



(86) **Date de dépôt PCT/PCT Filing Date:** 2006/12/21
(87) **Date publication PCT/PCT Publication Date:** 2007/06/28
(45) **Date de délivrance/Issue Date:** 2016/10/04
(85) **Entrée phase nationale/National Entry:** 2008/06/20
(86) **N° demande PCT/PCT Application No.:** CA 2006/002089
(87) **N° publication PCT/PCT Publication No.:** 2007/071045
(30) **Priorité/Priority:** 2005/12/21 (US60/752,018)

(51) **Cl.Int./Int.Cl. C12N 15/12** (2006.01),
A61K 31/105 (2006.01), **A61K 38/17** (2006.01),
A61P 25/00 (2006.01), **A61P 25/28** (2006.01),
C07K 14/47 (2006.01), **C07K 16/18** (2006.01),
C12Q 1/68 (2006.01), **G01N 33/50** (2006.01)

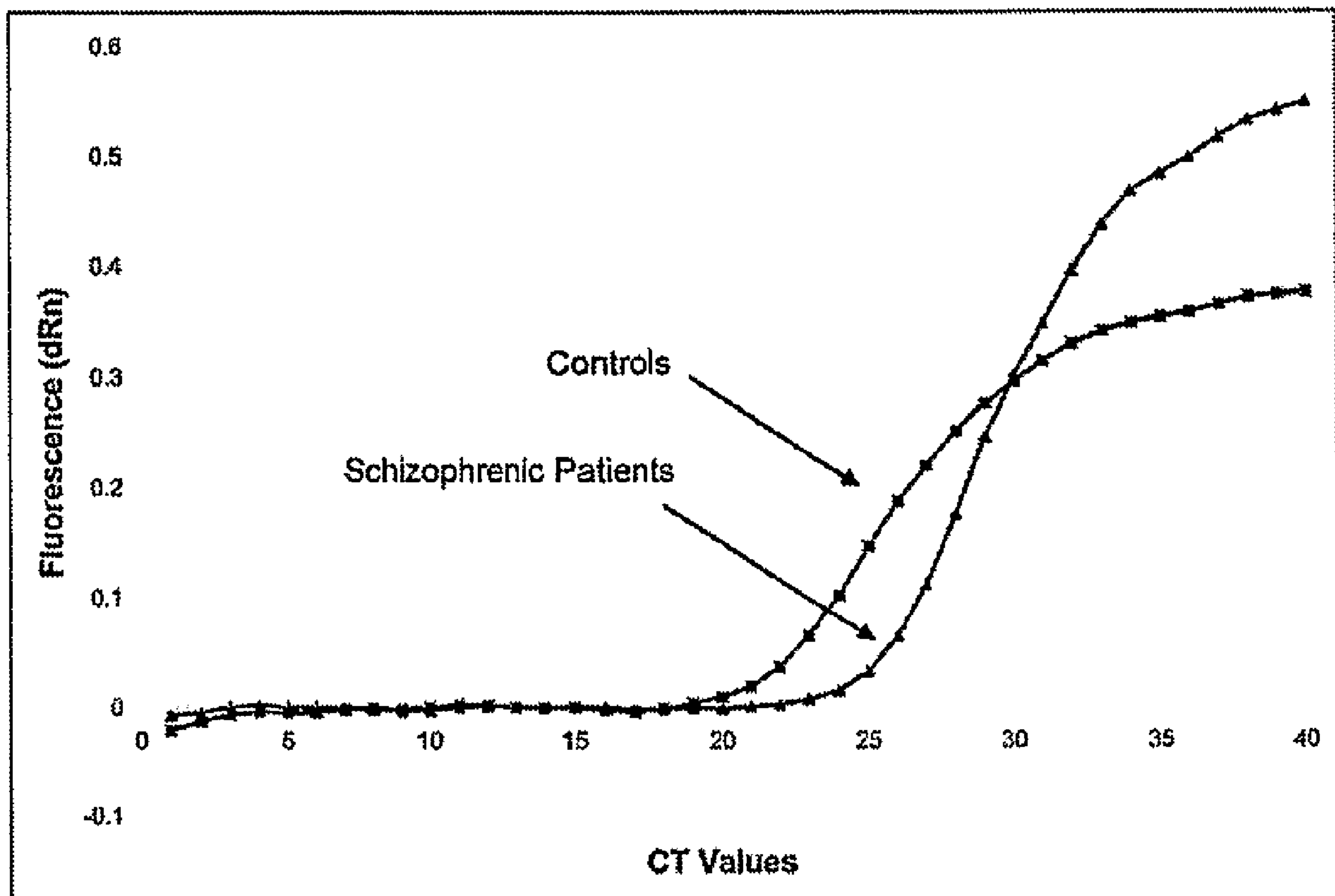
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(54) **Titre : PROTEINES REGULEES PAR CATECHOLAMINES**

(54) **Title: CATECHOLAMINE REGULATED PROTEIN**



(57) **Abrégé/Abstract:**

A novel family of mammalian catecholamine proteins is identified. These proteins are useful in methods of diagnosing neurological diseases, including schizophrenia and bipolar disease, as well as cardiovascular disease. This family of CRP40 proteins are also useful to treat neurological diseases.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 June 2007 (28.06.2007)

PCT

(10) International Publication Number
WO 2007/071045 A1

(51) International Patent Classification:

C12N 15/12 (2006.01) *C07K 14/47* (2006.01)
A61K 31/105 (2006.01) *C07K 16/18* (2006.01)
A61K 38/17 (2006.01) *C12Q 1/68* (2006.01)
A61P 25/00 (2006.01) *G01N 33/50* (2006.01)
A61P 25/28 (2006.01)

(21) International Application Number:

PCT/CA2006/002089

(22) International Filing Date:

21 December 2006 (21.12.2006)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/752,018 21 December 2005 (21.12.2005) US

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(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

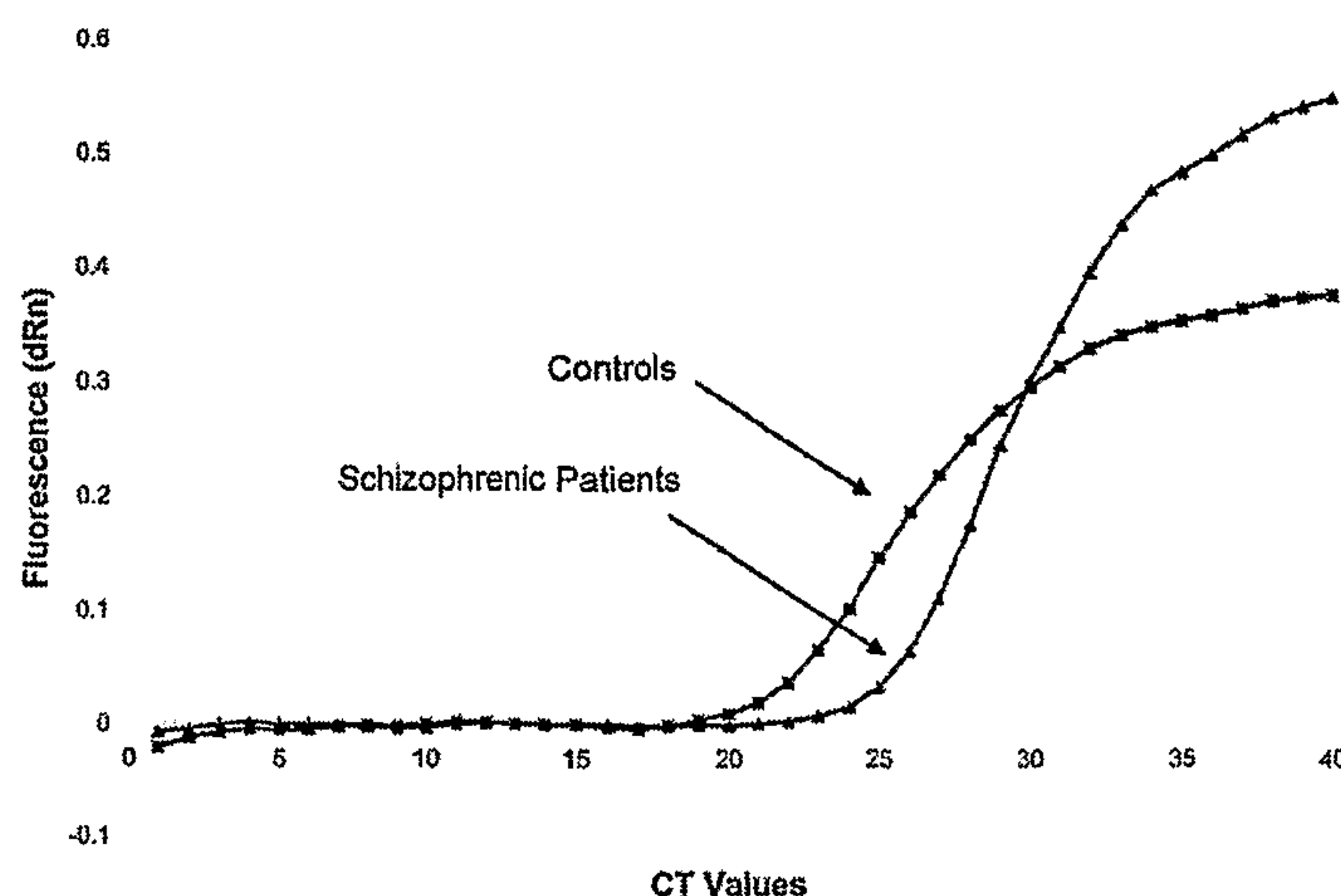
(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:— *of inventorship (Rule 4.17(iv))***Published:**

— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CATECHOLAMINE REGULATED PROTEIN



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WO 2007/071045 A1

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CATECHOLAMINE REGULATED PROTEIN

Field of the Invention

[0001]The present invention relates to a novel protein, and more particularly, the present invention relates to catecholamine-regulated proteins, including mortalin-2, which are useful as a biomarker and therapeutic for neurological disease, including neurodevelopmental and neurodegenerative diseases. In addition, these proteins are also useful as a biomarker of cardiovascular disease.

Background of the Invention

[0002]Molecular chaperone proteins also referred to as heat shock proteins, are ubiquitous highly conserved proteins that bind to unstable proteins and aid in their refolding to maintain proper and stable conformation (Macario and Conway 2002;Ohtsuka and Suzuki 2000;Sherman and Goldberg 2001;Soti and Csermely 2002b;Soti and Csermely 2002a). Furthermore, molecular chaperone proteins have additional functions such as: 1) shuttling proteins to the nucleus; 2) acting as a transcriptional factor in cell regulation; 3) and providing weak binding abilities to normal proteins within the cell in order to maintain elements of cellular networks (Sherman and Goldberg 2001;Soti and Csermely 2002b;Soti and Csermely 2002a). Environmental and oxidative stresses lead to the expression of molecular chaperone proteins, which bind to the hydrophobic surfaces of damaged and denatured proteins, allowing for their proper folding and prevention of precipitation and aggregation of these proteins, ultimately preventing cell death (Macario and Conway 2002).

[0003]As the organism ages, cells are prone to mutations, posttranslational aberrations, increased oxidative and environmental stresses, resulting in greater aggregation of proteins (Giffard et al. 2004;Muchowski and Wacker 2005). Ageing of the organism may also lead to deficiencies in the anti-stress mechanism such as decreased molecular chaperone synthesis and inefficiencies in the ubiquitin-proteasome and lysosome-mediated autophagy degradation pathways (Muchowski and Wacker 2005). Recent reports have implicated deficiencies in molecular chaperone recruitment to the progression of many neurodegenerative and neurodevelopmental diseases such as: Parkinsons, Alzheimers and schizophrenia (Ohtsuka and Suzuki 2000;Sherman and Goldberg 2001;Soti and Csermely 2002a). This concept is especially important in post-mitotic cells such as neurons, which leads to massive accumulation of aggregated proteins (Soti and Csermely 2002a).

[0004]The presence of a unique class of *brain-specific* proteins, which bind to DA and structurally related catecholamines has recently been reported. These proteins have been termed catecholamine-regulated proteins (CRPs) (Ross et al. 1993; Ross et al. 1995). Three species of CRP (with molecular weights of 26, 40, and 47-kDa, respectively) have been isolated. Pharmacological and biochemical studies have shown no similarity between these particular proteins, known catecholamine binding proteins or receptors present in the brain (Modi et al. 1996; Ross et al. 1993; Ross et al. 1995). However, these particular proteins have high homology with the heat shock protein family. For example, molecular cloning of bovine brain CRP40 (Genbank #AF047009) revealed that this protein is related to the heat shock protein 70kDa (Hsp70) family. As discussed earlier, heat shock proteins act as molecular chaperones and protect the cell from oxidative and other types of stresses (Ben Zvi and Goloubinoff 2001; Grover 2002; Soti and Csermely 2002b; Soti and Csermely 2002a).

[0005]Mortalin is a mitochondrial heat shock protein that was discovered in 1991 as a 66kDa protein in mouse embryonic fibroblasts (Wadhwa et al. 1991). In order to trace molecular mechanisms of cell immortalization, studies using mouse embryonic fibroblasts identified mortalin as a mortality marker. The protein was cloned and characterized as Mortalin-1 (mot-1) in the cytoplasm of murine fibroblast cells (Wadhwa et al. 1991). Transfected mot-1 cDNA in NIH3T3 cells was distributed in the cytoplasm and resulted in cell senescence. In contrast, the mot-2 cDNA isoform was encoded in the perinuclear region and resulted in the malignant transformation of NIH 3T3 cells ultimately inducing cell immortalization in normal human fibroblasts (Kaul et al. 2003). Interestingly, the mot-1 and mot-2 proteins differ from each other by only 2 amino acid residues and the 2 murine mortalin isoforms come from two separate genes (Xie et al. 2000).

[0006]The mot-2 protein has been described as a multifunctional protein due to its diverse functions (chaperone, anchoring protein and signal transduction). Recent reports have shown that the N-terminus of the mot-2 protein binds to the carboxyl end of the tumor suppressor gene p53 (Kaul et al. 2002; Wadhwa et al. 2002). This binding property inhibits the transactivation of p53 to the nucleus, resulting in cell immortalization. Mot-2 has been recognized as an important biological marker in cancer tumor research.

[0007]The human genome project has discovered approximately 32000 genes in the human species (Human Genome Sequencing Consortium, Nature, 2000). With the use of high-throughput techniques of sequencing the human genome and Established Sequence Tags

(EST's), the complexity of the human genome has increased dramatically (Graveley 2001; Modrek and Lee 2003; Wadhwa et al. 2002). EST's are derived from fully processed mRNA, which occur following the introduction of the 5' capping, splicing, and polyadenylation of the 3' end (Modrek and Lee 2003; Wadhwa et al. 2002). Alternate splicing involves the exon skipping of particular exons within a gene, which results in the generation of multiple transcripts encoding different proteins, with possibly different functions in 70-88% of the studies reported (Graveley 2001; Wadhwa et al. 2002). Alternate splicing occurs in the human genome with a frequency between 35-59%, which results in at least one splice alternative for each gene reported (Modrek and Lee 2003; Wadhwa et al. 2002).

[0008] With the aid of information gathered from the human genome project, it would be desirable to identify biomarkers effective to diagnose disease, such as neurological disease including neurodegenerative and neurodevelopmental disease, particularly since access to neuronal tissue to obtain this information is not possible.

