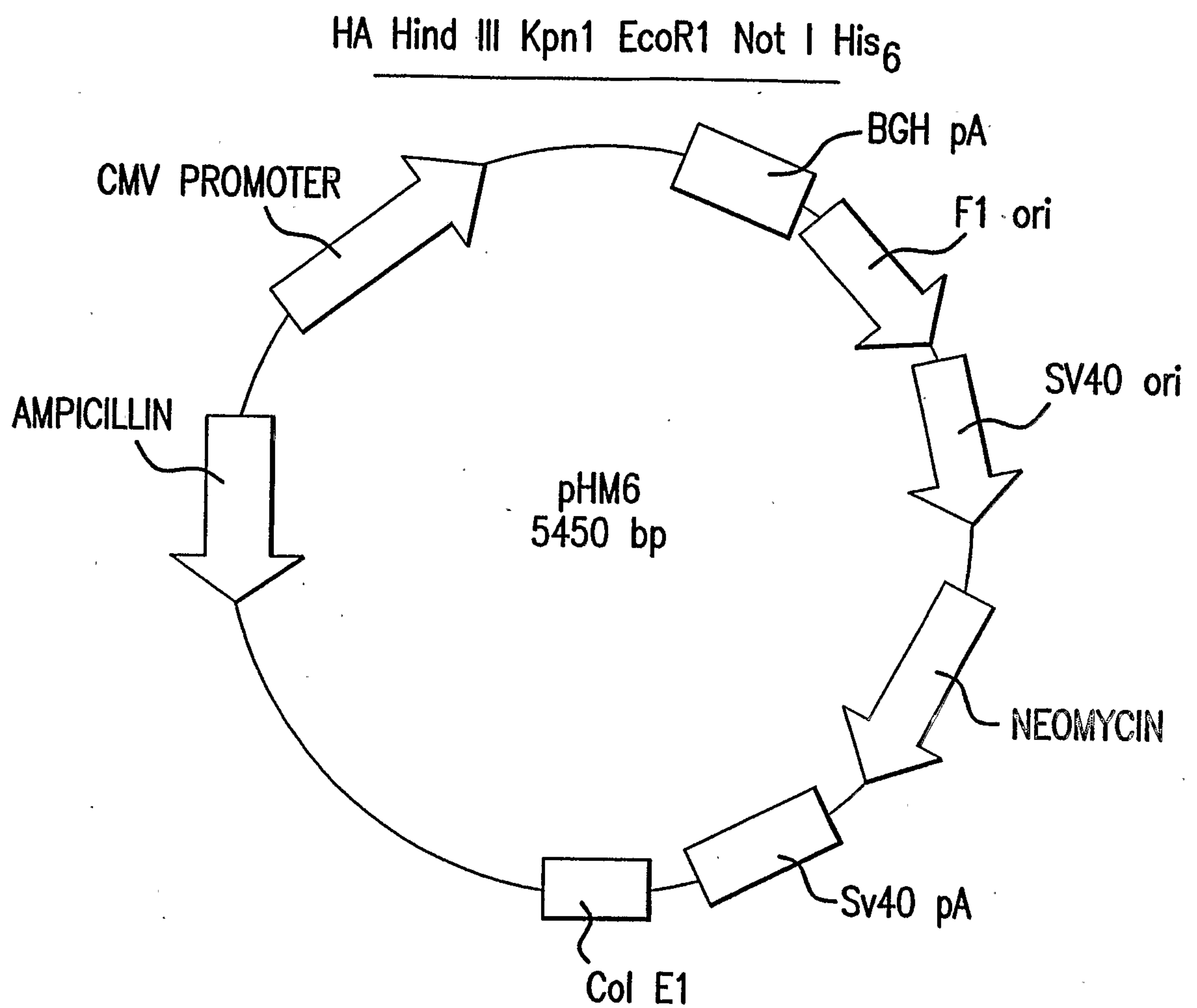


FIG.21

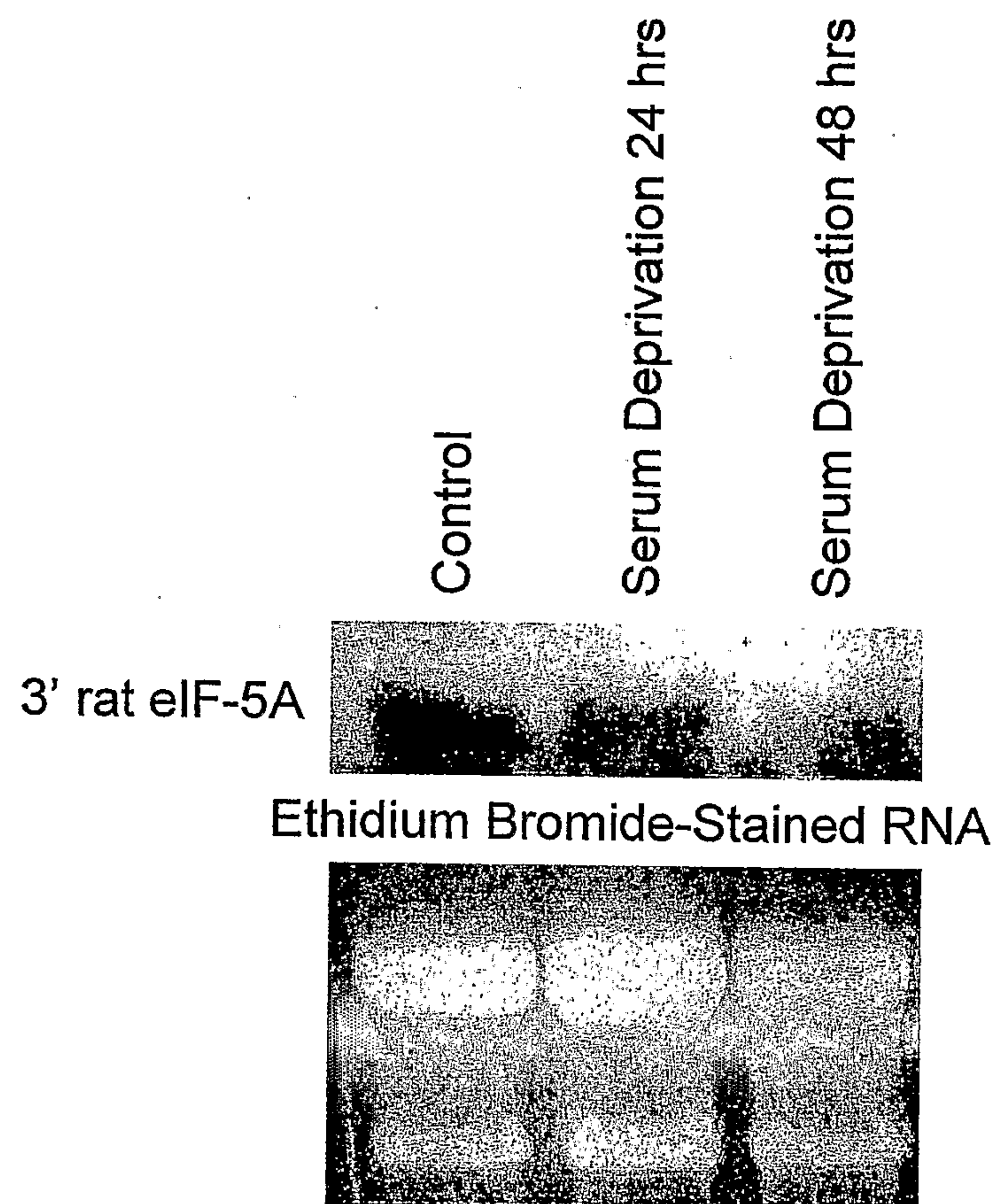


FIG.22

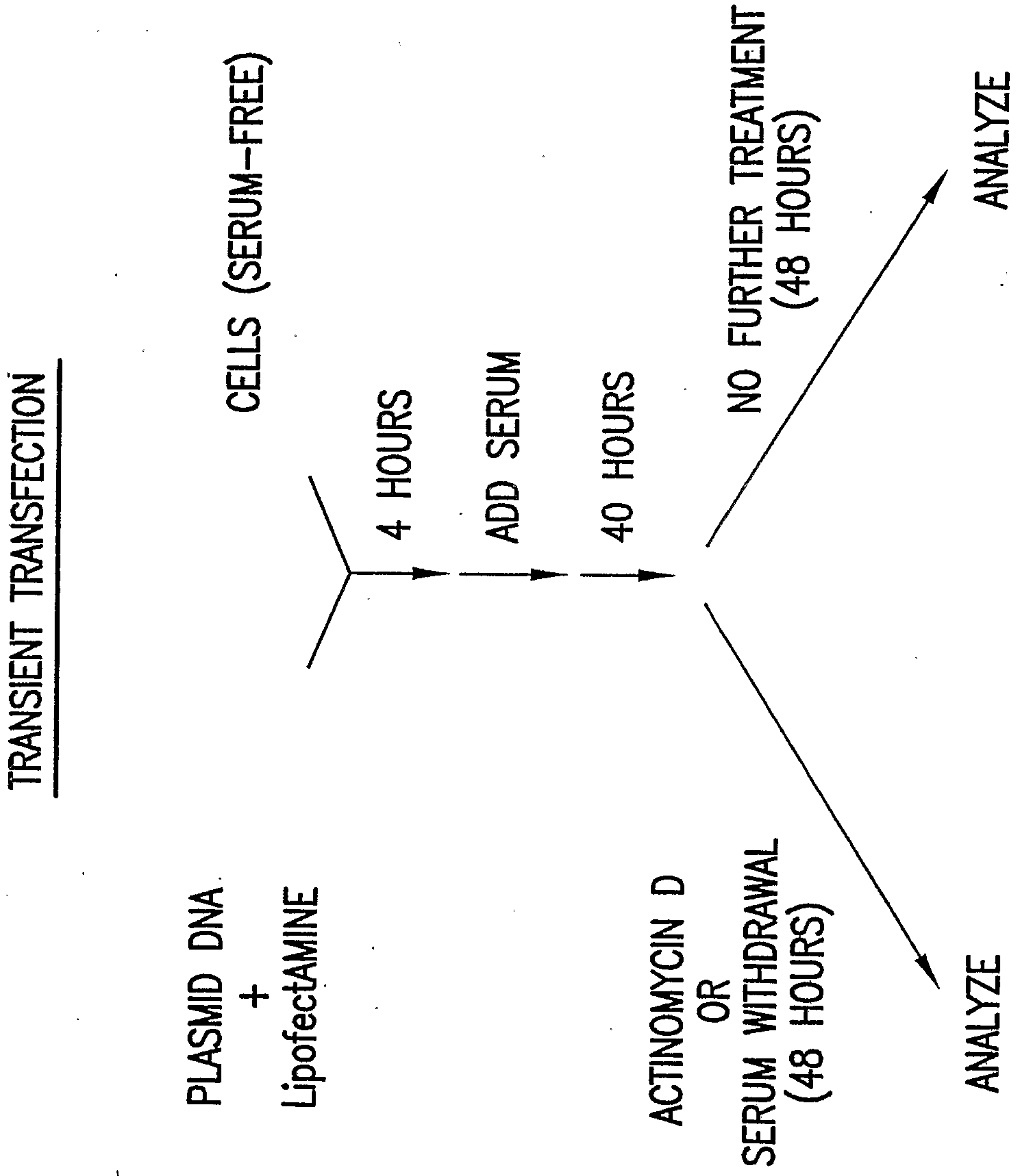


FIG.23

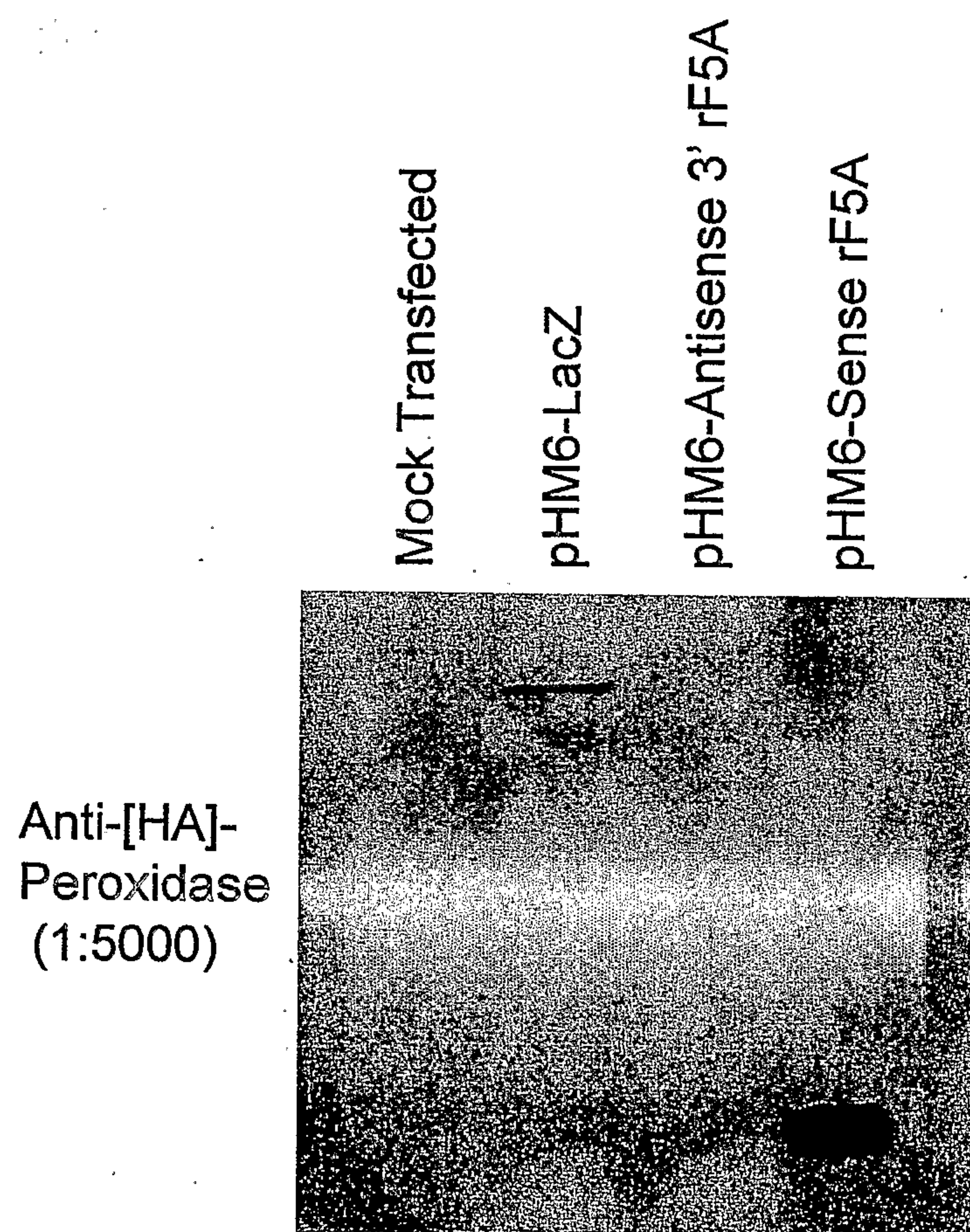


FIG.24

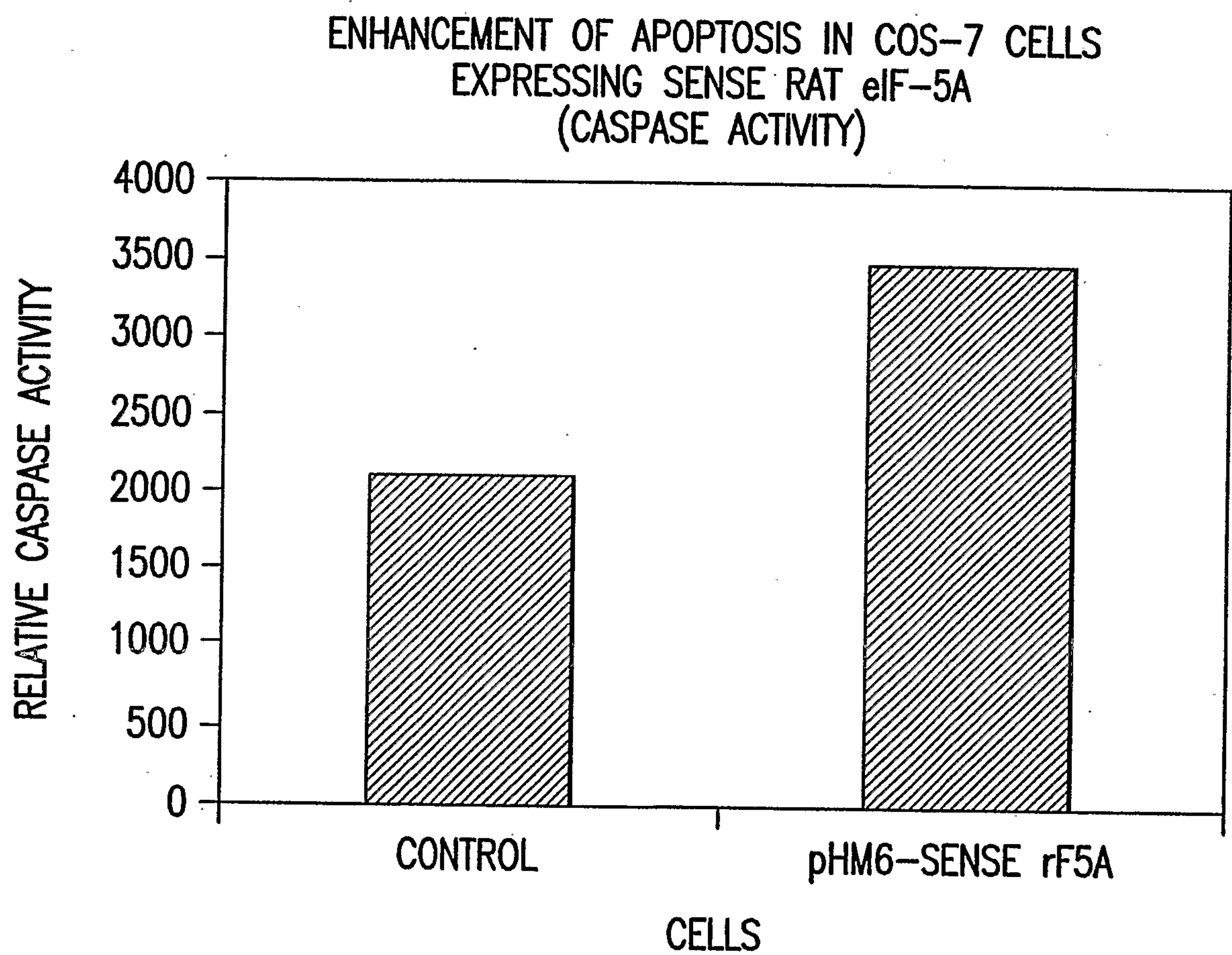


FIG.25

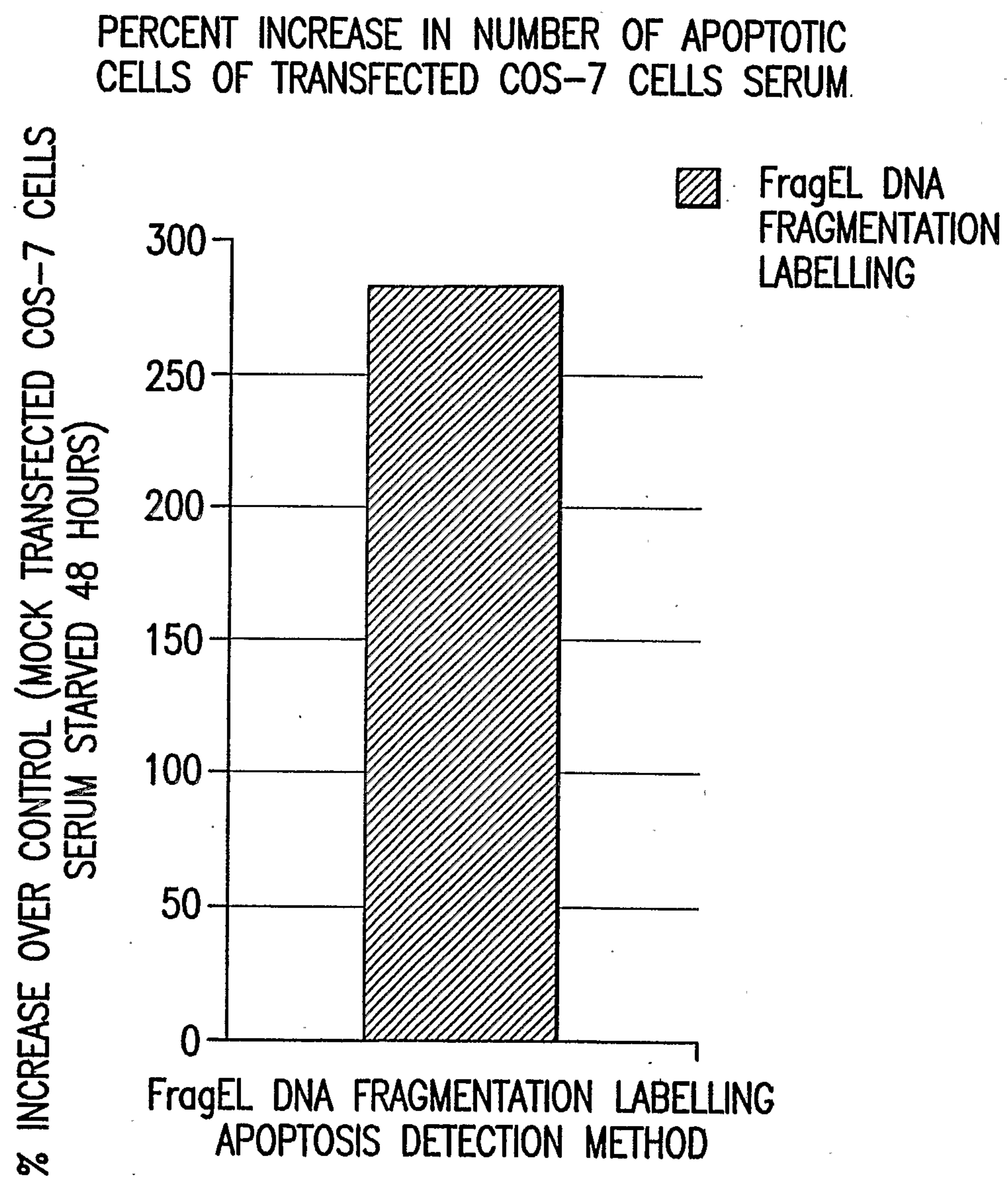
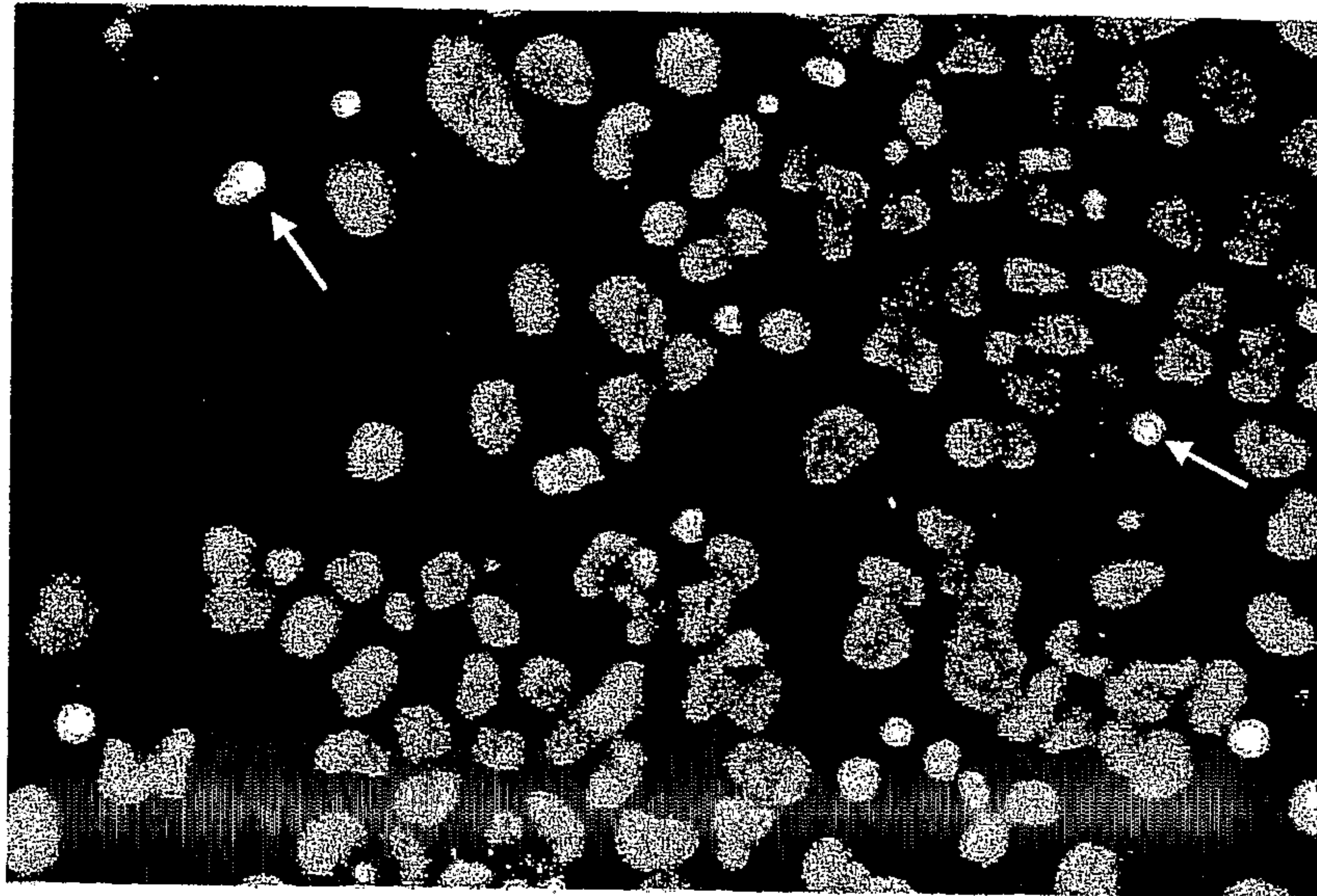
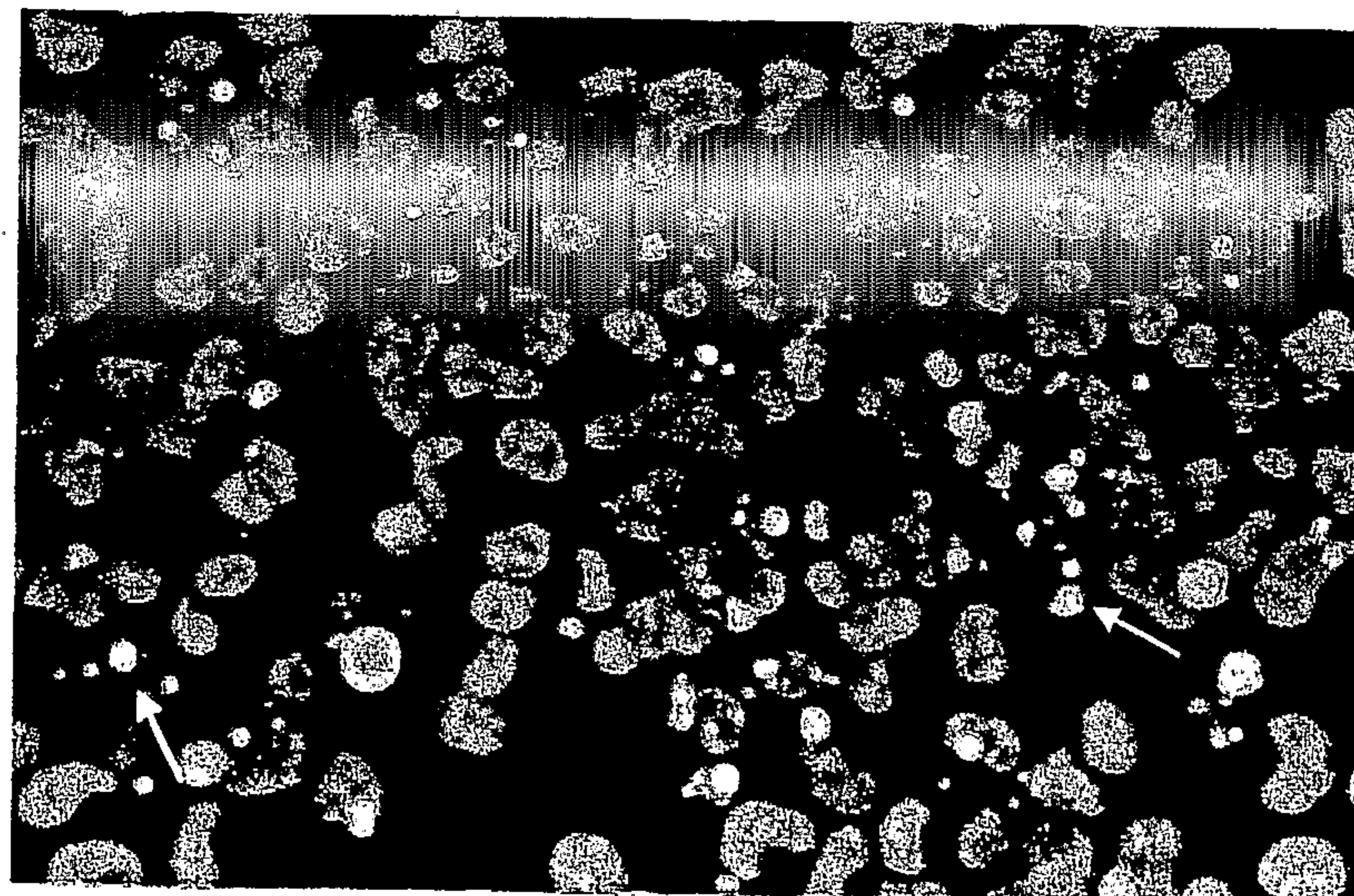