Summary of the Invention

[0009] A novel catecholamine regulated protein, referred to herein as CRP40, has been identified and determined to be useful in the diagnosis of neurological disease, including neurodegenerative and neurodevelopmental disorders, as well as cardiovascular disease. Reduced intracellular expression of CRP40 is indicative of neurological disease, while increased intracellular expression of CRP40 is indicative of cardiovascular disease. Mortalin-2 has also been found to have similar diagnostic uses.

[0010] Thus, in one aspect of the present invention, an isolated human CRP40 protein is provided or a functionally equivalent variant thereof.

[0011] In another aspect of the invention, a method of diagnosing neurological disease is provided which includes the steps of:

- a) obtaining a biological sample from a patient; and
- b) determining the amount of at least one of CRP40 and mortalin-2 protein present in the sample, wherein a decrease of at least about 10% in the amount of the protein as compared to a control amount present in a non-diseased patient is indicative of a neurological disease.

[0012]In another aspect of the invention, a method of diagnosing cardiovascular disease in a patient is provided which includes the steps of:

- a) obtaining a biological sample from the patient; and
- b) determining the amount of at least one of CRP40 and mortalin-2 protein present in the sample, wherein an increase of at least about 10% in the amount of the protein as compared to a control amount present in a non-diseased patient is indicative of cardiovascular disease.

[0013]In another aspect of the invention, a method of diagnosing neurological disease is provided which includes the steps of:

- a) obtaining a biological sample from a patient; and
- b) determining the amount of nucleic acid encoding at least one of CRP40 and mortalin-2 protein present in the sample, wherein a decrease of at least about 10% in the amount of CRP40 or mortalin-2 nucleic acid as compared to a control amount present in a non-diseased patient is indicative of disease.

[0014]In another aspect of the invention, a method of diagnosing cardiovascular disease is provided which includes the steps of:

- a) obtaining a biological sample from a patient; and
- b) determining the amount of nucleic acid encoding at least one of CRP40 and mortalin-2 protein present in the sample, wherein an increase of at least about 10% in the amount of CRP40 or mortalin-2 nucleic acid as compared to a control amount present in a non-diseased patient is indicative of disease.

[0015]A therapeutic composition is provided in another aspect of the invention. The composition comprises human CRP40, or a functionally equivalent variant thereof in combination with a pharmaceutically effective adjuvant.

[0016]A method of treating neurological disease is also provided in another aspect. The method comprises administering to a mammal in need of treatment a therapeutically effective amount of at least one of CRP40 or mortalin-2, or a functionally equivalent variant thereof.

[0017] A method of treating cardiovascular disease in a mammal is also provided in another aspect. The method comprises the step of inhibiting the expression of at least one of CRP40 and mortalin-2 in the mammal.

[0018] An article of manufacture is provided in another aspect of the invention. The article of manufacture includes a composition and packaging containing the composition. The composition includes at least one of CRP40 and mortalin-2, or a functionally equivalent fragment thereof, in combination with a pharmaceutically acceptable carrier. The packaging is labeled to indicate that the composition is effective to treat neurological disease.

[0019] An article of manufacture is provided in another aspect of the invention. The article of manufacture includes a composition and packaging containing the composition. The composition includes at least one of CRP40 and mortalin-2, or a functionally equivalent fragment thereof, in combination with a pharmaceutically acceptable carrier. The packaging is labeled to indicate that the composition is effective to treat cardiovascular disease.

[0019a] In one aspect, there is provided an isolated human CRP40 protein of approximately 40 kDa comprising the amino acid sequence as defined in SEQ ID NO: 8, or functionally equivalent variant thereof exhibiting at least about 98% sequence homology with the amino acid sequence of SEQ ID NO: 8.

[0019b] In another aspect, there is provided an isolated nucleic acid molecule encoding a human CRP40 protein of approximately 40 kDa comprising the amino acid sequence as defined in SEQ ID NO: 8, or functionally equivalent variant thereof exhibiting at least about 98% sequence homology with the amino acid sequence of SEQ ID NO: 8.

[0019c] In another aspect, there is provided a method of diagnosing neurological disease associated with hypo-dopamine levels in a human subject, wherein the neurological disease is bipolar disease, schizophrenia, or Parkinson's disease, comprising the steps of: a) obtaining a blood or blood-derived sample from the subject; b) measuring in the blood or blood-derived sample the level of a human CRP40 protein of approximately 40 kDa comprising the amino acid sequence as defined in SEQ ID NO: 8, or functionally equivalent variant thereof exhibiting at least about 98% sequence homology with the amino acid sequence of SEQ ID NO: 8 or nucleic acid encoding said CRP40 protein or

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functionally equivalent variant thereof exhibiting at least about 98% sequence homology with the amino acid sequence of SEQ ID NO: 8; and c) comparing the level of said CRP40 protein or said variant or the nucleic acid encoding said CRP40 protein or said variant against a normal control sample, wherein a reduction in the level of said CRP40 protein or said variant or the nucleic acid encoding said CRP40 protein or said variant by at least about 10% as compared to the normal control sample is indicative of disease.

[0019d] In another aspect, there is provided an isolated human CRP40 protein having a molecular weight of approximately 40 kDa comprising an amino acid sequence as defined in SEQ ID NO: 8 or a variant thereof exhibiting at least about 98% sequence homology to the amino acid sequence of SEQ ID No. 8 and which variant is capable of binding dopamine.

[0019e] In another aspect, there is provided the isolated human CRP40 protein or variant as defined herein, wherein the human CRP40 protein consists of the amino acid sequence as defined in SEQ ID No.8 or the amino acid sequence of SEQ ID No. 8 containing one or more conservative amino acid substitutions or additions or deletions.

[0019f] In another aspect, there is provided a method of diagnosing neurological disease in a biological sample, comprising the steps of determining the amount of CRP40 protein as defined herein or a nucleic acid as defined herein, present in the sample; wherein a reduction of the protein or the nucleic acid by at least 10% as compared to a control amount present in a non-diseased patient is indicative of a neurological disease.

[0020] These and other aspects of the invention will become apparent by reference to the accompanying description and drawings in which:

Brief Description of the Drawings

Figure. 1 illustrates the agarose gel electrophoresis results of a reverse transcriptase chain reaction performed with three combinations of forward and reverse primers generated from the BQ224193 EST;

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Figure. 2 illustrates that a 740bp band was identified by gel electrophoresis on a restriction enzyme digestion of the BQ224193 forward and reverse primers (pi and p5, respectively) which was performed prior to transfection of the BQ224193 fragment;

Figure. 3 illustrates by acrylamide gel electrophoresis results using Coomassie blue that, following the transformation and growth of the BQ224193 fusion protein fragment, growing the fusion protein at 12⁰C resulted in the largest portion of fusion protein in the soluble state;

Figure. 4 illustrates that pure, isolated 23kDa fusion protein was produced on cleavage with thrombin protease from the vector;

Figure. 5 graphically illustrates the results of protein binding assays which show that recombinant human CRP40 binds catecholamines in a manner similar to rat and bovine CRPs;

Figure. 6 shows RLM-RACE PCR results with BQ primers and Hela cells as a positive control;

Figure. 7 illustrates the results of a Northern Blot Analysis using 50 µg SHSY5Y Neuroblastoma Poly A RNA and a P³²cDNA probe with Human Ambion Brain RNA and p1, p5 primers. The blot was stripped and re-probed with Beta-Actin for proper size determination;

Figure. 8 is a standard curve by Real-Time PCR using BQ 2, 4 Primers, and SHSY5Y cells using Real-Time PCR;

Figure. 9 is an amplification plot of SHSY5Y cells transfected with the DA D2L receptor comparing HAL-treated cells versus normal controls with Real-Time PCR;

Figure. 10 graphically illustrates relative quantitation using the Housekeeping gene, human cyclophyllin with the same SHSY5Y cells;

Figure. 11 is an amplification plot of Real-Time PCR using 100ng cDNA from SHSY5Y RNA and cyclophyllin primers;

Figure. 12 graphically illustrates the results of localization studies using immunohistochemistry on postmortem brain specimens with human CRP40 as the primary antibody and FITC as the secondary florescent antibody;

Figure 13A illustrates the amino acid sequence of human CRP40 (SEQ ID NO: 8);

Figure 13B illustrates the amino acid sequence of human CRP40 (SEQ ID NO: 7);

Figure 13C illustrates the functional characteristics of the human CRP40 amino acid sequence;

Figure 13D illustrates a hypothetical 3D model of human CRP40;

Figure 14 graphically illustrates RNA expression of CRP40 in human post-mortem RNA of normal individuals, individuals with bipolar disease and individuals with schizophrenia;

Figure 15 graphically illustrates RNA expression of CRP40 in human post-mortem RNA of individuals with no drug treatment, and individuals with varying ranges of anti-psychotic drug treatment;

Figure 16 graphically illustrates prepulse inhibition in CRP40 knock-down animal study;

Figure 17 graphically illustrates the real time PCR significant difference between schizophrenic patients (n=6) and controls (n=4) with respect to CRP40 levels;

Figure 18 graphically illustrates the real time PCR significant difference between schizophrenic patients (n=4) and controls (n=2) with respect to CRP40 levels;

Figure 19 graphically illustrates the real time PCR significant difference between a drug naïve schizophrenic patient and a control with respect to CRP40 levels; and

Figure 20 graphically illustrates the real time PCR significant difference between a Parkinson's patient and a control with respect to CRP40 levels.

Detailed Description of the Invention

[0021] A novel isolated catecholamine regulated protein, referred to herein as human CRP40 or CRP40, is provided. CRP40 proteins, which include CRP40 and functionally equivalent variants thereof, are useful as both biomarkers in the diagnosis of neurological disease and as therapeutic agents in the treatment of such diseases. CRP40 proteins are also useful as biomarkers in the diagnosis of cardiovascular disease.

[0022] The term "isolated" is used herein to refer to CRP40 protein which are essentially pure and free from extraneous cellular material including other proteins or peptide fragments.

[0023]As used herein, the term “neurological disease” is meant to encompass neurodevelopmental disease such as schizophrenia, bipolar disease and autism, as well as neurodegenerative disease such as Parkinson’s and Alzheimer’s, and other autoimmune diseases and genetic diseases such as multiple sclerosis, amyotrophic lateral sclerosis, Huntington disease, Creutzfeldt-Jakob, ADHD, Tourettes Syndrome, Rett Syndrome, minimal brain dysfunction in children and related neurological and psychiatric disorders.

[0024]As used herein, the term “cardiovascular disease” is meant to encompass disease involving platelet aggregation that is greater than that which occurs in healthy individuals, such as atherosclerosis, obesity, ischemia hypoxia, stenosis, angina, diabetes and glucose dysregulation.

[0025]CRP40 is a 350 amino acid catecholamine-regulated protein, the specific amino acid sequence of which is identified in Figure 13A. The expression of CRP40 is inversely linked to neurological disease in mammals such that a decrease of at least about 10% in the expression of CRP40 is indicative of neurological disease. Thus, CRP40 is useful as a biomarker for neurological diseases. In addition, given that this protein is lacking in these diseases, CRP40, functionally equivalent variants of CRP40 and nucleic acid encoding these are also useful in the treatment of such diseases.

[0026]In contrast, increased levels of CRP40, i.e. to levels greater than that normally found in a healthy individual (a normal level), is indicative of cardiovascular disease. Increased levels of CRP40 results in platelet aggregation that leads to cardiovascular disease. Thus, CRP40 can also be used as a biomarker of cardiovascular disease wherein detection of an increase in CRP40 of at least about 10% from normal levels indicates a cardiovascular condition.

[0027]Mortalin-2 or mot-2 was determined to be related to CRP40, CRP40 being a spliced variant of mortalin-2. Mortalin-2 contains 679 amino acids. Mortalin-2 was also found to have utility as a biomarker of neurological disease, useful in the diagnosis of cardiovascular disease, and to have therapeutic utility. Although the overlapping regions of mot-2 and CRP40 are 98% homologous, the diagnostic and therapeutic utilities of mot-2 were not previously known. Given this functional overlap, reference to “CRP40/mortalin-2” is made to refer to either CRP40 or mortalin-2.

[0028]As will be appreciated by those of skill in the art, modified forms of CRP40 or mortalin-2, herein termed “functionally equivalent variants”, may exist or may be prepared

which retain CRP40 function and, thus, retain utility for use as biomarkers for neurological and cardiovascular disease, and for the treatment of neurological disease. The variant need not exhibit identical activity, but exhibit sufficient activity to be useful as a biomarker and/or for therapeutic uses.

[0029]Such modifications may, for example, result naturally from alternative splicing during transcription or from genetic coding differences. Such variants can readily be identified using established cloning techniques, as described in more detail in the specific examples that follow, employing primers derived from CRP40/mortalin-2. Additionally, such modifications may result from non-naturally occurring synthetic alterations made to CRP40/mortalin-2 to render a functionally equivalent variant which may have more desirable characteristics for use as a therapeutic, for example, increased activity or stability. Non-naturally occurring variants of CRP40 include analogues, fragments and derivatives thereof.

[0030]A functionally equivalent analogue of CRP40/mortalin-2 in accordance with the present invention incorporates 1 or more amino acid substitutions, additions or deletions. Commonly, amino acid additions or deletions are terminal additions or deletions at either end of the peptide to yield a functionally equivalent peptide; however, analogues incorporating internal amino acid insertion or deletion are within the scope of the present invention. Amino acid substitutions within CRP40/mortalin-2, particularly conservative amino acid substitutions, may also generate functionally equivalent analogues thereof. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as alanine, isoleucine, valine, leucine or methionine for another; the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glutamine and glutamic acid, between asparagine and aspartic acid, and between glycine and serine; the substitution of one basic residue such as lysine, arginine or histidine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

[0031]A functionally equivalent fragment in accordance with the present invention comprises a portion of the CRP40/mortalin-2 sequence. The fragment may comprise an interior portion of the CRP40/mortalin-2 sequence, or may comprise a terminal portion thereof.

[0032]A functionally equivalent CRP40 derivative in accordance with the present invention is CRP40/mortalin-2, or an analogue or fragment thereof, in which one or more of the amino

acid residues therein is chemically derivatized. The amino acids may be derivatized at the amino or carboxy groups, or alternatively, at the side "R" groups thereof. Derivatization of amino acids within the peptide may render a peptide having more desirable characteristics such as increased stability or activity. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form, for example, amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form, for example, salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form, for example, O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids, for example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Terminal modification of a peptide to protect against chemical or enzymatic degradation may also include acetylation at the N-terminus and amidation at the C-terminus of the peptide.

[0033]In the diagnostic aspects of the invention, a biological sample is obtained that is suitable to quantify either the level of CRP40/mortalin-2 protein (CRP40, mortalin-2 or a naturally occurring variant thereof) or the level of CRP40- or mortalin-2 -encoding nucleic therein. Suitable biological samples for this purpose include blood (e.g. platelets and lymphocytes), saliva, urine, semen, hair, skin and cerebrospinal fluid. The sample is obtained from the mammal using methods conventional for the sample type. Many of these samples can readily be obtained in a non-invasive manner. Cerebrospinal fluid is obtained using the spinal tap procedure. The amount of biological sample required must be sufficient to allow quantification of CRP40 protein or CRP40-encoding nucleic acid therein. For example, an amount of about 5 ug protein is generally needed for CRP40/mortalin-2 quantification, while about 10 ng nucleic acid is generally needed for CRP40/mortalin-2 nucleic acid quantification.