FIG.26

Hoescht Staining of Transformed
COS-7 Cells Deprived of Serum



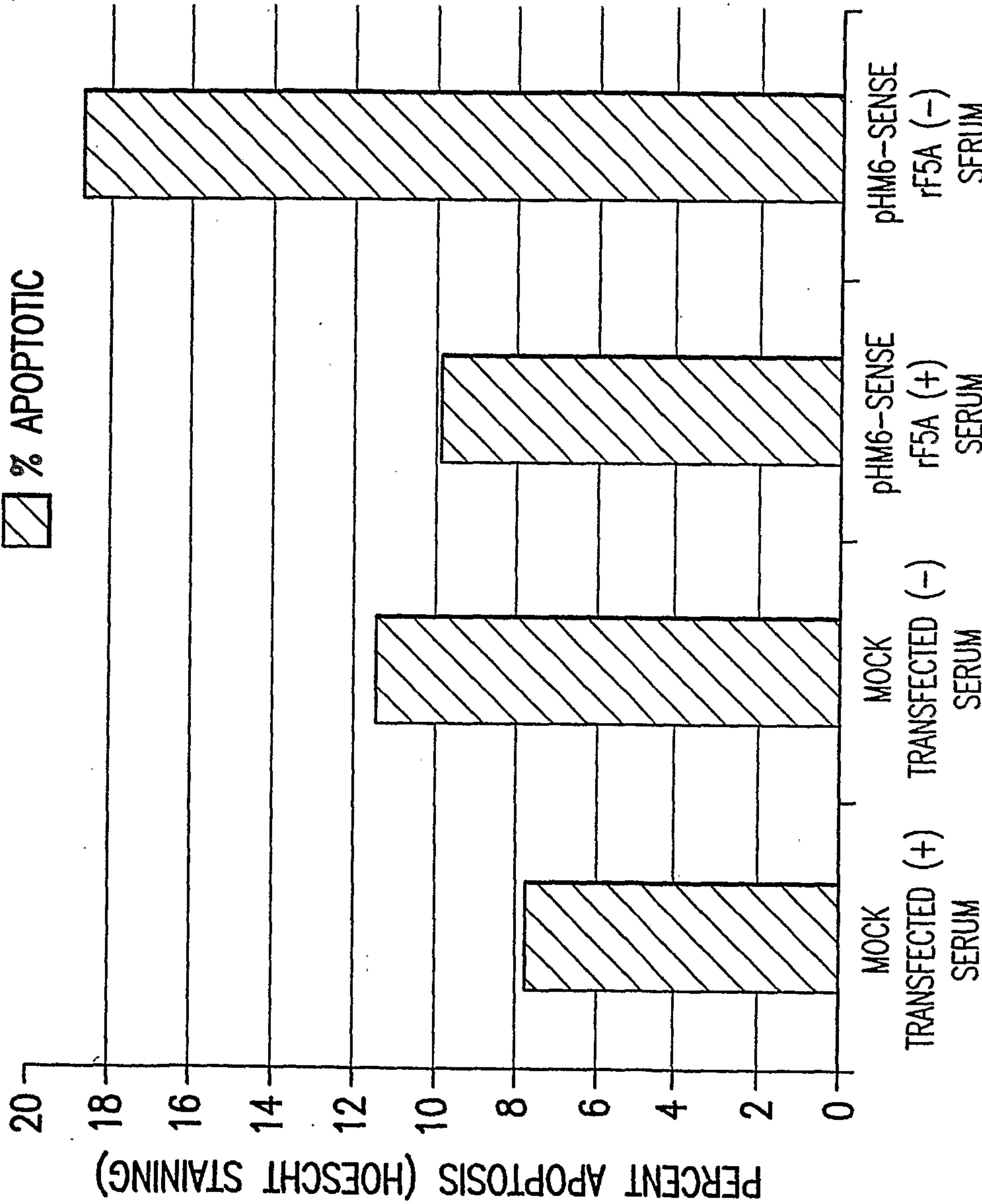
Mock Transformed



pHm6-Sense rat F5A

FIG.27

PERCENT APOPTOSIS OF TRANSIENTLY TRANSFECTED
COS-7 CELLS AFTER 48 HOURS OF SERUM STARVATION

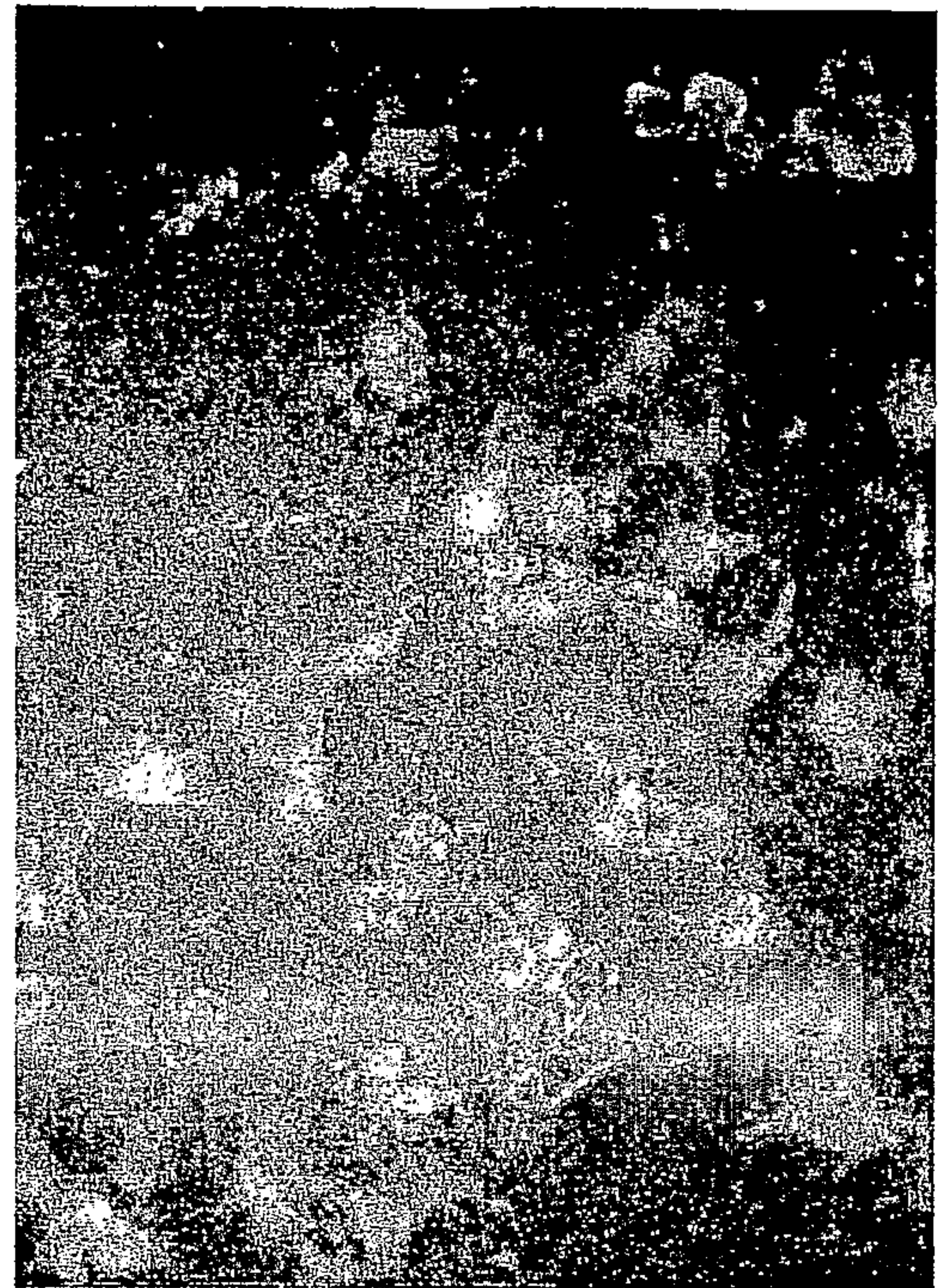


TRANSFECTION VECTOR

FIG. 28



Sense rF5A 20 X



Mock Transfected 20 X

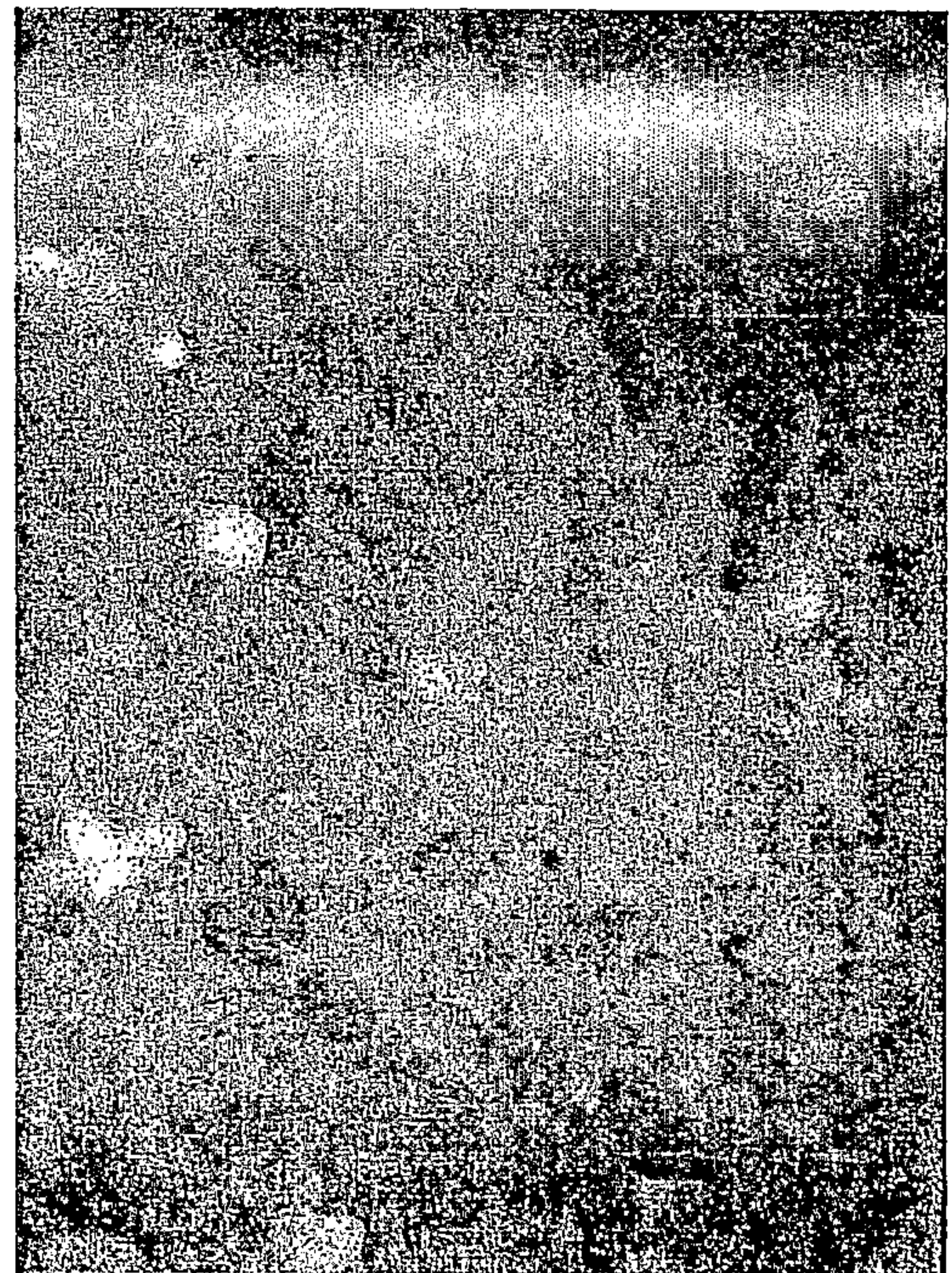


FIG. 29

PERCENT APOPTOSIS OF TRANSIENTLY TRANSFECTED
COS-7 CELLS AFTER 48 HOURS OF SERUM STARVATION

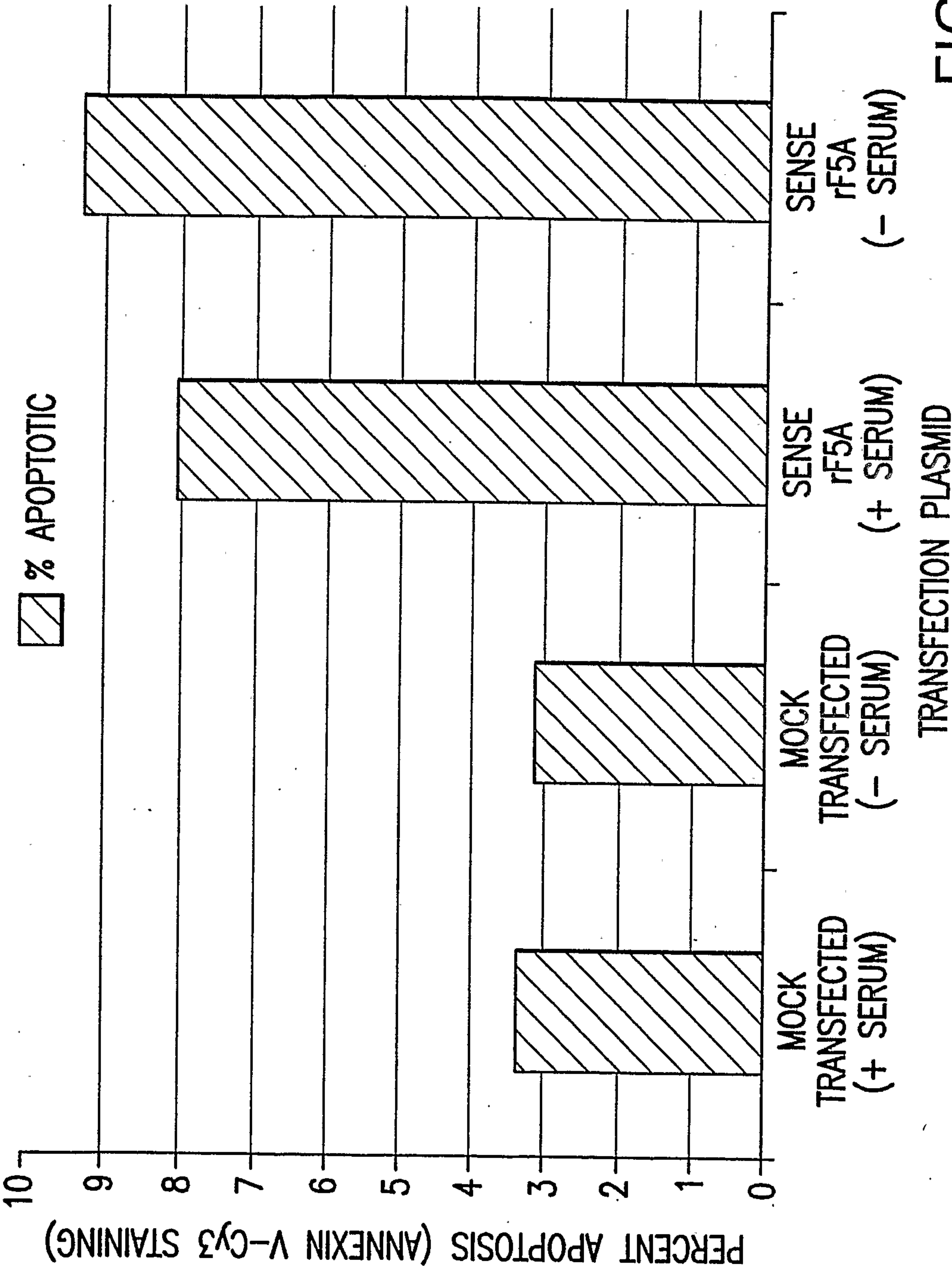


FIG.30

Condition	% Apoptosis (Hoescht Staining)
Mock Transfected Control	~5
Mock Transfected (+)	~11.5
pHM6-Sense rF5A	~11.5
pHM6-Sense rF5A (+)	~18.5

INCREASE IN PERCENT APOPTOSIS OF COS-7 CELLS TRANSIENTLY TRANSFECTED WITH pHM6--SENSE F5A OVER MOCK TRANSFECTED CONTROL CELLS

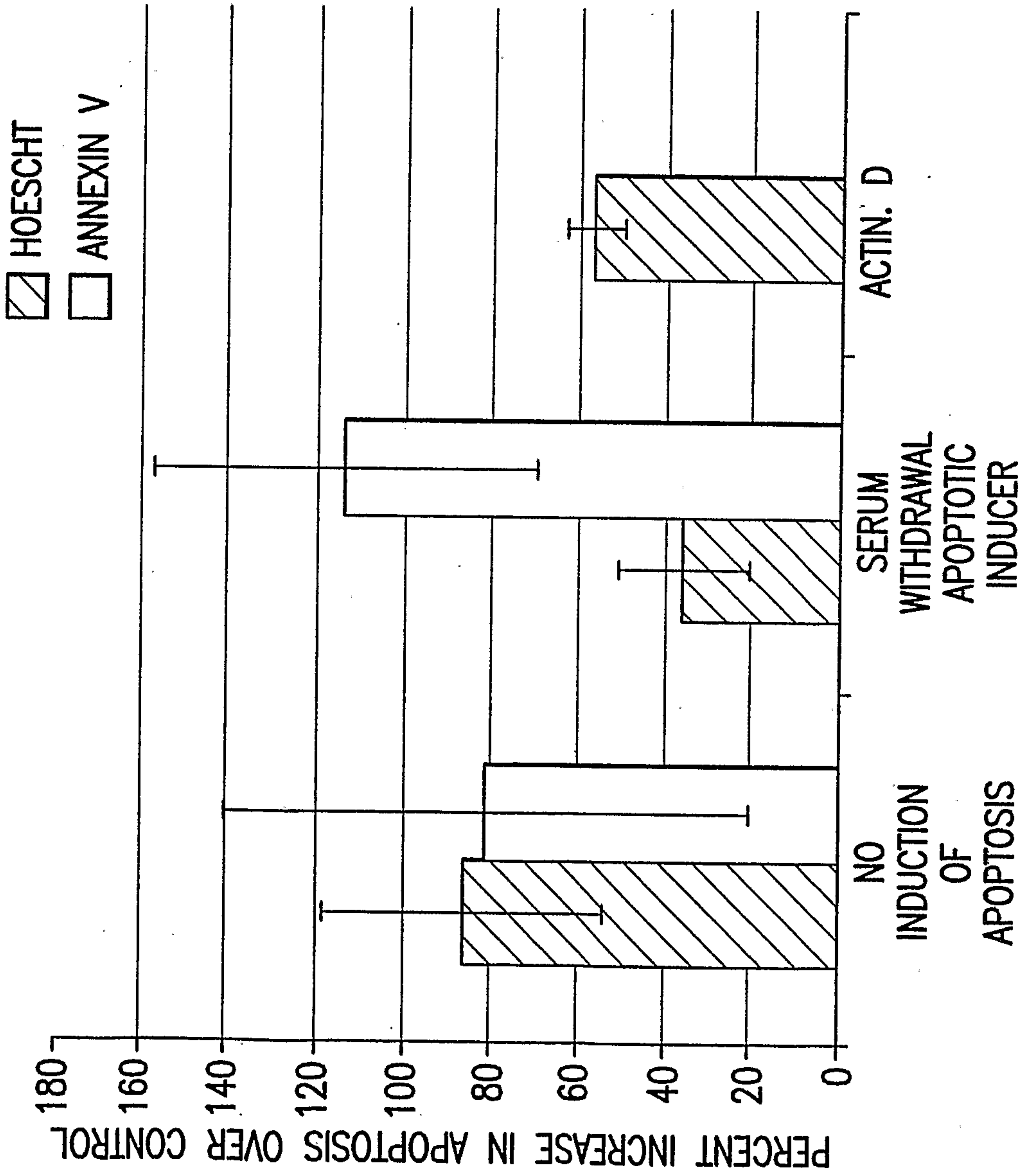


FIG.32

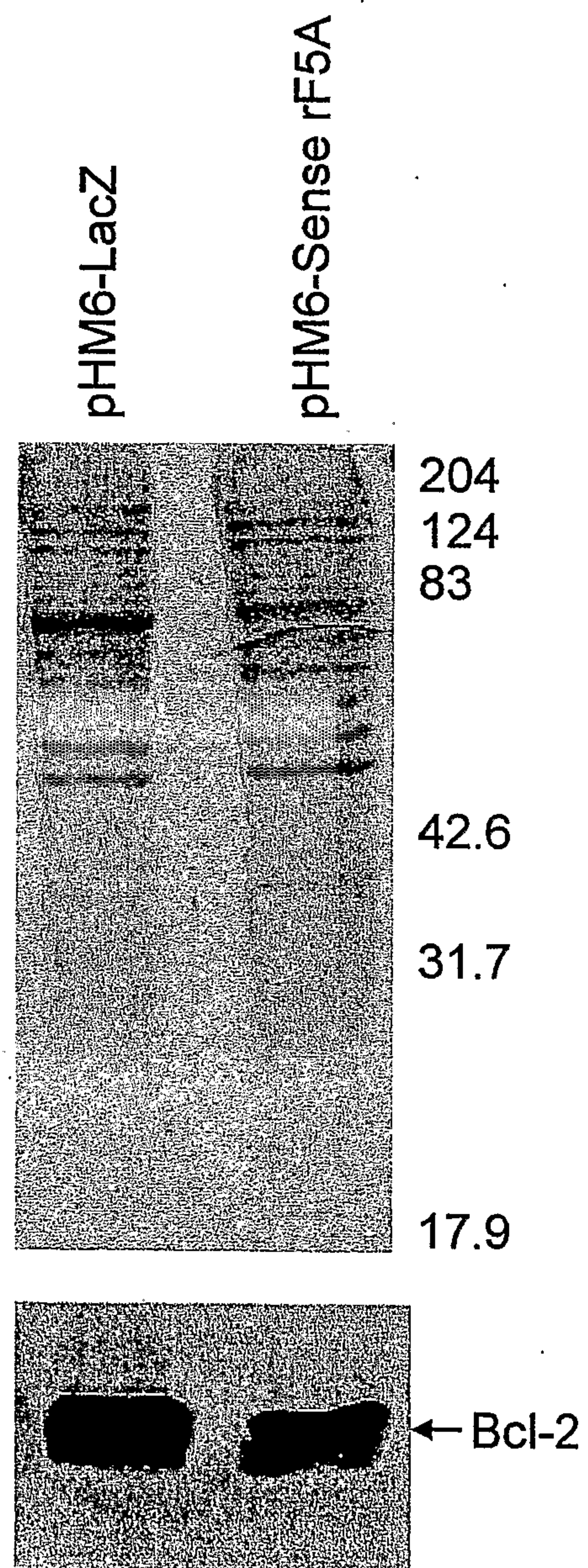


FIG.33

Bcl-2 Expression Levels in Transiently Transfected COS-7
Cells Serum-Starved for 48 Hours

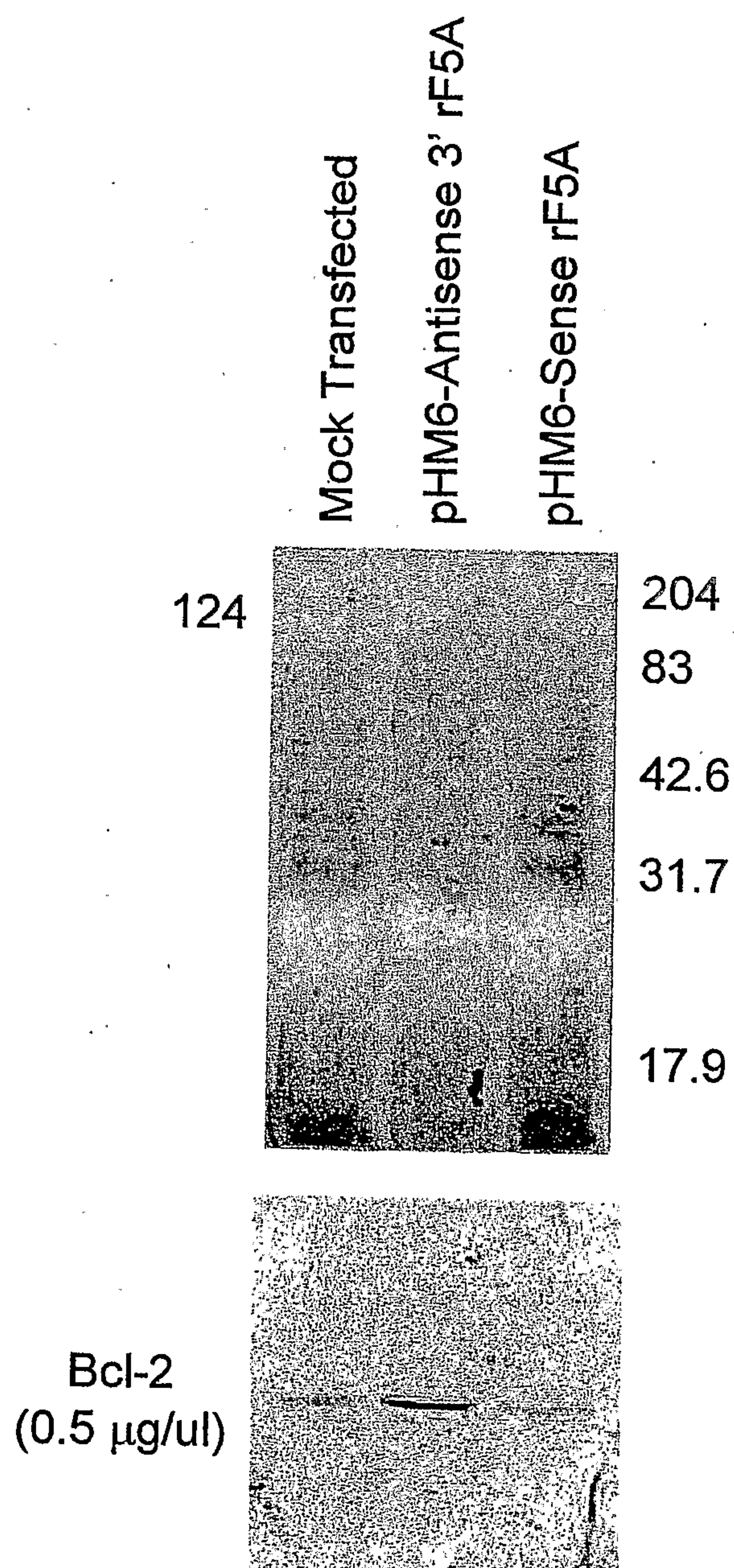


FIG.34

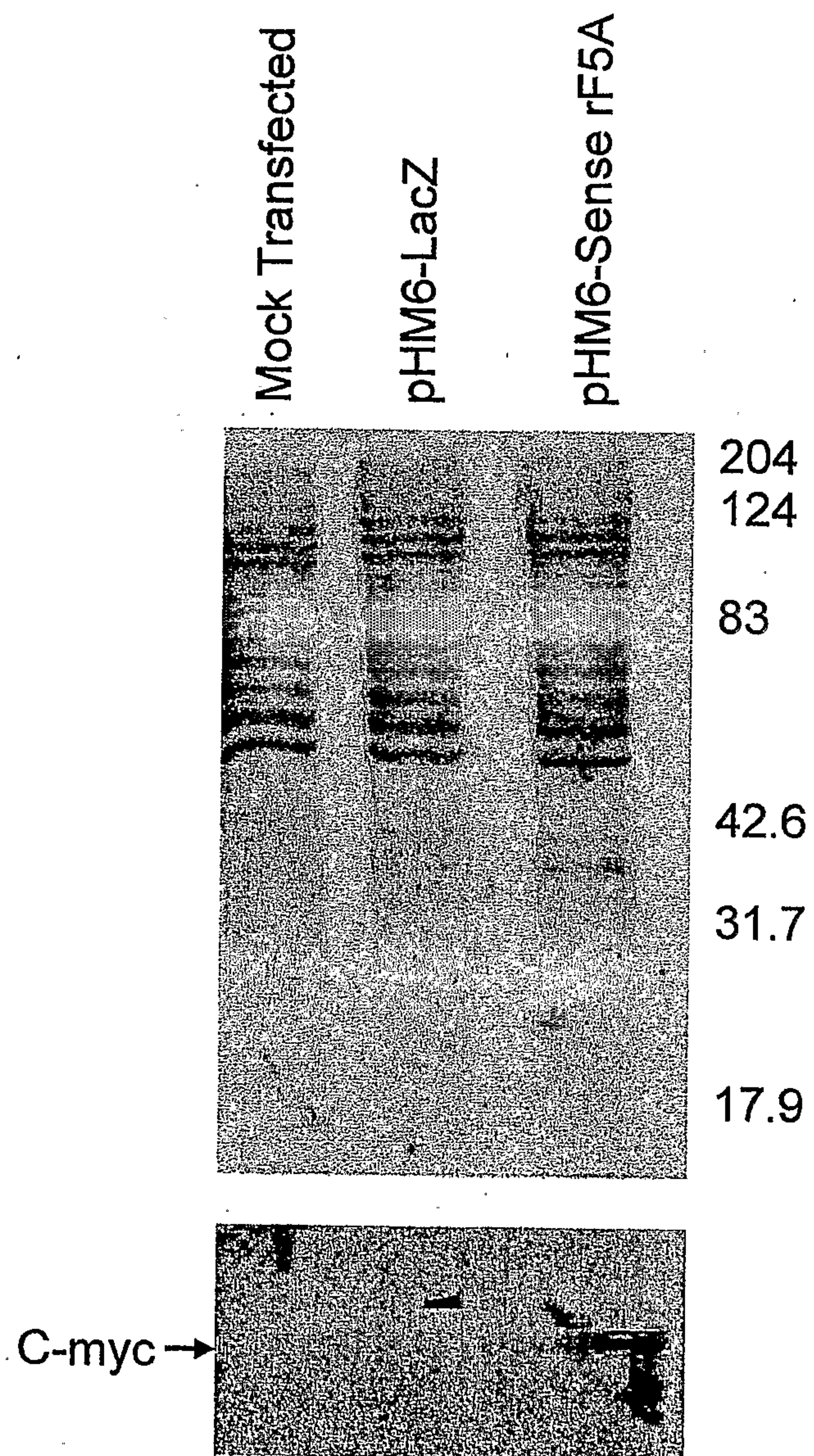


FIG.35

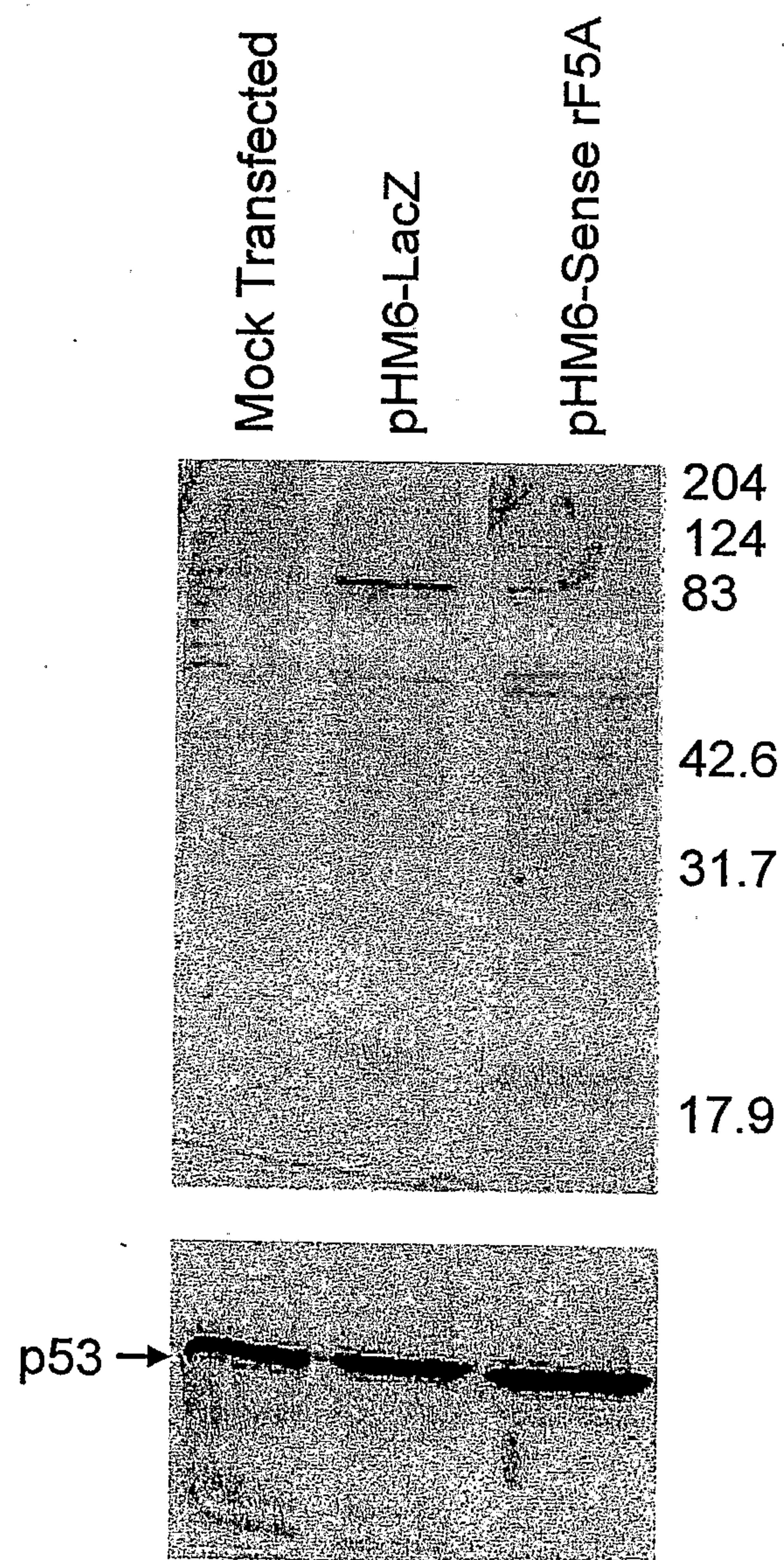


FIG.36

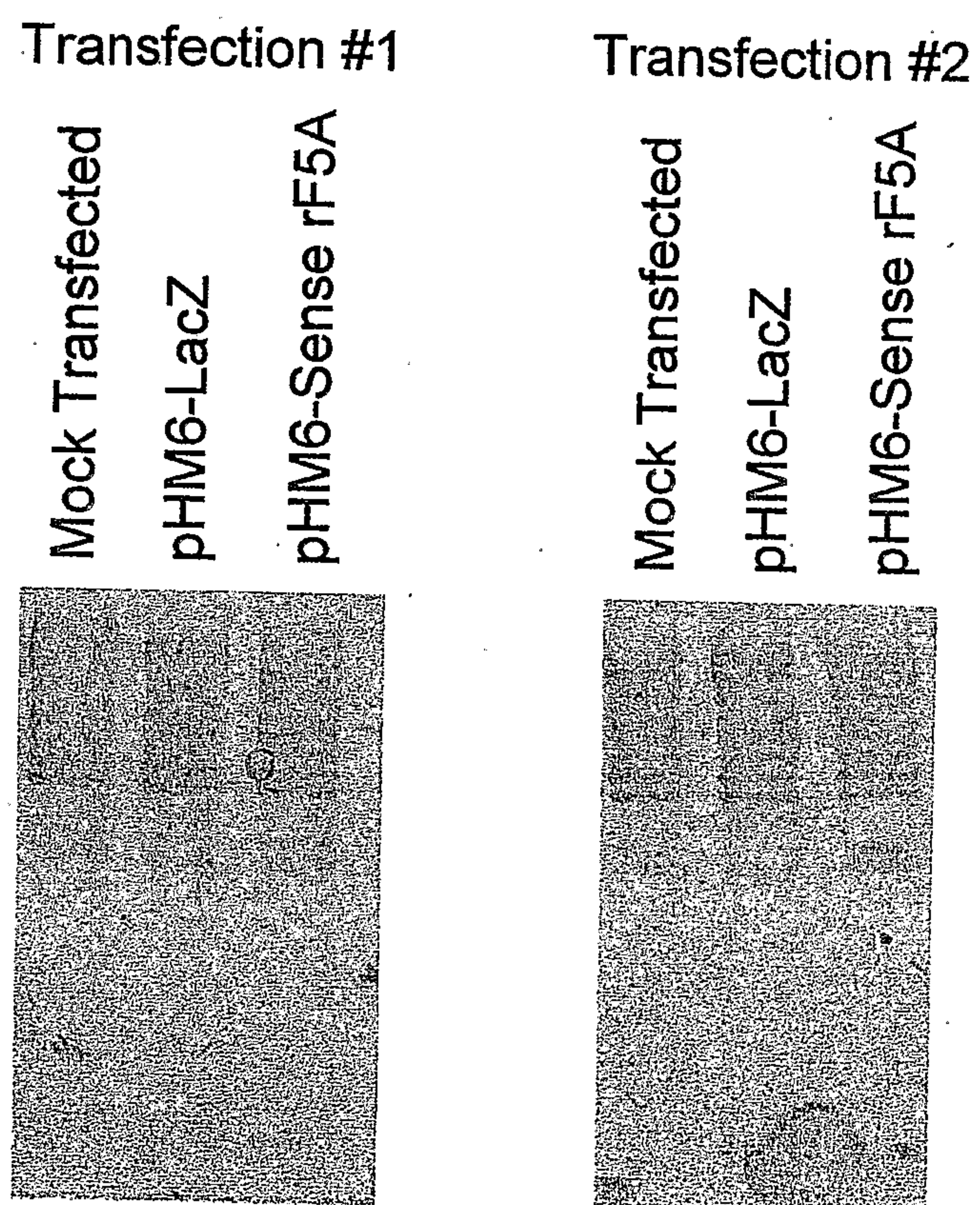


FIG.37A

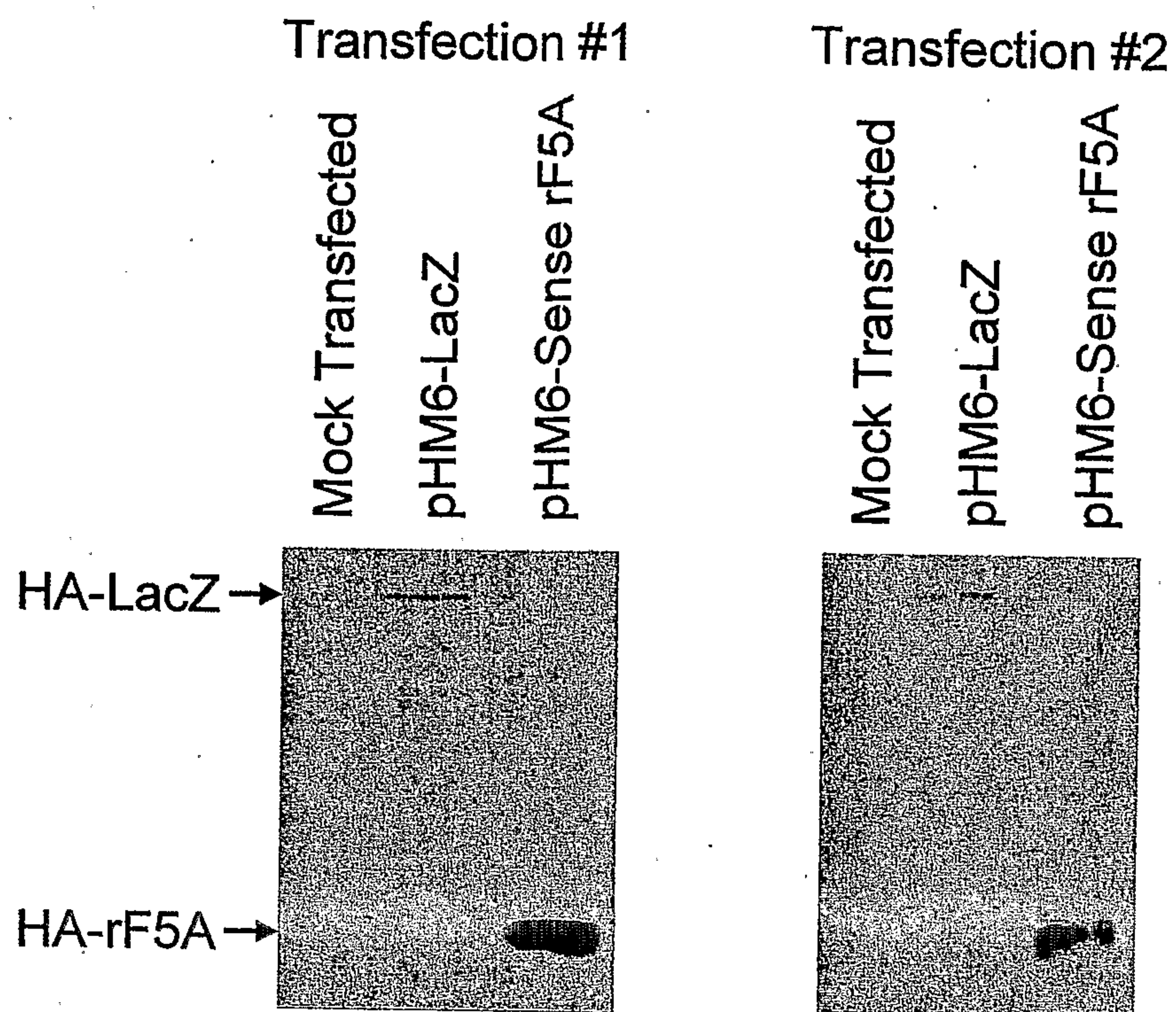


FIG.37B

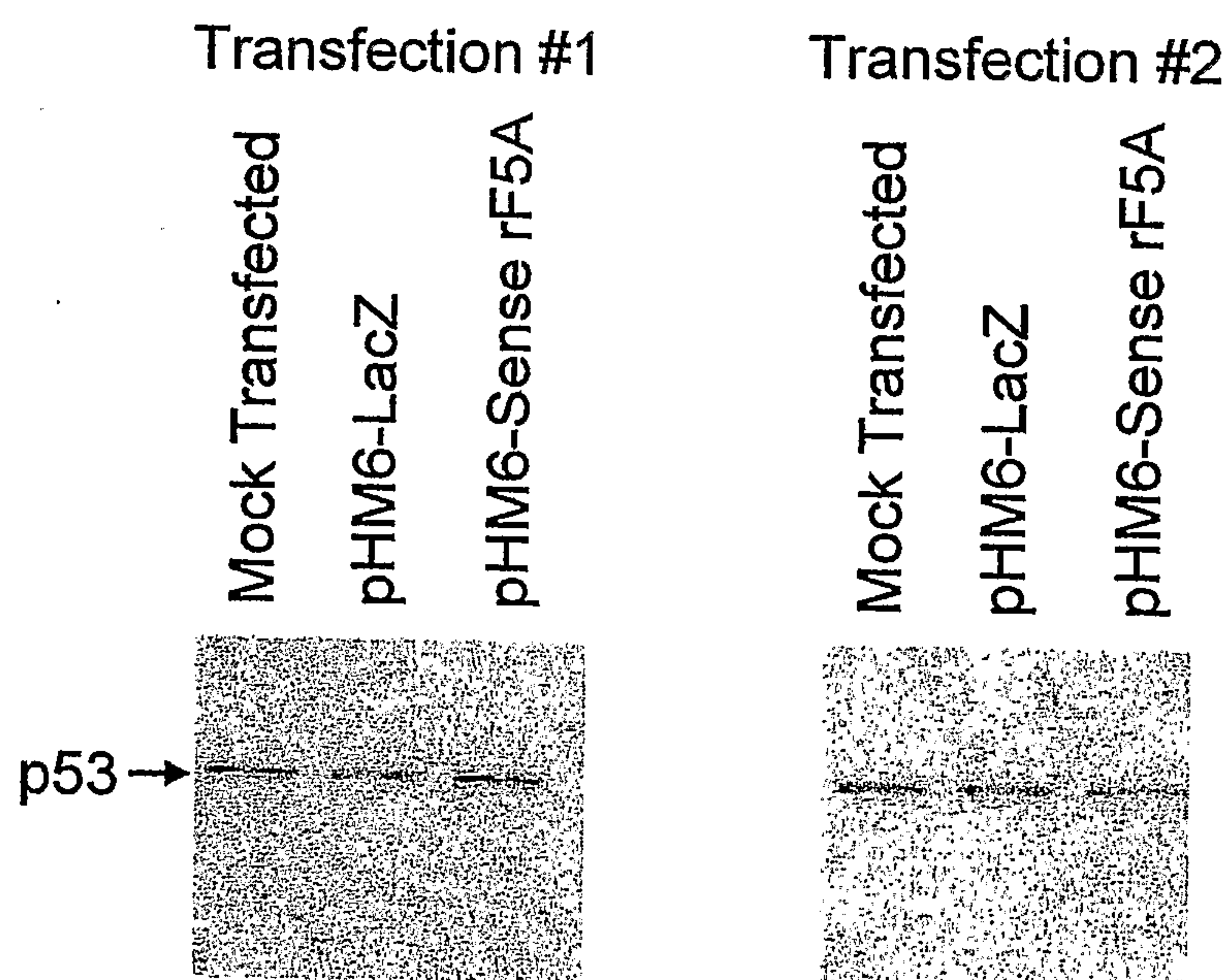


FIG.37C

Sequence and Alignment of human eIF5A2 isolated from RKO cells with
sequence of human eIF5A2 in Genbank (ACCESSION XM_113401)

XM_113401	MADEIDFTTG	DAGASSTYPM	QCSALRKNGF	VVLKGRPCKI	VEMSTSKTGK
PCR	MADEIDFTTG	DAGASSTYPM	QCSALRKNGF	VVLKGRPCKI	VEMSTSKTGK
Consensus	MADEIDFTTG	DAGASSTYPM	QCSALRKNGF	VVLKGRPCKI	VEMSTSKTGK
	51				100
XM_113401	HGHAKVHLVG	IDIFTGKKYE	DICPSTHNMD	VPNIKRNDYQ	LICIQDGYLS
PCR	HGHAKVHLVG	IDIFTGKKYE	DICPSTHNMD	VPNIKRNDYQ	LICIQDGCLS
Consensus	HGHAKVHLVG	IDIFTGKKYE	DICPSTHNMD	VPNIKRNDYQ	LICIQDGcLS
	101				150
XM_113401	LLTETGEVRE	DLKLPEGELG	KEIEGKYNAG	EDVQVSMCA	MSEYAVAIK
PCR	LLTETGEVRE	DLKLPEGELG	KEIEGKYNAG	EDVQVSMCA	MSEYAVAIK
Consensus	LLTETGEVRE	DLKLPEGELG	KEIEGKYNAG	EDVQVSMCA	MSEYAVAIK
	151				
XM_113401	PCK				
PCR	PCK				
Consensus	PCK				

FIG.38

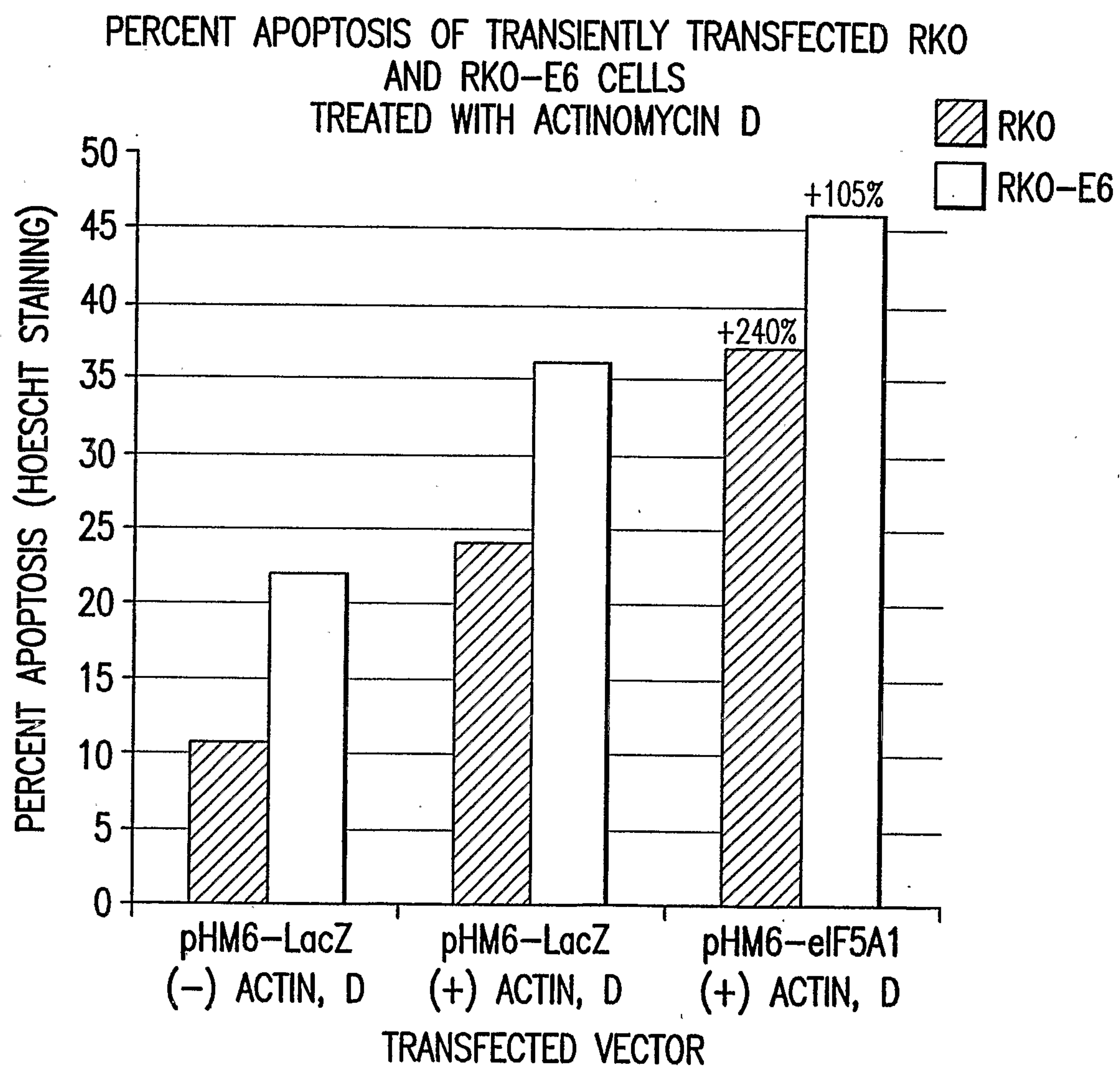


FIG.39

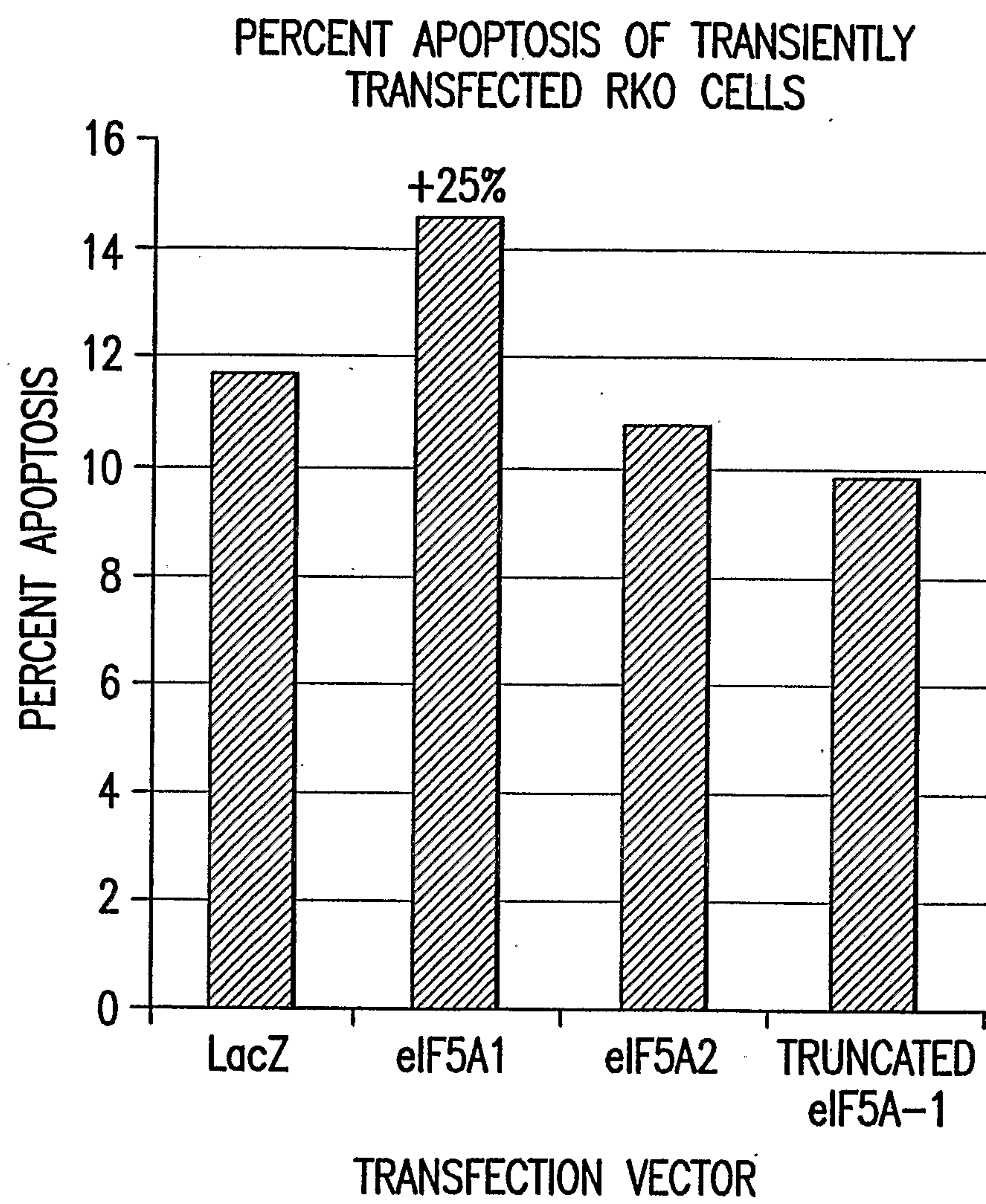


FIG.40

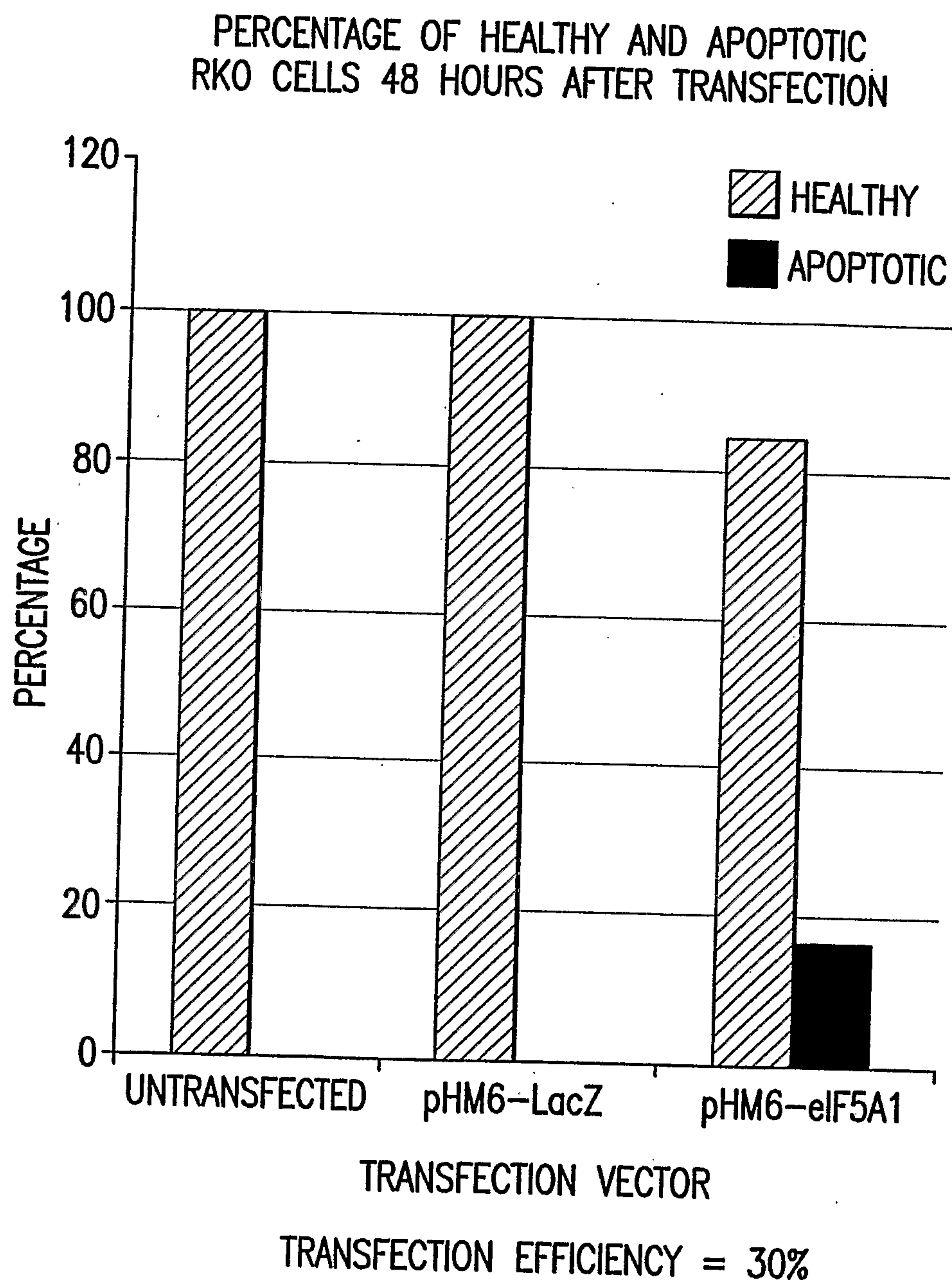


FIG.41

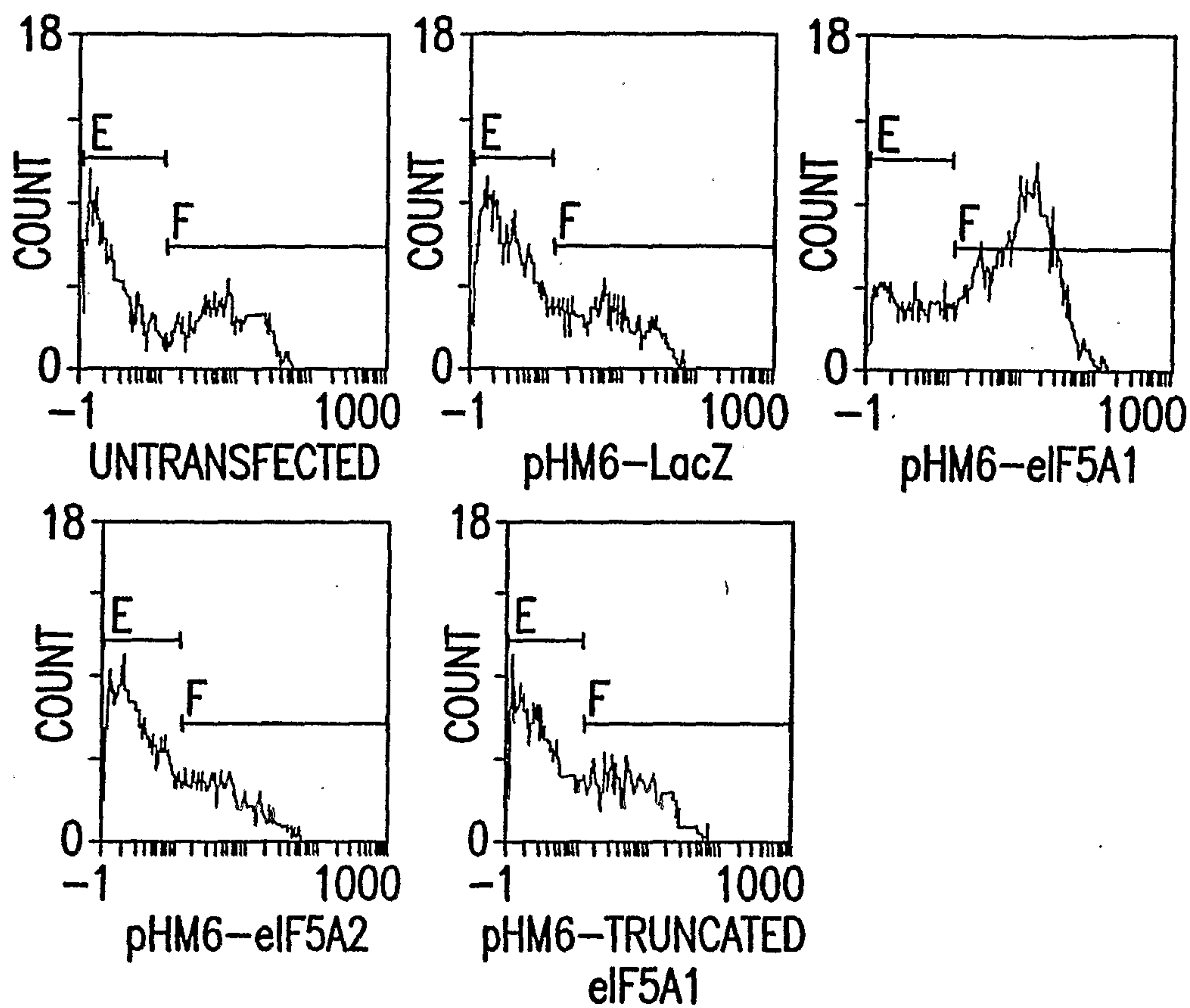
TRANSFECTION VECTOR

FIG.42A

TRANSFECTED PLASMID	PERCENT APOPTOSIS
UNTRANSFECTED	21.2%
pHM6-Lac Z	21.7%
pHM6-eIF5A1	60.7% (80%)*
pHM6-eIF5A2	20.5%
pHM6-TRUNCATED eIF5A1	24.1%

*CORRECTED FOR BACKGROUND APOPTOSIS IN UNTRANSFECTED CELLS AND FOR TRANSFECTION EFFICIENCY

FIG.42B

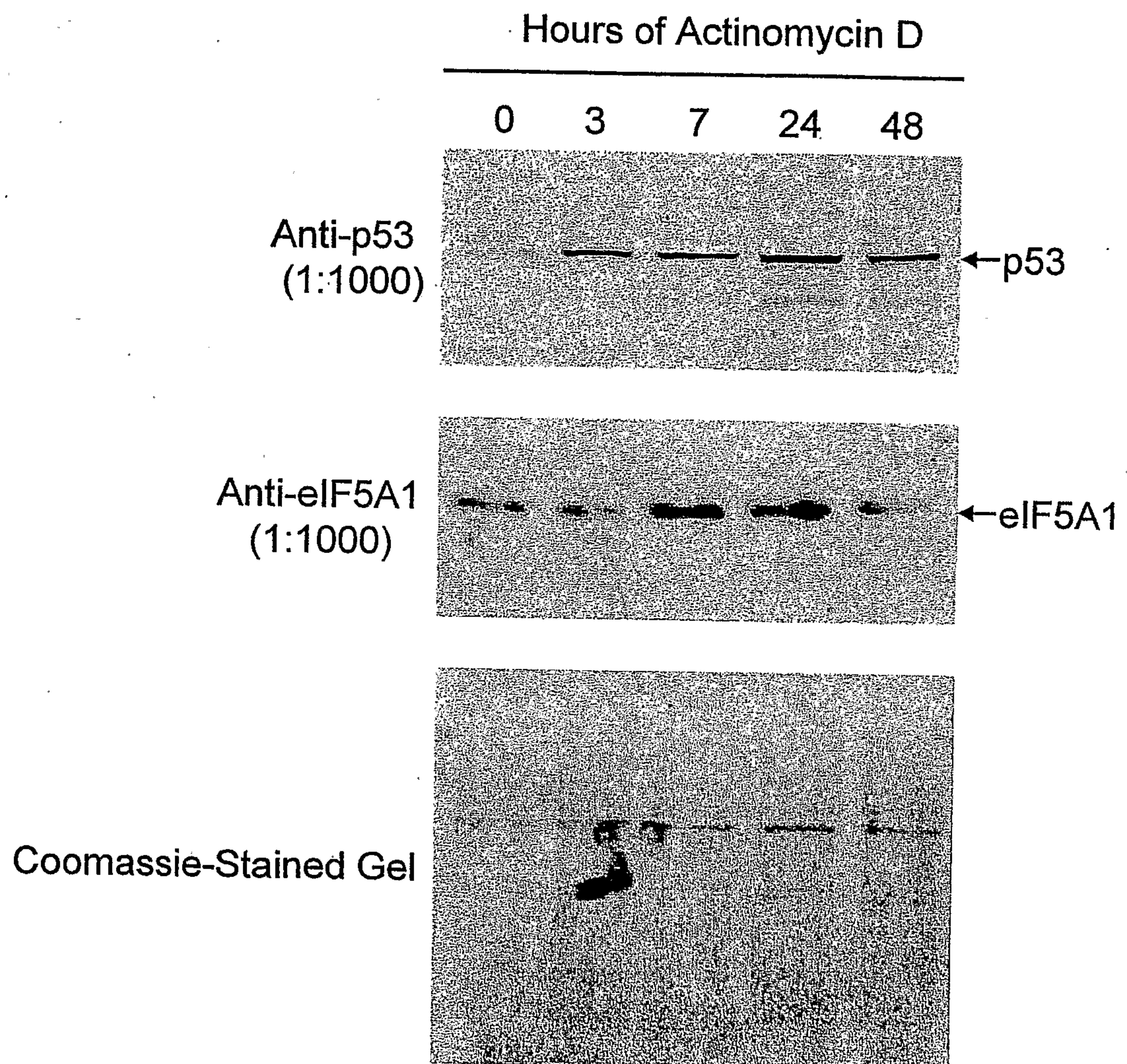


FIG.43

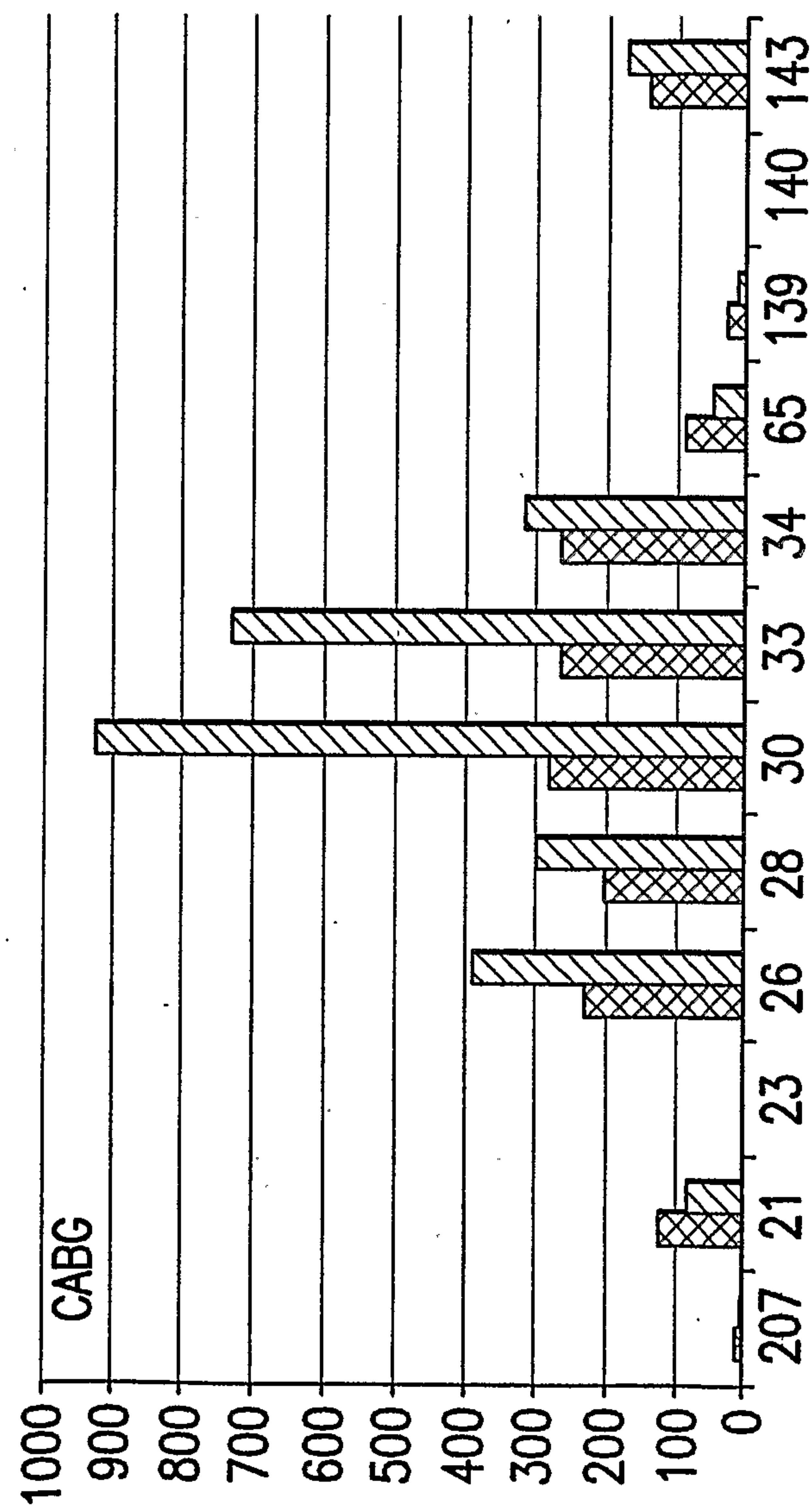


FIG. 44

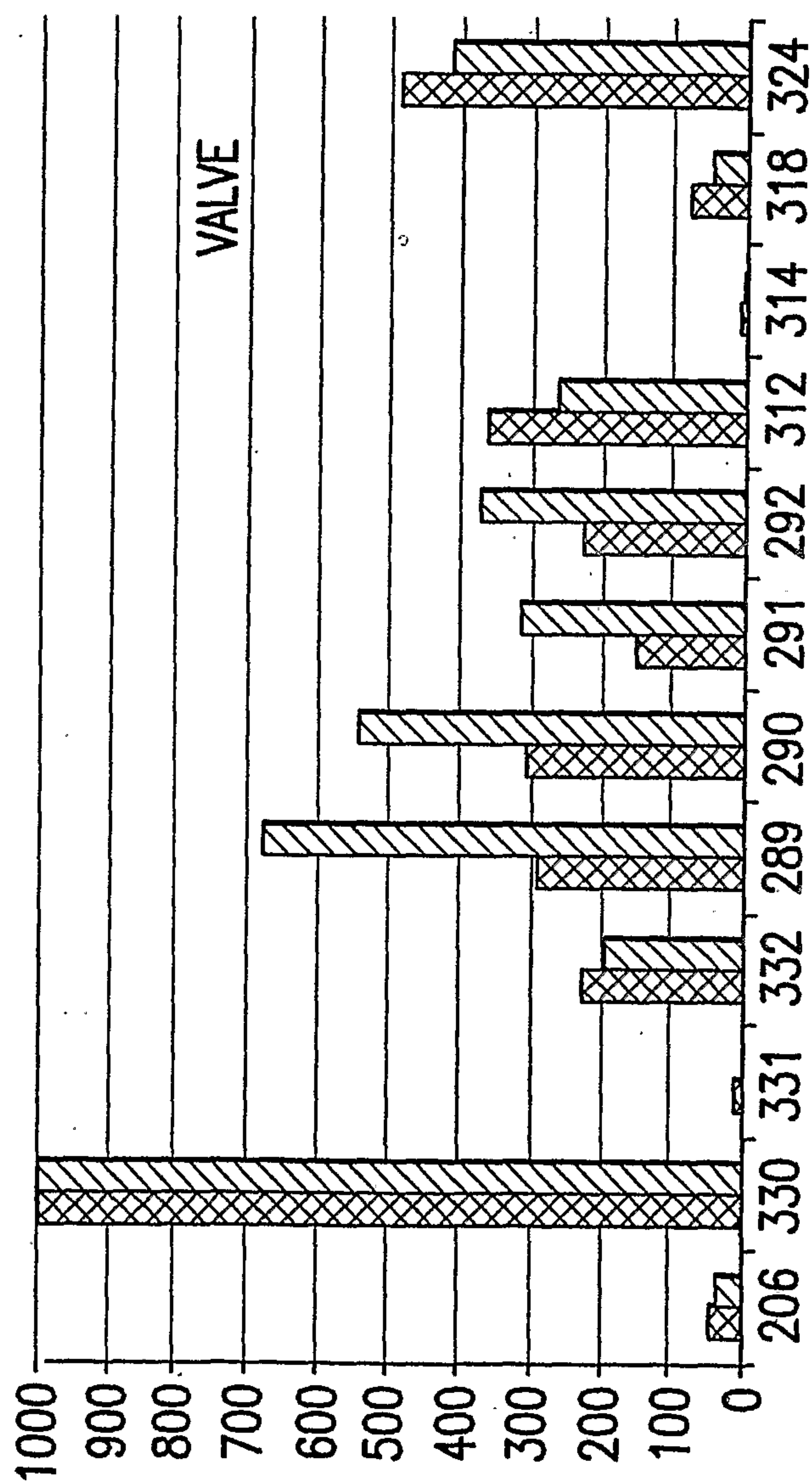


FIG.45

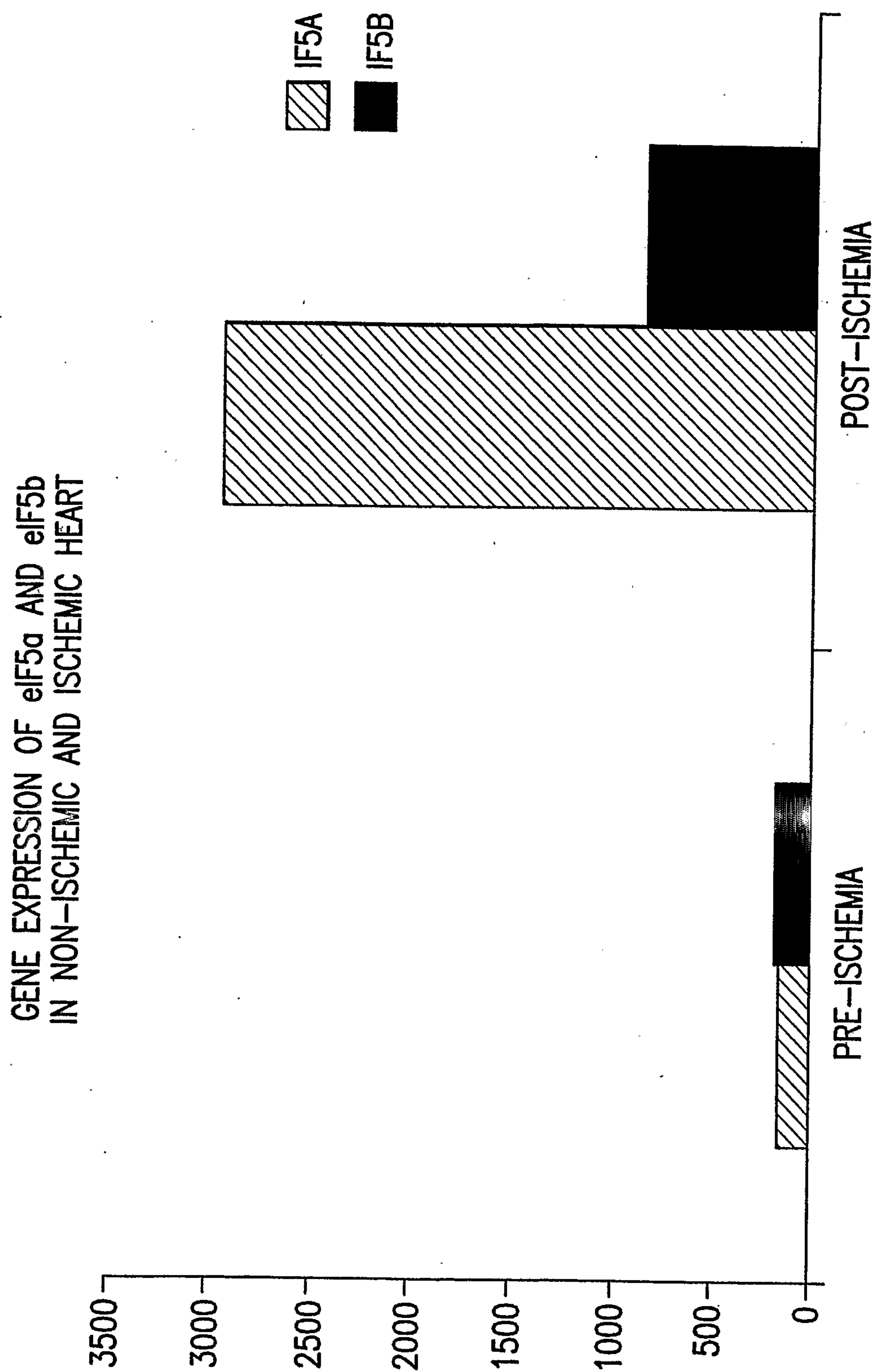
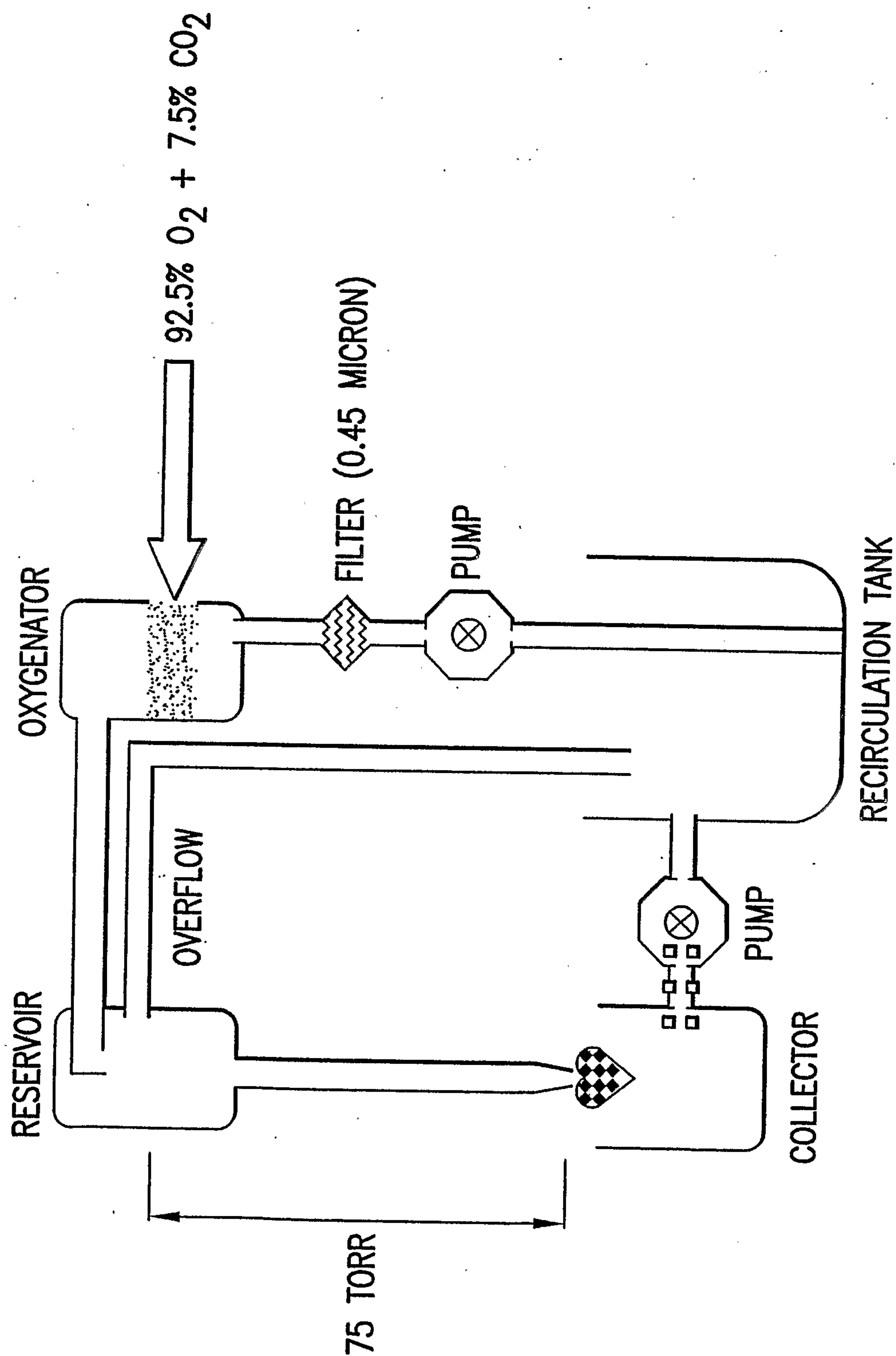


FIG. 46



HEATED TO 37°C

FIG. 47

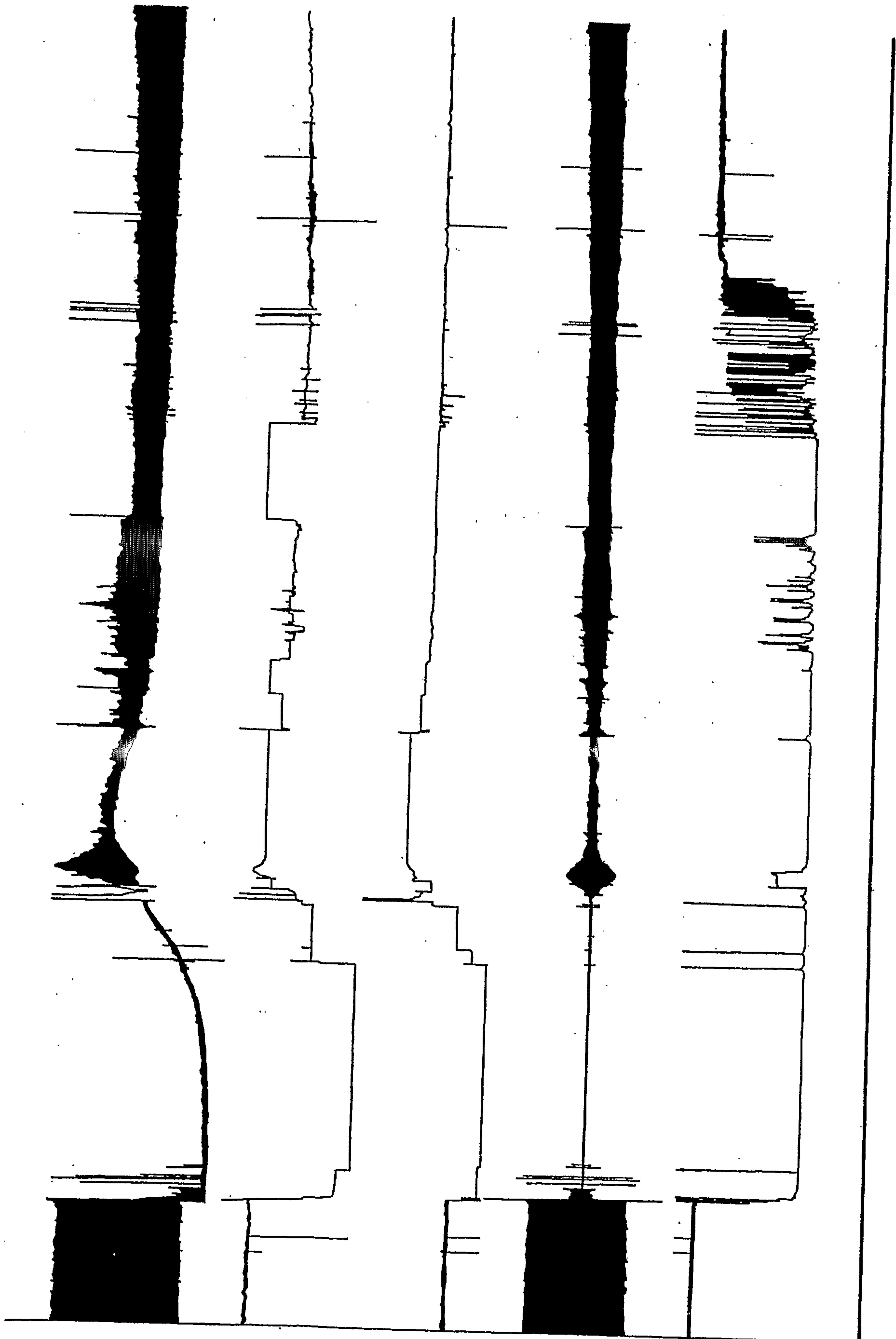


FIG. 48

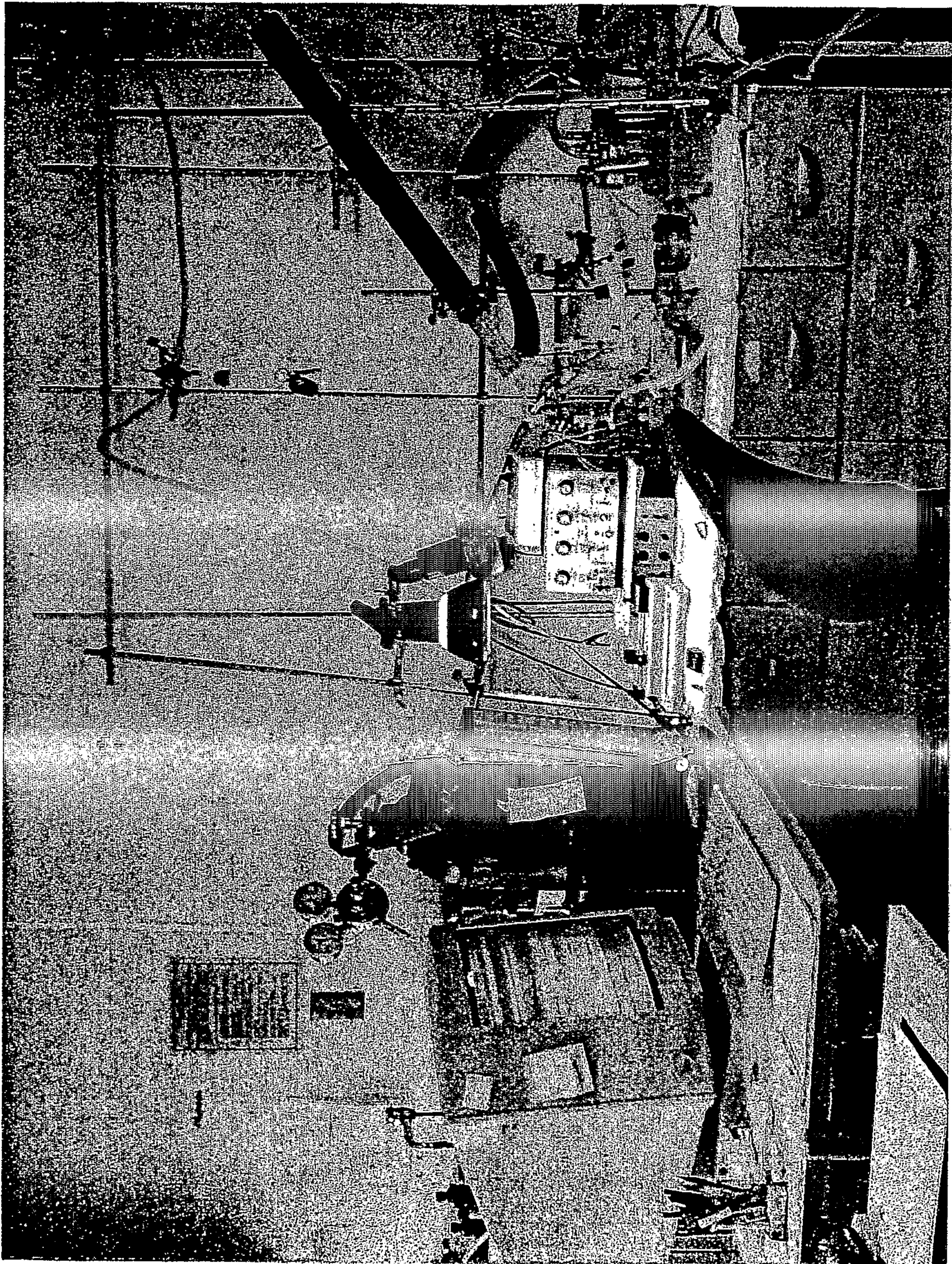


FIG. 49

PATIENT	Ds	IF5a1 pg/ng rRNA	IF5a2 pg/ng rRNA	0.5	2.0	IL-18 pg/ng rRNA	IL-1b pg/ng rRNA
207	CABG	9.0	2.4	3.8	0.3	10.1	7.2
21	CABG	123.2	81.6	1.5	0.7	405.9	41.5
23	CABG	0.1	0.0	11.0	0.1	0.0	0.0
26	CABG						
28	CABG	200.4	294.6	0.7	1.5	894.1	49.7
30	CABG	279.4	921.2	0.3	3.3	669.8	71.0
33	CABG	260.1	723.9	0.4	2.8	545.6	105.1
34	CABG	263.4	309.3	0.9	1.2	520.0	19.9
65	CABG	82.9	43.1	1.9	0.5	98.2	20.2
139	CABG	23.9	7.0	3.4	0.3	22.7	57.3
140	CABG	0.9	0.0	#DIV/0!	0.0	0.1	0.0
143	CABG	137.7	169.5	0.8	1.2	338.7	77.9