[0034]In order to quantify CRP40/mortalin-2 protein content in a biological sample, the protein fraction is first isolated therefrom using standard isolation and fractionation techniques including lysis/centrifugation, precipitation and separation using, for example,

electrophoresis and chromatography such as HPLC and affinity. Quantification of CRP40/mortalin-2 is then conducted in a number of ways as will be appreciated by one of skill in the art. CRP40/mortalin-2 can be isolated using a separation method and then quantified against standards. Immunological techniques, for example, can also be employed to identify and quantify CRP40/mortalin-2 either on its own or in conjunction with a separation technique. A CRP40/mortalin-2 primary antibody can be used in an affinity column to separate CRP40/mortalin-2 from a sample and a detectably labeled secondary antibody can be used for identification purposes. Also, detectably labeled (e.g. fluorescent, colorimetric, radioactive) CRP40/mortalin-2 antibody, or a related compound, can be linked to CRP40/mortalin-2 exposed in the sample or separated from a sample and quantified. Methods of making antibodies for use in the diagnostic methods are detailed below.

[0035]In another embodiment, CRP40/mortalin-2 in a sample can be quantified by measuring the amount of CRP40/mortalin-2 nucleic acid within the sample. For example, mRNA copy number can be measured by known techniques as described in detail in the examples that follow. Briefly, mRNA copy number can be determined using PCR, and specifically, the one-step real-time PCR protocol in which human CRP40/mortalin-2 forward and reverse primers were used in the protocol to amplify CRP40/mortalin-2 mRNA for quantity determination against pure CRP40/mortalin-2 mRNA standards.

[0036]Having determined the amount of CRP40/mortalin-2 in a biological sample obtained from a mammal, a comparison with a control value, determined to exist in a normal, undiseased state, is made. It has been determined that a decrease of at least about 10% in the amount of either of CRP40 or mortalin-2 protein or nucleic acid from normal is indicative of neurological disease. An increase of at least about 10% in the amount of either of CRP40 or mortalin-2 protein or nucleic acid from normal is indicative of cardiovascular disease.

[0037]For use as a therapeutic, CRP40/mortalin-2 in accordance with the present invention may be made using standard, well-established solid-phase *peptide* synthesis methods (SPPS). Two methods of solid phase *peptide* synthesis include the BOC and FMOC methods.

[0038]CRP40/mortalin-2 may also be made using any one of a number of suitable techniques based on recombinant technology. It will be appreciated that such techniques are well-established by those skilled in the art, and involve the expression of the CRP40/mortalin-2 encoding nucleic acid in a genetically engineered host cell.

[0039] Isolated nucleic acid encoding CRP40/mortalin-2, and variants thereof in accordance with the present invention, is also encompassed by the invention. Thus, nucleic acid, including DNA and RNA, encoding CRP40 having the amino acid sequence set out Figure 13A is provided. It will be appreciated that more than one nucleic acid sequence will encode CRP40 and its variants given the degeneracy that exists in the genetic code. A CRP40 coding sequence is provided in Figure 13B. The amino acid sequence of mortalin-2 is as set out in the GenBank under deposit ABF 50973, while the nucleotide gene sequence is as set out in deposit NM 004134 and the complete cDNA sequence is set out in deposit DQ 531046.

[0040] DNA encoding a CRP40/mortalin-2 protein may be synthesized de novo by automated techniques well-known in the art. Generally, gene synthesis may be conducted by the successive 3' to 5' coupling of appropriately protected nucleotide reagents in an automated synthesizer, followed by recovery of the deprotected polynucleotide. Alternatively, the block ligation methodology may be employed whereby oligonucleotide "blocks", including up to about 80 nucleotides, are ligated by overhang complementarity as described in Wosnick et al. in *Gene*, 1989, 76:153. Sequences obtained by de novo synthesis may be amplified using the polymerase chain reaction as described in United States Patent No. 4,683,195.

[0041] Upon obtaining CRP40 or mortalin-2 -encoding DNA, recombinant techniques for producing CRP40/mortalin-2 therefrom generally involve insertion of the DNA sequence into a suitable expression vector which is subsequently introduced into an appropriate host cell (such as Chinese hamster ovary cells (CHO cells) for example of K1 lineage (ATCC CCL 61), murine 3T3 cells (ATCC CRL 1658) or human embryonic kidney cells of the 293 lineage (ATCC CRL 1573)) for expression. Such transformed host cells are herein characterized as having the CRP40 DNA incorporated "expressibly" therein. Suitable expression vectors are those vectors which will drive expression of the inserted DNA in the selected host. Typically, expression vectors are prepared by site-directed insertion of a DNA construct therein. The DNA construct is prepared by replacing a coding region, or a portion thereof, within a gene native to the selected host, or in a gene originating from a virus infectious to the host, with CRP40/mortalin-2 DNA. In this way, regions required to control expression of the CRP40/mortalin-2 DNA, which are recognized by the host, including a region 5' of the PMCA CRP40 DNA to drive expression and a 3' region to terminate expression, are inherent in the DNA construct. To allow selection of host cells stably transformed with the expression vector, a selection marker is generally included in the vector

which takes the form of a gene conferring some survival advantage on the transformants such as antibiotic resistance.

[0042] Cells stably transformed with a CRP40/mortalin-2 DNA-containing vector are grown in culture media and under growth conditions that facilitate the growth of the particular host cell used. One of skill in the art would be familiar with the media and other growth conditions required by the particular host cell chosen, as such information is well-documented in the art. Recombinant CRP40/mortalin-2 protein may be isolated from the host culture media by any one of a number of acceptable methods, such as the use of affinity columns or immunogenic methods using antibodies directed specifically to CRP40/mortalin-2, and then purified using techniques also well-known in the art such as gel electrophoresis. For therapeutic use, the oligopeptide compounds of the invention are desirably of "pharmaceutical grade" purity, a term used herein to denote an oligopeptide preparation which has been shown to migrate as a single peak on HPLC, to exhibit uniform and authentic amino acid composition and sequence upon analysis thereof, and which otherwise meets standards set by the various national bodies which regulate quality of pharmaceutical products.

[0043] Once prepared and suitably purified, CRP40, mortalin-2 and functional variants thereof in accordance with the invention, may be utilized to treat neurological disease. Generally, a pharmaceutical composition comprising the CRP40/mortalin-2 protein and at least one pharmaceutically acceptable adjuvant is used. The expression "pharmaceutically acceptable" means acceptable for use in the pharmaceutical and veterinary arts, i.e. not being unacceptably toxic or otherwise unsuitable. Examples of pharmaceutically acceptable adjuvants are those used conventionally with peptide-based drugs, such as diluents, excipients and the like. Reference may be made to "Remington's: The Science and Practice of Pharmacy", 21st Ed., Lippincott Williams & Wilkins, 2005, for guidance on drug formulations generally. The selection of adjuvant depends on the intended mode of administration of the composition. In one embodiment of the invention, the compounds are formulated for administration by infusion, or by injection either subcutaneously or intravenously, and are accordingly utilized as aqueous solutions in sterile and pyrogen-free form and optionally buffered or made isotonic. Thus, the compounds may be administered in distilled water or, more desirably, in saline, phosphate-buffered saline or 5% dextrose solution. Compositions for oral administration via tablet, capsule or suspension are prepared using adjuvants including sugars, such as lactose, glucose and sucrose; starches such as corn

starch and potato starch; cellulose and derivatives thereof, including sodium carboxymethylcellulose, ethylcellulose and cellulose acetates; powdered tragacanth; malt; gelatin; talc; stearic acids; magnesium stearate; calcium sulfate; vegetable oils, such as peanut oils, cotton seed oil, sesame oil, olive oil and corn oil; polyols such as propylene glycol, glycerine, sorbitol, mannitol and polyethylene glycol; agar; alginic acids; water; isotonic saline and phosphate buffer solutions. Wetting agents, lubricants such as sodium lauryl sulfate, stabilizers, tableting agents, anti-oxidants, preservatives, colouring agents and flavouring agents may also be present. Creams, lotions and ointments may be prepared for topical application using an appropriate base such as a triglyceride base. Such creams, lotions and ointments may also contain a surface active agent. Aerosol formulations, for example, for nasal delivery, may also be prepared in which suitable propellant adjuvants are used. Other adjuvants may also be added to the composition regardless of how it is to be administered, for example, anti-microbial agents may be added to the composition to prevent microbial growth over prolonged storage periods.

[0044] In accordance with the invention, a therapeutically effective amount of a CRP40/mortalin-2 protein is administered to a mammal in the treatment of a neurological disease. As used herein, the term "mammal" is meant to encompass, without limitation, humans, domestic animals such as dogs, cats, horses, cattle, swine, sheep, goats and the like, as well as wild animals. The term "therapeutically effective amount" is an amount of the CRP40 or mortalin-2 protein indicated for treatment of the disease while not exceeding an amount which may cause significant adverse effects. Dosages of the CRP40 or mortalin-2 protein will vary with many factors including the condition and individual being treated. Appropriate dosages are expected to be in the range of about 1 ug-100mg.

[0045] In another aspect of the present invention, an article of manufacture is provided. The article of manufacture comprises packaging material and a pharmaceutical composition. The composition comprises a pharmaceutically acceptable adjuvant and a therapeutically effective amount of a CRP40 or mortalin-2 protein, wherein the packaging material is labeled to indicate that the composition is useful to treat a neurological disease.

[0046] The packaging material may be any suitable material generally used to package pharmaceutical agents including, for example, glass, plastic, foil and cardboard.

[0047]Antibodies to CRP40 proteins are also provided in another aspect of the invention. The antibodies are useful in the diagnostic method of the invention as described above. Conventional methods can be used to prepare the antibodies including polyclonal antisera or monoclonal antibodies. To produce polyclonal antibodies, a mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the protein which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide are well known in the art and include, for example, conjugation to carriers. The peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess antibody levels. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

[0048]To produce monoclonal antibodies, antibody-producing cells (lymphocytes) are harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures to form immortal hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497(1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a selected CRP40 peptide and the monoclonal antibodies can be isolated.

[0049]The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a CRP40 protein according to the invention. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, fragments can be generated by treating an antibody with pepsin. The resulting fragment can be further treated to reduce disulfide bridges.

[0050]Chimeric antibody derivatives, i.e., antibody molecules resulting from the combination of a variable non-human animal peptide region and a constant human peptide region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species

with a constant human peptide region. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a CRP40 protein of the invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452(1985), Cabilly et al., U.S. Pat. No. 4,816,567; Boss et al., U.S. Pat. No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B).

[0051] Monoclonal or chimeric antibodies specifically reactive with a CRP40 protein of the invention as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al, Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great. Britain).

[0052] CRP40 or mortalin-2 -encoding nucleic acid molecules or oligonucleotide may also be used in a gene therapy method of treating a neurological disease. Administration of oligonucleotide that encodes CRP40/mortalin-2 to a patient suffering from such a disease will function to increase cellular CRP40/mortalin-2, thereby alleviating, at least in part, symptoms of the disease.

[0053] The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligonucleotides comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

[0054]The therapeutic oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-tri-fluoromethyl uracil and 5-trifluoro cytosine.

[0055]Other therapeutic oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. For example, phosphorothioate bonds may link only the four to six 3'-terminal bases, may link all the nucleotides or may link only 1 pair of bases.

[0056]The therapeutic oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue in accordance with the invention is a peptide nucleic acid (PNA) in which the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyimide backbone which is similar to that found in peptides (P.E. Nielson, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also form stronger bonds with a complementary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotide analogues may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotide analogues may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also incorporate sugar mimetics as will be appreciated by one of skill in the art.

[0057]Nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art as previously described. The nucleic acid molecules of the invention may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene, e.g. phosphorothioate derivatives and acridine substituted nucleotides.

CRP40/mortalin-2 may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which the CRP40/mortalin-2 sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

[0058]Once prepared, the oligonucleotides may be introduced into tissues or cells using techniques in the art including vectors (retroviral vectors, adenoviral vectors and DNA virus vectors) or physical techniques such as microinjection. The oligonucleotides may be directly administered *in vivo* or may be used to transfect cells *in vitro* which are then administered *in vivo*.

[0059]In the treatment of cardiovascular disease, it is desirable to downregulate the expression of CRP40/mortalin-2 to reduce the occurrence of platelet aggregation which leads to a diseased condition. As one of skill in the art will appreciate, CRP40/mortalin-2 expression can be inhibited at either the protein or nucleic acid levels. Synthetic inhibitors of CRP40 or mortalin-2 can be determined, for example, using assays designed to detect reduced CRP40 activity such as flow cytometry for platelet aggregation as described in detail in the specific examples.

[0060]CRP40 and mortalin-2 can also be inhibited at the nucleic acid level, for example, using anti-sense, snp or siRNA technologies. CRP40/mortalin-2-encoding nucleic acid molecules may be used to prepare antisense oligonucleotides against CRP40/mortalin-2 which may be therapeutically useful to inhibit CRP40 or mortalin-2. Accordingly, antisense oligonucleotides that are complementary to a nucleic acid sequence encoding CRP40/mortalin-2 according to the invention are also provided. The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complementary to a target CRP40 or mortalin-2 nucleic acid sequence.

[0061]The term “oligonucleotide” refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

[0062]The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-tri-fluoromethyl uracil and 5-trifluoro cytosine.

[0063]Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. For example, phosphorothioate bonds may link only the four to six 3'-terminal bases, may link all the nucleotides or may link only 1 pair of bases.

[0064]The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) in which the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polymide backbone which is similar to that found in peptides (P.E. Nielson, et al Science 1991, 254, 1497). PNA

analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also form stronger bonds with a complementary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotide analogues may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotide analogues may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also incorporate sugar mimetics as will be appreciated by one of skill in the art.

[0065]Antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art based on CRP40 amino acid sequence information such as that provided. The antisense nucleic acid molecules of the invention, or fragments thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene, e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

[0066]The antisense oligonucleotides may be introduced into tissues or cells using techniques in the art including vectors (retroviral vectors, adenoviral vectors and DNA virus vectors) or physical techniques such as microinjection. The antisense oligonucleotides may be directly administered *in vivo* or may be used to transfect cells *in vitro* which are then administered *in vivo*.

[0067]In another embodiment, siRNA technology can be applied to prevent expression of CRP40 or mortalin-2. Application of nucleic acid fragments such as siRNA fragments that correspond with regions in CRP40/mortalin-2 and which selectively target the CRP40/mortalin-2 gene may be used to block CRP40/mortalin-2 expression resulting in a reduction of platelet aggregation. Such blocking occurs when the siRNA fragments bind to

the CRP40/mortalin-2 gene thereby preventing translation of the gene to yield functional CRP40/mortalin-2.