FIG.50A

PATIENT	Ds	IF5a1 pg/ng rRNA	IF5a2 pg/ng rRNA	0.5	2.0	IL-18 pg/ng rRNA	IL-1b pg/ng rRNA
206	VALVE	44.4	33.1	1.3	0.7	50.0	67.2
330	VALVE						
331	VALVE	11.3	0.2	68.9	0.0	0.1	0.0
332	VALVE	224.5	194.0	1.2	0.9	510.5	37.2
289	VALVE	286.3	674.1	0.4	2.4	888.0	167.5
290	VALVE	303.9	540.4	0.6	1.8	545.8	20.6
291	VALVE	148.1	311.8	0.5	2.1	229.2	31.1
292	VALVE	221.1	367.2	0.6	1.7	280.8	55.0
312	VALVE	361.1	262.5	1.4	0.7	198.0	44.2
314	VALVE	6.5	1.6	4.0	0.2	74.7	18.6
318	VALVE	83.3	48.5	1.7	0.6	69.3	13.3
324	VALVE	485.9	414.1	1.2	0.9	584.9	98.6

FIG.50B

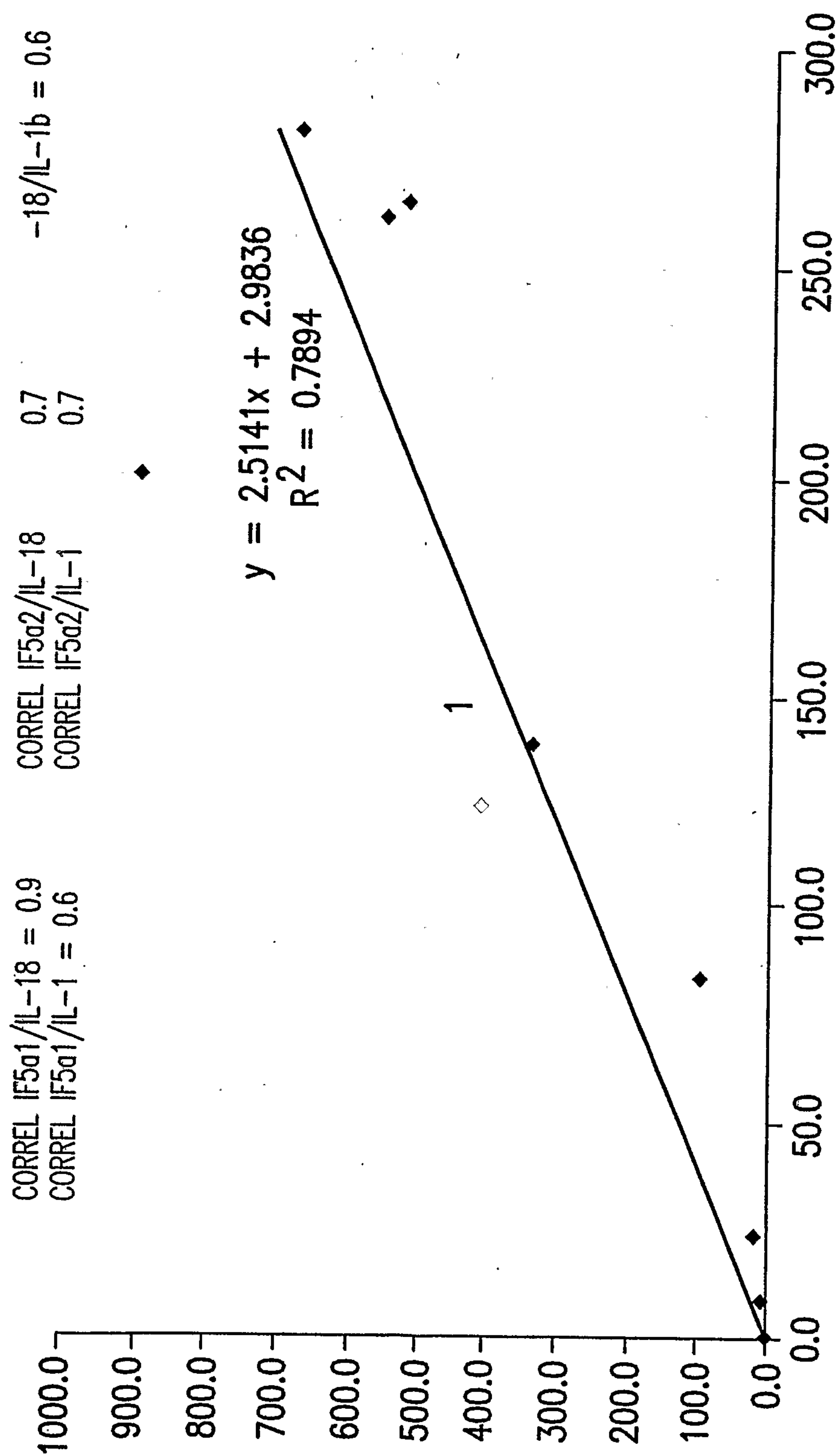


FIG. 50C

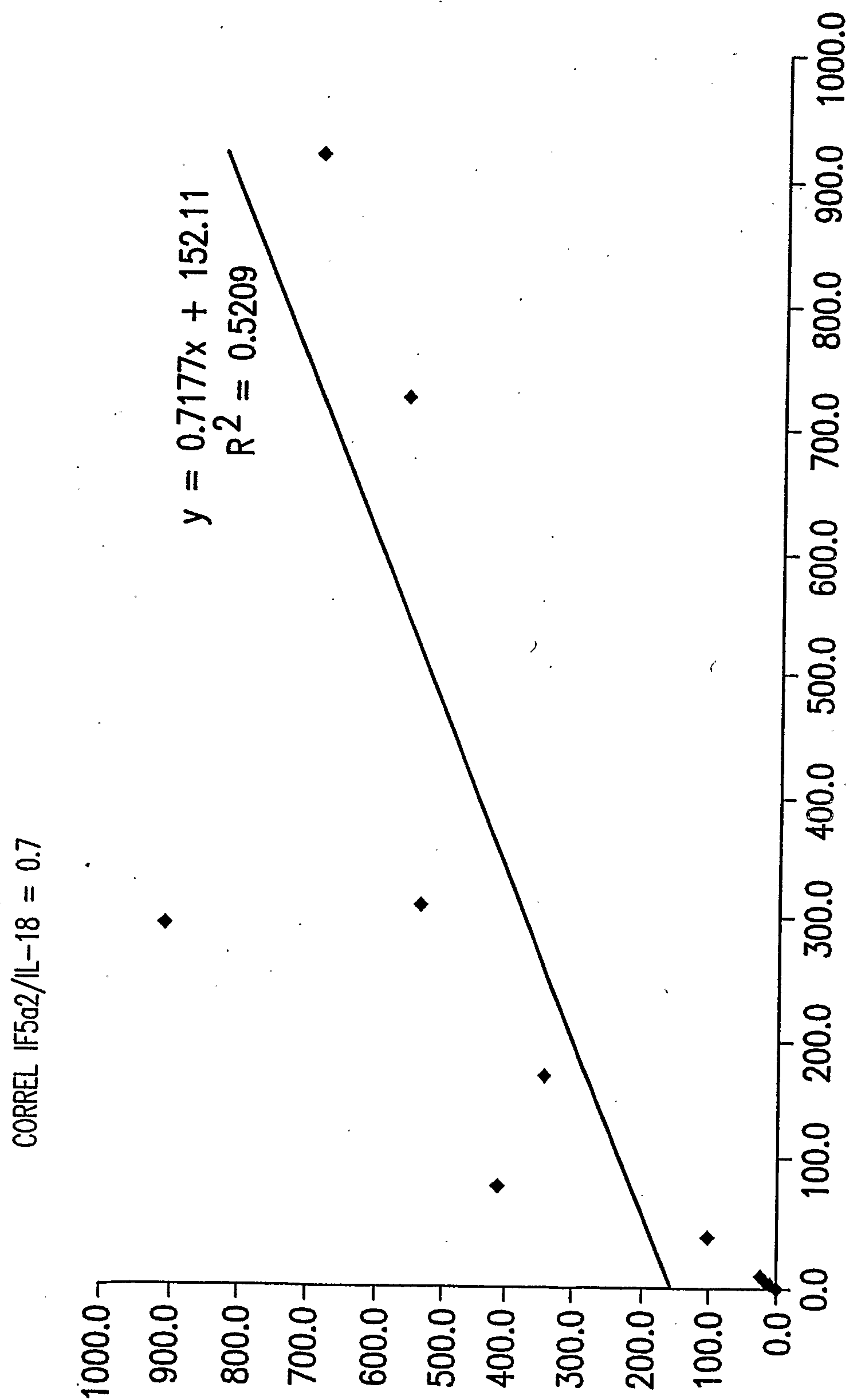


FIG. 50D

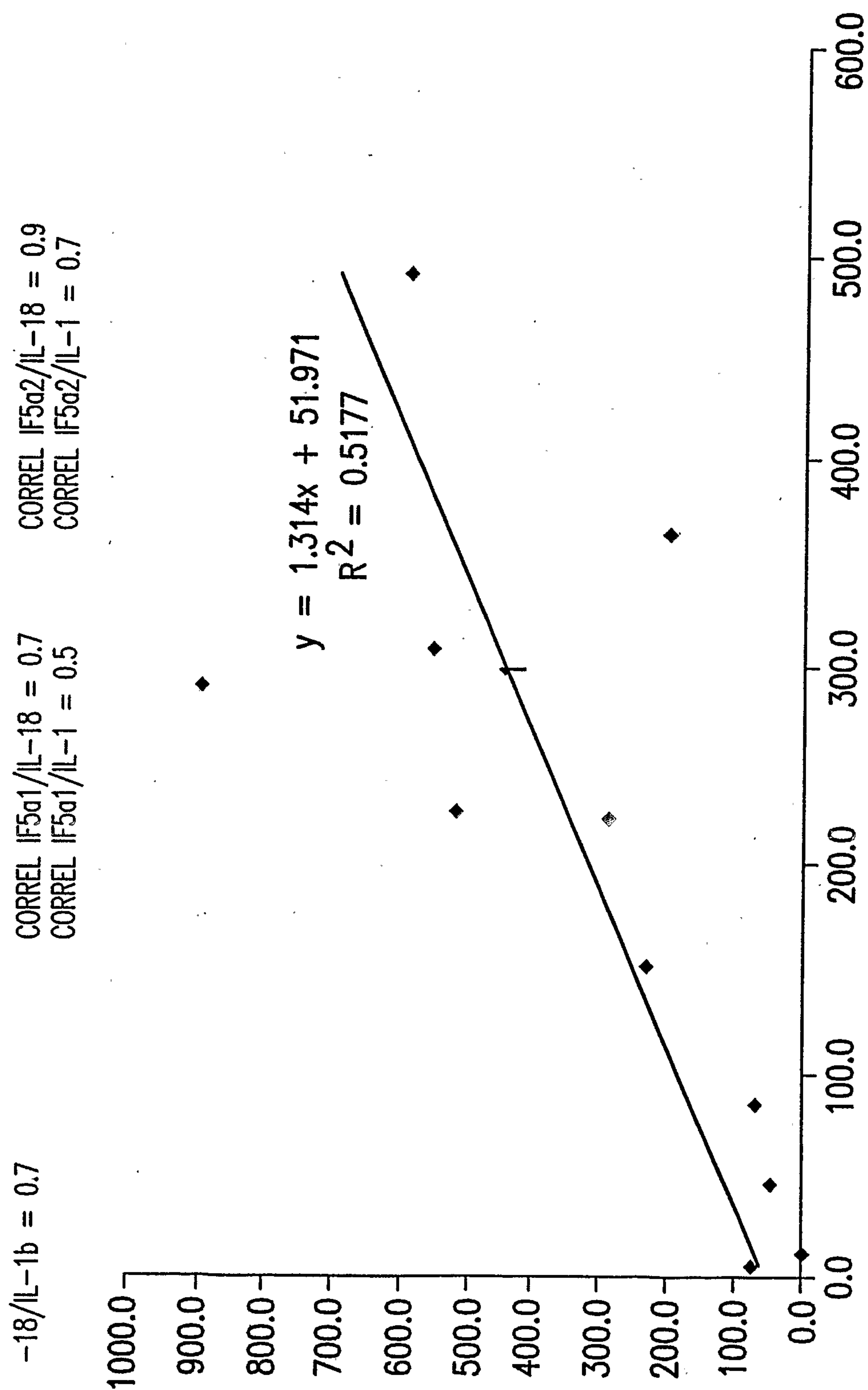


FIG. 50E

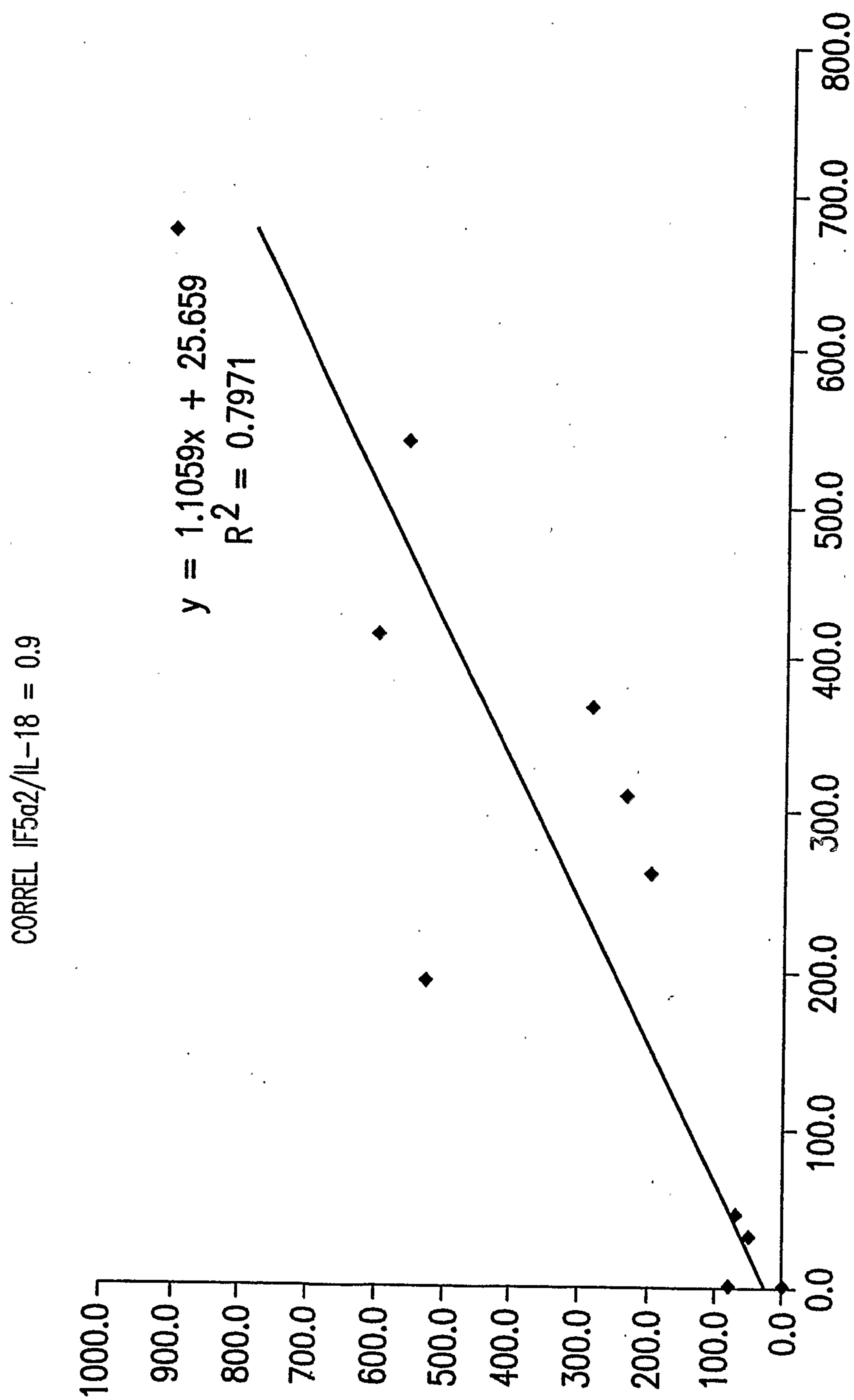


FIG. 50F

Expt #	Age	Sex	DM	Unstable engine	Operation	Misc....	date RNA
19	61	M	Y	N	CABG	Metoprolol, Lovastatin, NTG	
20	72	M	Y	N	CABG	Metoprolol, Lipitor	
21	55	M	N	N	CABG	CYA, Imuran, Prednisone, Lasix, INS, Metoprolol	7/30/02
23	71	M	N	N	CABG	Imipramine, Isordil, NTG, Metoprolol	7/30/02
24	75	M	Y	Y	CABG	Lisinopril, Glipizide, Metoprolol, NTG	
?	61	M	N	N	CABG	Lisinopril, digoxin, Lasix, NTG, ASA	
26	57	M	N	N	CABG	Atenolol, Norvasc, HCTZ, NTG, ASA	7/30/02
28	48	F	N	N	CABG	Atenolol, INS, Lisinopril	9-Aug
29	64	M	N	N	MVR	Lasix, Lisinopril	
30	76	M	N	N	CABG		9-Aug
31	65	M	Y	N	CABG		
33	54	M	N	N	CABG	Simvastatin, metoprolol	9-Aug
34	63	M	N	N	CABG	Lisinopril, Felodipine, NTG	9-Aug
35	74	M	Y	N	CABG	Amlodipine, Glyburide, Prazosin	
36	62	M	Y	N	CABG	Simvastatin, INS, Felodipine, Spironolactone, Metoprtolol, Lisinopril,	
37	63	M	N	N	CABG	Famotidine, Atenolol, Fosinopril, Procardia, Zocor, NTG	
38	63	M	N	N	CABG	A-fib, Dig, Atenolol, Zocor, Coumadin	

FIG. 51

CONT'D ON FIG.51A

CONT'D FROM FIG.51

39	77	M	N	N	CABG	Coumadin, Cozar, Zocor, HCTZ, Cortioarone	
41	74	F	N	Y	AVR/CABG	Dig, Zestril	
42	59	M	Y	N	CABG	Statin, Atenolol, Glucotrol, Lisinopril	
44	64	M	Y	N	CABG	Atenolol, NTG, Lisinopril, Simvastatin, Verapamil	
47	64	M	N	N	CABG	NTG, Lisinopril, Synthroid, simvastatin	
48	56	M	Y	Y	CABG	NTG, Metoprolol, Glipizide, Simvastatin	
52	70	M	Y	N	CABG	Fosinopril, Glipizide, HCTZ, Felodipine, Metoprolol,	
53	55	M	Y	N	CABG	Lasix, Lisinopril, Glyburide, Nifedipine	
54	48	M	Y	Y	CABG	Lisinopril, Metoprolol, NTG, Lasix, ASA<Simvastatin, INS	
55	67	M	N	N	CABG	ASA, prednisone, Atenolol	
56	71	M	Y	N	CABG	ASA, Simvastatin, Atenolol, Lisinopril, NTG	
58	70	M	Y	N	CABG	Coumadin, Synthroid, NTG, Glyburide	
59	55	M	Y	N	CABG	Lisinopril, NTG, Glyburide, metoprolol, Simvastatin	
60	55	M	Y	N	CABG	Metformin, Glyburide, ASA, Atenolol, Lisinopril, Simvastatin	
61	68	M	Y	N	CABG	Atenolol, Lasix, Fosinopril, Simvastatin, Glyburide	
61	68	M	Y	N	CABG	Atenolol, Lasix, Fosinopril, Simvastatin, Glyburide	
62	76	M	N	N	CABG	Gemfibrozil, ASA, Metoprolol, NTG	
64	61	M	N	N	MVR		

CONT'D ON FIG.51B

FIG.51A

CONT'D FROM FIG.51A

64	61	M	N	N	N	MVR		
65	72	M	N	N	Y	CABG	ASA, NTG	12-Sep
67	69	M	Y	N	N	CABG		
138	75	M	Y	N	N	CABG	Lisinopril, Simvastatin, metoprolol, Metformin, glyburide	
139	71	M	N	N	Y	CABG	Metoprolol, Dyazide, Lipitor, Cardura, Lotrol	12-Sep
140	86	M	N	N	Y	CABG	Metoprolol, Amlodipine, NTG, Heparin	12-Sep
141	65	M	Y	N	N	CABG	ASA, Clopidogril, Glipizide, Lisinopril, INS,	
142	75	F	Y	N	N	CABG	Glyburide, Atenolol, Lisinopril, Maxide, Methyl dopa	
141	44	M	N	N	N	CABG	Atenolol, Lisinopril, Simvastatin,	12-Sep
144	57	M	N	N	N	AVR	Allopurinol, Colchicine, Minoxidil, Atenol, Lasix, Prednisone, Simvastatin	
145	67	M	N	N	N	CABG	Lipitor, Metoprolol, NTG, ASA	
146	42	M	N	N	N	CABG	Atenolol, Norvasc, NTG	
147	71	M	N	N	N	MVR	Dig, Verapamil, Coumadin	
148	71	F	N	N	N	CABG		
150	70	M	Y	N	N	CABG	Felodipine, HCTZ, INS, Synthroid, Losartin, Simvastatin, Atenolol,	
151	78	F	Y	N	N	CABG	Dig, NTG, Lisinopril, Cavedolol, INS	

CONT'D ON FIG.51C

FIG.51B

CONT'D FROM FIG.51B

152	50	M	N	N	CABG	Lisinopril, NTG, Lasix, Metoprolol	
153	48	M	N	N	AVR		
154	67	M	N	N	CABG	Simvastatin, Lisinopril, Atenolol	
155	59	M	N	N	CABG	Lisinopril, ASA, Metoprolol, Simvastatin	
156	62	M	Y	N	CABG	NTG, Glyburide, Atorvastatin, Atenolol	
161	56	M	Y	N	MVR	INS, Metoprolol, NTG, Simvastatin, Lisinopril	
162	23	F	N	N	MVR		
163	72	M	N	N	AVR	Simvastatin, Felodipine, Cimetidine	
164	65	F	N	N	MVR	Multi valve Dz. A-fib Metoprolol, DIG, Coumadin	
166	57	F	N	N	MVR	Synthroid	
173	60	M	N	N	AVR		
177	66	F	N	N	MVR	Severe MR, Lisinopril	
184	46	M	N	N	MVR	Vasotec	
185	75	M	N	Y	CABG, MVR	Tropolol, Capoten, Plavix, Synthroid	
201	50	M	N	N	AVR	Lasix, Incubated in ICE prior to D/C	

CONT'D ON FIG.51D

FIG.51C

CONT'D FROM FIG.51C

206	58	M	N	N	AVR	Vasotec		
207	68	M	N	N	CABG	Simvastatin, Atenolol		
211	51	M	N	N	AVR	EF<20%		
289	45	M	N	N	AVR		9-Aug	
290	77	M	N	N	MVR		9-Aug	
291	55	F	N	N	AVR		9-Aug	
292	39	M	N	N	MVR		9-Aug	
310	67	F	N	N	AVR/MVY			
312	72	F	N	N	AVR		12-Sep	
314	75	F	N	N	AVR		12-Sep	
318	62	M	N	N	AVR		12-Sep	
329	70	M	N	N	AVR		12-Sep	
330	67	M	N	N	AVR		7/30/02	
331	58	M	N	N	AVR		7/30/02	
339	69	M	N	N	AVR		7/30/02	

FIG.51D

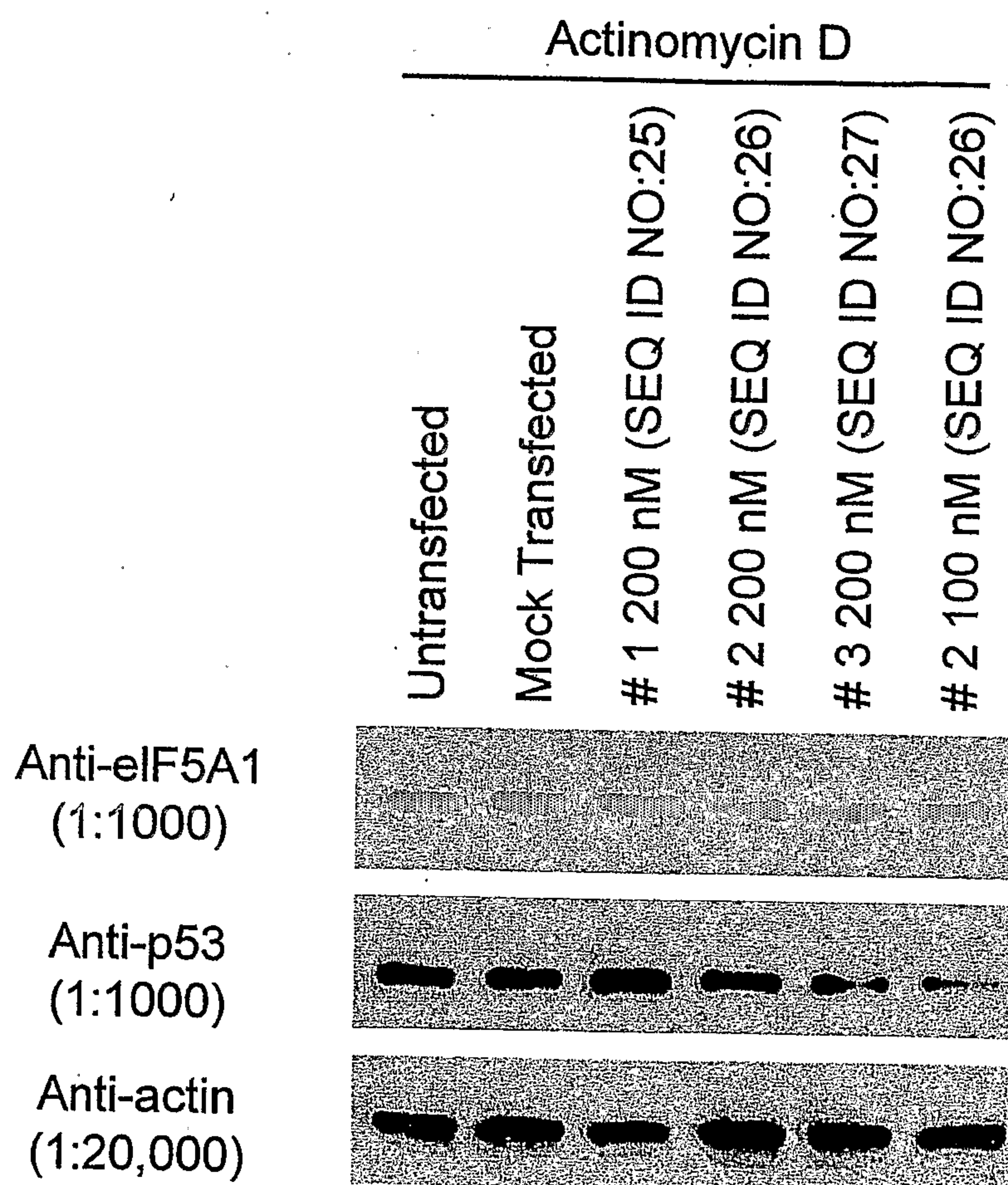


FIG.52

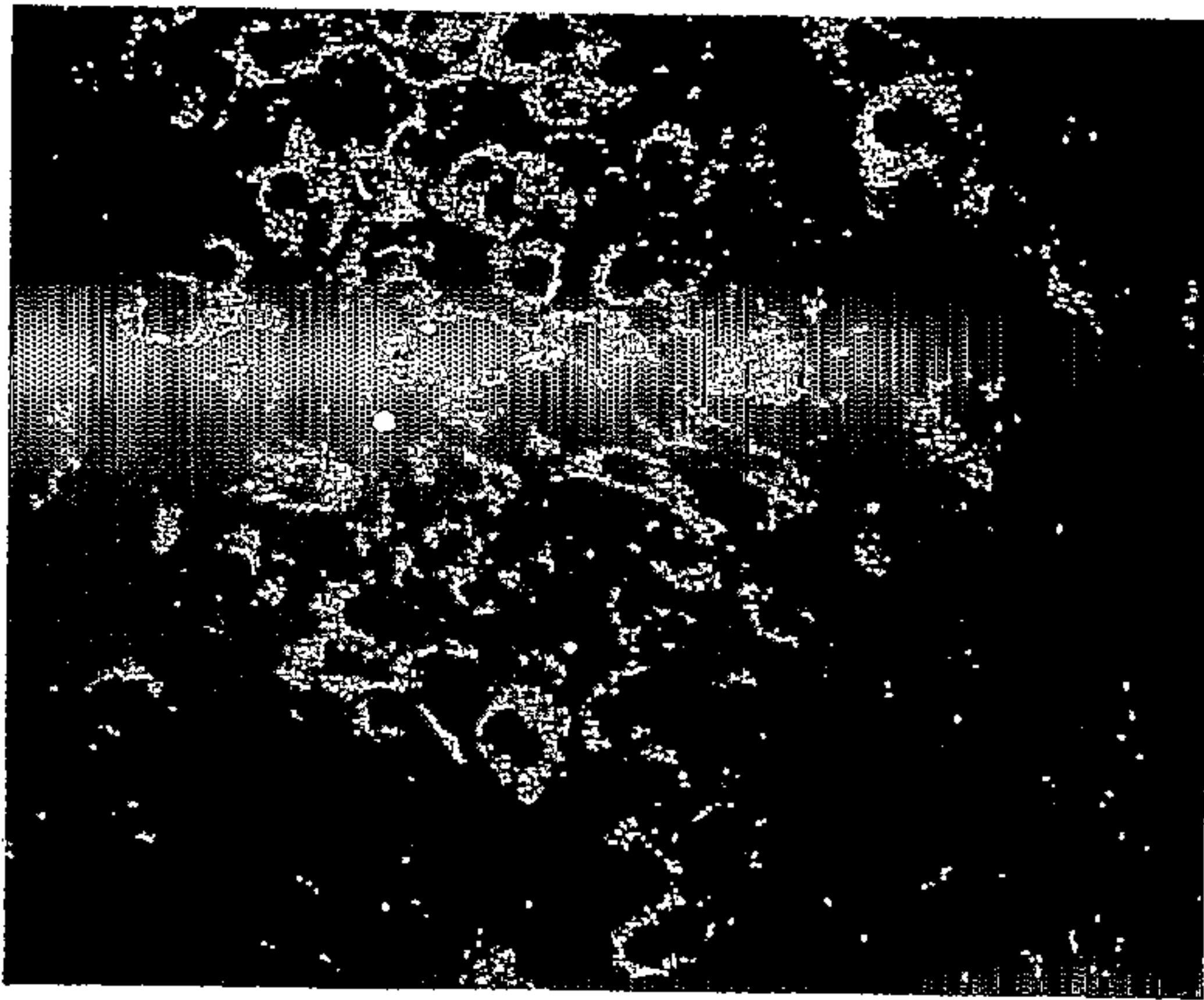


FIG. 53A

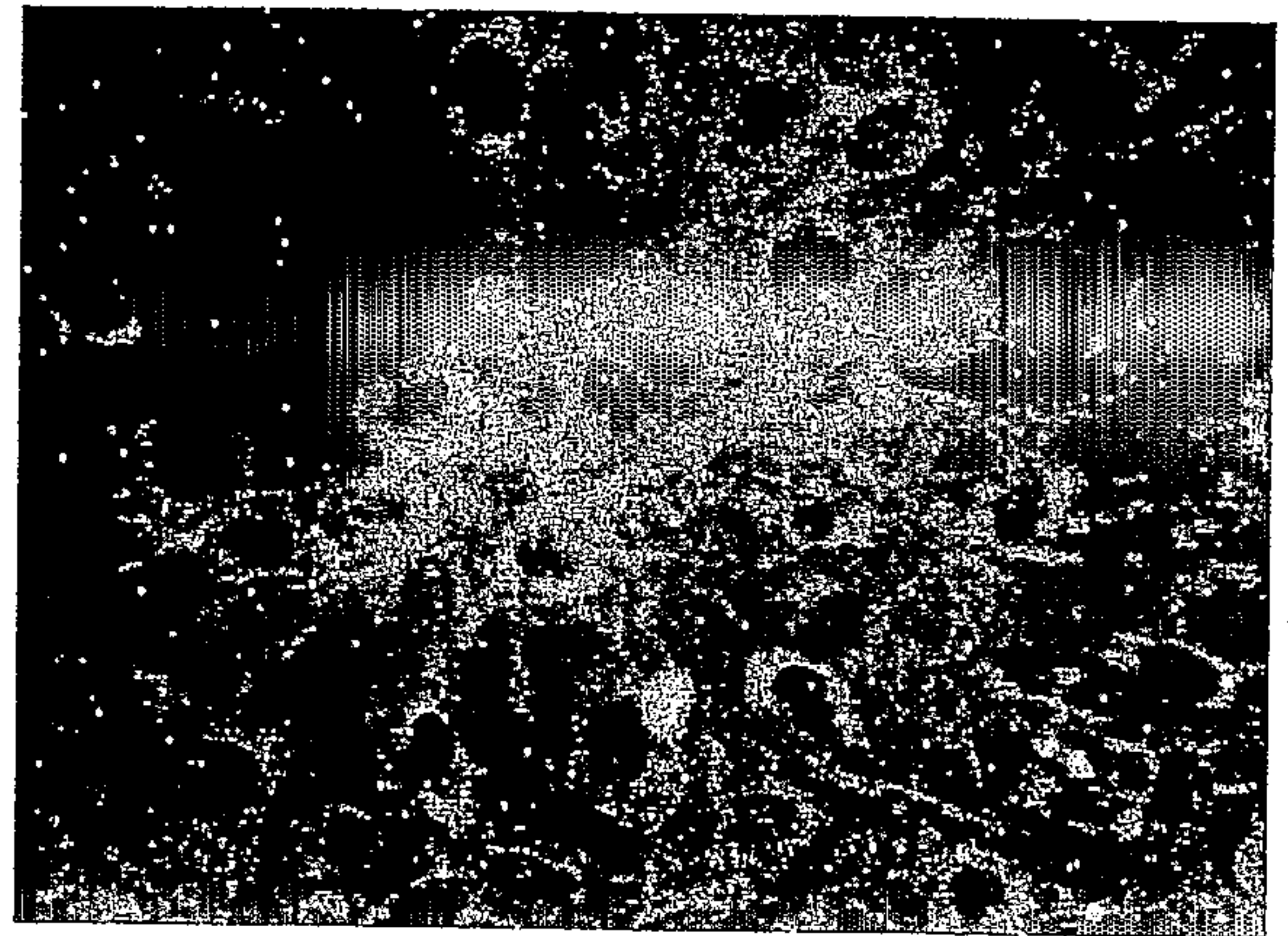


FIG. 53B

PERCENT APOPTOSIS OF # 506 LAMINA CRIBOSA CELLS AFTER TREATMENT WITH ANTISENSE OLIGONUCLEOTIDES FOR 4 DAYS AND CAMPTOTHECIN FOR 2 DAYS

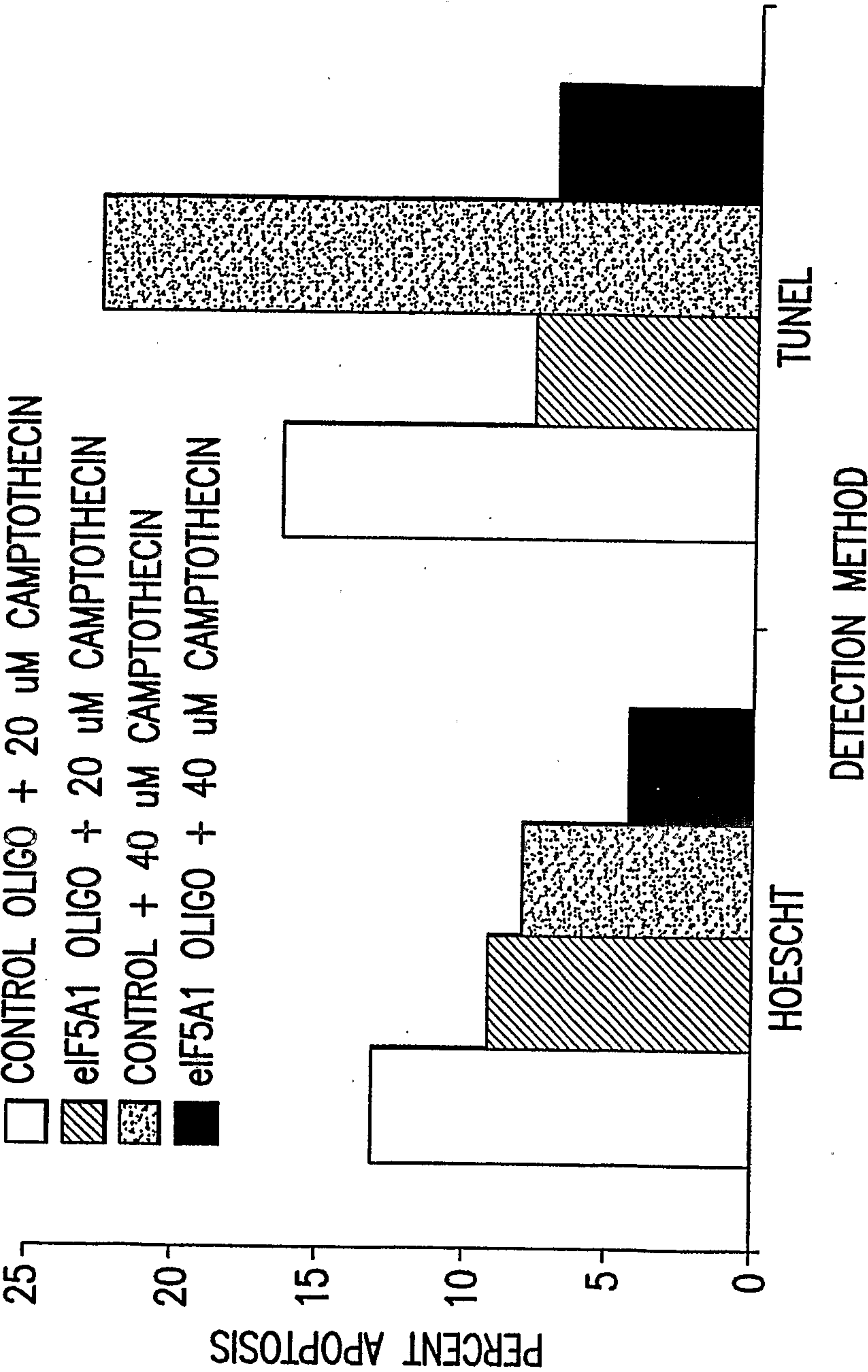


FIG. 54

PERCENT APOPTOSIS OF # 506 LAMINA CRIBOSA CELLS FOLLOWING
TREATMENT WITH ANTISENSE OLIGONUCLEOTIDES FOR 2 DAYS AND
CAMPTOTHECIN FOR 3 DAYS

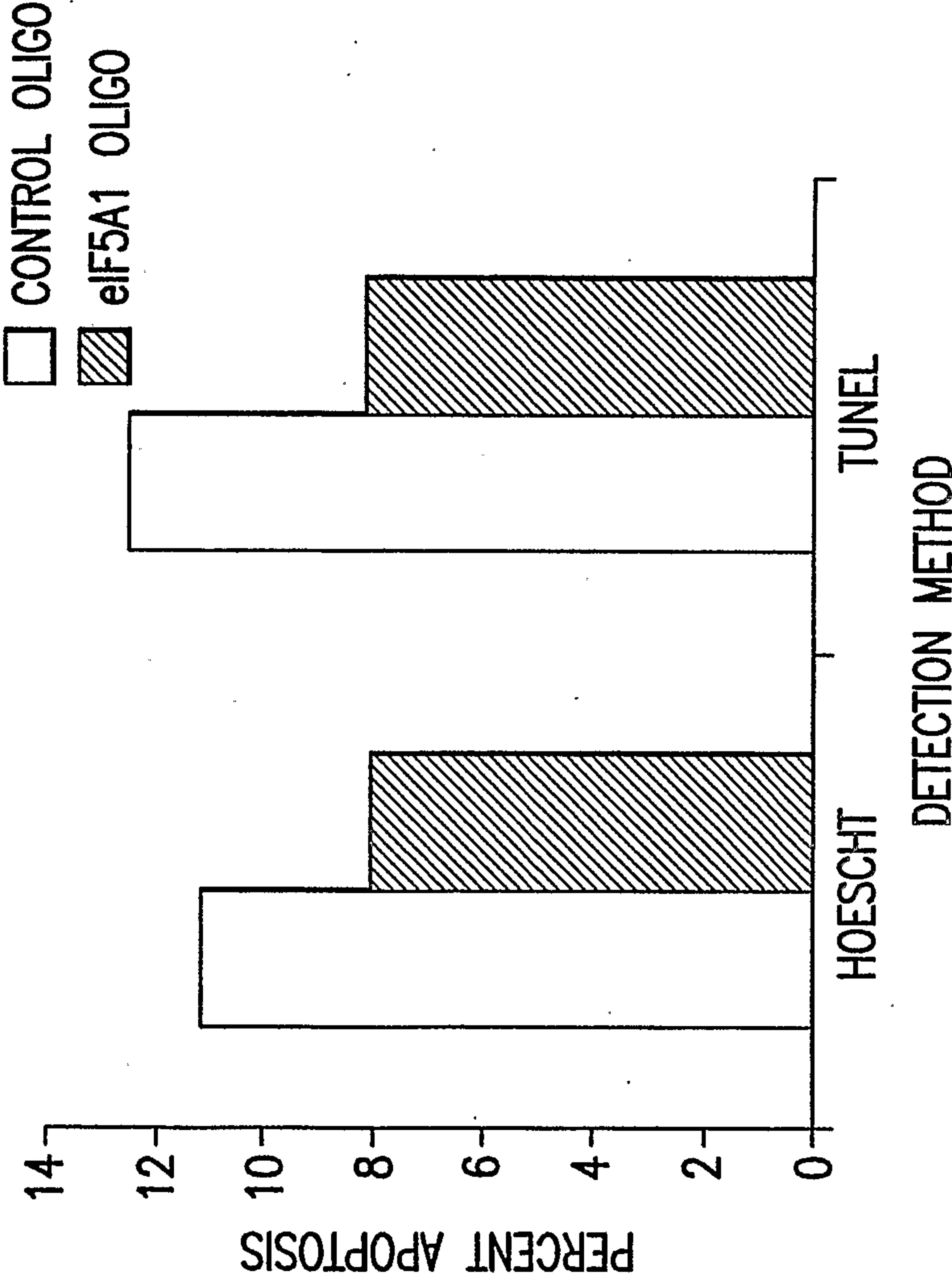


FIG.55

PERCENT APOPTOSIS OF # 517 LAMINA CRIBOSA CELLS AFTER TREATMENT WITH
ANTISENSE OLIGONUCLEOTIDES FOR 5 DAYS AND CAMPTOTHECIN FOR 3 OR 4 DAYS

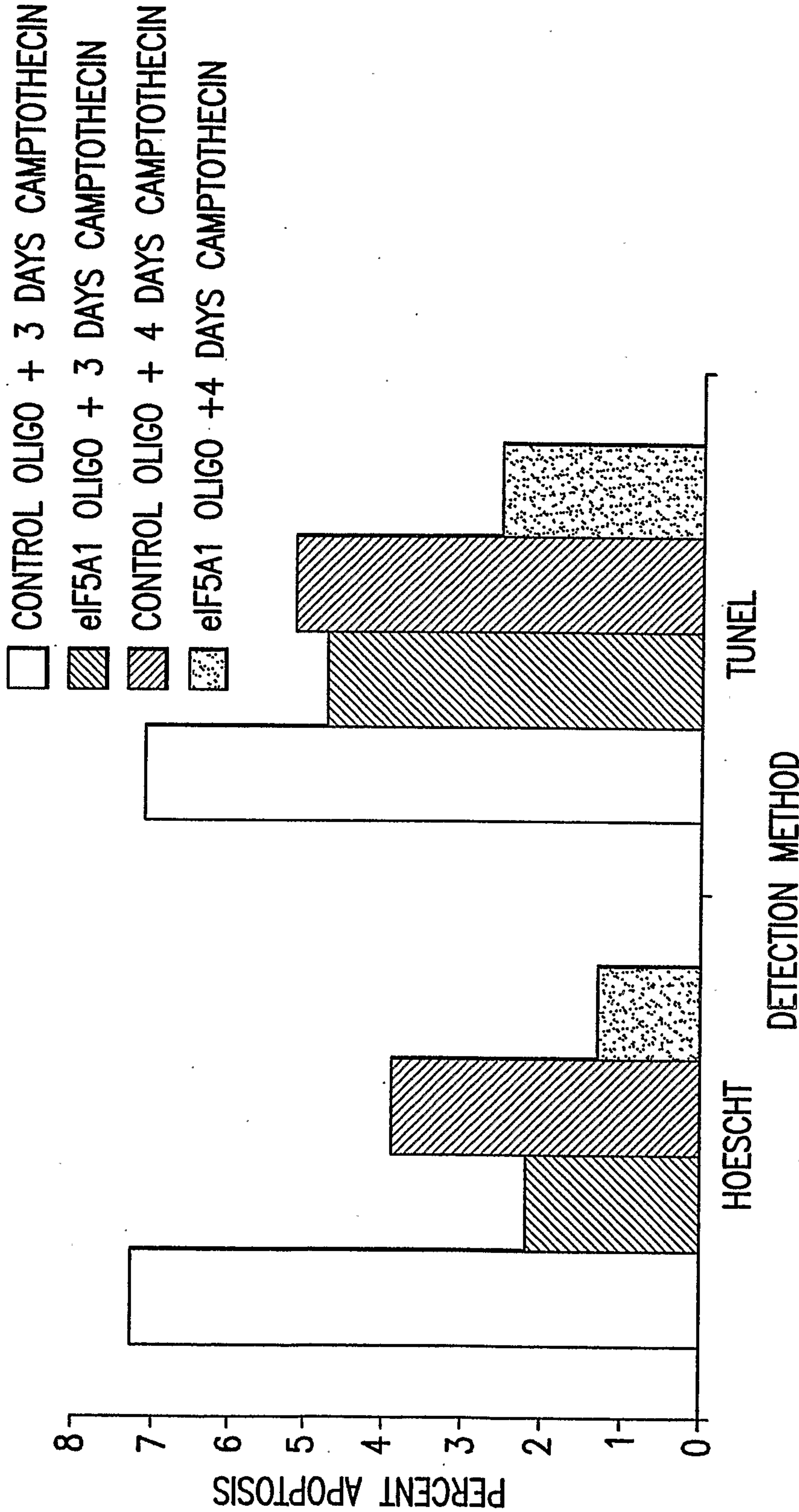


FIG. 56

PERCENT APOPTOSIS OF # 517 LAMINA CRIBOSA CELLS AFTER TREATMENT WITH
ANTISENSE OLIGONUCLEOTIDE FOR 5 DAYS AND CAMPTOTHECIN FOR 3 DAYS

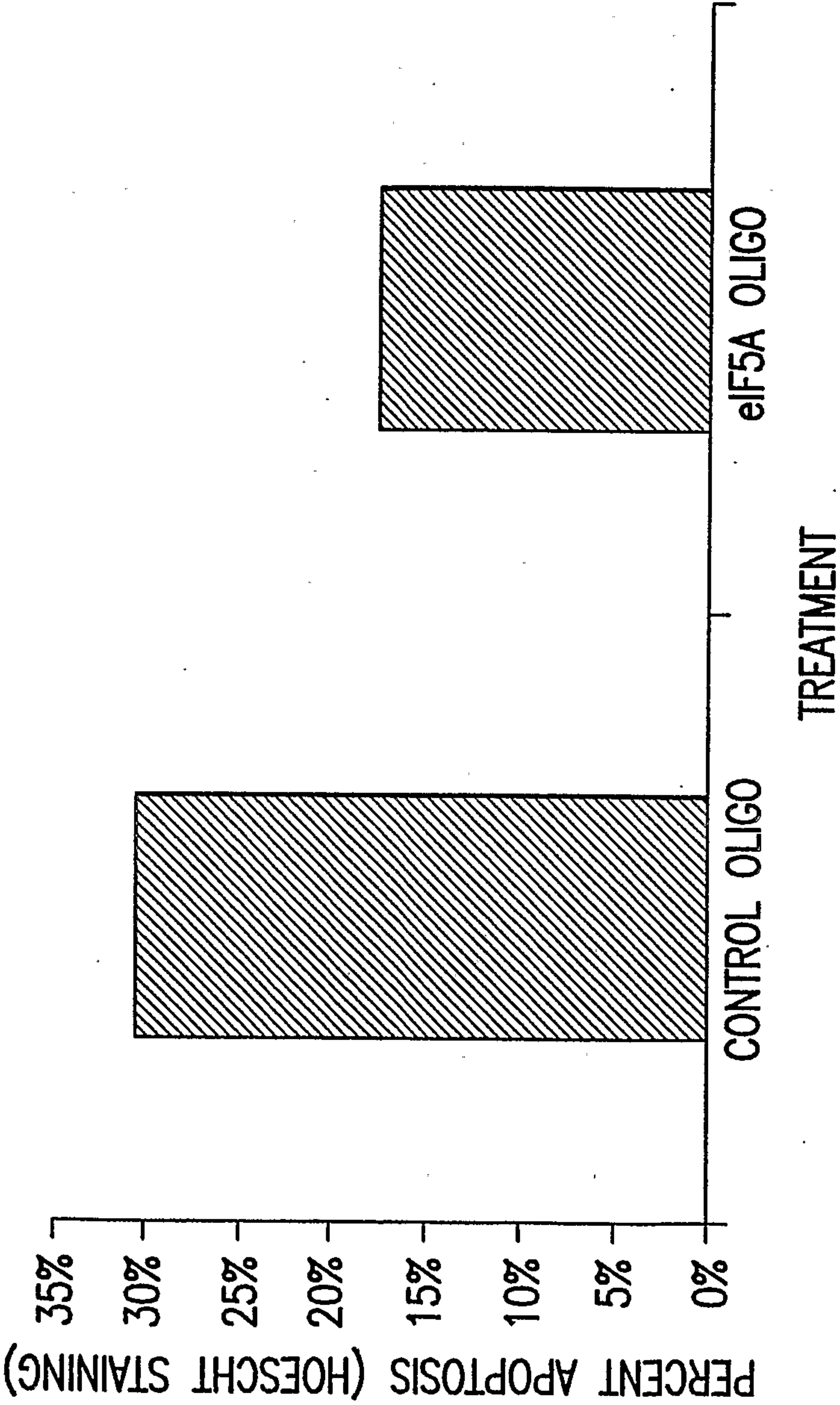


FIG. 57

Figure 58

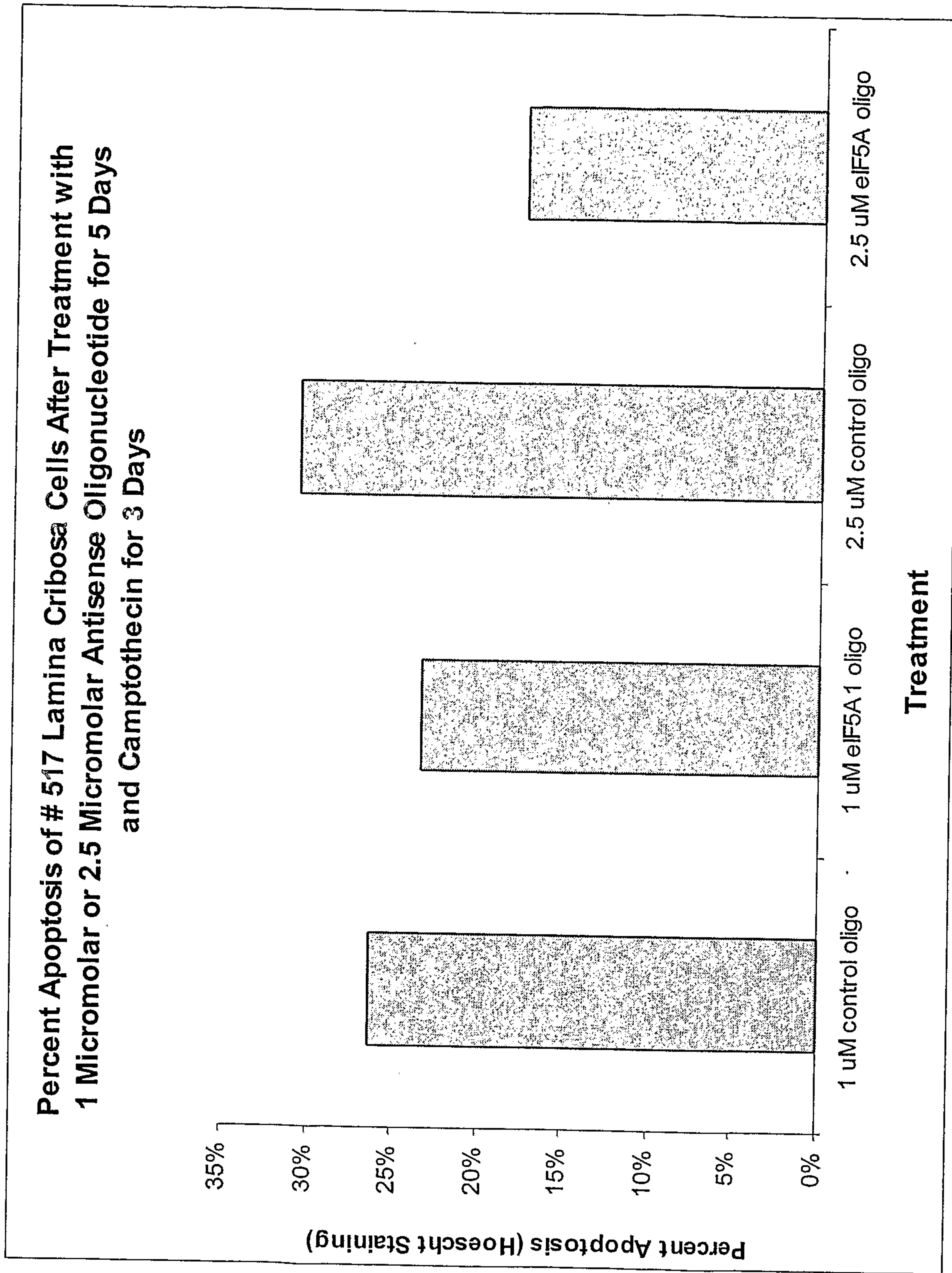


Figure 59

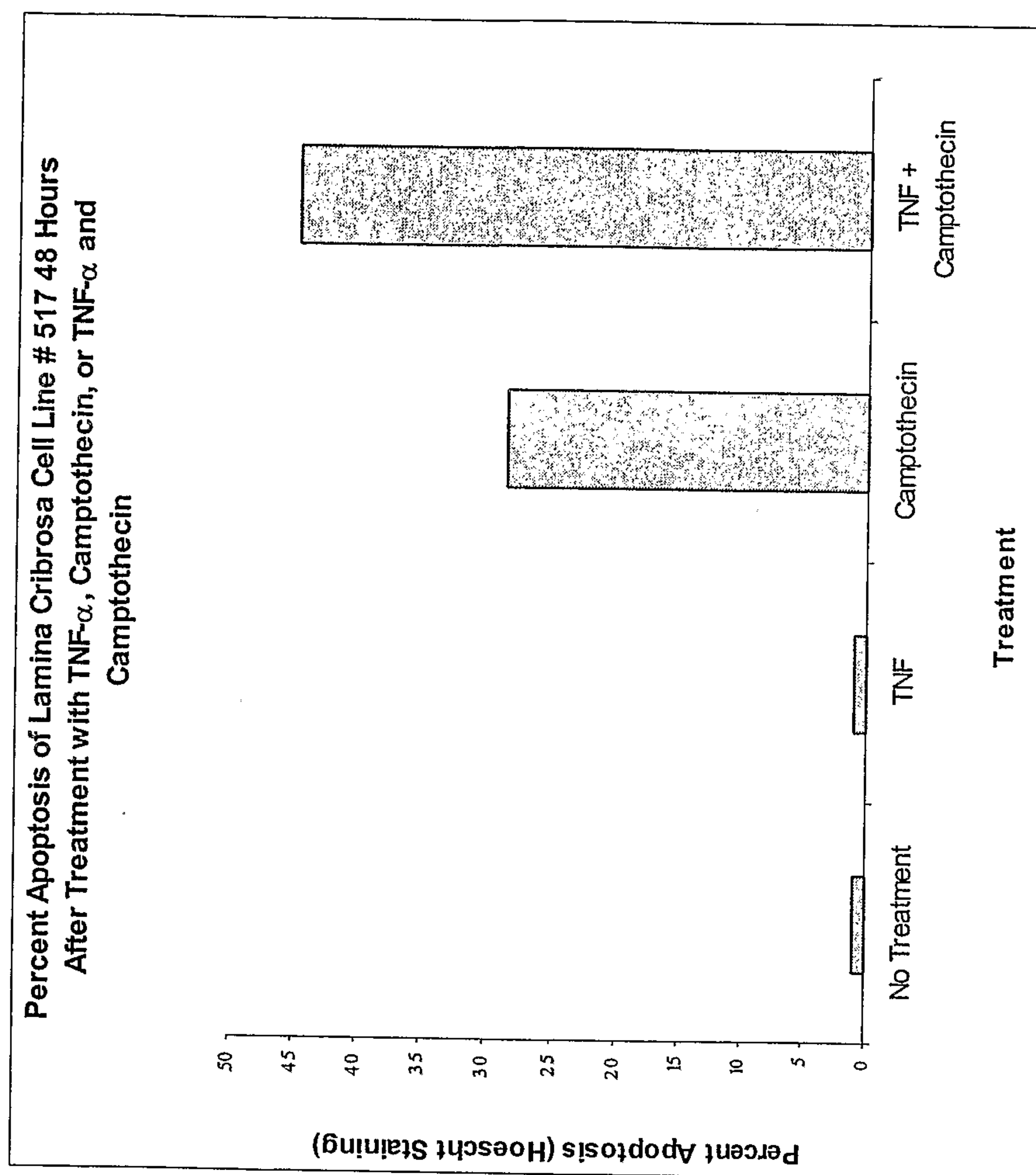


Figure 40

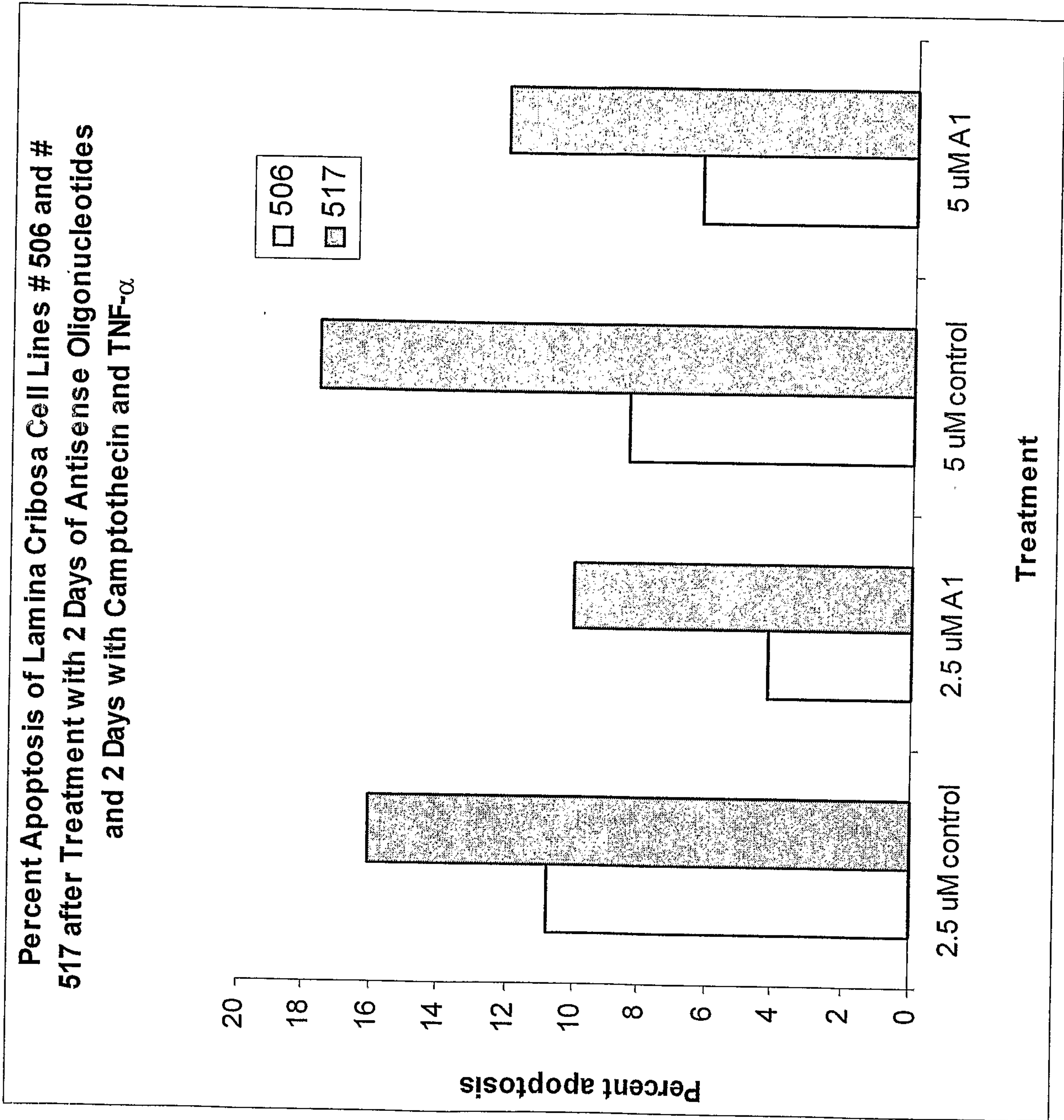


Figure 6

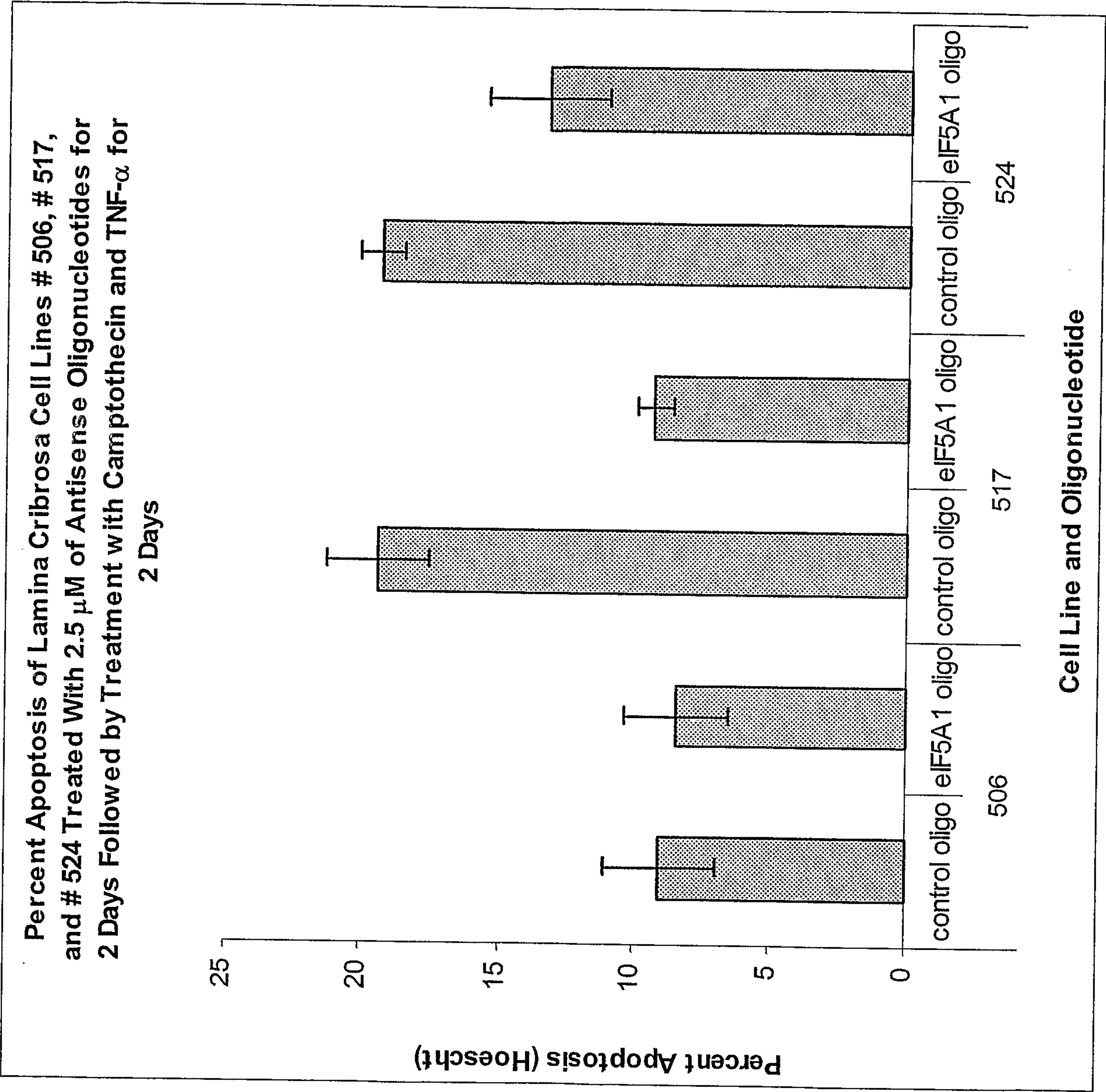
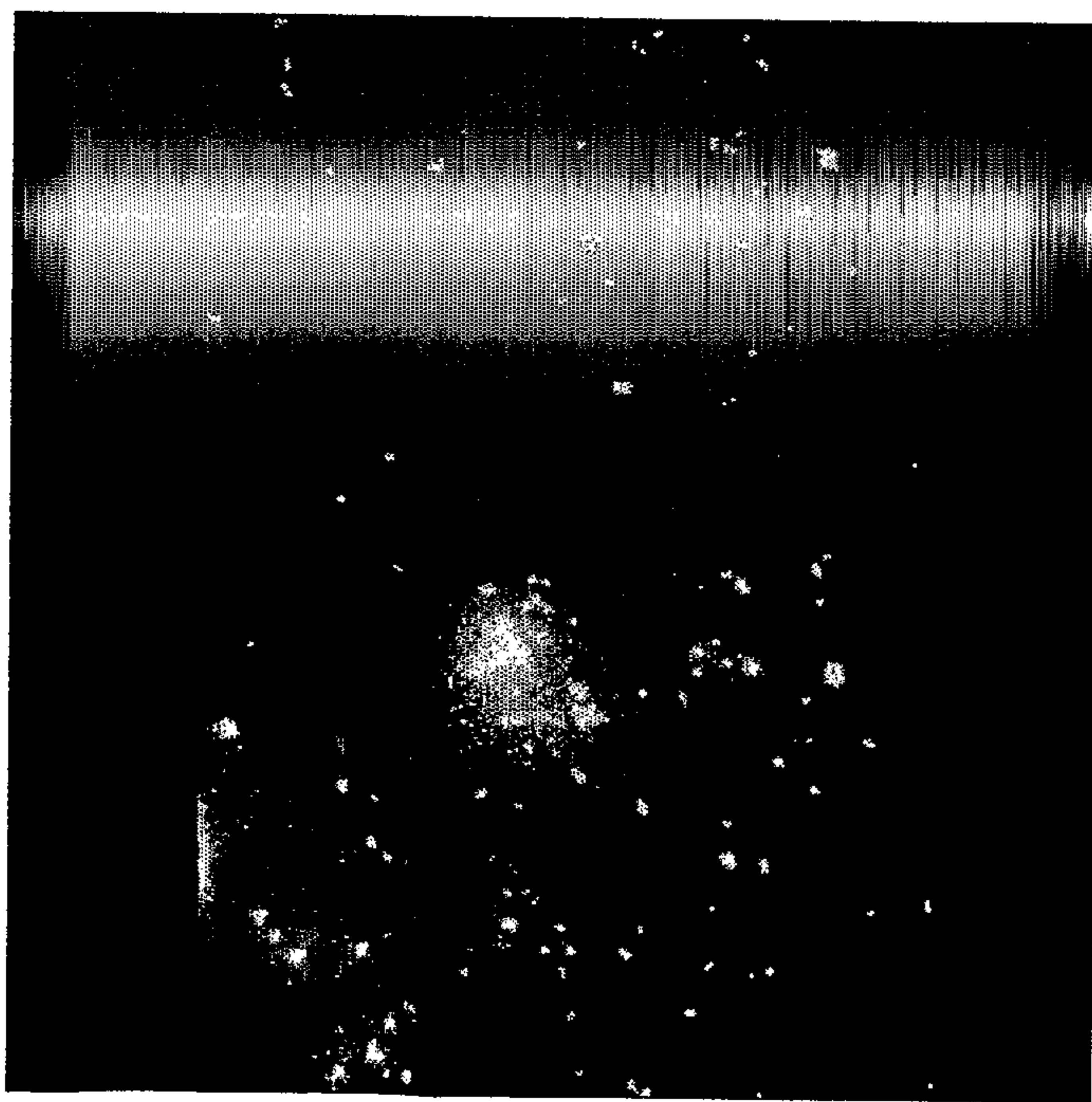


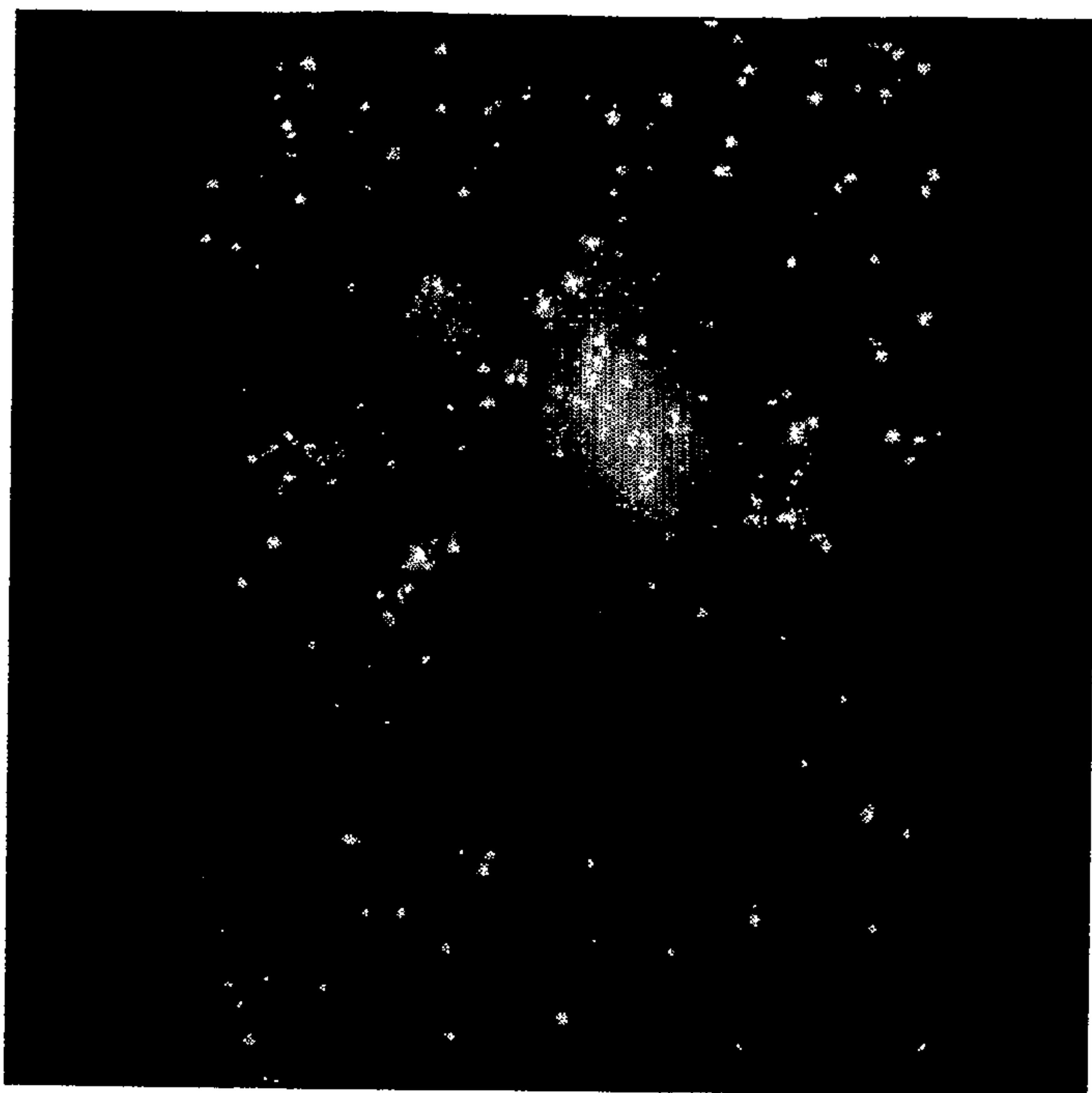
Figure 6a

A)



+ serum

B)



- serum

Figure 63

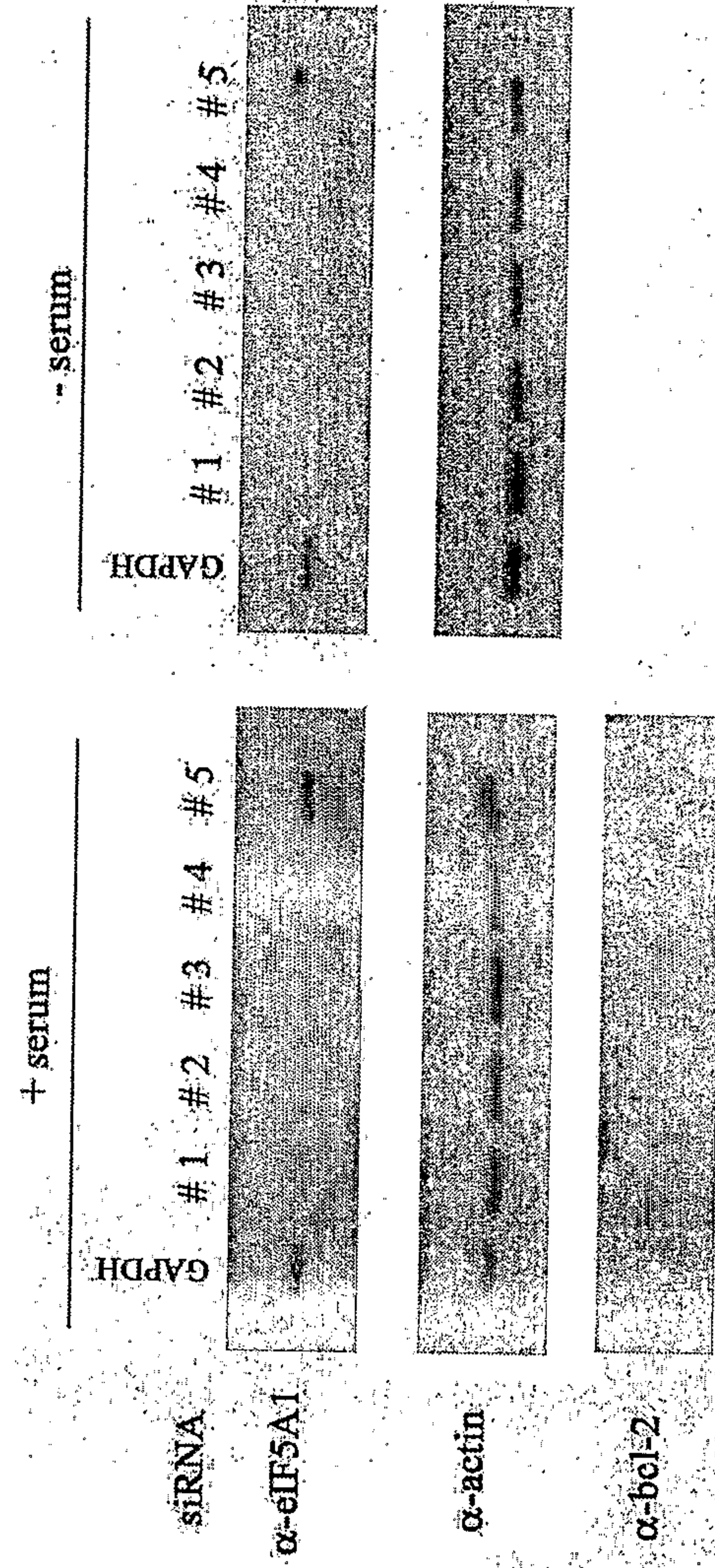


Figure 6c4

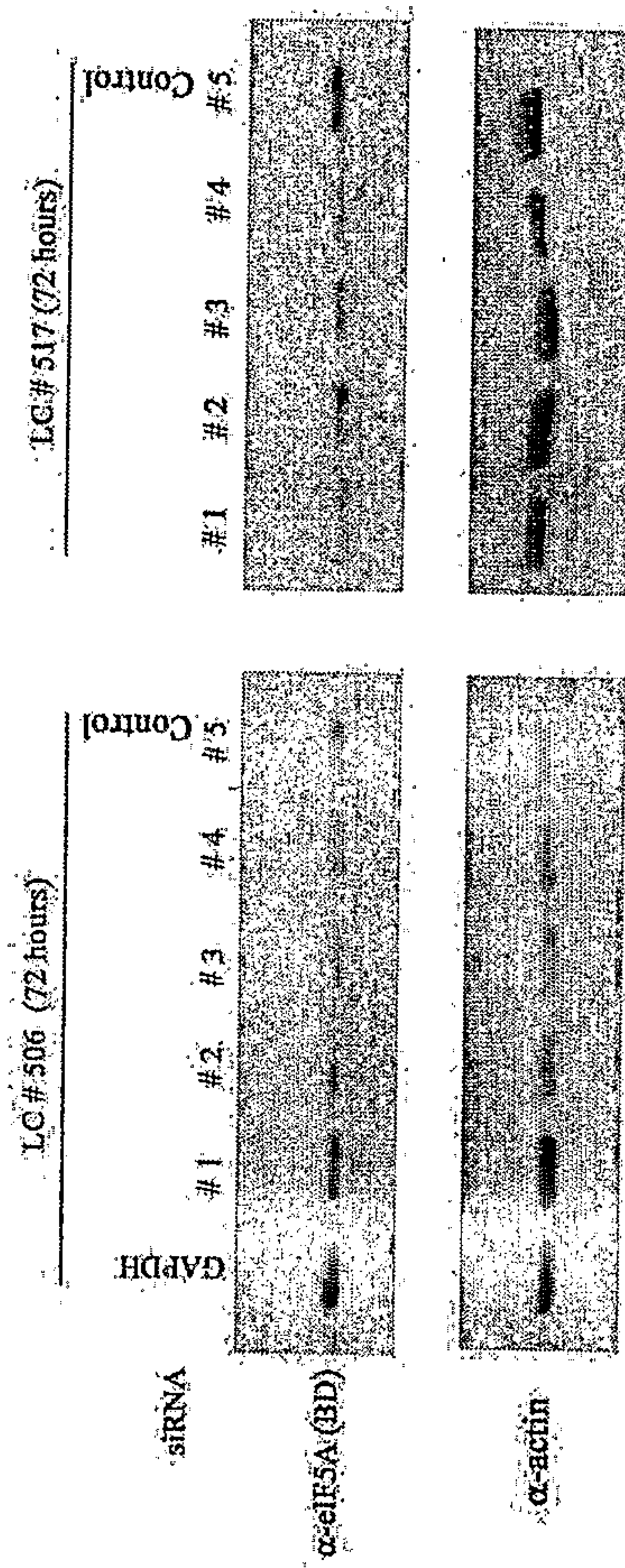


Figure 65

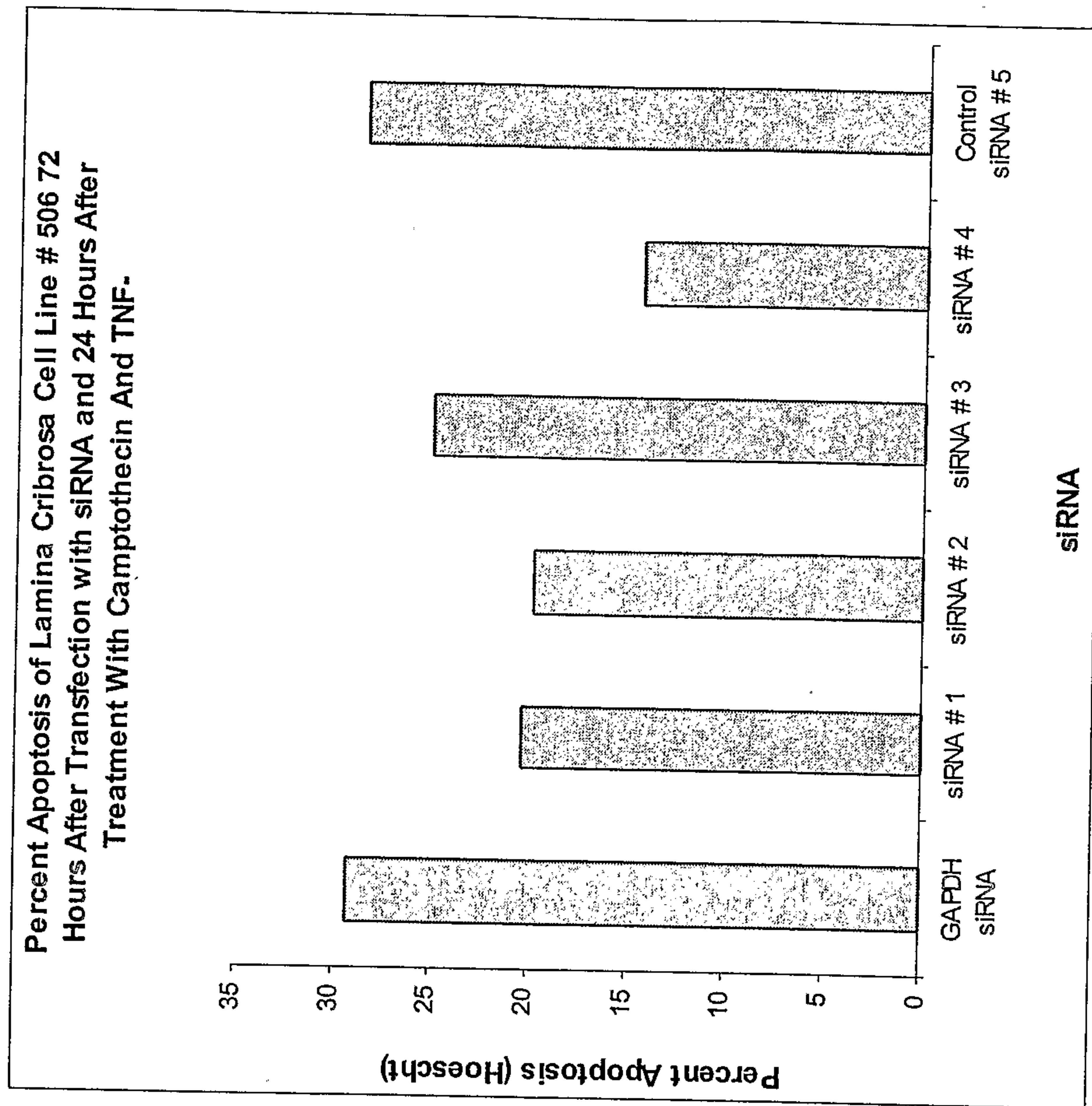


Figure 66

Percent Apoptosis of Lamina Cribrosa Cell Line # 506 96
Hours After Transfection with siRNA and 24 Hours After
Treatment With Camptothecin And TNF- α