[0068]SiRNA, small interfering RNA molecules, corresponding to CRP40/mortalin-2 are made using well-established methods of nucleic acid syntheses including automated systems. Since the structure of the CRP40/mortalin-2 gene is known, fragments of RNA that correspond therewith can readily be made as outlined above with respect to antisense oligonucleotides. The effectiveness of selected siRNA to block CRP40/mortalin-2 activity can be confirmed using a CRP40/mortalin-2 -expressing cell line. Briefly, selected siRNA is incubated with a CRP40/mortalin-2 -expressing cell line under appropriate growth conditions. Following a sufficient reaction time, i.e. for the siRNA to bind with CRP40/mortalin-2 DNA to result in decreased expression of the CRP40/mortalin-2 DNA, the reaction mixture is tested to determine if such decreased expression has occurred. Suitable siRNA will prevent processing of the CRP40/mortalin-2 gene to yield functional CRP40/mortalin-2. This can be detected by assaying for CRP40/mortalin-2 function in the reaction mixture, for example, CREB activity.

[0069]It will be appreciated by one of skill in the art that siRNA fragments useful in the present method may be derived from specific regions of CRP40 or mortalin-2 -encoding nucleic acid. Moreover, suitable modifications include, for example, addition, deletion or substitution of one or more of the nucleotide bases therein, provided that the modified siRNA retains its ability to bind to the targeted CRP40/mortalin-2 gene. Selected siRNA fragments may additionally be modified in order to yield fragments that are more desirable for use. For example, siRNA fragments may be modified to attain increased stability in a manner similar to that described for antisense oligonucleotides.

[0070]Embodiments of the present invention are described by reference to the following specific examples which are not to be construed as limiting.

[0071]The following experimental work is described by reference to the methods and materials used, as well as by reference to the results obtained.

Example 1 – Characterization of human CRP40

METHODS AND MATERIALS

Generation of BQ224193 primers synthesized by MOBIX, McMaster University:

[0072] Human Brain RNA (Ambion) was reverse transcribed using MuLV Reverse Transcriptase (Applied Biosystems) and a fragment of 720 base pairs (**Genbank #BQ224193**) was amplified using 5' atg gat tct tct gga ccc aag cat 3' (Sense primer) (SEQ ID NO: 3) and 5' tcg ttc ctt ctt tgg ccg gtt ttt t 3' (Antisense primer) (SEQ ID NO: 4) designed by MOBIX , McMaster University. The conditions used were: 95°C, 2.25 minutes (min), 95°C-15 sec, 60°C-30-sec, 72°C-55-sec, 40 cycles 72°C-7.0-min. The PCR product was run on a 1% agarose gel with 1XTAE and 0.05% ethidium bromide (EtBr). The band at 720 relative to a 100 bp marker (Biorad) was cut out, purified using a Qiagen gel extraction kit, and sequenced.

Cloning BQ224193

[0073] Bam H1 and EcoR1 restriction enzyme sites were introduced 5' of sense and antisense primers, respectively, to facilitate cloning. Subsequent RT-PCR and analysis of the products revealed a 740bp fragment, which was purified from an agarose gel as described above. The PGEX-2T vector (Invitrogen) and the 740bp fragment were digested using the restriction enzymes as below: 0.5µg (1ul of 500ug/ul) vector, 4.0µl of 10X Y+/Tango buffer, 1.0 µl BamH1 (5 U), EcoR1, 1.0µl (5.0 U), 13.0µl DH₂O, 32µl BQ224193-740bp fragment (0.6ug), 8 µl 10X Y+/Tango buffer, 2.0 µl BamH1 (10 U), 2.0 µl EcoR1 (10 U); mixed and incubated at 37°C for 1 hour (h) and enzymes were inactivated at 75°C, 15 min. The digested vector and human CRP40 fragments were run on a 0.7% agarose gel with 0.05% EtBr in separate lanes with 1KB ladder as marker. The bands at 1.8Kb and 740bp were cut out separately and purified using gel Extraction kit. (Qiagen). The 740bp fragment was ligated to PGEX-2T vector as follows: cut 740bp BQ224193 DNA, 14.0µl, cut pGEX-2T, 2.0µl, 10X ligase buffer, 2.0µl, T4 DNA ligase (Fermentas), mixed and incubated at 22°C for 1h, and enzyme inactivated at 65°C for 10 min. The products were frozen at -20°C until transformation.

Preparation of BL21 E.coli cells and Transformation

[0074] Competent cells were prepared as follows. BL-21 *E.coli* (Invitrogen) lyophilized powder was resuspended in 1.0 ml LB medium in a shaker at 37°C, 250rpm overnight. The *E.coli* was then plated on LB agar plates. Following a 24hr incubation, 1ml of LB was inoculated using a single colony in a 15ml Falcon tube and incubated overnight at 37°C, 250 rpm (New Brunswick Scientific Co. Inc, Series 25D). Following an 18hr incubation, 100ml of LB medium was inoculated with 200µl of overnight culture and incubated at 37°C at 200rpm till OD 600 was 0.4-0.5 (approx- 3.5hrs). The culture was spun down at 2000 rpm for

5 min and the pellet was re-suspended in 1.0 ml of CaCl₂ solution (50mM CaCl₂, 10mM Tris-HCL, pH 8.0) and 24ml CaCl₂ solution. The solution was then incubated on ice for 15 min, spun at 2000 rpm for 5 min and the pellet re-suspended in 3ml of CaCl₂ solution. The competent cells were used fresh for subsequent transformation. Transformation: 20µl ligated mix was mixed with 200 µl competent cells, incubated on ice for 45 min, then in a 42°C water-bath for 2 min. and chilled briefly on ice. Transformed cells (100µl) were mixed with 900ul LBG (20mM glucose in LB medium) and incubated at 37°C for 1hr without shaking. Uncut vector was also transformed side by side. Following 1hr, 100ul of BQ transformed cells, 100ul of uncut vector transformed cells and competent cells were plated on LB agar plates with ampicillin and incubated overnight at 37°C.

Preparation of BQ protein:

[0075]The following day, colonies were picked, inoculated (1.5 ml eppendorf tubes) and placed on a shaker (250 rpm) and the next day, 3.0 ml LB ampicillin media were mixed with 50µl of overnight grown culture until OD600 was 0.5. IPTG (100mM) was added to each tube which were then incubated for 22hrs h at 14 °C and spun down at 13000 rpm for 30 sec. The supernatant was discarded. The pellet was re-suspended in 300µl of 1X-PBS and centrifuged at 13000 rpm for 30 sec. It was then washed with 1X-PBS 2x. Glutathione elution buffer (10µl) was added to the beads, incubated for 5 min, centrifuged 13000 rpm, 5 min. The supernatant was transferred to fresh tubes to which was added 10 µl of loading buffer. All above fractions were loaded on SDS-PAGE.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

[0076]Protein concentration was determined using the Bradford method. Briefly, 10µg of protein was mixed with sample buffer (0.625 M Tris, 2% SDS, 0.05% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, pH 6.8) and boiled for 4min (Bradford 1976). SDS-PAGE (12% acryl amide) was used with the following procedure: running buffer (0.025 M Tris, pH 8.3, 0.3 M glycine, 0.1% SDS) and 65V was applied. The pellet was re-suspended in 20µl 1X-PBS for SDS-PAGE analysis. The supernatant was mixed with 50µl of 50% Glutathione Sepharose 4B at room temperature for 5 min. 100µl 1X-PBS was added and then the mixture was centrifuged at 13000 rpm, 30 sec. The pellet was washed with 1X-PBS 2x and Glutathione elution buffer (10µl) was added to the beads, incubated for 5 min,

centrifuged 13000 rpm, 5 min. Lastly, the supernatant was transferred to fresh tubes and 10 μ l of loading buffer was added. All above fractions were then loaded onto SDS-PAGE.

Production of Polyclonal Antibody Using BQ Fusion Protein

[0077] 0.5 μ g of the pure BQ protein was run in each lane of 10% acrylamide gel and transferred onto a nitrocellulose filter paper (binding capacity of 26 μ g/cm²). Ponceau-S stained BQ bands were cut out and stored at -20°C. Approximately 30 μ g BQ protein was homogenized and required for the first injection. Two New Zealand rabbits were used and the following protocol was employed: injected BQ slurry subcutaneously (s.c.) on the nape of the neck at 4 injection sites (250 μ g each injection site) on the following injection days, 0, 14, 42, 134 and bleeds were performed on days 13, 32, 50, 89, and 146. Approximately 100 ml of blood was collected from each rabbit and the serum was collected and centrifuged and 0.2% sodium azide was added to the aliquots.

Purification of BQ antibody

[0078] Protein A antibody purification kit from Sigma was used and is briefly described. 10ml of the crude BQ serum was used for antibody purification. 10 fractions of 1.0ml were collected from the column, checked OD 280nm and purified BQ antibody was aliquoted and stored at -80°C.

Functional Studies- Protein-Binding Assays

[0079] Tritiated N-propylnorapomorphine ([³H]-NPA) binding was performed with BQ-fusion protein in the presence of cold dopamine (DA). Briefly, the assays were performed in triplicate and consisted of the following solutions and protocol: 50mM TRIS, 5.0 mM MgCl₂, 1.0 mM EDTA, 0.1mM DTT, 0.1mM PMSF, 100 mg/ml Bacitracin and 5.0 mg/ml soybean trypsin; DA (mw= 189.6) was dissolved in 0.1% ascorbic acid; each tube was vortexed briefly and incubated in a shaking water bath at 37°C, for 2h; receptor binding equipment (Brandel, U.S.A.) washed 3 times with distilled water; then repeated washes in assay buffer, 50nM TRIS, 1 mM EDTA, pH 7.4; 18 test-tubes were set on the binding platform and fiberglass filter paper was put on the platform. The samples were passed through the filter paper with 3 consecutive washes; placed in the scintillation tubes and 5.0 ml Biodegradable Counting Scintillant was added and radioactive counts were measured (Amersham, U.S.A.).

Studies on DRD2 Transfected SH-SY5Y Human Neuroblastoma Cells

[0080]The SH-SY5Y cell line (obtained from ATCC) used in this study has been extensively characterized. This cell line does not express endogenous dopamine receptors, therefore the cells were transfected with dopamine D2 long isoform using the following method. The dopamine DRD2 receptor was subcloned into a mammalian expression vector (pcDNA 3.1(+)), which was generously donated by Guthrie cDNA Resource centre (Pennsylvania, U.S.A.). This plasmid DNA was prepared in bulk using TOPO10 chemically competent *E.coli* from Invitrogen. Briefly, 1 colony was inoculated with 5 ml of LB Ampicillin and incubated at 37°C, at 250 rpm for 8h. Culture (0.5ml) was transferred to 250 ml LB Ampicillin in 500 ml flasks, incubated at 37°C in shaker unit (275 rpm, 24h) (New Brunswick Scientific Co. Inc, Series 25D). Culture was removed, transferred and centrifuged and remaining pellet was processed for the extraction of DNA as previously described (QIAGEN Plasmid Purification Protocol). The DNA was quantified by spectrophotometry (Beckman 640), aliquoted and stored at -80°C.

Transfection of SH-SY5Y cells

[0081]The pcDNA 3.1(+) vector was introduced into SH-SY5Y cells by lipofection method (Invitrogen Life Science technology. U.S.A.). Briefly, 24µg of DRD2 plasmid DNA was mixed with 1.5 ml of Opti MEM media and 60 µl of lipofectamine 2000 reagent was added and mixed with 1.5ml of Opti MEM media and SH-SY5Y cells. Geneticin-resistant clones stably expressing the D2L receptor were screened by a [³H] spirerone-binding assay and was maintained in RPMI medium (RPMI contained 10% fetal bovine serum, 1mM glutamate, 50 U/ml of penicillin, and 50 U/ml of streptomycin) with 200ug/ml of geneticin. Cells were grown to confluency (5-6 days following sub-culturing) in fresh RPMI medium.

Homogenizing subcellular fractions: nucleus, mitochondria, supernatant (cytosol)

[0082]SH-SY5Y cells were scraped off and this procedure was repeated a second time. The cells were incubated 5 min, centrifuged at 2000rpm for 5 min and the supernatant was discarded. The pellet was re-suspended in 0.5ml 0.35M sucrose buffer (wPMSF), homogenized and centrifuged at 1000g for 10 min. The supernatant was centrifuged and the pellet was re-suspended in a 0.32M sucrose buffer (wPMSF), homogenized, and centrifuged. The pellet was re-suspended 0.053ml Tris-EDTA PMSF and labeled as **nuclear fraction 1**. The supernatant was centrifuged and the resulting supernatant was stored and labeled

cytosolic fraction 1. The remaining pellet was re-suspended in 0.150 ml Tris-EDTA-PMSF and labeled **mitochondrial fraction 1.**

[0083] Each cell fraction was exposed to: 1) 100 μ M DA; 2) 100 μ M Haloperidol; 3) both 100 μ M DA and 100 μ M Haloperidol; 4) and a negative control. The cells were exposed to the neurotransmitter and/or the neuroleptic (antipsychotic) and left overnight. Western immunoblotting with BQ primary antibody was performed, followed by immunoblotting with polyclonal conjugated horseradish peroxidase secondary antibody.

RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE PCR)

[0084] Total RNA from human brain was purchased from Ambion and used as the cDNA library to amplify BQ gene specific primers. The RLM-RACE-PCR kit was purchased from Invitrogen (Invitrogen life technologies, California, U.S.A.). First, human brain total RNA was treated with calf intestinal phosphatase to remove the 5' phosphates from truncated mRNA and all other non-mRNA. Briefly, 2.5 μ l total RNA, 1.0 μ l 10x CIP buffer, 1.0 μ l RNase out, 1.0 μ l CIP enzyme, and 4.5 μ l DEPC water were mixed in 1.0 μ l microcentrifuge tube, vortexed and centrifuged and incubated in a 50°C water bath for 1h, then centrifuged and put on ice; precipitated RNA, added 90 μ l DEPC water and 100 μ l phenol:chloroform, vortexed 30 sec, centrifuged 5 min at RT and to the transferred aqueous phase was added 2 μ l 10mg/ml mussel glycogen and 10 μ l 3M sodium acetate, pH 5.2, followed by mixing. Then, 220 μ l 95% ethanol was added, followed by vortexing. The mixture was placed on dry ice for 10 min, centrifuged and the supernatant was removed. Then, 500 μ l 70% ethanol was added to solubilize RNA pellet. The solution was then centrifuged for 2 min, 4°C. The supernatant was removed and the pellet was re-suspended in 7 μ l DEPC water. The next major step involved was to treat the dephosphorylated RNA with tobacco acid pyrophosphatase to remove the 5' cap structure from full length mRNA. Briefly, added and mixed 7 μ l dephosphorylated RNA, 1 μ l 10X TAP buffer (40U/ μ l), 1 μ l RNaseOut, 1 μ l TAP (0.5u/ μ l), mixed, vortexed and centrifuged, incubated at 37°C 1h. Repeated exactly as above precipitation procedure. Following decapping of the mRNA, the next step was to ligate a gene specific Gene Racer RNA Oligo to the 5' end of the Ambion human brain total RNA. Briefly, added 7 μ l of the decapped RNA into a tube containing the pre-aliquoted lipophilized GeneRacer RNA Oligo (0.25 μ g), mixed and centrifuged; then incubated in water bath at 65°C and placed mixture on ice for 2 min. The following reagents were then added to the

centrifuged mixture: 1µl, 10X lysate buffer, 1µl 10mM ATP, 1µl RNaseOut (40 U/µl), 1µl T4 RNA ligase (5U/µl), incubated for 1h at 37°C, and briefly centrifuged and placed on ice. Repeated exactly as above precipitation procedure. The 5' ligation step provided the GeneRacer RNA Oligo ligated to the decapped mRNA, which required reverse transcription into complementary cDNA. Briefly, added 1µl hexamer primer and 1µl dNTP, mixed to ligate RNA, incubated at 65°C for 5 min, followed by placing mixture on ice for 2 min and centrifuging. Next, the following reagents were added: 12µl ligated RNA and primer mixture; 4µl 5X First strand Buffer, 2µl 0.1 M DTT, 1µl RNaseOut (40U/µl), 1µl Superscript III (200U/µl), then mixed solution and incubated at 50°C for 50 min. RT-reaction was inactivated at 70°C, and centrifuged. Then 1µl RNase H (2U) was added to the RT-reaction at 37°C, 30 min. The protocol for PCR was as follows: 94°C, 2.0 min, 1 cycle; 94°C, 30 sec, 5 cycles; 72°C, 2.0 min, 5 cycles; 94°C, 30 sec, 5 cycles; 70°C, 2.0 min, 5 cycles; 94°C, 30 sec, 20 cycles; 65°C, 30 sec, 20 cycles; 68°C, 2.0 min, 1 cycle; 68°C, 10 min, 1 cycle (Invitrogen Life Science Technologies).