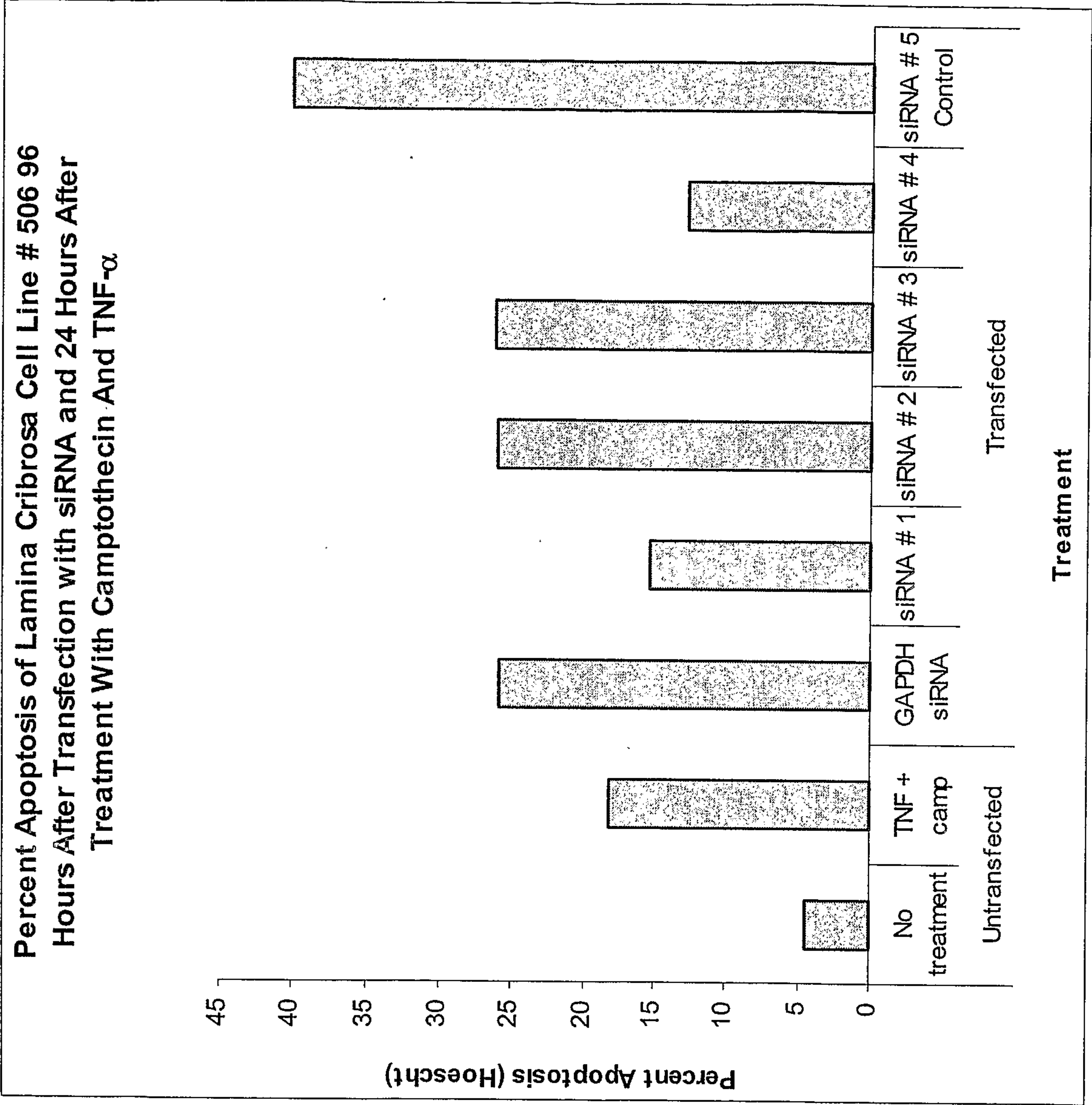


Figure 67

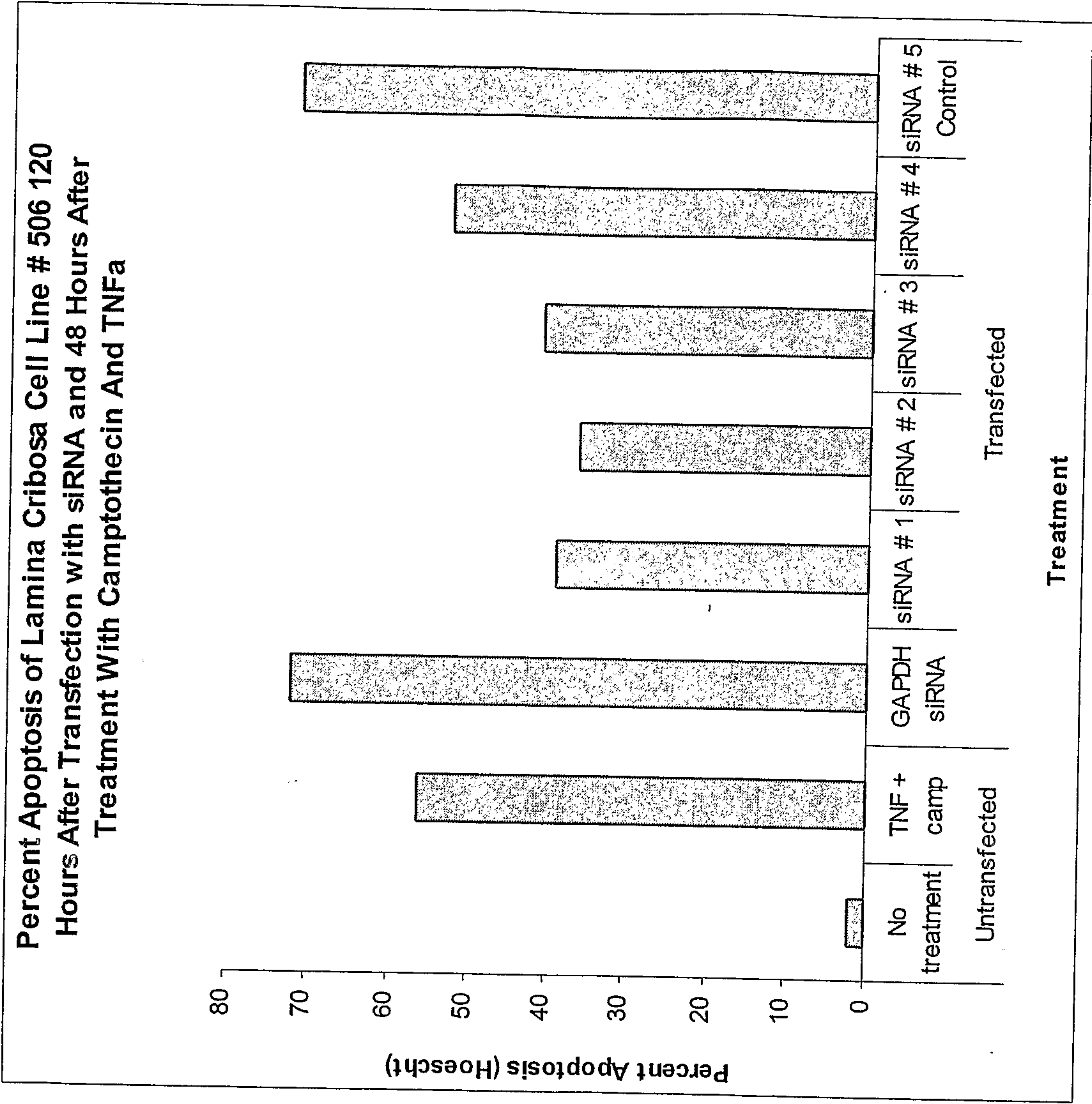


Figure 68

Hoescht Staining of siRNA-Transfected Lamina
Cribrosa Cells Treated With Camptothecin and TNF- α

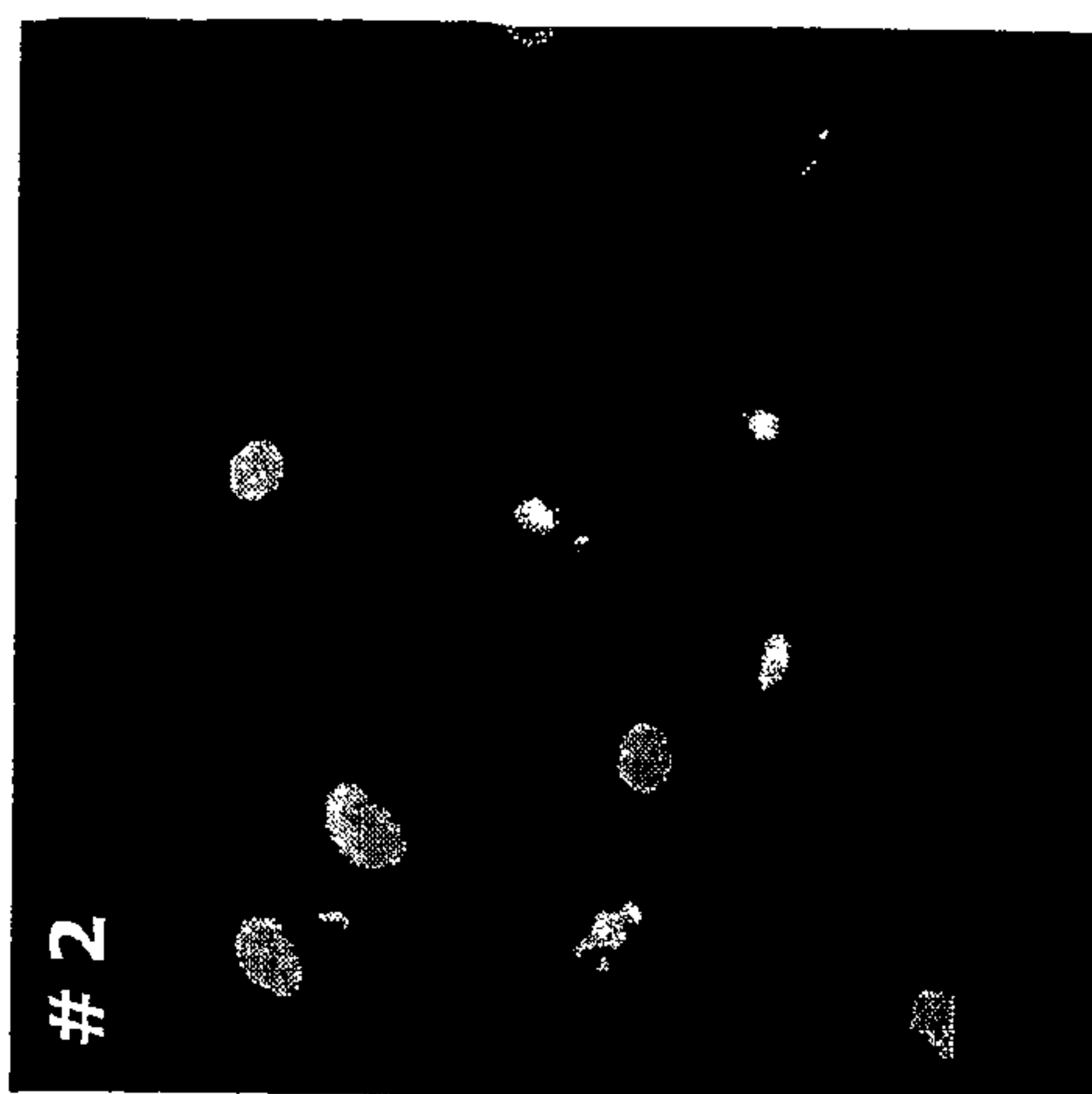
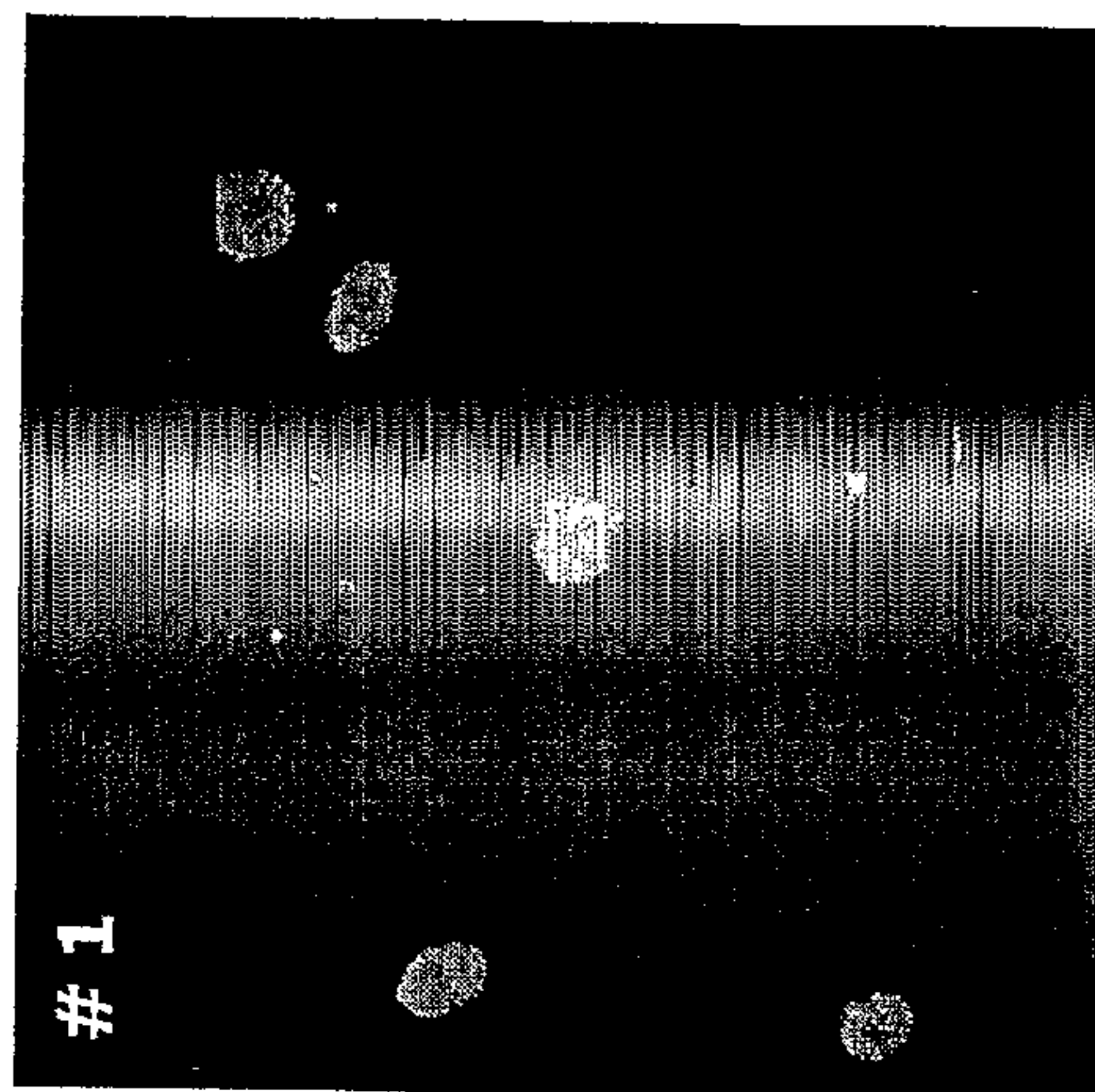
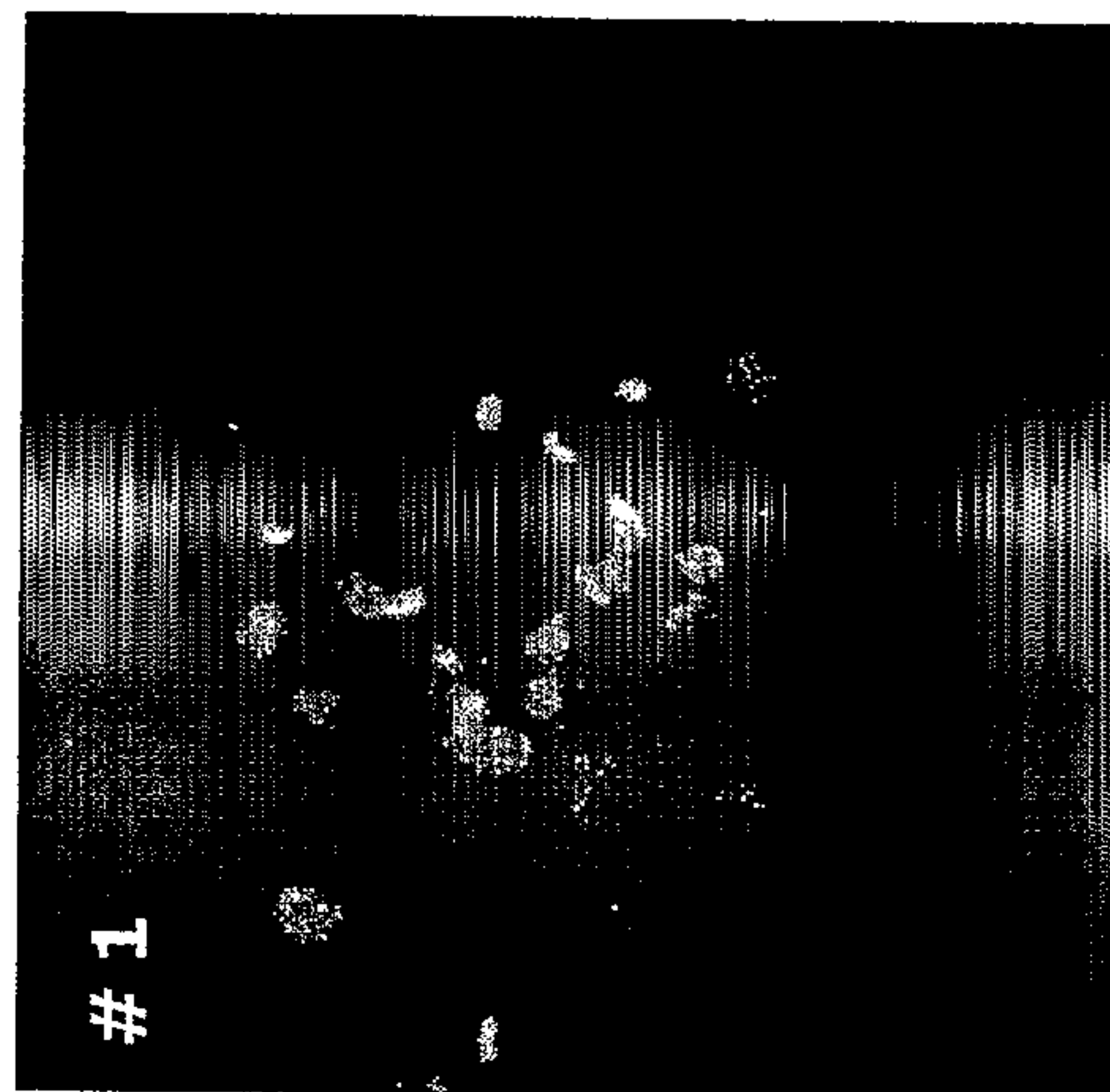
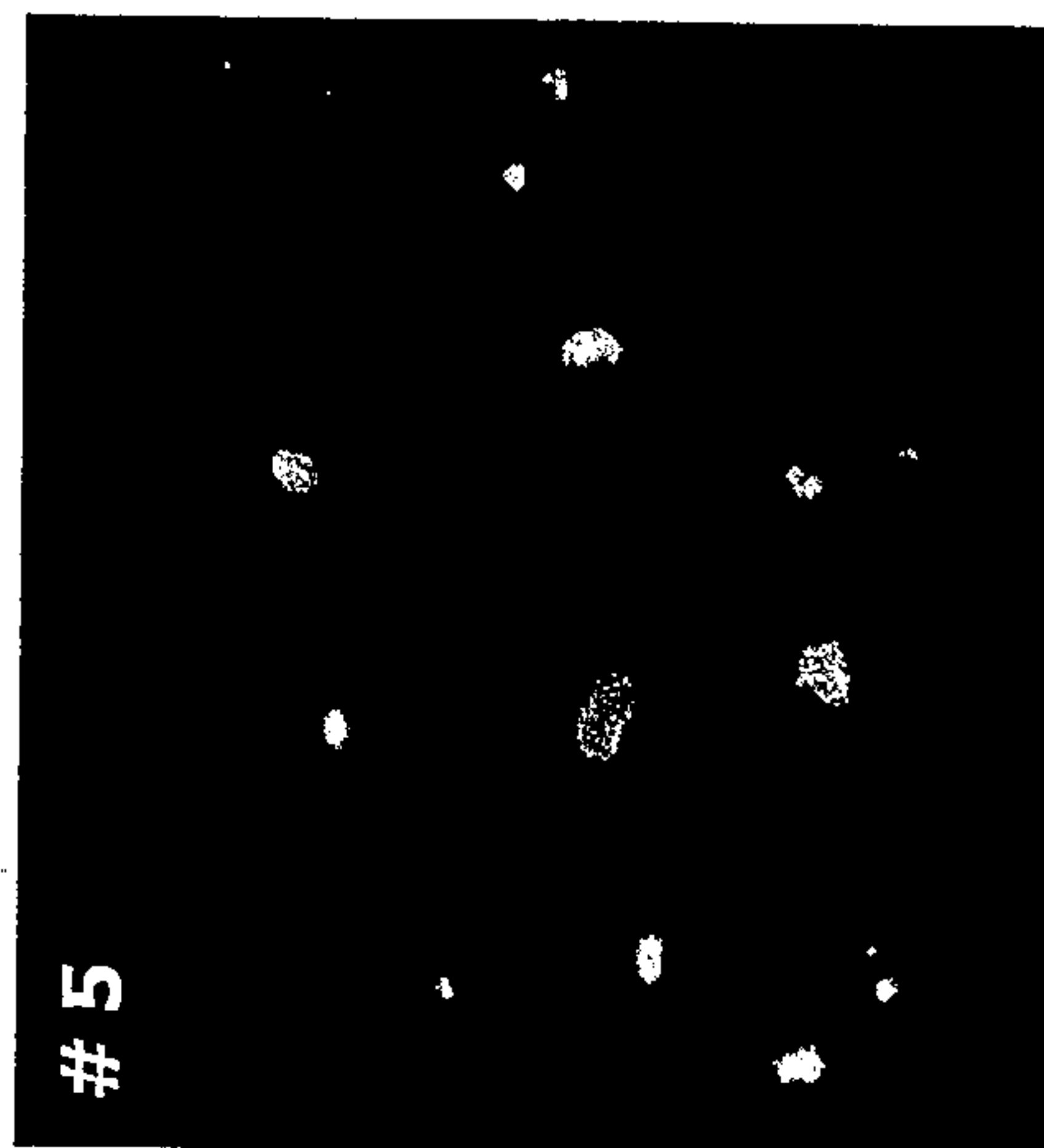
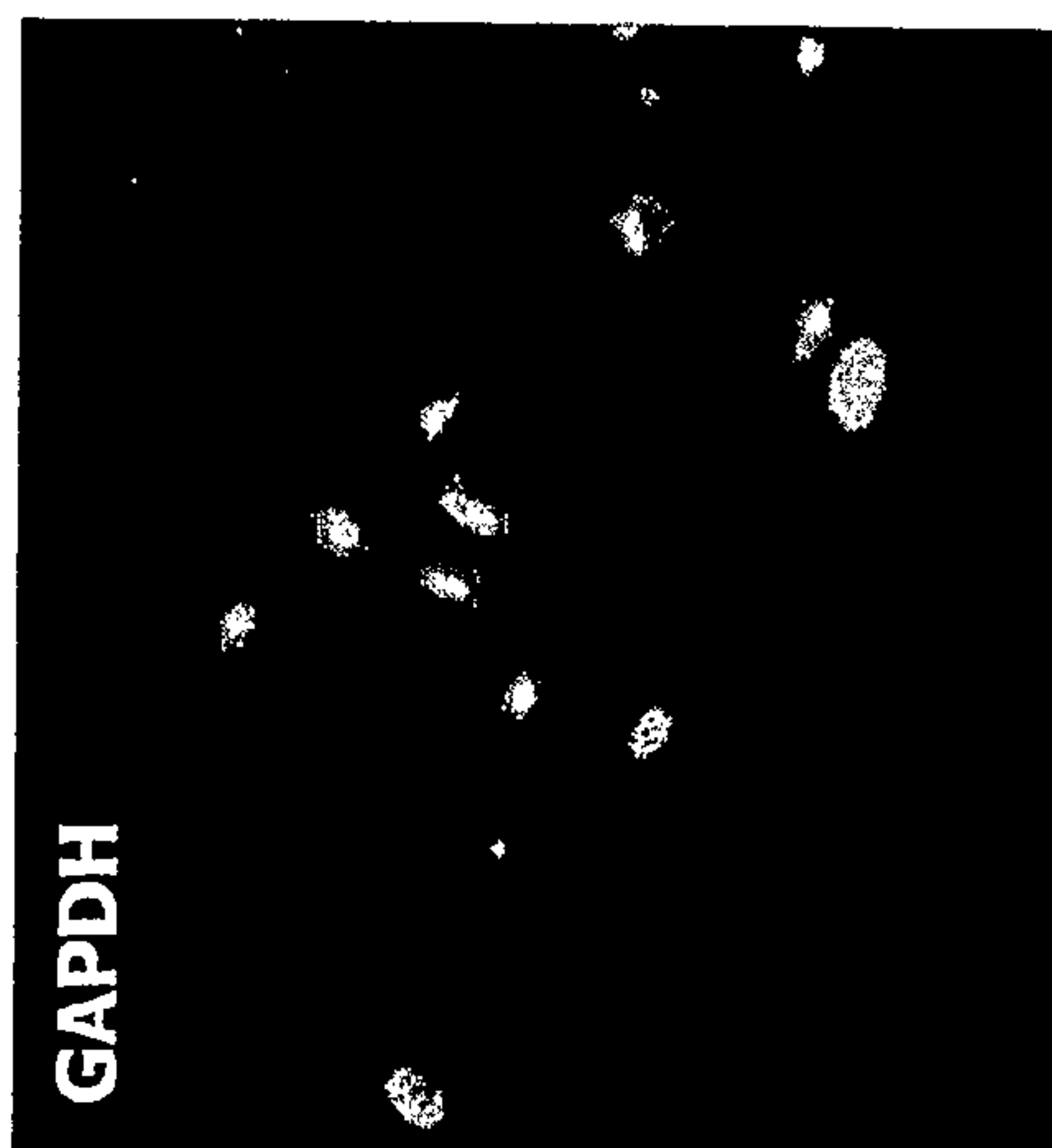
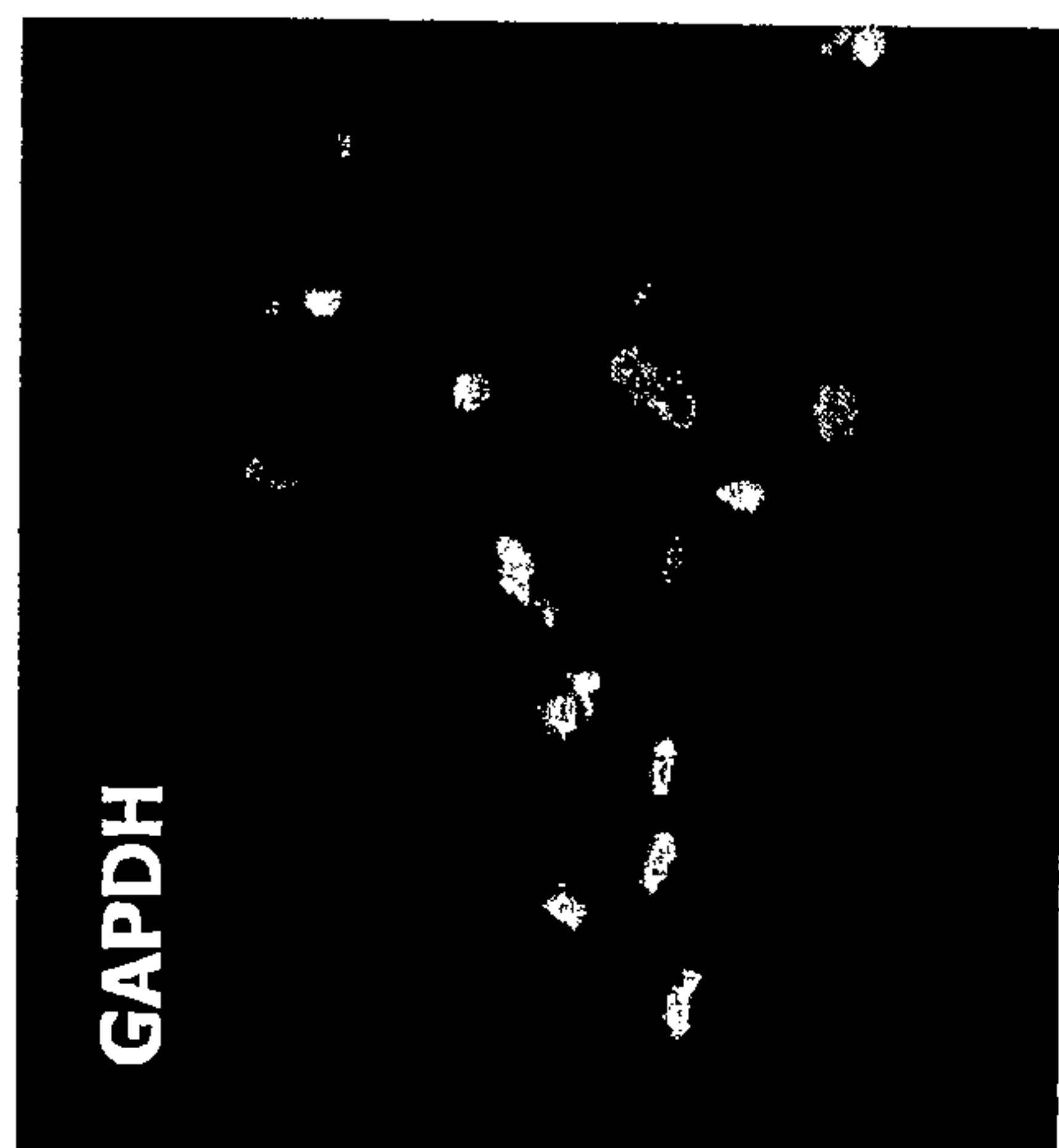
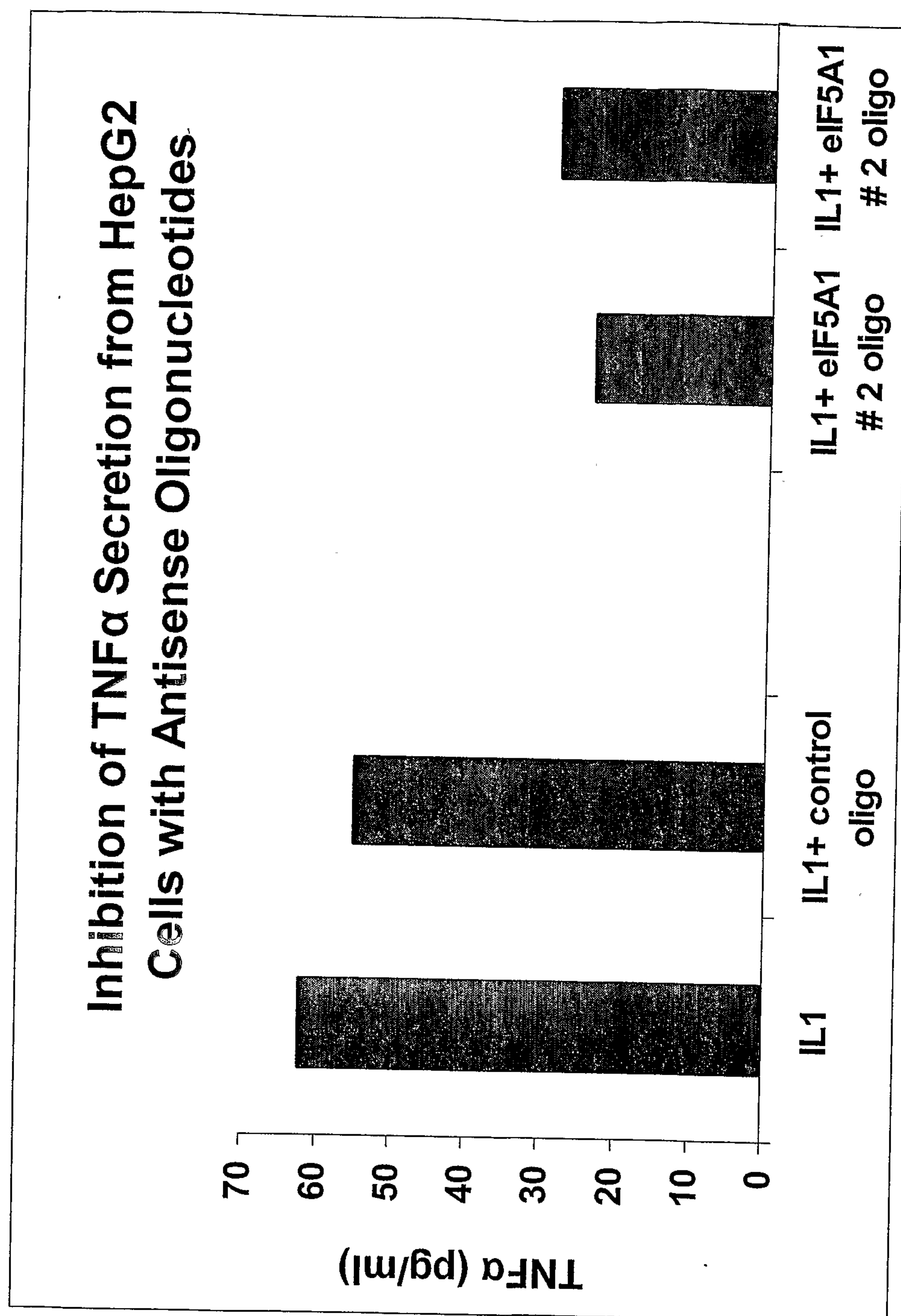


Figure 69



target 5' AA (AGGAATGACTTCCAGCTGA)TT 3' SEQ ID NO: *1
 target 5' AA (GATCGTCGAGATGTCTACT)TC 3' SEQ ID NO: *2
 target 5' AA (GGTCCATCTGGTTGGTATT)GA 3' SEQ ID NO: *3
 target 5' AA (GCTGGACTCCTCCTACACA)AT 3' SEQ ID NO: *4
 siRNA #5 reverse of siRNA #1 5' AA (AGTCGACCTTCAGTAAGGA)TT 3' SEQ ID NO: *5

```

1. ggcacgaggg tagaggcggc ggcggcggcg gcagcgggct cggaggcagc ggttgggctc
61. gcggcgagcg gacggggctcg agtcagtgcg ttgcgcgag ttggaatcga agcctcttaa
121. aatggcagat gacttggact tcgagacagg agatgcaggg gcctcagcca ccttcccaat
181. gcagtgtca gcattacgta agaattggctt tgttgtgtc aaaggccggc catgt
241. ggcagcagcga agactggcaa gcacggccac gcc
301. ccatc tttactggga agaaatatga agatatctgc ccgtcaactc ataatatgga
361. tgtccccaac atca ggc atccaggatg ggtacctatc
421. actgtccag gacagcgggg aggtacgaga ggaccttcgt ctccctgagg gagaccttg
481. caaggagatt gagcagaagt acgactgtgg agaagagatc ctgatcacgg tgcgtgtctc
541. catgacagag gaggcagctg ttgcaatcaa ggccatggca aaataactgg ctcccaggat
601. ggcggtggtg gcagcagtga tcctctgaac ctgcagaggc cccctccccg agcctggcct
661. ggctctggcc cggctct
721. gttttcccca cccctcaat ctgtcgggga gccctgccc ttcacctagc tcccttggcc
781. aggagcgagc gaagctgtgg ccttgggtgaa gctgccctcc tcttctcccc tcacactaca
841. gccctggtgg gggagaagg ggtgggtgct gcttgtggt tagtcttttt ttttttttt
901. tttttttttt aaattcaatc tggaatcaga aagcgggtgga ttctggcaaa tggctctgt
961. gccctcccca ctcatccctg gtctggtccc ctgttgccca tagccttta cctgagcac
1021. caccccaaca gactggggac cagccccctc gcctgcctgt gtctctcccc aaacccttt
1081. agatggggag ggaagaggag gagaggggag gggacctgcc cctcctcag gcctctggga
1141. gggccctgcc cccatgggct ttacccttcc ctgcgggctc tctccccgac acatttgta
1201. aaatcaaacc tgaataaaac tacaagtta atatgaaaa aaaaaaaaaa aaaaaaaaaa
1261. aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa SEQ ID *AA

```

Figure 70



target

5'- CCT GTC TCG AAG TCC AAG TC -3' (SEQ ID NO: *6)
 5'- GACTTGGACTTCGAGACA GG -3'



Target

5'- GGA CCT TGG CGT GGC CGT GC -3' (SEQ ID NO: *7)
 5'- GCACGGCC ACGCCAAGGTCC -3'



target

5'- CTC GTA CCT CCC CGC TCT CC 3' (SEQ ID NO: *8)
 5'- GGACAGCGG GGAGGTACGA -3'

```

1  ggcacgaggg tagaggcggc ggcggcggcg gcagcgggct cggaggcagc ggttgggctc
61  gcggcgagcg gacggggtcg agtcagtgcg ttcgcgcgag ttggaatcga agcctcttaa
121  aatggcagat gacgagcagc agtcagtgcg agatgcaggg gcctcagcca ccttcccaat
181  gcagtgtctc gcattacgta agaatggcctt tgtgggtgctc aaaggccggc catgtaagat
241  cgtcgagatg tctacttcga agactggcaa gacgagcagc atctggttgg
301  tattgacatc tttactggga agaaatatga agatatctgc ccgtcaactc ataatatgga
361  tgtccccaac atcaaaagga atgacttcca gctgattggc atccaggatg ggtacctatc
421  actgctccac gacgagcagc agtcagtgcg gacgagcagc ctccctgagg gagaccttgg
481  caaggagatt gagcagaagt acgactgtgg agaagagatc ctgatcacgg tgctgtctgc
541  catgacagag gaggcagctg ttgcaatcaa ggccatggca aaataactgg ctcccaggat
601  ggcgggtggtg gcagcagtgga tcctctgaac ctgcagaggc cccctccccg agcctggcct
661  ggctctggcc cggtcctaag ctggactcct cctacacaat ttatttgacg ttttattttg
721  gttttcccca cccctcaat ctgtcgggga gccctgccc ttcacctagc tcccttggcc
781  aggagcgagc gaagctgtgg ccttgggtgaa gctgccctcc tcttctcccc tcacactaca
841  gccctggtgg gggagaaggg ggtgggtgct gcttgtgggt tagtcttttt tttttttttt
901  tttttttttt aaattcaatc tggaatcaga aagcgggtgga ttctggcaaa tggtccttgt
961  gccctcccca ctcatcctg gtctgggtccc ctgttgccca tagcccttta cctgagcac
1021  caccccaaca gactggggac cagccccctc gcctgcctgt gtctctcccc aaaccctttt
1081  agatggggag ggaagaggag gagaggggag gggacctgcc cctcctcag gcactctggga
1141  gggccctgcc cccatgggct ttacccttcc ctgcgggctc tctccccgac acatttggtt
1201  aaatcaaacc tgaataaaac tacaagttta atatgaaaaa aaaaaaaaaa aaaaaaaaaa
1261  aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa

```

Figure 71

Antisense eIF5A1 # 1 (SEQ ID NO:25) - binds to GACTTGGACTTCGAGACAGG

Antisense eIF5A1 # 2 (SEQ ID NO:26) - binds to GCACGGCCACGCCAAGTC

Antisense eIF5A1 # 3 (SEQ ID NO:27) - binds to GGACAGCGGGAGGTACGAG

[illegible]

FIG. 72.