RNA Isolation Using TRIzol Method

[0085] Approximately 50-100 mg of bovine, rat tissue, lymphocytes and neuroblastoma SH-SY5Y cells were used in the RNA isolation. The tissue was mixed with 1000µl Tri-pure reagent and homogenized. Next, the solution was poured into a 1.5ml micro-centrifuge tube and incubated at room temperature for 5 min. Immediately following, 200µl of chloroform was added, shaken vigorously for 15 sec and allowed to sit 20 min. The samples were then centrifuged 12000 for 15 min, 4°C, centrifuged and colorless phase was removed and transferred to a new tube to which was added 500µl isopropanol. The solution was allowed to precipitate for 10 min at room temperature, then centrifuged for 10 min at 12000g and the supernatant was discarded. The pellet was re-suspended in 75% ethanol, centrifuged 5 min and allowed to dry. It was then suspended in 30µl DEPC-treated RNase- free H₂O. The RNA was incubated for 15 min at 55°C and analyzed with a Beckman spectrophotometer DU-640 for RNA concentration and purity.

Isolation of Poly (A)⁺ RNA

[0086]Bovine tissues and neuroblastoma SH-SY5Y cells were removed and treated with TRIzol reagent (as previously described) to isolate total RNA. 200 µg total RNA was used to isolate the polyA RNA with Oligo Dt cellulose containing a capacity of 10 mg RNA per gram of resin. The following reagents were used in the polyA RNA isolation: 1) Binding buffer (pH final solution to 7.5), 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA (pH 7.5), 0.5% SDS; 2) 2X Binding Buffer (pH final solution to 7.5), 20 mM Tris-HCl (pH 7.5), 1 M NaCl, 2 mM EDTA (pH 7.5), 1% SDS; 3) Wash Buffer (pH final solution to 7.5), 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA (pH 7.5); 4) Elution Buffer (pH final solution to 7.5), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The eluted poly(A)⁺ RNA was added to five 0.5-mL aliquots elution buffer, and a Beckman U-640 spectrophotometer was used to read A₂₆₀ to determine aliquot with max Poly A.

Northern Blot

[0087]Twenty micrograms of polyA RNA from neuroblastoma SH-SY5Y cells, human tissue and bovine tissue were separated on 1.0% formaldehyde agarose gels and transferred to Hybond nylon filters (Amersham Pharmacia Biotech, England) which were baked at 80°C for 2h. Prehybridization involved 70 µl of salmon sperm DNA at 95°C and Express-Hyb and rotated for 2h at 68 °C. The blot was hybridized to α-³²P-dCTP-labelled cDNA probes for 22h at 45°C, and washed with 2XSSC. The blots were exposed to Kodak X-ray film. A hybridization probe was prepared from a 720bp fragment from BQ224194 primers. The pair of primers used consisted of the forward primer: 5' atg gat tct tct gga ccc aag cat 3' (SEQ ID NO: 3) and reverse primer 5' tcg ttc ctt ctt tgg ccg gtt ttt t 3' (SEQ ID NO: 4). The same filters were hybridized with a β-actin probe as an internal control of both the RNA integrity and amount.

Generation of the Standard Curve For Real-Time PCR Experiments:

[0088]A standard curve was used for absolute quantitation of the unknown SH-SY5Y cDNA samples with 6 concentrations (1pg-10ag) of cDNA. The following protocol was used to produce the first and second PCR reaction to make a pure cDNA amplicon with SH-SY5Y cDNA and forward BQ primer, 2 and reverse primer, 4: **1st PCR reaction**, 50mM MgCl₂, 0.75µl, 10X Buffer, 2.5µl, 2,4 Primer mix., 2.0µl, SH-SY5Y cDNA sample #1, 2.5µl, Platinum Taq, 0.2µl, DEPC water, 14.55µl; 95°C, 2.25 min, 95°C, 15 sec, 60°C, 30 sec, 72°C, 1.0 min, 72°C, 7.0 min; **2nd PCR reaction**, 50mM MgCl₂, 1.5µl, 10X Buffer, 5.0µl,

2,4 Primer mix, 4 + 4µl, SH-SY5Y cDNA sample #1, 5.0µl, Platinum Taq, 0.4µl, DEPC water, 29.10µl, and ran PCR with the same conditions. Purification of SH-SY5Y cDNA PCR Product QIAGEN Minielute PCR Purification Kit.

Calculations in Copy Number per microgram of RNA for Real-Time PCR Experiment:

[0089]The following formula was used to determine copy number for the standard curve:

$$\# \text{ Copies}/\mu\text{l} = \frac{\text{Concentration (g}/\mu\text{l}) \times 6.032 \times 10^{23}}{\text{Length in base pairs} \times 660}$$

Real-Time PCR Protocol with D2L- Transfected SH-SY5Y cDNA:

[0090]D2L-transfected SH-SY5Y cells, Haloperidol-treated were compared to the same Neuroblastoma cells not treated with HAL, which acted as a control. All samples were performed in duplicate, and samples were also prepared in duplicate, as a No Template Control (NTC) and No Reverse Transcriptase Control (NRT). The following protocol was used: 2X SYBRgreen, 10.0µl, Forward primer, P2, 1.2µl, Reverse primer, P4, 1.2µl, Reverse Transcriptase Mix, 0.2µl, DEPC H2O, 6.4µl, SH-SY5Y cDNA, 1.0µl; Real-Time PCR Conditions, 50°C 30 sec (1 cycle), 95°C 15 min (1 cycle), 95°C 15 sec (40 cycles), 60°C 30 sec (40 cycles), 72°C 40 sec (40 cycles).

Localization Studies of Human CRP40- Immunohistochemistry Protocol with Post-Mortem Brain Slide Samples of the Nucleus Accumbens from The Stanley Foundation Neuropathology Consortium:

[0091]Three slides were chosen from the 60 samples generously donated by The Stanley Foundation. The slides consisted of: 1) control; 2) schizophrenia patient-drug free; 3) schizophrenic patient-15000K, HAL-treated. The slides were incubated with 4% formaldehyde and diluted in PBS for 30 min. Immediately following, washed sections 3 times with PBS for 5 min, each time and dried off each slide as close as possible to the tissue and outlined the section with the hydrophobic pen. The sections were then blocked by incubating sections with 3% normal goat serum* (NGS) (diluted in PBS containing 0.6% Triton X-100) for 1h. Next, incubated slides with primary human CRP40 polyclonal antibody diluted in PBS containing 0.6% Triton X-100 (and 3% NGS if necessary) overnight at 4°C

(wrapped in aluminum foil). The following day, the slides were washed 3 times with PBS for 5 min, each time, and incubated slides with FITC secondary antibody diluted in PBS containing 0.6% Triton X-100 for 4h. Next, the sections were washed 6 times in PBS and slides were mounted with appropriate cover-slips and fixed with nail polish.

RESULTS

RT-PCR showed combinations of BQ224193 primers produced 3 discrete bands, all containing significant homology to the mot-2 sequence

[0092]RT-PCR with Ambion human brain cDNA was performed utilizing 2 forward primers and 3 reverse primers from the BQ224193 sequence in different combinations, resulting in 3 distinct bands (see Figure. 1). The largest band consisting of 720bp was used in the cloning part of the experiment due to the sharpness of the band and its increased size compared to other primer mixtures with 1.2% agarose gel and 15 μ l EtBr (5mg/ml). The bands were eluted out and sent for sequencing at MOBIX, McMaster University. Sequencing analysis results consistently showed 96% nucleotide homology to mot-2.

RT-PCR fragment using modified primers containing restriction enzymes BamH 1 and EcoR1, ligation and transformation into BL-21 Ecoli cells

[0093]The following primers were synthesized in order to incorporate the restriction enzyme sites into the known 720bp BQ fragment, which resulted in a slightly larger nucleotide fragment of 740bp (see Figure. 2). The following 5' primer including a BamH1 site: tag gga tcc atg gat tct tct gga ccc aag cat (SEQ ID NO: 1); and 3' primer including an EcoR1 site, cta gaa ttc tca tcg ttc ctt ctt tgg ccg gtt ttt (SEQ ID NO: 2) were used to perform the RT-PCR reaction.

[0094]The glutathione S-transferase (GST) gene fusion system was used in these experiments because integration of the BQ transgene, expression, purification and detection of the fusion protein in *E.coli* is reliable, fast and reproducible. The pGEX plasmids function as being inducible with high expression of genes and transgene fusions with *Schistosoma japonicum* GST. Furthermore, the pGEX-2T vector contains a Taq promoter site in order to chemically induce high expression of the LacI gene. Isopropyl β D-thiogalactosidase (IPTG), a lactose analog, was used to induce expression of the Lac gene, ultimately translating high quantities of BQ fusion protein.

[0095]The digestion of the 740 BQ fragment and pGEX-2T was performed using the specific restriction enzymes noted above, in order to provide the sticky ends for the ligation into the vector. Following ligation and transformation into BL-21 *E.coli* cells, the transformants were grown on Ampicillin resistant agar plates at 37°C overnight. The colonies were picked and grown in LB media with Ampicillin overnight. Producing fusion protein involved using 500 ml LB media, 10 ml overnight culture, 500µl Ampicillin in baffled flask. Translation was initiated following the addition of 500µl IPTG. The mixture was placed in a refrigerated shaker, 12°C, 250rpm for 23 h.

[0096]The optimal temperature for the BQ fusion protein was 12°C, in order to allow proper protein folding thereby allowing the fusion protein to remain in its soluble form and allowing it to be actively functional. The next day, the mixture was centrifuged and the pellet of cells was re-suspended in 1XPBS including a mini-C protease inhibitor tablet. The cells were lysed using a French press. The released fusion protein was allowed to bind to the matrix of Glutathione Sepharose 4B beads overnight. The bound fusion protein-Glutathione Sepharose matrix was added to an affinity chromatography column. As can be seen in Figure. 3, the majority of the fusion protein was in soluble form, which indicates that the protein was in its proper active state. Fractions were eluted out through the column and protein concentration was determined by spectrophotometry

[0097]Following washes, the bound fusion protein was cleaved from the Glutathione Sepharose beads at specific sites by adding the enzyme thrombin protease, and SDS PAGE electrophoresis was performed on cleaved product. Coomassie staining was also performed on the gel (see Figure 4).

Protein Binding Studies

[0098]The first protein binding study involved [³H]NPA and 3 different concentrations of displacement molecules (DA, NPA), 100 µM, 10 µM and 1 µM, respectively. Following protein binding, DPM counts were performed in a Beckman scintillation counter. The amount of displacement for each concentration was as follows: 100 µM DA-78.6%, 10 µM DA-74.6%, 1 µM DA-55% and 100 µM NPA-76.3%, 100 µM NPA-75%, 100 µM 64.3% (see Figure 5).

[0099]The second binding study involved [³H]DA and 4 different concentrations of displacement molecule DA, 1000 µM, 100 µM, 10 µM and 1 µM, respectively. Following

protein binding, DPM counts were performed in a Beckman scintillation counter. The amount of displacement for each concentration was as follows: 1000 μ M DA- 75%, 100 μ M DA- 65%, 10 μ M DA- 60%

Production of human CRP40 Polyclonal Antibody with BQ Fusion Protein

[00100]34 μ l of pure BQ protein was loaded on a 12% acrylamide gel and electrophoresis proceeded. The protein was then transferred to nitrocellulose paper and distinct single bands at approximately 23 kDa were identified using Ponceau-S staining. The bands were carefully cut out as close to the bands as possible with sharp scissors. Next, the bands were cut into very small pieces, placed in a 1.5ml dounce hand homogenizer and crushed in 1ml 1XPBS. The solution was a milky white mixture that was quite consistent. Approximately 30 μ g was injected into each of 2 New Zealand rabbits. Following 4 injections and numerous bleeds (described in the methods and materials), the animals were sacrificed and the serum was removed and purified. Following Western immunoblotting with numerous BQ antibody concentrations, a 1:4000 BQ antibody concentration showed the best results. Western Immunoblots demonstrated a strong distinct band at approximately 40kDa and a less distinct band at 70kDa (see Figure. 5).

RLM-RACE PCR

[00101]cDNA isolated from the human brain cDNA library (Ambion) using the Invitrogen RACE kit resulted in a successful sequencing of the 3' end of the BQ gene. Results of sequencing by MOBIX showed that this stretch of PCR product was 96% homologous with mot-2 gene. Optimization of the PCR conditions, such as: 1) decreasing annealing temperatures; 2) increasing number of cycles; 3) addition of reducing agents, DMSO; 4) addition of betaine in order to straighten out secondary structures; 5) and making RACE cDNA with a thermal reverse transcriptase (superscript III) all failed to produce a positive band for the 5' end of the BQ gene.

RLM-RACE PCR Results with BQ primers and Hela cells as a positive control.

[00102]The DA D2 receptor was transfected into the SH-SY5Y Neuroblastoma cells because they are not found endogenously in this particular cell-line. Addition of either 100 μ M DA, 100 μ M Haloperidol or a combination of both resulted in a significant increase of BQ expression compared to controls in the SH-SY5Y cells (see Figure 6).

Northern Blot

[00103]The first attempt of the northern blot involved the use of total RNA from: rat STR; bovine STR; human STR; human heart; human liver and SH-SY5Y cells. The subsequent blot showed a distinct band at the 2.8kb level relative to the standard, and a slight band could be seen at the 1.9kb level representing the BQ transcript (see Figure 7). The northern was repeated using 20 µg polyA RNA and the results showed two distinct bands at 2.8kb representing mortalin and 1.9kb representing the human CRP40 protein, especially evident in SH-SY5Y cells. This was the first quantitative evidence that human CRP40 is alternative spliced variant from the mortalin gene. Furthermore, the northern provided information that the spliced variant is expressed in lower amounts than mortalin and it is specific in the CNS.

Real-Time PCR

[00104]Two step Real-Time PCR (RT-PCR) was performed for quantification of mRNA in DAD2L transfected SH-SY5Y cells treated with HAL relative to normal SH-SY5Y cells. The two step RT-PCR was performed in duplicate for each sample using MX3000P Real-Time PCR (Stratagene). The primers used were forward: 5' TTGGCCGGCGATGTCACGGATGTG-3' (SEQ ID NO: 5) and reverse: 5' - ACACACTTTAATTTCCACTTGCGT-3' (SEQ ID NO: 6). No primer dimers were detected and transcripts showed optimal PCR efficiencies. An absolute standard curve (Figure 8) was constructed with the use of corresponding purified cDNA from control SH-SY5Y RNA sample in the range of 1pg-10ag. MX3000P Real-Time PCR were optimized to ensure the amplifications were in the exponential phase and the efficiencies remained during the course of PCR. The results of the amplification showed that HAL-treated SH-SY5Y cells had a lower CT-value, which corresponded to approximately a 100% increase in initial template copies relative to the control cells (Figure 9). These sensitive results show that HAL-treated cells, which result in increased DA release directly modulate the Human CRP40 transcript. Relative quantitation was performed using the same SH-SY5Y cells as the previous experiment; however, human cyclophyllin was used as the housekeeping gene (Figures 10 and 11). The results showed that the CT-values of the DAD2L-treated SH-SY5Y RNA were the same relative to the control SH-SY5Y RNA.

Localization of human CRP40 in Postmortem NA brain specimens

[00105]Three slides containing post mortem brain specimens of the NA were used in immunohistochemistry studies. The primary antibody directed to human CRP40 was added in a dilution of 1:200 and the FITC secondary antibody was used as the fluorescent probe. The next day, the slides were analyzed using a confocal microscope (McMaster University). The results showed that human CRP40 is densely located in the perinuclear region in the control and schizophrenic specimen naïve of drug treatment (Figure 12). However, the slide containing the specimen that was treated with large doses of HAL, showed the antibody to be located almost exclusively within the nucleus.

Discussion

[00106]A human EST (Genbank Accession # BQ224193) has been used to identify a human brain CRP40 protein. Nucleic acid primers derived from this EST were used in RT-PCR to amplify a cDNA, which was cloned into the pGEX-2T bacterial expression vector. Functional studies of the recombinant fusion protein using [³H] propylnorapomorphine and [³H]DA clearly demonstrated that human CRP40 binds catecholamines. Furthermore, RACE-PCR was employed and the full-length gene sequence of this human CRP40 protein was elucidated. Northern blotting confirmed two discrete mRNA bands with human-CRP40 specific primers giving evidence that human CRP40 is an alternative spliced variant of the mot-2 gene. Real-Time PCR experiments were conducted with neuroblastoma SH-SY5Y cells treated with the typical neuroleptic, haloperidol (HAL) and CRP40 mRNA copy numbers were significantly expressed relative to normal control cells. Last, human CRP40 expression is found (at least) in both the lymphocytes and platelets of human blood.

[00107]Reverse transcriptase PCR was employed using specific BQ224193 primers with human, rat, and bovine striatal cDNA, and following agarose electrophoresis, 3 discrete bands were elucidated and sequencing analysis (MOBIX) of the PCR fragments showed 97% homology to the mitochondrial heat shock protein 70kda, mortalin-2 (mot-2) (HSPA9) (see **Figure.1**). Mot-2 is a 66kDa protein, known to contain multifunctional properties. Most importantly, this protein is known to bind to the c-terminus of the tumor suppressor gene p53, preventing its transactivation to the nucleus, ultimately causing cell immortalization.

[00108]In order to determine if the BQ224193 fragment had catecholamine functional properties, cloning experiments were designed and performed using a pGEX-2T bacterial expression vector system, which were transformed in BL-21 *E.coli* cells. Following the

production of 23kDa recombinant fusion protein, protein sequence analysis from the University of Calgary confirmed the BQ224193 fusion protein to have 97% homology to the mot-2 protein.

[00109]Preliminary functional studies using protein binding assays were performed using [³H] propylnorapomorphine and [³H]DA with the recombinant BQ224193 fusion protein. The results in these experiments showed this novel protein fragment to significantly displace the tritiated compounds with different concentrations of cold DA; giving direct evidence that this novel fusion protein fragment, referred to as human CRP40, has the ability to bind catecholamines with low affinity and with high capacity (see **Figure. 5**).

[00110]At this point, polyclonal antibodies were produced using the purified human CRP40 fusion protein. Following numerous injections of fusion protein and bleeds in two New Zealand rabbits, the animals were sacrificed on day 150. The polyclonal antibody produced a strong discrete band at approximately 40kDa, and a weaker diffuse band at approximately 70kDa in both rat and human striatal tissue, evidenced by Western immunoblotting.

[00111]Additional functional studies were conducted using DAD2L transfected SH-SY5Y cells that were homogenized in subcellular fractions, which included: 1) the cytoplasm; 2) the mitochondria; 3) and the nuclear fractions. Western blotting using SH-SY5Y tissue sub-fractions showed tissues exposed to elevated temperature of 42°C resulted in a significant increase in human CRP40 levels, specifically in the nuclear fraction. The nuclear fraction tissue showed the most dramatic increase in human CRP40 protein, indicating that the CRP40 proteins translocate to the nucleus in the presence of stress such as heat or increased catecholamine exposure. Additional functional studies were conducted with SH-SY5Y cells that were exposed to 100µM HAL and 100µM DA, resulting in a significant increase in human CRP40 protein expression in the nuclear fraction relative to SH-SY5Y control cells.

[00112]Following the cloning and characterization of the novel protein human CRP40, RLM-RACE PCR confirmed the complete sequence of human CRP40 protein. Sequencing results revealed that the entire BQ sequence to have 97% homology with the mot-2 gene at the C-terminus. From this point, it was hypothesized that human CRP40 is an alternative spliced variant from the 17 exon mot-2 gene (2.8kb transcript). Using the Swiss-Prot ExPASy program allowed the conversion of the human CRP40 nucleotide sequence to the translated

protein sequence. The results showed the novel human protein to have a molecular weight of 37.5kDa and theoretical P.I. of 6.21.

[00113]Northern blotting experiments were performed using the same primers as the cloning and RACE PCR experiments utilizing different total RNA and polyA RNA samples from different species (bovine, rat, human, SHSY5Y cells), without success. It was suggested that the novel spliced variant CRP40 may be expressed in low amounts and the Northern was repeated with increased concentrations of poly A RNA and confirmation of two discrete bands were elucidated in bovine striatal tissue, human striatal tissue and SH-SY5Y cells (see **Figure. 7**). The bands were normalized against the beta-actin transcript and measured following gel electrophoresis. The Northern blot demonstrated 2 discrete bands at approximately 2.8kb and 1.9kb, corresponding to the 66 kDa mortalin protein and Human CRP40 (40kDa) protein, respectively.

[00114]Additional functional studies were conducted at a transcriptional level with the neuroblastoma SHSY5Y cells, due to its extensive characterization in CNS function. The DA-D2L receptor was transfected in the SHSY5Y by lipofection. The typical antipsychotic, HAL, was added to the cell culture and the RNA was extracted from these cells along with SH-SY5Y control cells. Real-Time PCR using Human CRP40 primers revealed this novel alternative spliced variant of mortalin is differentially up-regulated in the HAL-treated cells versus control cells, by absolute quantitation. Furthermore, relative quantitation and normalization was used with the housekeeping gene, human cyclophyllin, which showed no significant change between the treated and un-treated cells.

[00115]Localization studies were also conducted using immunohistochemistry on postmortem brain samples of the Nucleus Accumbens (NA) from the Stanley Foundation Consortium (SFNC). The results demonstrated that human CRP40 antibody was localized in the perinuclear region of neurons within the NA region in normal controls. Furthermore, similar staining was seen in schizophrenia specimen slides that were drug naive. However, immunostaining in HAL-treated schizophrenia postmortem samples showed that the human CRP40 antibody was clearly seen within the nuclear region.

[00116]Real-Time PCR was performed on 105 Postmortem Brain RNA brain samples of the prefrontal cortex (PFC) region, that were generously donated from SFNC in a coded fashion, and on 50 ng DNase-treated RNA using the QIAGEN one-step method in a double paradigm

approach. This study showed that ANOVA examination indicated age, sex and PMI had no significant effects on the PFC Human CRP40 expression among the SFNC. However, a significant decrease in Human CRP40 mRNA copy numbers was seen in bipolar patients (n=35), relative to control specimens (n=35). Furthermore, dividing the groups according to amounts of lifetime antipsychotic treatment, showed that a significant decrease in human CRP40 mRNA copy numbers was seen between control specimens and specimens that had a history of consuming the lowest doses of neuroleptics (0-50K). In addition, an increased trend in human CRP40 mRNA expression was seen in patients that consumed the largest lifetime dose of antipsychotics (>400K) versus specimens with the lowest antipsychotic use (0-50K), showing a human CRP40 mRNA normalizing effect with larger antipsychotic use. The above results demonstrate that human CRP40 mRNA expression behaves dysfunctionally in both schizophrenic and bipolar patients within the PFC brain region and this novel protein is differentially modulated by DA-activity. Genetic mapping studies have shown that mot-2 and human CRP40 are on chromosome 5 band q31, which has been shown to be a putative susceptible schizophrenia and bipolar gene locus.

[00117]Human CRP40 primers were used with cDNA made from both lymphocytes and platelets, and RT-PCR was performed. The experiment demonstrated distinct bands in both lymphocytes and platelets and sequencing revealed the exact human CRP40 sequence. This finding is important for the use of CRP40 as a biomarker since access to neuronal tissue to study the pathophysiological changes in psychiatric disorders is not possible.

[00118]In summary, the cloning, characterization, and localization of human CRP40 protein have demonstrated that this novel protein has catecholamine-regulated functions. Human CRP40 is an alternative spliced variant from the mot-2 gene and has been shown to be down-regulated in neurological disease states.

Example 2 – Utility of CRP40 as Biomarker in Neurological Diseases

[00119]The present study was undertaken to examine human CRP40 expression in healthy control, schizophrenic and bipolar postmortem brain specimens. A similar study was conducted using primers directed to mortalin-2 and the results were similar.

METHODS AND MATERIALS

Post-mortem RNA PFC sample

[00120]Post-mortem DNase-treated RNA specimens of the prefrontal cortex were provided by the Stanley Foundation Neuropathology Consortium. Microscopic examination was performed on all samples by 2 independent neuropathologists trained at the Stanley Foundation in order to provide consistent results. Extensive records of patients were available and were reviewed by two psychiatrists. Diagnoses were established using DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition) criteria (demographics shown in **Table 1**) as follows. The control patients were confirmed to be free of psychiatric illness and free of substance abuse, and were assessed by the same method.

Table 1. Overview of Stanley Foundation Patient Profile

Characteristics	Bipolar (<i>n</i> = 35)		Normal (<i>n</i> = 35)		Schizophrenia (<i>n</i> = 35)	
Sex	Male 17	Female 18	Male 26	Female 9	Male 26	Female 9
Age (mean±S.D., years)	44 ± 11.7 (range 19-64)	48.5 ± 9.29 (range 26-63)	47 ± 7.82 (range 31-60)	39 ± 5.10 (range 33-49)	42.5 ± 8.2 (range 19-53)	47 ± 8.54 (range 32-59)
Race						
white	15	18	26	9	25	9
native american	1	0	0	0	0	0
black	1	0	0	0	0	0
hispanic	0	0	0	0	1	0
Lifetime Antipsychotics (mean ± S.D., h)	1600 ± 32336.2 (range 0-130000)	3500 ± 8881.3 (range 0-30000)	0 (range 0 – 0)	0 (range 0 – 0)	65000 ± 91937.9 (range 50-350000)	20000 ± 126622.6 (range 600 – 400000)
PMI (mean ± S.D., h)	32 ± 19.9 (range 12 – 84)	37.5 ± 17.2 (range 17–77)	27 ± 12.1 (range 9 – 52)	29 ± 14.7 (range 10 – 58)	29.5 ± 17.0 (range 9 – 80)	35 ± 11.0 (range 13 – 52)

Abbreviations: PMI Postmortem interval

[00121]Data regarding cause of death, substance abuse history, antipsychotic intake data and other medication intake at time of death were also provided (**Tables 2-5**). Matched set RNA brain specimens from 105 patients were obtained, 35 being diagnosed with schizophrenia, 35 being diagnosed with familial bipolar disorder, and 35 being normal controls, and immediately stored under dry ice. Each sample was matched for patient age, gender, mRNA quality and brain pH.

**Table 2 - Subject Profile for Normal Control Subjects
From Stanley Foundation**

Age (years/sex)	PMI (h)	CNS medications at time of death
31/M	11	N/A
32/M	13	N/A

34/M	22	N/A
35/M	52	N/A
35/M	24	N/A
37/M	13	N/A
40/M	38	N/A
42/M	37	N/A
45/M	29	N/A
45/M	18	N/A
46/M	31	N/A
47/M	21	N/A
47/M	11	N/A
47/M	36	N/A
48/M	31	N/A
48/M	24	N/A
49/M	46	N/A
49/M	23	N/A
50/M	49	N/A
51/M	31	N/A
51/M	22	N/A
53/M	9	N/A
53/M	28	N/A
55/M	31	N/A
57/M	26	N/A
60/M	47	N/A
33/F	29	N/A
34/F	24	N/A
38/F	33	N/A
38/F	28	N/A
39/F	58	N/A
41/F	50	N/A
44/F	28	N/A
44/F	10	N/A
49/F	45	N/A

Abbreviations: M, male; F, female

Table 3			Subject Profile for Bipolar Subjects From Stanley Foundation
Age (years/sex)	PMI (h)	CNS medications at time of death	
19/M	12	Quetiapine, Topiramate	
29/M	48	Risperidone, Lithium	
29/M	60	None	
35/M	35	Haloperidol, Lithium	
35/M	22	None	
41/M	39	None	
41/M	70	Lithium, Valproate	
42/M	32	None	
44/M	19	Valproate	
		Thioridazine, Risperidone, Lithium, Valproate, Paroxetine,	
45/M	28	Benzotropine	
45/M	35	Thioridazine, Olanzapine, Gabapentin	
48/M	23	Lithium, Fluoxetine	
51/M	23	Lithium, Carbamazepine & Valproate	

54/M	44	Valproate, Paroxetine
56/M	23	Olanzapine, Carbamazepine, Fluoxetine, Doxepin
59/M	84	Valproate, Gabapentin, Trazodone, Zolpidem
64/M	16	Clozapine, Valproate
29/F	62	Trazodone
33/F	24	Risperidone, Lithium, Fluoxetine
35/F	17	Olanzapine, Amitriptyline
41/F	28	Risperidone, Valproate, Trazodone
42/F	49	Risperidone, Trazodone
43/F	39	Quetiapine, Carbamazepine, Gabapentin, Venlafaxine
43/F	57	Quetiapine, Carbamazepine, Fluoxetine, Venlafaxine
44/F	37	Quetiapine, Olanzapine, Valproate, Venlafaxine
48/F	18	Fluoxetine & Trazodone, Doxepin
49/F	19	Perphenazine, Lithium
49/F	38	Amitriptyline, Venlafaxine
50/F	62	Amitriptyline
51/F	77	Risperidone, Valproate, Paroxetine
55/F	41	Thiothixene
56/F	26	Valproate, Gabapentin, Trazodone, Sertraline
58/F	35	Haloperidol, Lithium
59/F	53	Valproate, Paroxetine, Trazodone
63/F	32	Mirtazapine