Nucleotide Alignment of Human eIF5A1 (ACC. NM 001970) and Human eIF5A2 (ACC. NM 020390)

eIF5a1	1	ATGGCAGATG	ACTTGGACTT	CGAGACAGGA	GATGCAGGGG	CCTCAGCCAC
eIF5a2	1	ATGGCAGACG	AAATTGATTT	CACTACTGGA	GATGCCGGGG	CTTCCAGCAC
eIf5a1	51	CTTCCCAATG	CAGTGCTCAG	CATTACGtAA	GAATGGCTTT	GTGGTGCTCA
eIF5a2	51	TTACCCTATG	CAGTGCTCGG	CCTTGCGcAA	AAACGGCTTC	GTGGTGCTCA
eIf5a1	101	AAGGCCGGCC	ATGtAAGATC	GTCGAGATGT	CTACTTCGAA	GACTGGCAAG
eIF5a2	101	AAGGACGACC	ATGcAAAATA	GTGGAGATGT	CAACTTCCAA	AACTGGAAAG
eIf5a1	151	CACGGCCACG	CCAAGGTCCA	TCTGGTTGGT	ATTGACAICT	TtACTGGGAA
eIF5a2	151	CAtGGTCATG	CCAAGGTTCA	CCTTGTTGGA	ATTGATAATT	TcACGGGCAA
eIf5a1	201	GAAATATGAA	GATATCTGCC	CGTCAACTCA	tAATATGGAT	GTCCCCAACA
eIF5a2	201	AAAATATGAA	GATAITTTGTC	CTTCTACTCA	CAACATGGAT	GTTCCAAATA
eIf5a1	251	TCAAAAGGAA	TGACTtcCAG	CTGATTGGCA	TCCAGGATGG	GTACCTATCA
eIF5a2	251	TTAAGAGAAA	TGATTatCAA	CTGATAIGCA	TTCAAGATGG	TTACCTTTCC
eIf5a1	301	CTGCTccagG	ACAGCGGGGA	GGTACGAGAG	GACCTTCGTC	TcCCTGAGGG
eIF5a2	301	CTGCTgacaG	AAACTGGTGA	AGTTCGTGAG	GATCTTAAAC	TgCCAGAAGG
eIf5a1	351	AGACCTTGGC	AAGGAGATTG	AGcagAAGTA	CGACTGTGGA	GAAGAGATCC
eIF5a2	351	TGAACTAGGC	AAAGAAATAG	AGggaAAATA	CAATGCAGGT	GAAGATGTAC
eIf5a1	401	TGATCACGGT	GCTGTctGCC	ATGACAGAGG	AGGCAGCTGT	TGCAATCAAG
eIF5a2	401	AGGTGTCTGT	CATGTgTGCA	ATGAGTGAAG	AATATGCTGT	AGCCATAAAA
eIf5a1	451	GCCatgGCAA	AATAa			
eIF5a2	451	CCct--GCAA	ATAA-			

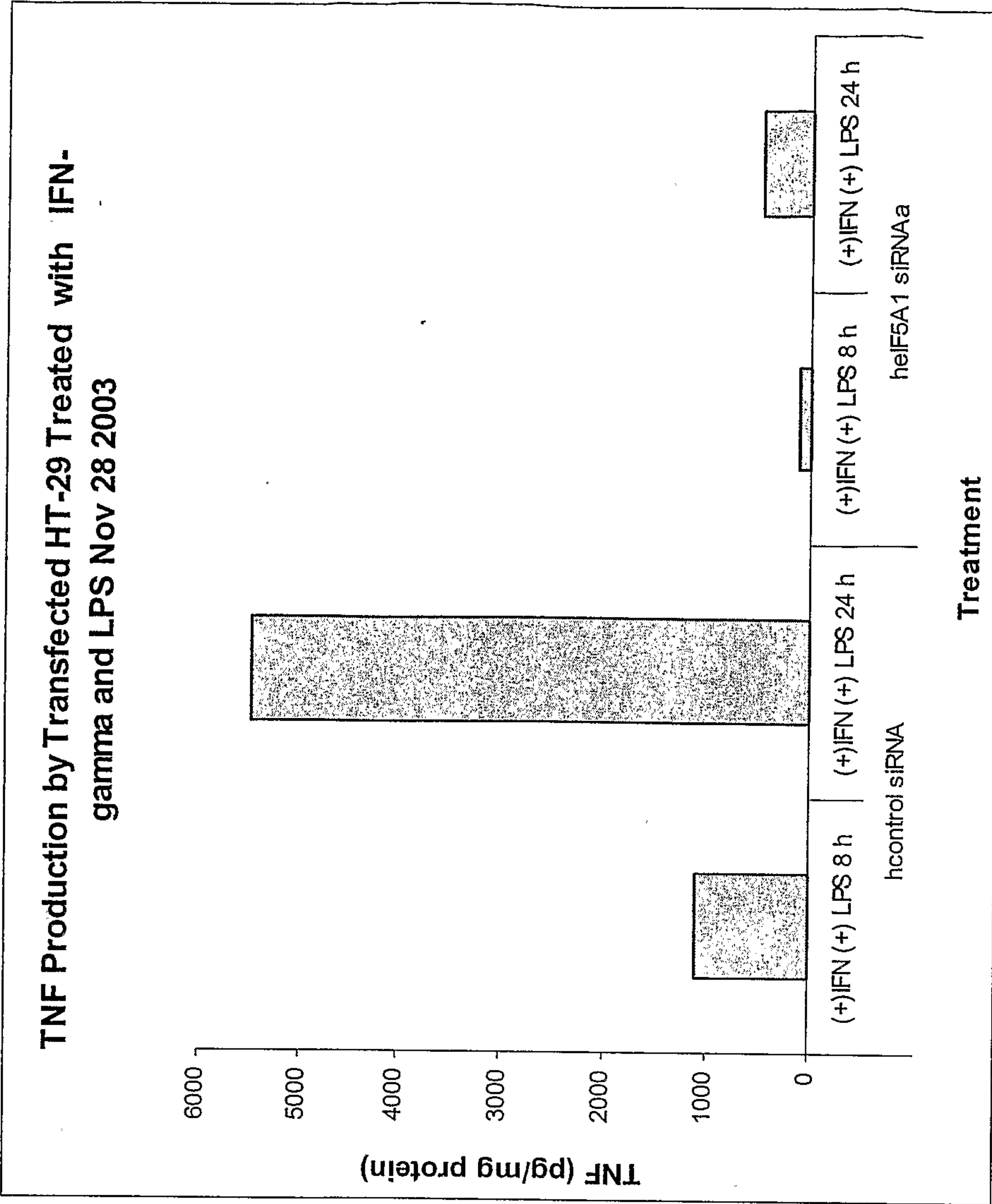
FIG. 73A

Amino Acid Alignment of Human eIF5A1 (ACC. NM 001970) and Human eIF5A2 (ACC. NM 020390)

eIF5a1		MADDLDFETG	DAGSATFPM	QCSALRKNGF	VVLKGwPCKI	VEMSASKTGK
eIF5a2	1	MADEIDFTTG	DAGASSTYPM	QCSALRKNGF	VVLKGwPCKI	VEMSTSKTGK
eIf5a1	51	HGHAKVHLVG	IDIFTGKKYE	DICPSTHNMD	VPNIKRNDYQ	LIGIQDGYLS
eIF5a2	51	HGHAKVHLVG	IDIFTGKKYE	DICPSTHNMD	VPNIKRNDYQ	LICIQDGYLS
eIf5a1	101	LLQDSGEVPE	DLRLPEGDLG	KEIEQKYDCG	EEILITLLSA	MTEEAATAIK
eIF5a2	101	LLTETGEVRE	DLKLPEGELG	KEIEGKYNAG	EDVQVSVPCA	MSEYAYATAIK
eIf5a1	151				amak	
eIF5a2	151				pck-	

FIG. 73B

B) ELISA



A) Western

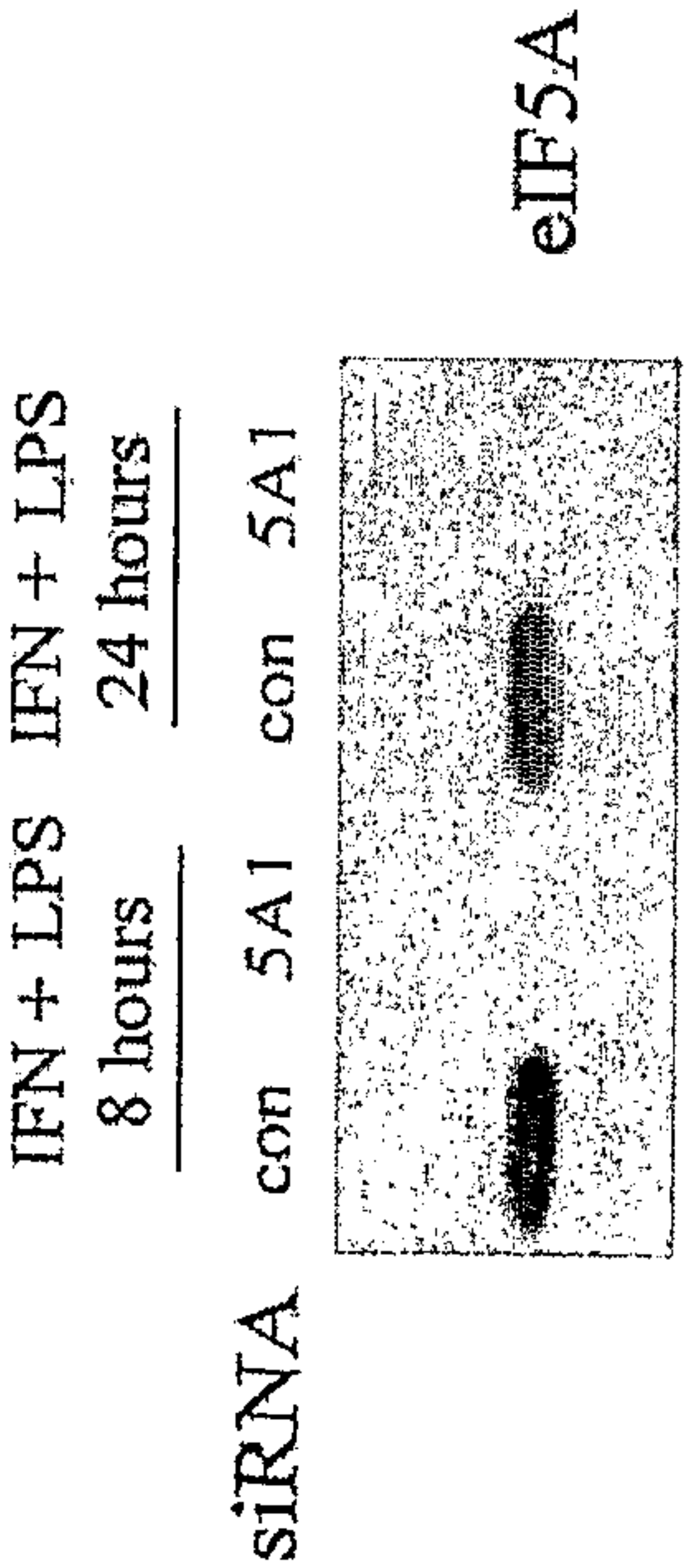


Figure 74

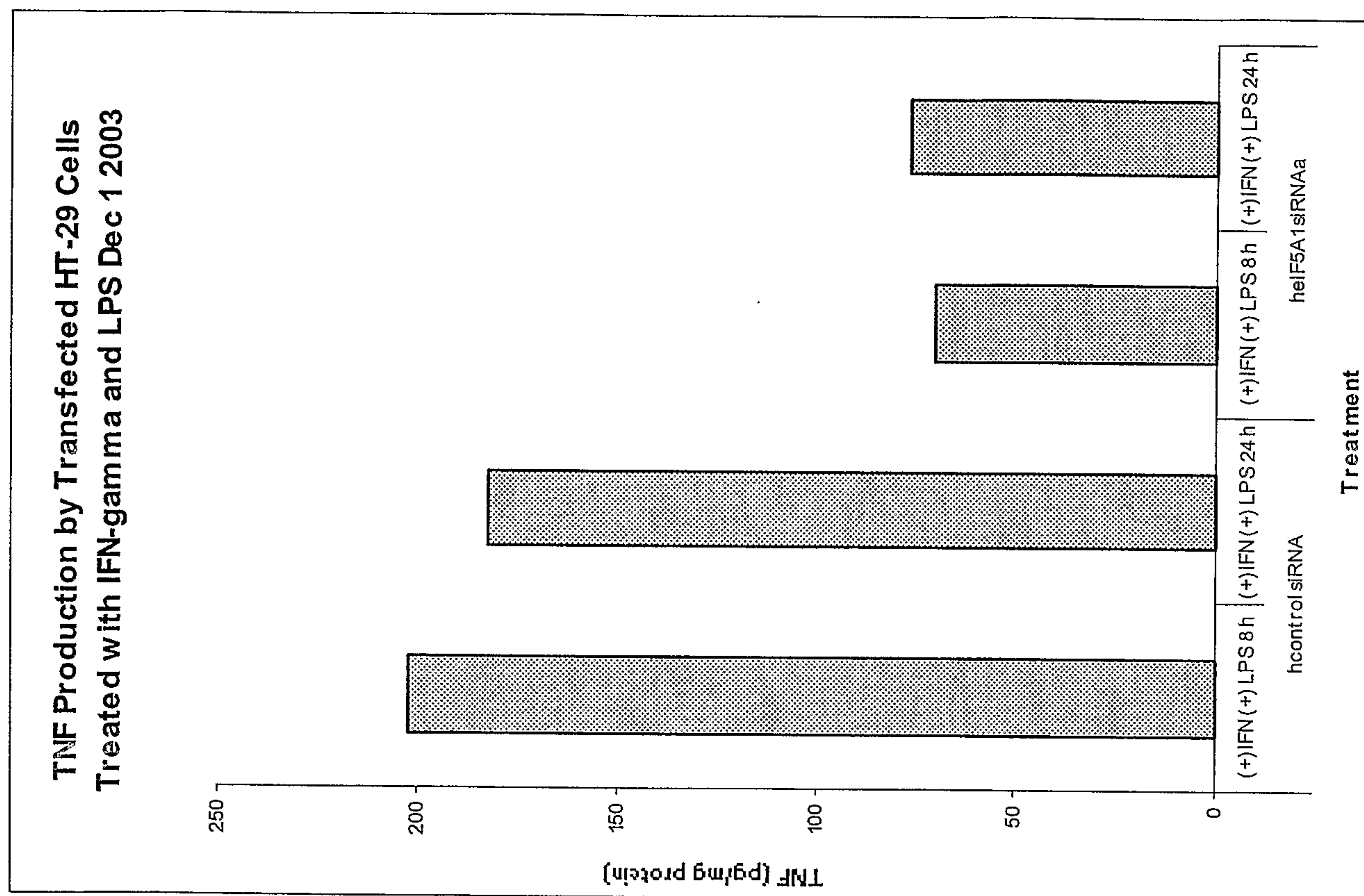


Figure 75

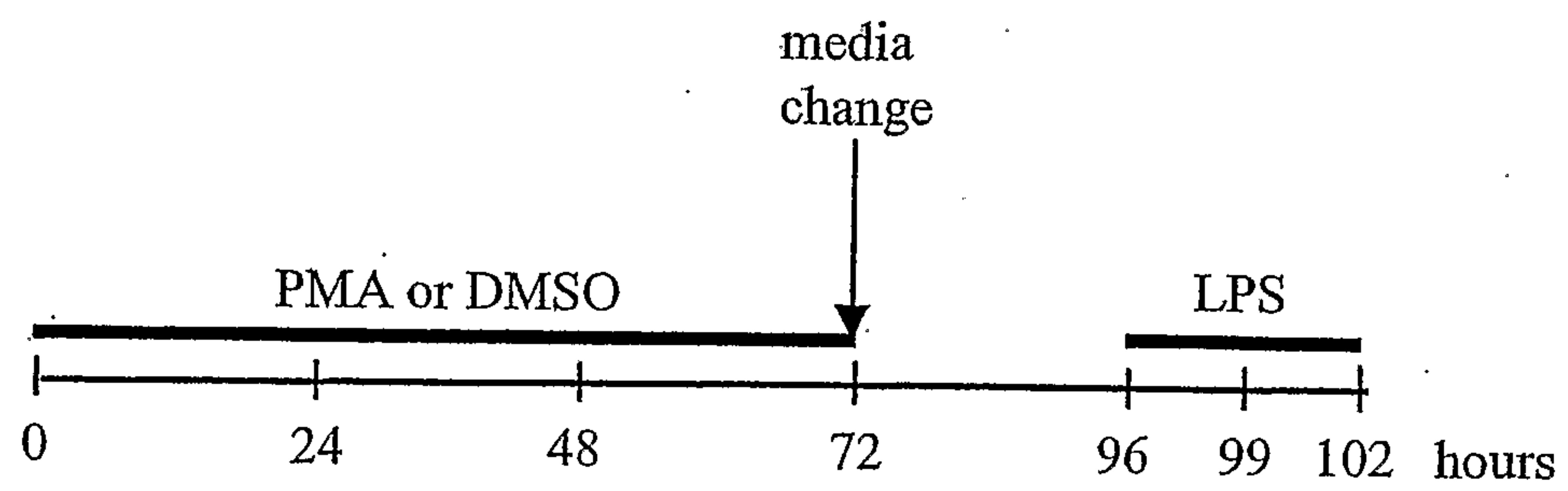


Figure 76 Time course of U-937 differentiation experiment.

eIF5A1 is Up-Regulated During Monocyte (U937) differentiation and subsequent TNF- α Secretion

WO 2004/078940

CA 02517974 2005-09-02

PCT/US2004/006598

87/100

Western Blot

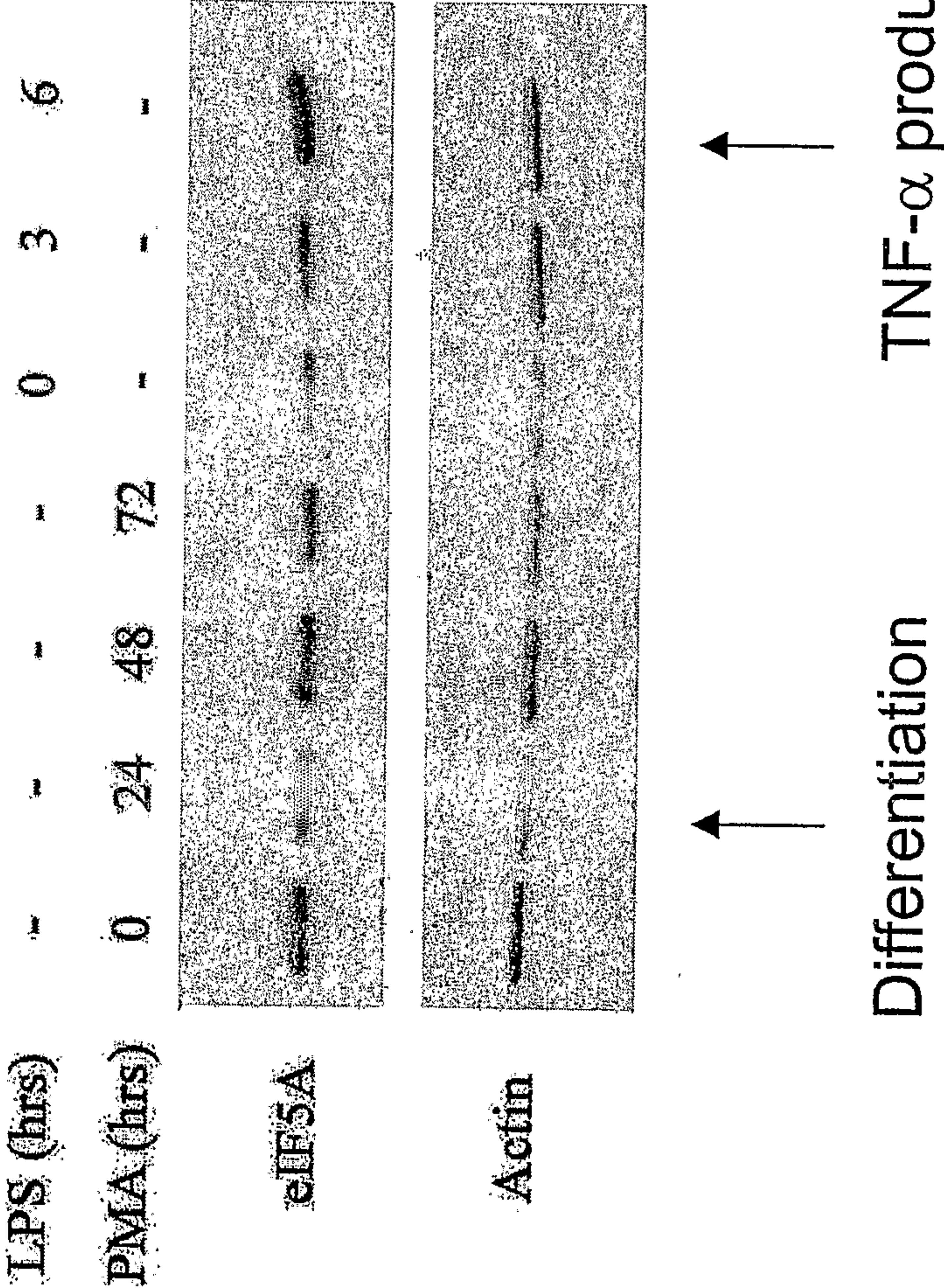


Fig. 7A

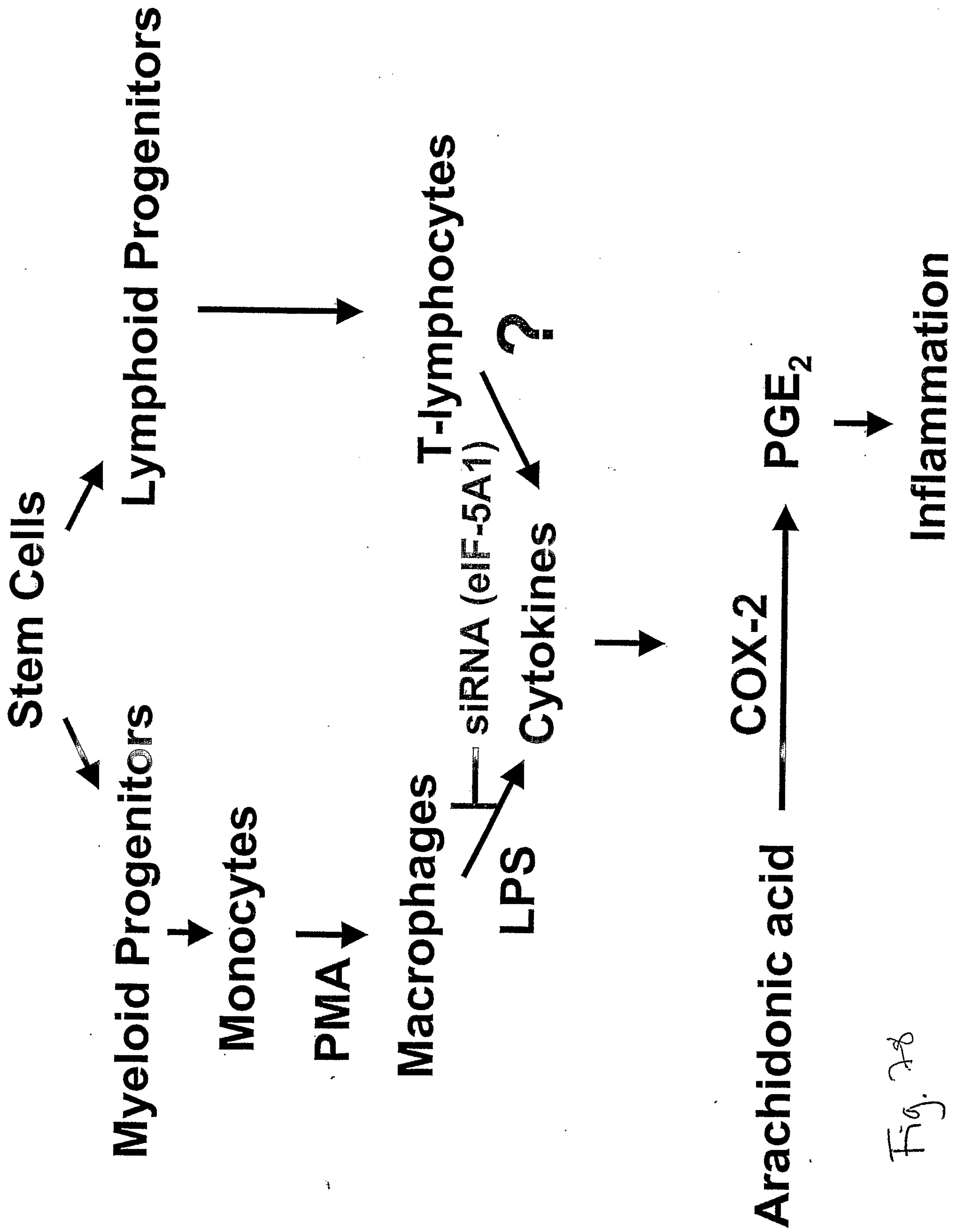


Fig. 28

Fig. 79

IL-8 Production of Transfected HT-29 Primed with IFN-gamma and Stimulated with TNF- α Jan 23 2004

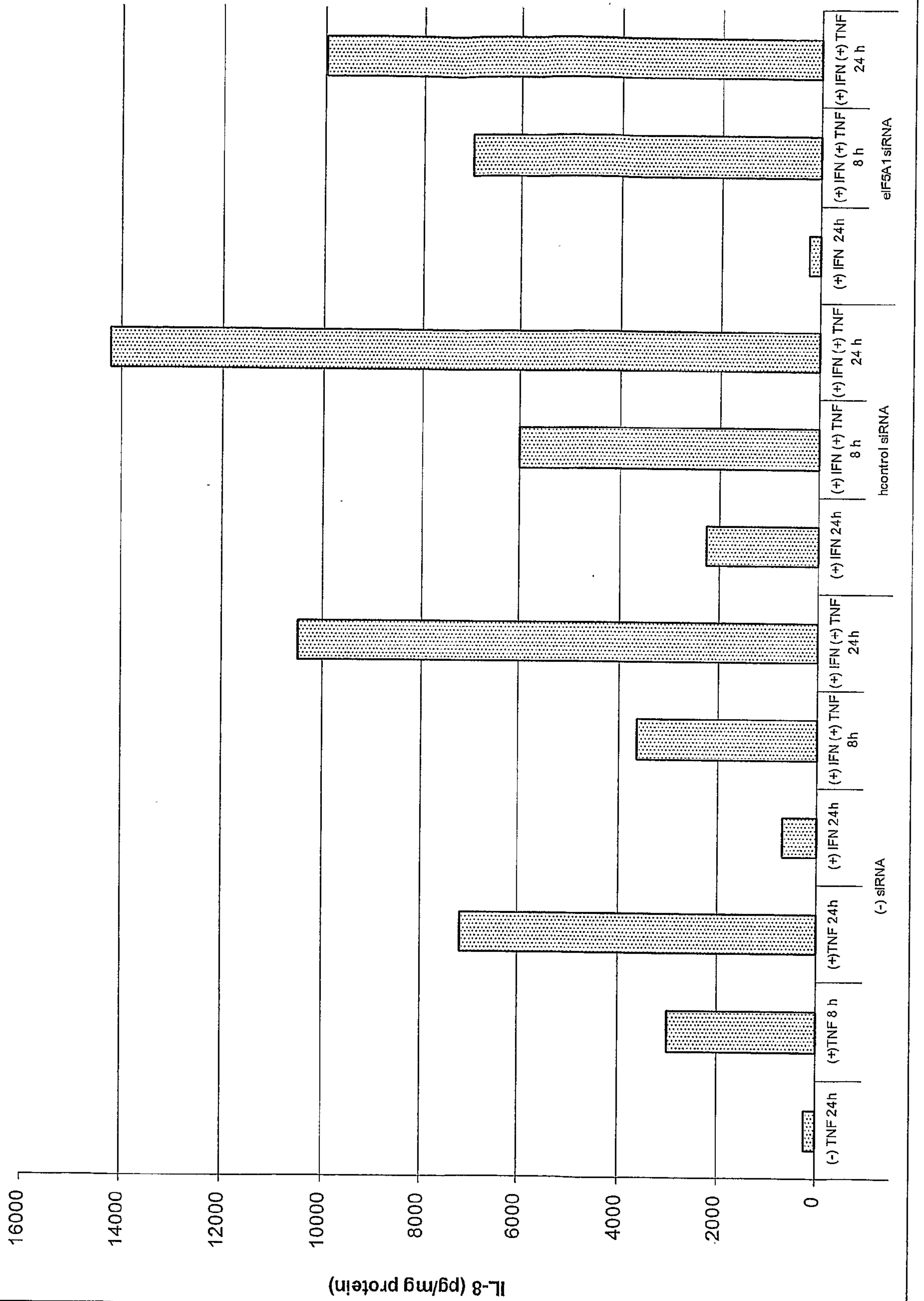
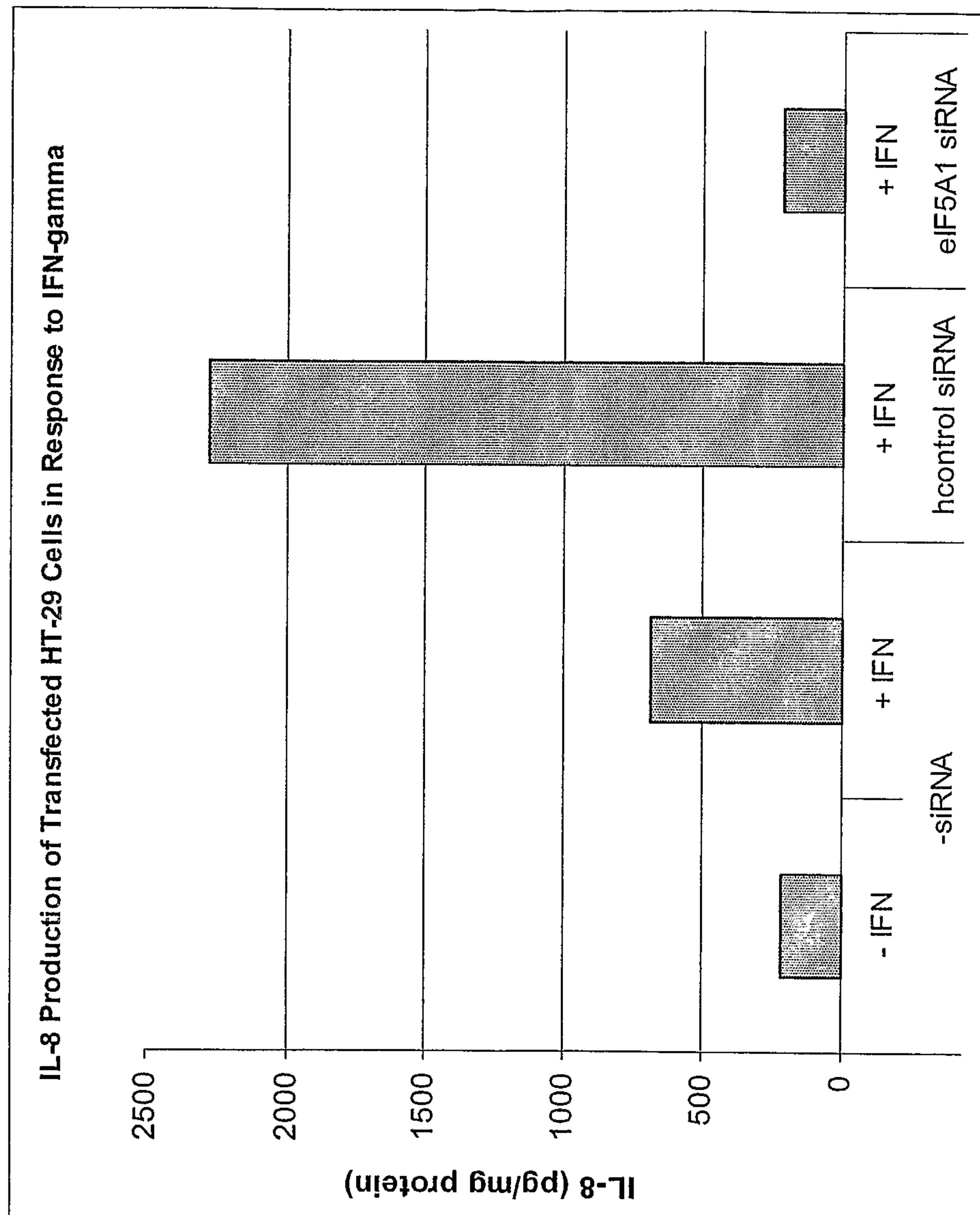