Abbreviations: M, male; F, female

Table 4 - Subject Profile for Patients With Schizophrenia from The Stanley Foundation

Age (years/sex)	PMI (h)	CNS medications at time of death
19/M	28	Thioridazine, Olanzapine, Valproate
24/M	15	Olanzapine, Valproate
31/M	33	Clozapine, Benztropine
33/M	29	Haloperidol, Lithium
35/M	47	Fluphenazine, Trihexyphenidyl
37/M	30	Thioridazine, Thiothixene, Fluoxetine
38/M	35	Quetiapine, Haloperidol, Gabapentin, Trazodone
39/M	80	Fluphenazine, Benztropine
39/M	26	Ziprasidone, Risperidone, Olanzapine, Haloperidol
40/M	34	Thiothixene, Clozapine, Valproate, Benztropine
41/M	54	Risperidone, Quetiapine, Lithium, Procyclidine
42/M	26	Olanzapine, Paroxetine, Buspirone, Clonazepam
42/M	19	Fluphenazine
43/M	26	Fluphenazine, Valproate, Benztropine
43/M	18	Haloperidol, Benztropine
43/M	65	Haloperidol
44/M	9	Haloperidol
44/M	32	Risperidone, Fluvoxamine
45/M	35	None
46/M	30	Haloperidol, Risperidone, Carbamazepine
47/M	13	Haloperidol, Olanzapine, Valproate
50/M	9	Thiothixene
51/M	43	Fluphenazine
52/M	10	Haloperidol, Benztropine, Diphenhydramine
52/M	16	Thiothixene, Fluoxetine, Benztropine

53/M	38	Risperidone
32/F	36	Risperidone
36/F	27	Risperidone, Paroxetine, Trihexyphenidyl
44/F	26	Thiothixene
45/F	52	None
47/F	30	Quetiapine, Valproate, Mirtazapine, Bupropion & Amitriptyline
47/F	35	Risperidone, Haloperidol
53/F	13	Haloperidol, Lithium
54/F	42	Haloperidol, Chlorpromazine, Benztropine
59/F	38	Risperidone, Trazodone

Abbreviations: M, male; F, female

Table 5- Categorical Summary of Data used in Statistical Analysis

Age (years)	Sex	Race	PMI (h)	Disease	CNS medications at time of death (Schizophrenic subjects only)
19-28	Male	White	9-18	Normal Control	Psychotic
29-38	Female	Black	19-38	Bipolar	Antipsychotic
39-48		Hispanic	39-48	Schizophrenia	
49-58		Asian	49-58		
59-68		Native American	59-68		
		Mixed Race	69-78		
			79-88		

One-Step Real-Time PCR Protocol with Human Post-Mortem Brain RNA Samples in the PFC region:

[00122]The RNA samples consisted of 10 µg DNase-treated RNA, and the concentration and purity (260/280) of each sample was also provided. Real-Time RT-PCR was performed in triplicate for each sample using 50 ng RNA in an MX-3000P Real-Time PCR Machine (Stratagene, U.S.A.). The following Human CRP40 primers were used for all Real-Time PCR experiments: 5'-TTGGCCGGCGATGTCACGGATGTG-3' (Forward, sense primer) (SEQ ID NO: 5) and 5'-ACACACTTTAATTTCCTTGCCT-3' (Reverse, antisense primer) (SEQ ID NO: 6). Previous studies using these primers with human RNA samples demonstrated no primer-dimers, and the transcripts showed optimal Real-Time RT-PCR efficiencies. An absolute standard curve was run along with sample using purified DNA fragment (Sample #1 from The Stanley Foundation) in the range of 1pg-10pg, and initial

template copy numbers were calculated. MX-3000P Real-Time RT-PCR conditions were optimized to ensure that amplifications were in the exponential phase and that efficiencies remained constant during the course of the Real-Time RT-PCR reaction. This experiment was conducted using the QIAGEN One-step RT-PCR method, which contained the QuantiTect SYBR Green with the QuantiTect RT-mix in one tube. Advantages of the One-step method, versus the Two-step method are as follows: 1) fewer pipetting steps minimizing error and contamination; 2) improved sensitivity and specificity at higher temperatures to eliminate problems with secondary RNA structures; 3) and minimal time requirements. All samples were performed in triplicate and a NRT and NTC were also run as controls. The experiment was repeated with the same samples in duplicate using Human Cyclophyllin, a house-keeping gene, to normalize the samples in relative quantitation. The components used in the Real-Time RT-PCR were as follows: 2X SYBRgreen, 10 μ l; Forward primer (300nM), P2, 1.2 μ l (5 μ M); Reverse primer, (300nM), P4, 1.2 μ l (5 μ M); Reverse Transcriptase Mix, 0.2 μ l; DEPC H₂O, 6.4 μ l; Coded Stanley RNA, 1 μ l (50ng). The Real-Time PCR conditions were as follows: 50°C 30 sec (1 cycle); 95°C 15 min (1 cycle); 95°C 15 sec (40 cycles); 60°C 30 sec (40 cycles); 72°C 40 sec (40 cycles).

Statistical analyses

[00123]GraphPad Prism, SPSS and MiniTab softwares were used for statistical analyses. Pearson product moment correlation coefficients were determined for all pairs of the measured variables (i.e. mRNA copy numbers, age, sex, post mortem interval (PMI), disease and drug type) and two-tailed tests of the correlations were performed to measure degrees of linear relationships. Statistical analysis was carried out on copy numbers of RNA molecules with respect to the other variables (see **Table 1-5**) using analysis of variance (ANOVA) methods followed by Tukey's post hoc comparison test.

RESULTS

a) Age, sex and post-mortem interval have no effect on Human CRP40 expression

[00124]No significant correlation was found between any pairs of the variables. Thus, ANOVA procedures described in materials section were performed. There were no significant differences in Human CRP40 expression in the PFC with respect to age [$F=0.142$ $df(1,34)$, $p > 0.05$], sex [$F = 1.56$ $df(1,34)$, $p > 0.05$], or post-mortem interval [$F=0.3$ $df(2,34)$, $p > 0.5$] between, control, schizophrenic, and bipolar subjects.

b) Medial Prefrontal Cortex Human CRP40 expression is specifically reduced in bipolar patients

[00125]ANOVA analysis showed significant differences in Human CRP40 expression in the PFC with respect to bipolar disorder [$F=8.89$, $df(2,104)$, $p < 0.01$] (see Figure.14). Post hoc analysis revealed a significant reduction in PFC Human CRP40 expression in bipolar specimens of approximately 31% relative to normal control specimens ($n=35$).

c) Prefrontal Cortex Human CRP40 expression is increased according to the quantities of Life-Time Antipsychotics administered in schizophrenic and bipolar patients.

[00126]ANOVA analysis showed a significant difference was detected in human CRP40 values ($F 6.062$, $df(3,104)$, $P= 0.008$) with respect to lifetime cumulative drug-intake dose. Therefore, post-hoc analysis was performed with a Tukey's test. Post-hoc analysis showed a significant difference between the control and the lowest lifetime cumulative antipsychotic drug-intake dose ($P < 0.001$). Furthermore, a definite trend was also found between the lowest treated group and the highest-treated antipsychotic group ($>150K$), displaying an increased normalizing effect of human CRP40 expression with increased antipsychotic use; however, not statistically significant.

Discussion

[00127]Postmortem Brain RNA brain samples of the PFC region were generously donated from the Stanley Foundation Neuropathology Consortium (SFNC) in a coded fashion. Real-Time PCR was performed on 105 PFC RNA samples using the QIAGEN one-step method and 50 ng DNase-treated RNA. The present study showed that ANOVA examination indicated age, sex and PMI had no significant effects on the PFC Human CRP40 expression among the SFNC. However, a significant decrease in Human CRP40 mRNA copy numbers was seen in bipolar patients ($n=35$), relative to control specimens ($n=35$) (see Figure 14). Furthermore, dividing the groups according to amounts of lifetime antipsychotic treatment, showed that a significant decrease in human CRP40 mRNA copy numbers were seen between control specimens and specimens that had a history of consuming the lowest doses of neuroleptics (0-50K). In addition, an increased trend in Human CRP40 mRNA expression was seen in patients that consumed the largest lifetime dose of antipsychotics ($>150K$) versus specimens with the lowest antipsychotic use (0-50K), showing a human CRP40 mRNA normalizing effect with larger antipsychotic use (see Figure 14). The above results demonstrate that Human CRP40 mRNA expression behaves dysfunctionally in both

schizophrenic and bipolar patients within the PFC brain region and this novel protein is differentially modulated by DA-activity.

[00128] Since both schizophrenia and bipolar disorder are known to have hypo-DA function in the PFC, and the fact that CRP40 is modulated by DA-activity, may explain why there was a significant decrease of Human CRP40 mRNA copy numbers, evidenced by Real-Time PCR in the bipolar specimen samples. Furthermore, the bipolar group consisted of 1 patient out of 35 that was treated with a substantial lifetime amount of neuroleptics, explaining the low CRP40 copy numbers in these DA-depleted PFC brain specimens. In contrast, the entire schizophrenic patient profile had a broad administration of antipsychotic use, from less than 50K to > 400K. The increased antipsychotic use in the schizophrenic specimens are known to cause increased DA release, which may explain the normalizing effect of Human CRP40 mRNA copy numbers in the schizophrenic group (see. **Figure.15**).

[00129] To further assess the observed decrease in CRP40 expression levels in these patients, the entire patient profile was separated according to ranges of lifetime antipsychotic administration and the mRNA copy numbers were used for statistical analysis. The results showed that patients administered low doses of antipsychotic drugs (0-50 K) displayed a significantly lower initial copy number expression of Human CRP40 than controls (see **Figure. 15**). These results correlate well with the original hypothesis that human CRP40 expression is directly related to the amount of antipsychotic use and increased DA release. A normalized effect was seen with the increased lifetime antipsychotic treatment.

Example #3 – Affect of Reduced CRP40 Expression

[00130] This experiment was conducted to determine whether or not reduced CRP40 in the prefrontal cortex is linked to the development of schizophrenia-like behavioural abnormalities or whether reduction in CRP40 in patients with schizophrenia is a consequence of the disease process.

[00131] To establish whether reduced expression of CRP40 in the medial prefrontal cortex leads to the development of behavioural abnormalities in a putative animal model, the following experimental approach was used.

[00132]Five groups of rats each consisting of 4 subgroups (n=12/subgroup, totaling 240 rats) were implanted with bilateral 26-gauge stainless steel guide cannulae (Plastics One Cat#3280PD-2.0) above the prefrontal cortex (stereotaxic coordinates: 3.0mm anterior to bregma, 0.7mm lateral to midline and 2.5mm below the surface of the skull). ADONS solutions were infused continuously for 3 weeks via S.C. implanted osmotic mini-pumps (Alzet model #2004) connected by separate catheters (PVC60 cannula tubing, Plastics One Cat#C312VT) to respective cannulae. **Group A** (Subgroups: A₁, A₂, A₃, A₄; n=12/subgroup) received Human CRP40 antisense deoxyoligonucleotides, 5' - tcg ttc ctt ctt tgg ccg gtt ttt t - 3' (SEQ ID NO: 4). **Group B** (Subgroups: B₁, B₂, B₃, B₄; n=12/subgroup) received sense deoxyoligonucleotide, 10nmol/day via osmotic pump. **Group C** (Subgroups: C₁, C₂, C₃, C₄; n=12/subgroup) received ADONS 10nmol/day via osmotic pump. **Group D** (Subgroups: D₁, D₂, D₃, D₄; n=12/subgroup) received missense (random) deoxyoligonucleotides similar to Group B. **Group E** (Subgroups: E₁, E₂, E₃, E₄; n=12/subgroup) served as a normal control. At the end of the infusion period, all groups of rats were monitored for development of **behavioural abnormalities: 1. PPI of acoustic startle response (76)**. These behavioural abnormalities were recorded on a weekly basis, up to 4 weeks, without challenge with any dopaminergic drug (since schizophrenia patients display abnormalities at baseline as well as in response to pharmacological challenges (6;76;80;81)). One subgroup from each group was sacrificed every week (up to 4 weeks) so that CRP40 mRNA levels could be estimated by Real-Time RT-PCR and protein levels by western blotting. The percent reduction of prefrontal cortex CRP40 levels was correlated with the intensity of behavioural abnormalities displayed by various groups of rats at weekly intervals.

[00133]**Determination of Prepulse Inhibition:** This technique measures the sensorimotor gating deficit observed in patients with schizophrenia by assessing a reflex startle response to a brief stimulus as a behavioural measure in rats. In this model, a weak sub-threshold acoustic stimulus (prepulse) was presented to a rat before a strong acoustic or tactile stimulus (pulse) and functions to inhibit the startle to the latter one. A disruption of PPI was found in schizophrenic patients and in animals by dopaminomimetics and antagonized by APDs. PPI in all groups was recorded using the SR-Lab Startle Response System (San Diego Instruments) as described by Tenn et al. in 2003 (64). Briefly, rats were placed in the startle apparatus and allowed to acclimatize for 10 minutes with a background noise [65 dB]. The

rats were then presented with a series of 5 startle pulses without any prepulse to control for habituation of the startle response. This series of stimuli was followed by 60 randomized trials consisting of no pulse [0 dB], a startle pulse [110 dB, 40 ms] or three prepulse intensities [70, 75 and 80 dB, 20ms] presented alone or 100 ms preceding the startle pulse. At the end, another series of five startle pulse-alone trials was presented. The startle response was measured every 1 ms for a 100 ms period from the onset of the startle stimulus. The % PPI was calculated as $100 - [(P + S)/S]100$. P + S is the mean response amplitude for prepulse trials and S is the mean response amplitude for the startle pulse-trial alone (64).

[00134]As shown in Fig. 16, significant pre-pulse inhibition (PPI) resulted from the foregoing CRP40 knock-down study. PPI is a standard test used to identify schizophrenia.

Example #4 – Platelet Aggregation by CRP40

[00135]CRP40 was also determined to cause aggregation of platelets. The following protocol was followed to study this affect:

- 1) isolated platelets from whole blood using established methodology;
- 2) washed platelets (500000/ul) with F agonist;
- 3) stopped reaction in 1% paraformaldehyde- 50 ul Rx mixture + 200ul PF;
- 4) washed cells with boton (4.5 ml), then centrifuged at 2200 rpm for 15 minutes; resuspended cells in isoton + 1mg/ml BSA 250 ul;
- 5) Staining- 50 ul platelets + 20ul CD62 PE (1/10 dilution)- incubated 30 minutes in dark at room temperature;
- 6) washed with 4.5 ml isoton and centrifuged 2200 rpm for 15 minutes; and
- 7) resuspended in 250 ul isoton.

[00136]Flow cytometry was then conducted.

[00137]The results showed that the addition of 10 ul pure CRP40 fusion protein caused a 80% increase in platelet aggregation relative to control platelets.

[00138]This activity of CRP40 as a platelet aggregator renders CRP40 a target in the treatment of cardiovascular disease.