Fig. 80



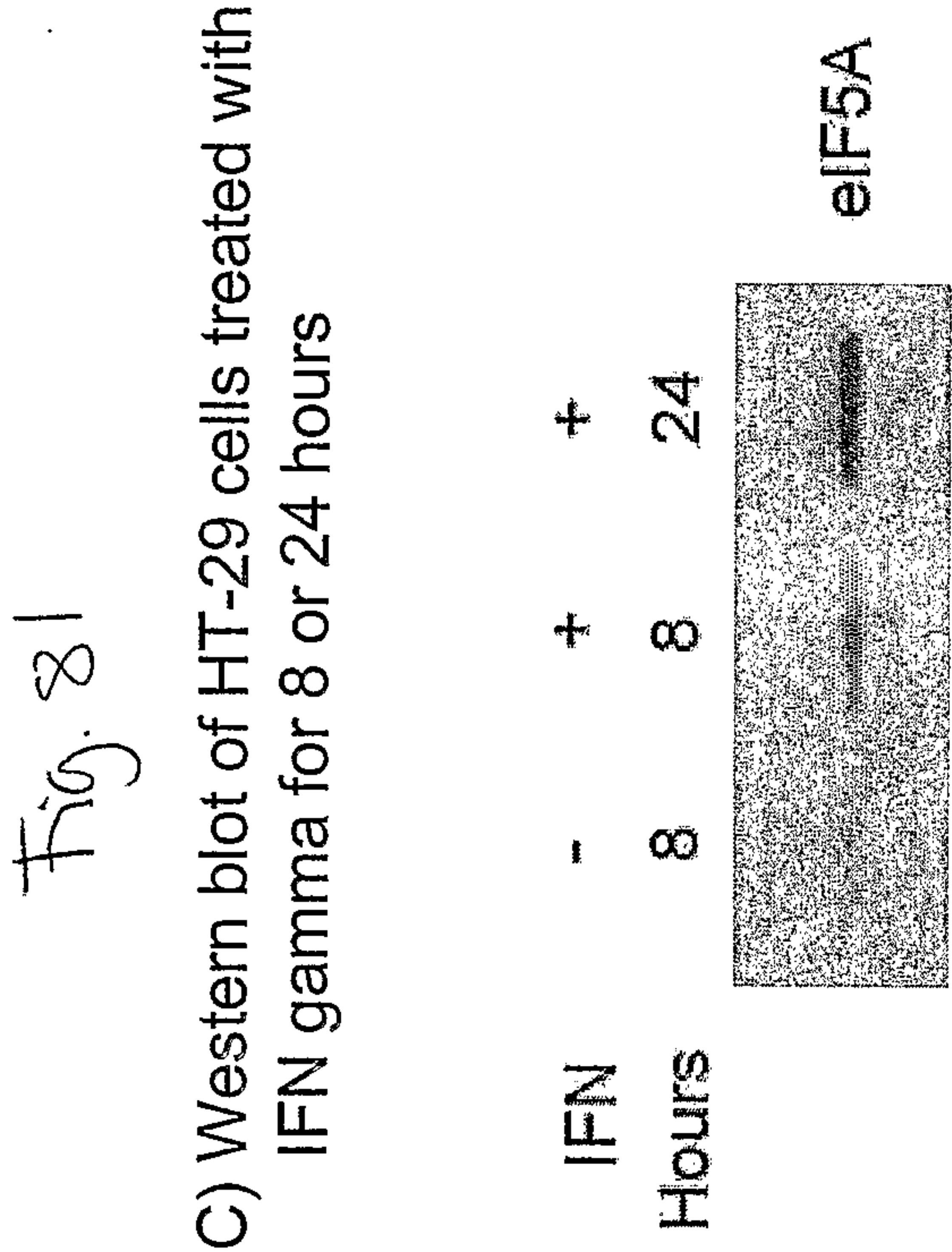


Figure 82

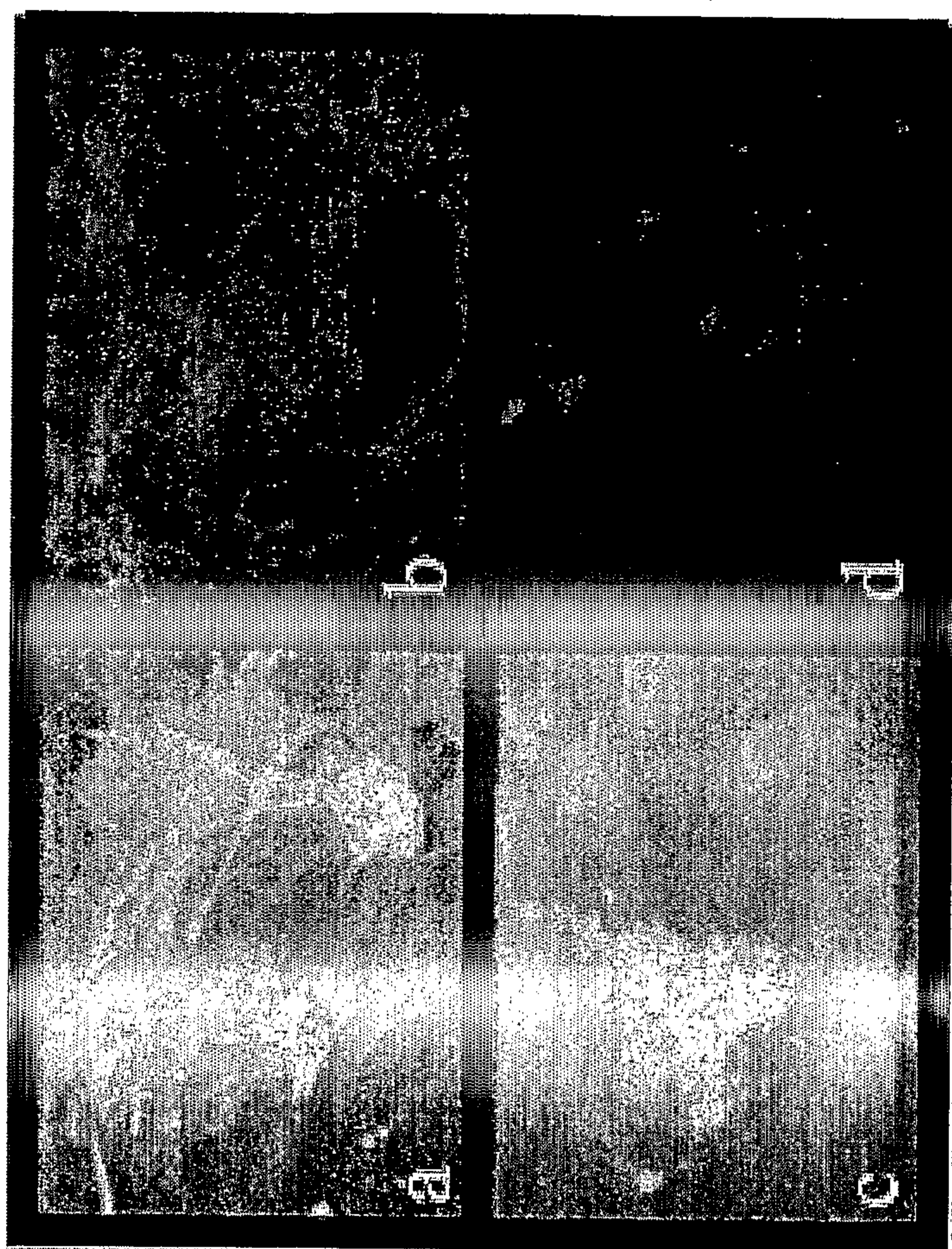


Figure 83

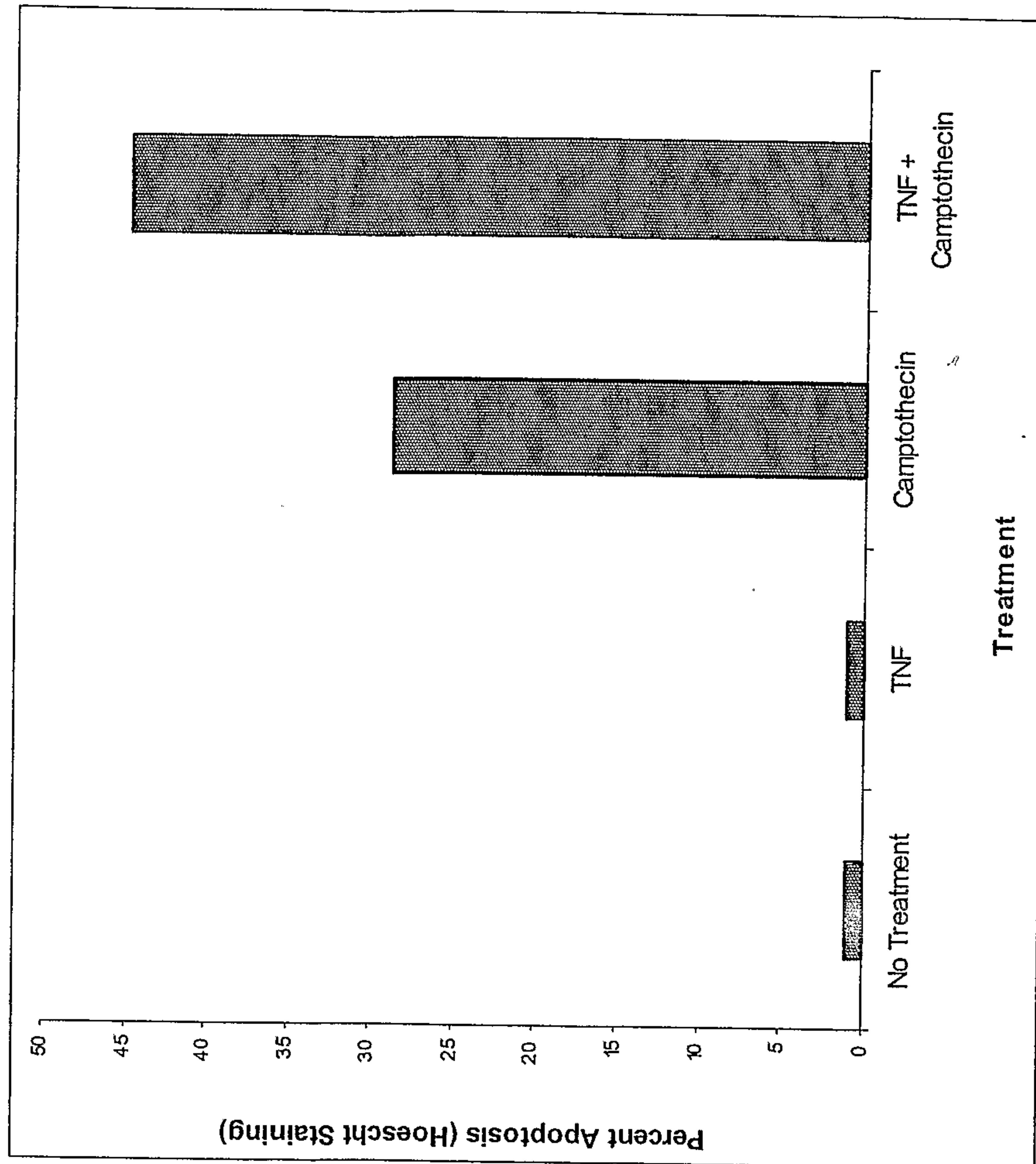


Figure 84

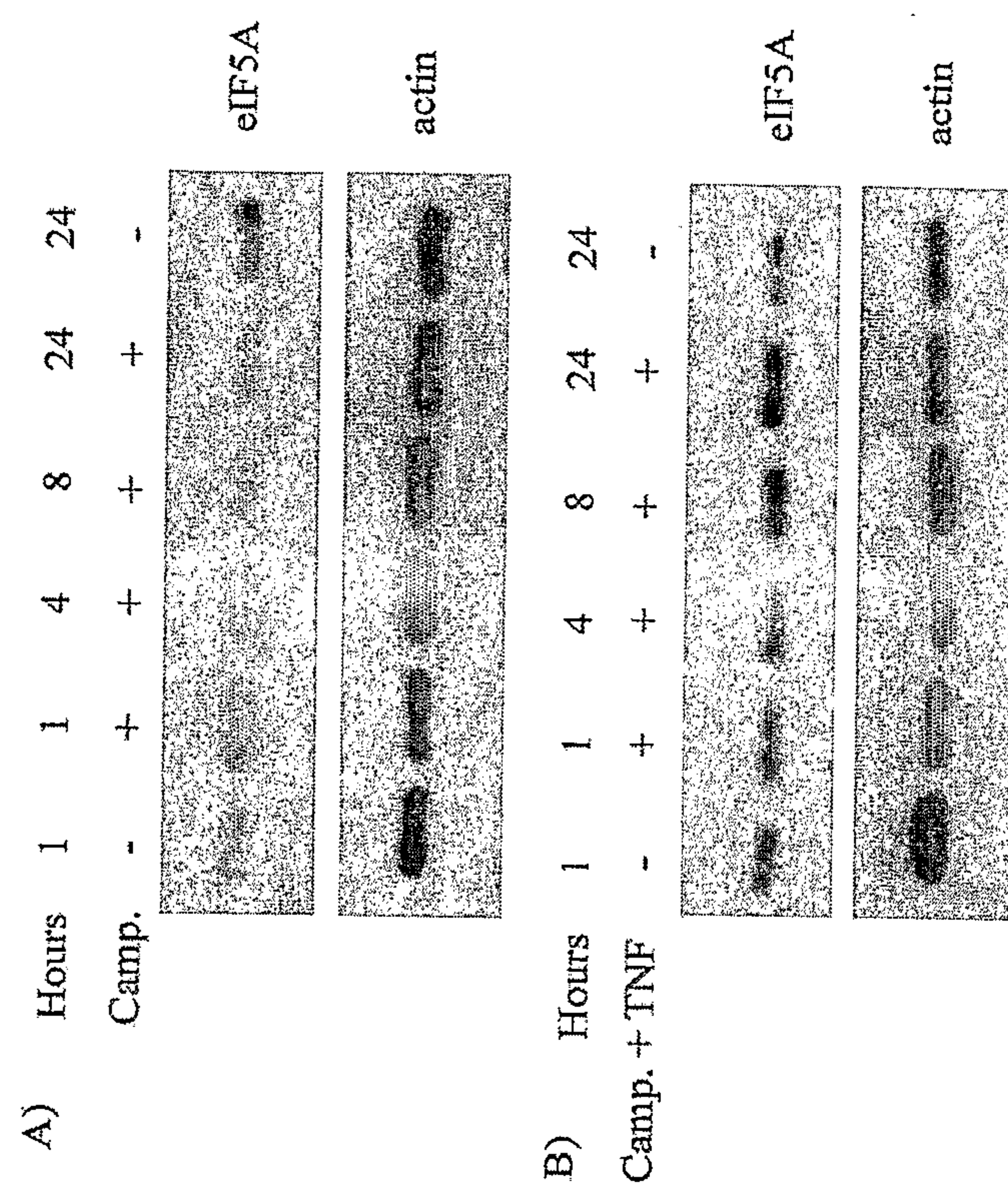


Figure 85

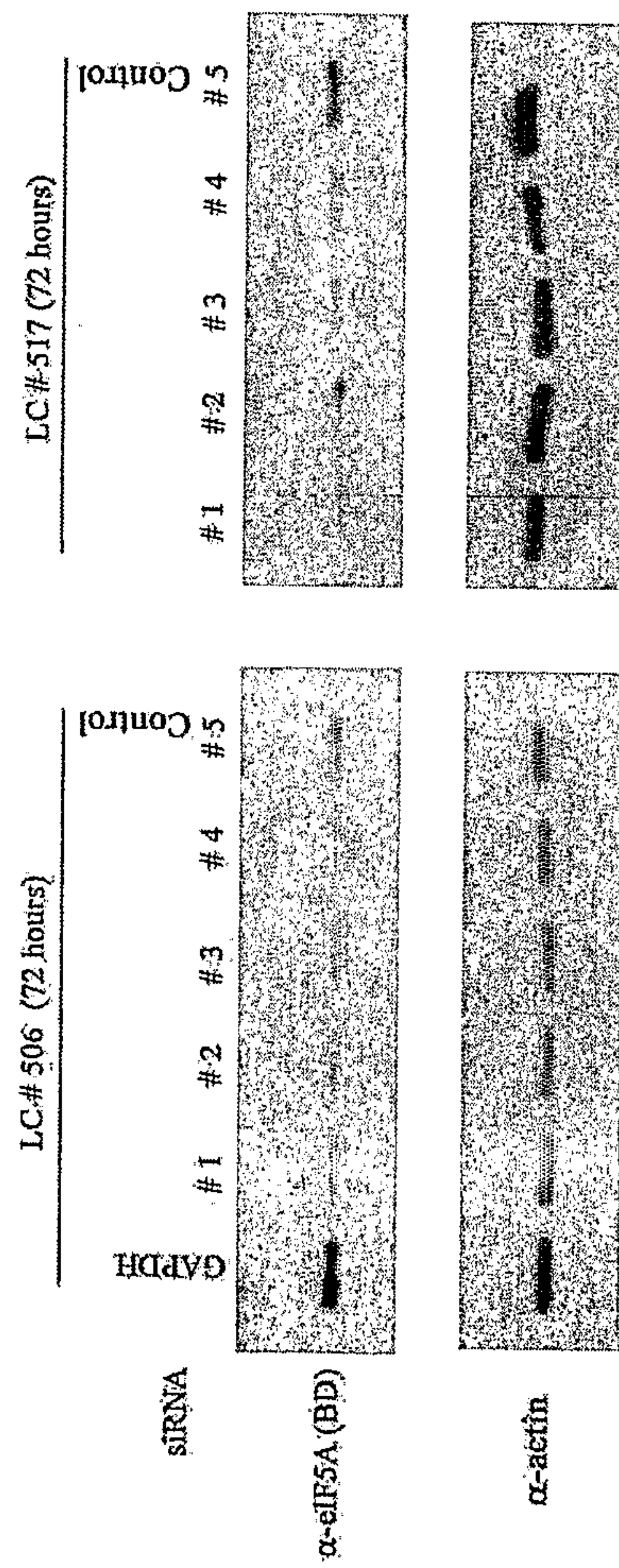


Figure 8c

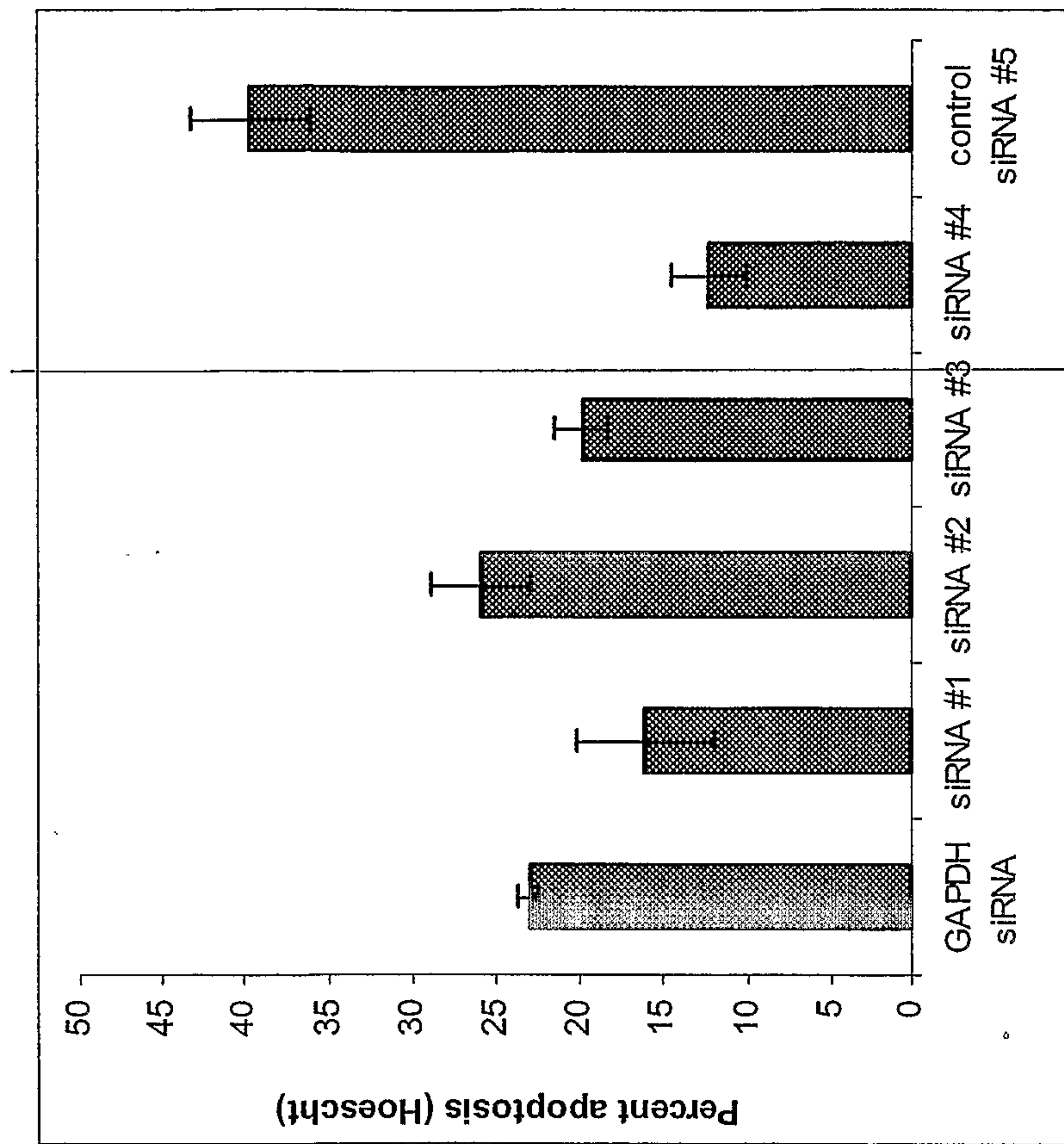


Figure 87

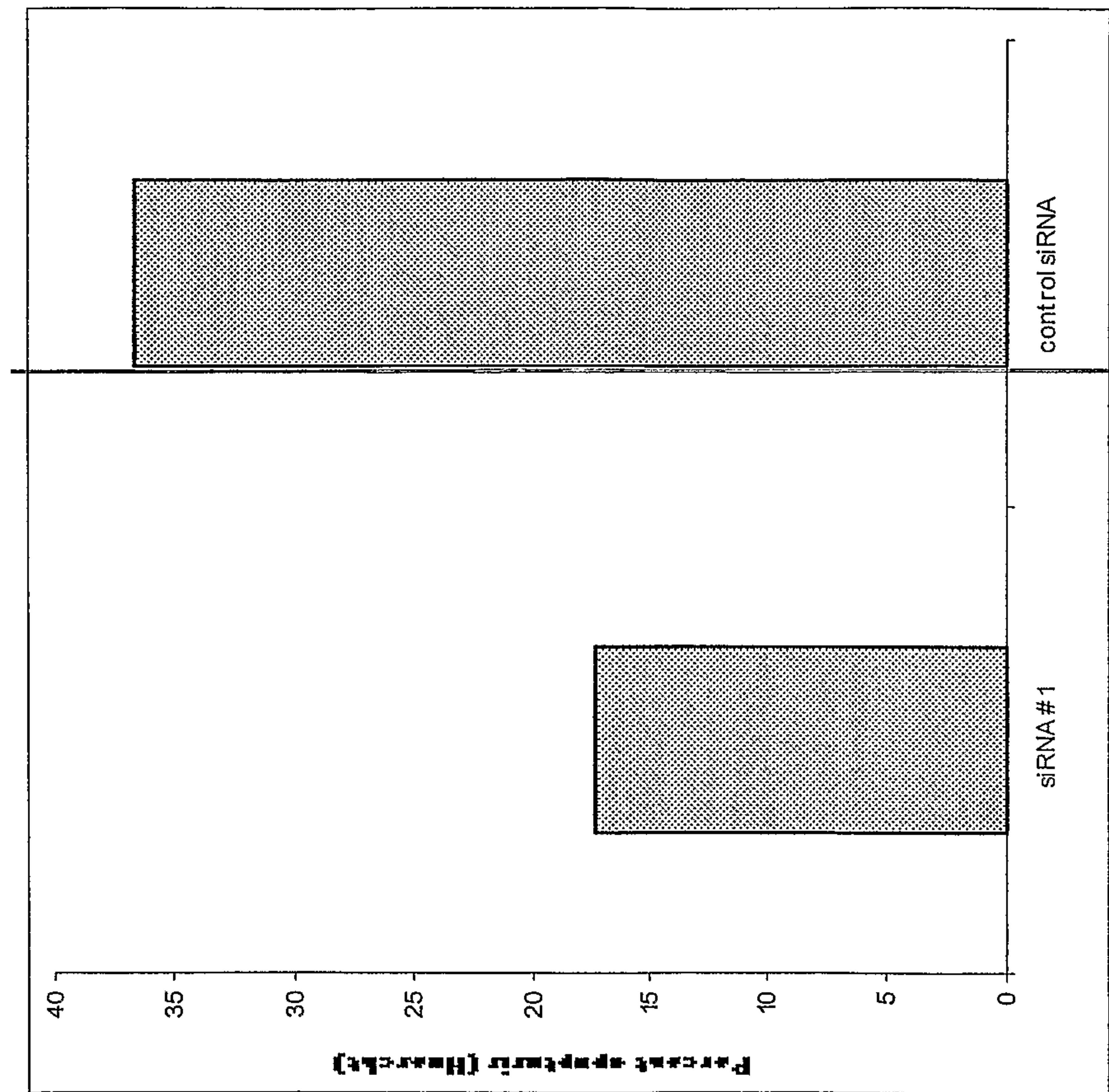
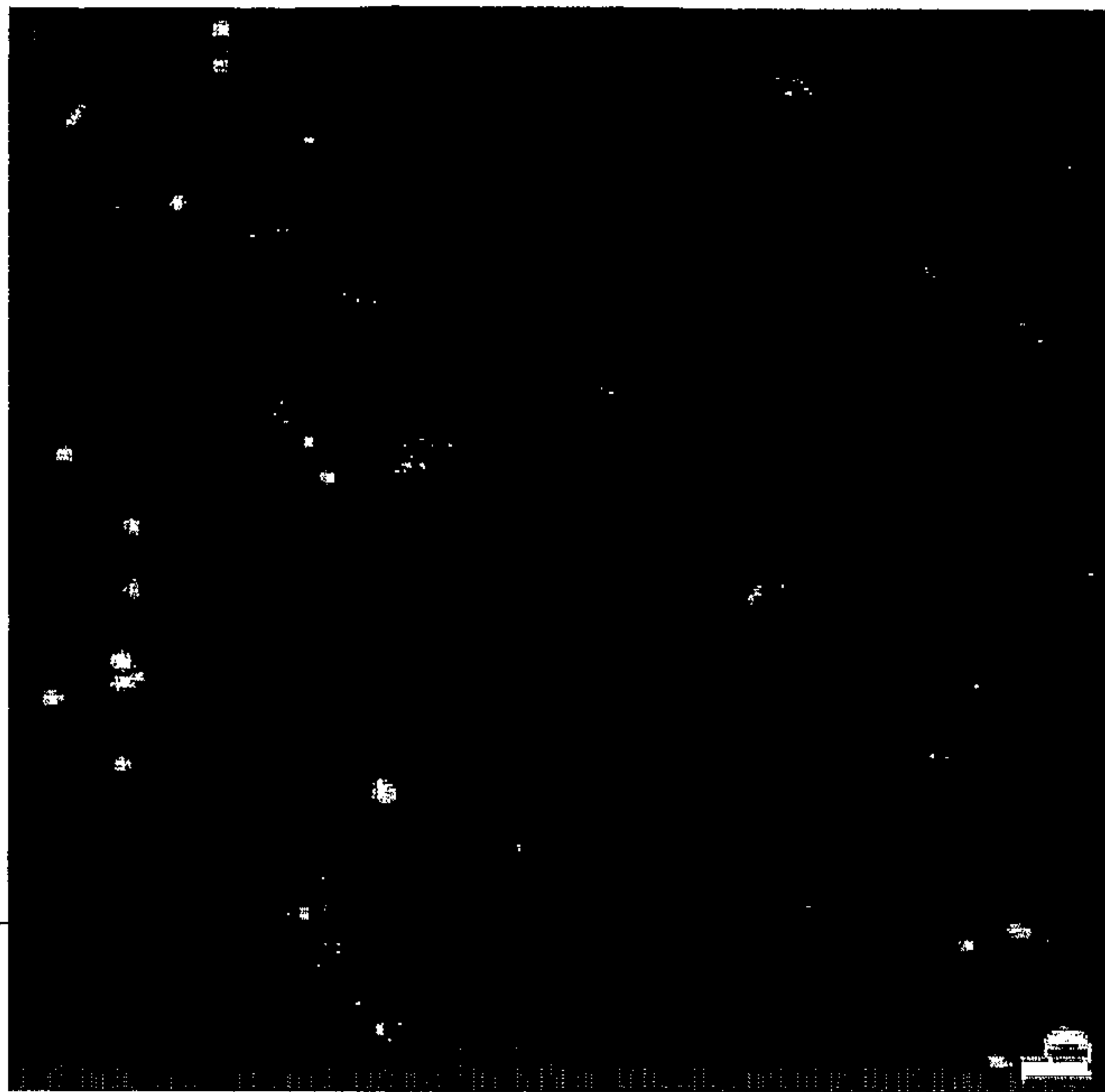
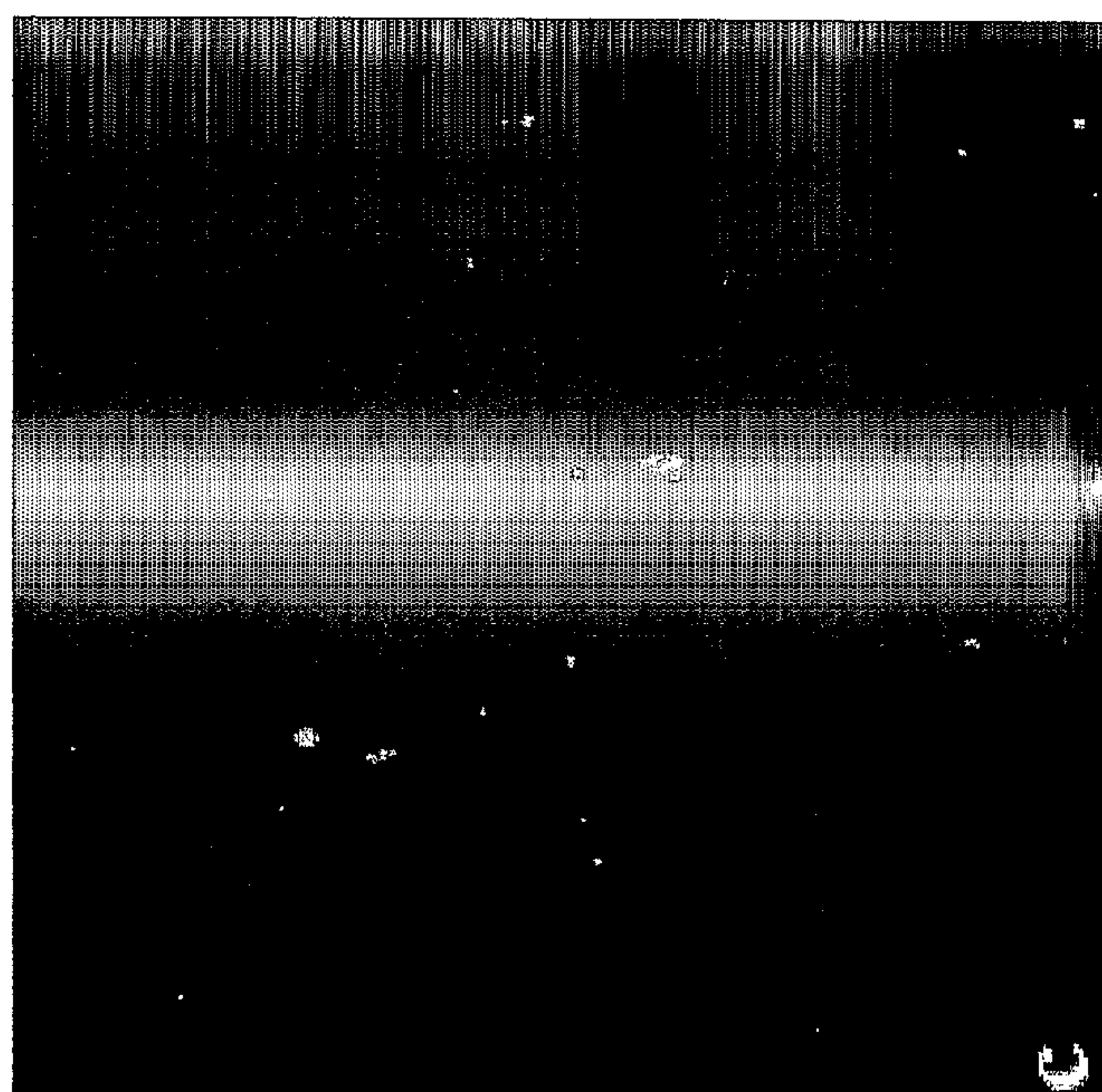
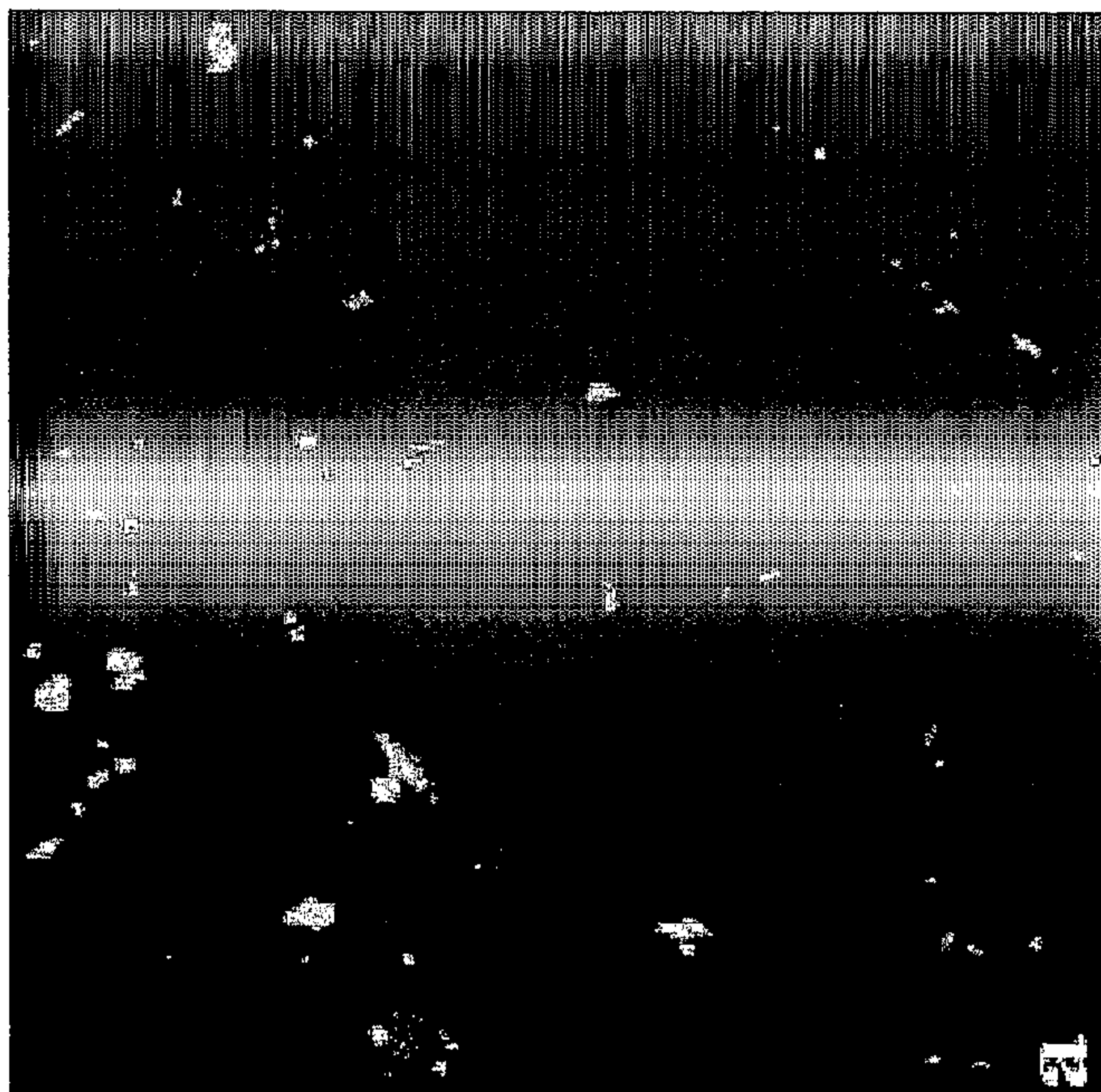


Figure 88

B



A



Control siRNA # 5

eIF5A siRNA # 1

Fig 89

Design of siRNAs against eIF5A1**Position of siRNAs within eIF5A1 mRNA**

* translation start and stop are bold and underlined

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1  ggcacgaggg tagagggcggc ggcggcggcg gcagcgggct cggagggcagc ggttgggctc
61  gcggcgagcg gacgggggtcg agtcagtgcg ttcgcgcgag ttggaatcga agcctcttaa
121 aatggcagat gacttggact tcgagacagg agatgcaggg gcctcagcca ccttcccaat
181 gcagtgtctca gcattacgta agaatggctt tgtggtgctc aaaggccggc catgtaagat
241 cgtcgagatg tctacttcga agactggcaa gcacggccac gccaaaggctc atctggttgg
301 tattgacatc tttactggga agaaatatga agatatctgc ccgtcaactc ataatatgga
361 tgtccccaac atcaaaagga atgacttcca gctgattggc atccaggatg ggtacctatc
421 actgctccag gacagcgggg aggtacgaga ggaccttcgt ctccctgagg gagaccttgg
481 caaggagatt gagcagaagt acgactgtgg agaagagatc ctgatcacgg tgctgtctgc
541 catgacagag gaggcagctg ttgcaatcaa ggccatggca aaataaactgg ctcccaggat
601 ggcggtggtg gcagcagtga tcctctgaac ctgcagaggc cccctccccg agcctggcct
661 ggctctggcc cggctcctaa ctggactccc cctacacaaat ttatttgacg ttttattttg
721 gttttcccca cccctcctcaat ctgtcgggga gccctgccc ttcacctagc tcccttggcc
781 aggagcgagc gaagctgtgg ccttggtgaa gctgccctcc tcttctcccc tcacactaca
841 gccctggtgg gggagaaggg ggtgggtgct gcttggtggt tagtcttttt tttttttttt
901 tttttttttt aaattcaatc tggaaatcaga aagcgggtgga ttctggcaaa tggtccttgt
961 gccctcccca ctcatccctg gtctggtccc ctggtgccc tagcccttta ccctgagcac
1021 caccccaaca gactggggac cagccccctc gcctgcctgt gtctctcccc aaaccctttt
1081 agatggggag ggaagaggag gagaggggag gggacctgcc ccctcctcag gcactctggga
1141 gggccctgcc cccatgggct ttacccttcc ctgcgggctc tctccccgac acatttggtta
1201 aaatcaaacc tgaataaaac tacaagttta atatgaaaaa aaaaaaaaaa aaaaaaaaaa
1261 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa

```

siRNA # 1 target

siRNA # 2 target

siRNA # 3 target

siRNA # 4 target

Position of siRNA targets and sequences and BLAST results**siRNA # 1** target position 375 to 395 bp (position 254 to 274 bp relative to start) % G/C = 39.1 BLAST = none but eIF5A

target 5' AA(AGGAATGACTTCCAGCTGA) 3'

siRNA 5' AAAGGAAUGACUCCAGCUGAdTdT 3'
3' dTdTUUUCUUACUGAAGGUCGACU 5'**siRNA # 2** target position 236 to 256 (115 to 135 bp relative to start) % G/C = 43.4 BLAST = identical to hypothetical protein in rat

target 5' AA(GATCGTCGAGATGTCTACT) 3'

siRNA 5' AAGAUCGUCGAGAUGUCUACUdTdT 3'
3' dTdTUUCUAGCAGCUCUACAGAUGA 5'**siRNA # 3** target position 284 to 304 (163 to 183 bp relative to start) % G/C = 43.4 BLAST = none but eIF5A

target 5' AA(GGTCCATCTGGTTGGTATT) 3'

siRNA 5' AAGGUCCAUCUGGUUGGUAUdTdT 3'
3' dTdTUUCAGGUAGACCAACCAUAA 3'**siRNA # 4** target position 678 to 698 (3' UTR; position 557 to 577 bp relative to start) % G/C = 48 BLAST = none but eIF5A

target 5' AA(GCTGGACTCCTCCTACACA) 3'

Fig 89
continued

siRNA

5' AAGCUGGACUCCUCCUACACAdTdT 3'
3' dTdT UUCGACCUGAGGAGGAUGUGU 5'

siRNA # 5 Control ; reverse sequence of siRNA # 1

% G/C = 39.1 BLAST = good, no more than 17/23 % identity

target

5' AA(AGTCGACCTTCAGTAAGGA) 3'

siRNA 5' AAAGUCGACCUUCAGUAAGGAdTdT 3'
3' dTdTUUUCAGCUGGAAGUCAUCCU 5'