Example #5 – Diagnostic Blood Test for Neurological Disease

Isolation of Platelets

[00139]2.0 ml of fresh rat blood was collected in eppendorf tubes. The blood was centrifuged at 1000 rpm (190 g) (Beckman TJ-6 bench centrifuge) for 10 min. Platelet rich plasma (PRP) was carefully transferred to a 15 ml conical tube (carefully transferred 0.75 % of the supernatant to avoid contamination). The platelets were centrifuged 2750 rpm (1600g) for 7 min. and the supernatant was decanted. The platelet button was resuspended in 1 ml of PBS (GIBCO) with 1% Na²⁺EDTA, 0.1% BSA. Platelet suspension was then transferred to a 20 ml Falcon tube and filled completely with PBS buffer (pipette mix) and centrifuged at 2750 rpm (1600g) for 7 min. The supernatant was decanted and washing repeated one more time.

Isolation of Lymphocytes from Rat Blood

[00140]2.0 ml fresh rat blood was obtained, transferred to a sterile 50 ml Falcon tube and 15 ml 1XPBS was added. Next, 5 ml Ficoll Paque sample was added to the mixture through a Pasteur pipette. The sample was centrifuged in a Beckman TJ-6 centrifuge at 1700 rpm for 20 min. Immediately following, the buffy coat was removed and transferred to a new 50 ml Falcon tube. The tube was filled to the 50 ml mark and centrifuged at 1800 rpm for 12 min. Following centrifugation, the aqueous layer was removed. Isopropyl alcohol (0.5 ml) was added to remaining suspension and incubated at room temperature for 10 min. Again, the solution was centrifuged at max. speed for 15 min and the pellet was retained. The pellet was resuspended with 1 ml 75% ethanol and centrifuged at 4°C for 8 min. The supernatant was then drawn off and the pellet was allowed to dry for 5 min. The dried pellet was re-suspended in 30 ml DEPC H₂O. RNA was isolated by TRIzol method and reverse transcribed into cDNA. Real-time PCR was performed as previously described.

Preparation of RNA using QIAGEN RNeasy Mini Kit

[00141]To the platelet pellet, 300 µl of RLT buffer was added and the mixture was homogenized well by passing the lysate through a 21 gauge needle fitted to an Rnase-free syringe (5 times). The suspension was transferred to a 1.5 ml eppendorf tube. An equal

volume of 70 % ethanol was added and mixed by pipetting. The full amount was applied to the QIAGEN RNeasy mini-column, placed in a 2 ml collection tube. The tube was closed gently and centrifuged at 10000 rpm (8000g) for 15 sec. The flow-through was discarded and the same collection tube used again. 700 µl of RW1 buffer was then added. The tube was closed gently and centrifuged at 10000 rpm (8000g) for 15 sec. Flow-through was again discarded. 500 µl of RPE was added onto QIAGEN RNeasy mini-column and centrifuged at 10000 rpm (8000g) for 15 sec. Flow-through was discarded, another 500 µl of RPE was added on QIAGEN RNeasy mini-column and centrifuged for 2 min at 10000 rpm (8000 g). The QIAGEN RNeasy mini-column was placed over a new collection tube and centrifuged at maximum speed for 1 min to ensure that no ethanol was carried over during elution. To elute, the QIAGEN RNeasy mini-column was transferred to a new 1.5 ml collection tube and 30 µl RNase free H₂O was pipetted directly on to the RNeasy silica-gel membrane. The tube was closed gently and centrifuged at 10000 rpm (8000 g) for 1 min. To increase RNA yield, the column with re-eluted with the first eluate. Check OD at 260 and 280. The platelet RNA was reverse transcribed into cDNA, and Realtime PCR was performed as previously described.

Results

[00142]Following Real-time PCR using specific human CRP40 primers (as identified in Experiment #2 above), the product was run on a 1% agarose gel, the band was cut out and sent for sequencing to MOBIX at McMaster University. The sequence results for both the platelets and lymphocytes were identical to the brain tissue RNA previously reported.

Example #6 – Diagnostic Blood Test for Neurological Disease

[00143]The blood test described above was also conducted using human blood. Platelets and lymphocytes were collected in the manner described in Example 5 from 20 ml human blood, and CRP40 DNA quantified as described.

[00144]The results of the real time PCR CRP40 quantification are shown in Figures 17 – 19 and each illustrate statistically significant differences between patients with neurological disease and control patients without neurological disease. Specifically, Figure 17 graphically illustrates the detection of reduced CRP40 levels in schizophrenic patients (n=6) versus non-schizophrenic control patients (n=4). cDNA was obtained from lymphocytes. Figure 18

graphically illustrates reduced CRP40 levels between schizophrenic patients (n=4) and controls (n=2) using cDNA obtained from platelets. Figure 19 graphically illustrates reduced CRP40 levels between a drug naïve schizophrenic patient with symptoms of glucose dysregulation and a control. cDNA was obtained from platelets. Figure 20 graphically illustrates reduced CRP40 levels between patients (n=3) with Parkinson's and controls (n=3) using cDNA obtained from platelets.

Example 7 – Preparation of a CRP40 therapeutic composition

[00145]Previous reports have shown that nanoparticles via nasal delivery reach numerous regions of the brain (Gao et al. 2006; Zhang et al. 2006). Accordingly, the following protocol is used to formulate CRP40 nanoparticles for nasal spray.

[00146]**Preparation of Nanoparticles:** The mortalin gene (human CRP40 alternative splice variant) is cloned in a mammalian expression plasmid, known to induce stable and strong transgene expression (Gomez-Vargas et al. 2004) driven by a ubiquitous promoter (such as #946;-actin which has been shown to be a stable genetic element driving antigen transcription *in vivo* (Broome et al. 2006). Chitosan nanoparticles, for use in delivering this construct, are synthesized by complexing high-molecular-weight (about 390,000 Da) chitosan with plasmid DNA to obtain uniform particles. Thus, 0.02% chitosan, pH 5.7, at 55 °C is added to plasmid DNA (50 mg/ml in 50 mM sodium sulfate) during high-speed vortexing. Transmission and scanning electron microscopy are run to test that the particle size does not exceed 150-300 nm in size (Roy et al. 1999). The plasmid is partially protected from DNase degradation in this formulation and its gel migration properties are unchanged by the process of forming complexes.

[00147]The foregoing description and examples describe embodiments of the invention. As will be appreciated by one of skill in the art, other embodiments of the invention exist which are encompassed by the appended claims.

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DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS:

1. An isolated human CRP40 protein of approximately 40 kDa comprising the amino acid sequence of SEQ ID NO: 8, or functionally equivalent variant thereof exhibiting about 98% or greater sequence identity with the amino acid sequence of SEQ ID NO: 8 across the full length of the sequence, wherein the functionally equivalent variant binds dopamine.
2. An isolated nucleic acid molecule encoding a human CRP40 protein of approximately 40 kDa comprising the amino acid sequence of SEQ ID NO: 8, or functionally equivalent variant thereof exhibiting about 98% or greater sequence identity with the amino acid sequence of SEQ ID NO: 8 across the full length of the sequence, wherein the functionally equivalent variant binds dopamine.
3. The isolated human CRP40 protein of claim 1, wherein the protein has the amino acid sequence of SEQ ID NO: 8.
4. The isolated nucleic acid molecule of claim 2, wherein the nucleic acid encodes a human CRP40 protein having the amino acid sequence of SEQ ID NO: 8.
5. A method of diagnosing neurological disease associated with hypo-dopamine levels in a human subject, wherein the neurological disease is bipolar disease, schizophrenia, or Parkinson's disease, comprising the steps of:
 - a) obtaining a blood or blood-derived sample from the subject;
 - b) measuring in the blood or blood-derived sample the level of a human CRP40 protein of approximately 40 kDa comprising the amino acid sequence of SEQ ID NO: 8, or functionally equivalent variant thereof exhibiting about 98% or greater sequence identity with the amino acid sequence of SEQ ID NO: 8 across the full length of the sequence, or nucleic acid encoding said CRP40 protein or functionally equivalent variant thereof exhibiting about 98% or greater sequence identity with the amino acid sequence of SEQ ID NO: 8 across the full length of the sequence and which binds dopamine; and

c) comparing the level of said CRP40 protein or said variant or the nucleic acid encoding said CRP40 protein or said variant against a normal control sample,

wherein a reduction in the level of said CRP40 protein or said variant or the nucleic acid encoding said CRP40 protein or said variant by about 10% or greater as compared to the normal control sample is indicative of disease.

6. The isolated nucleic acid molecule of claim 2 having the nucleic acid sequence defined in SEQ ID NO: 7.

7. The method of claim 5, wherein the human CRP40 protein comprises the amino acid sequence of SEQ ID NO: 8 or nucleic acid encoding the human CRP40 protein comprising the amino acid sequence of SEQ ID NO: 8.

8. The method of claim 5, wherein the blood-derived sample comprises a blood component selected from the group consisting of platelets and lymphocytes.

9. The method of claim 5, wherein the neurological disease is schizophrenia.

10. The method of claim 5, wherein the neurological disease is bipolar disease.

11. The method of claim 5, wherein the neurological disease is Parkinson's disease.

12. An isolated human CRP40 protein having a molecular weight of approximately 40 kDa comprising the amino acid sequence of SEQ ID NO: 8 or a variant thereof exhibiting about 98% or greater sequence identity to the amino acid sequence of SEQ ID NO: 8 across the full length of the sequence and which variant binds dopamine.

13. The isolated human CRP40 protein or variant according to claim 12, wherein the human CRP40 protein consists of the amino acid sequence of SEQ ID NO: 8 or the amino acid sequence of SEQ ID NO: 8 containing one or more conservative amino acid substitutions

or additions or deletions, wherein said additions or deletions are terminal additions or deletions at either end of the protein or internal amino acid deletions or insertions, and wherein said substitutions, additions or deletions that yield a variant of the CRP40 protein of amino acid sequence SEQ ID NO: 8 which binds dopamine.

14. An isolated nucleic acid molecule encoding a human CRP40 protein or a variant thereof as claimed in any one of claims 1, 3, 12 or 13.

15. An anti-CRP40 antibody that binds to a human CRP40 protein or a variant thereof as defined in any one of claims 1, 3, 12 or 13.

16. A pharmaceutical composition comprising the human CRP40 protein or variant thereof as defined in any one of claims 1, 3, 12 or 13 and a pharmaceutically acceptable carrier.

17. The isolated human CRP40 protein of any one of claims 1, 3, 12 or 13 for use as a biomarker for neurological disease, wherein the neurological disease is schizophrenia, bipolar disorder or Parkinson's disease.

18. The use of claim 17, wherein the neurological disease is schizophrenia.

19. The use of claim 17, wherein the neurological disease is bipolar disorder.

20. The use of claim 17, wherein the neurological disease is Parkinson's disease.

21. The isolated human CRP40 protein of any one of claims 1, 3, 12 or 13 for use in the treatment of a neurological disease, wherein the neurological disease is schizophrenia, bipolar disorder or Parkinson's disease.

22. The use of claim 21, wherein the neurological disease is schizophrenia.

23. The use of claim 21, wherein the neurological disease is bipolar disorder.
24. The use of claim 21, wherein the neurological disease is Parkinson's disease.
25. A method of diagnosing a neurological disease in a human, comprising the steps of determining the amount of CRP40 protein according to any one of claims 1, 3, 12 or 13 or a nucleic acid according to claim 14, present in a biological sample obtained from the human;
wherein a reduction of the protein or the nucleic acid by at least 10% as compared to a control amount present in a non-diseased patient is indicative of a neurological disease, wherein the neurological disease is schizophrenia, bipolar disorder or Parkinson's disease.
26. The method of claim 25, wherein the neurological disease is Parkinson's disease.
27. The method of claim 25, wherein the neurological disease is schizophrenia.
28. The method of claim 25, wherein the neurological disease is bipolar disorder.
29. The method of any one of claims 25 to 28, wherein the biological sample is a blood sample.

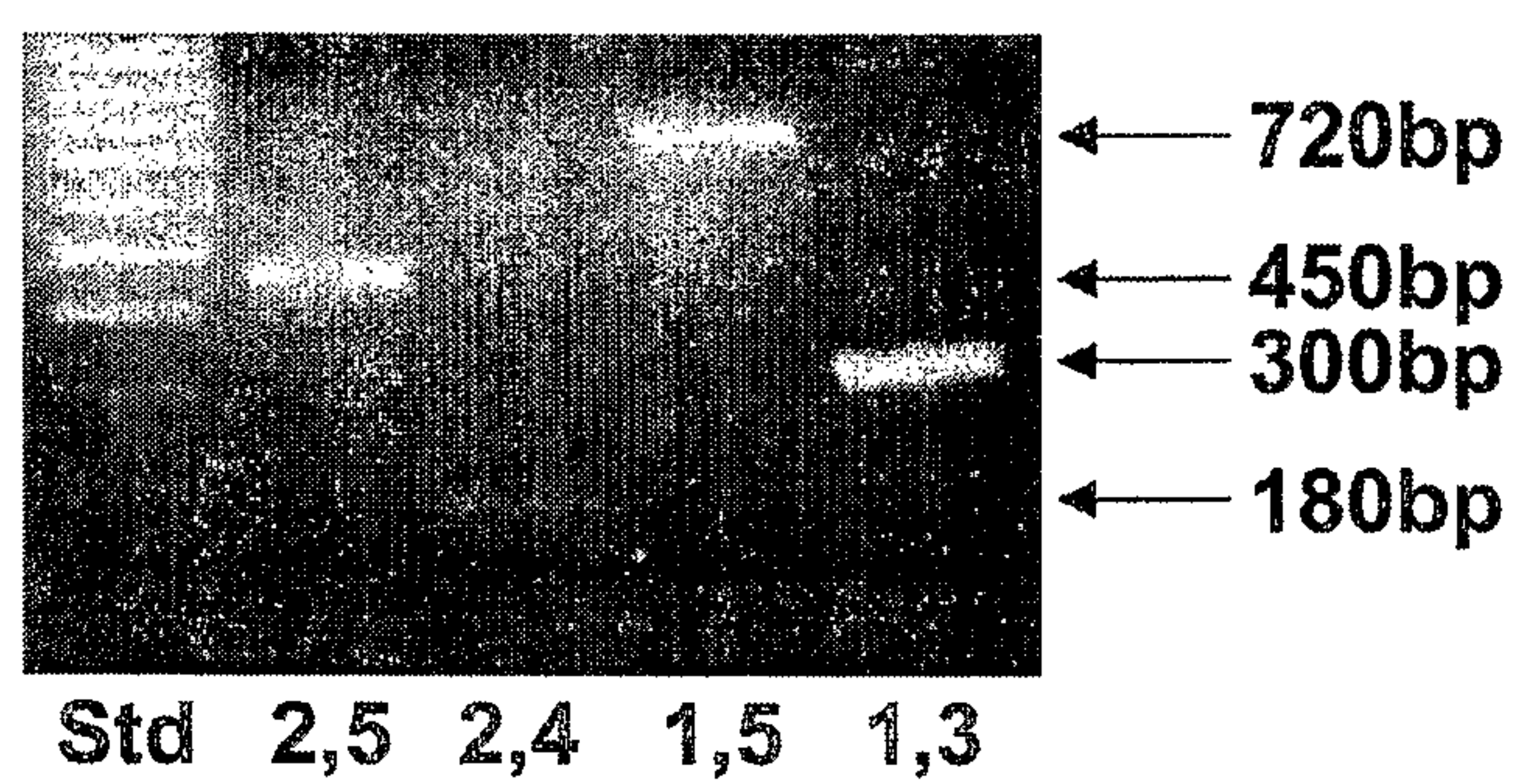


Figure 1

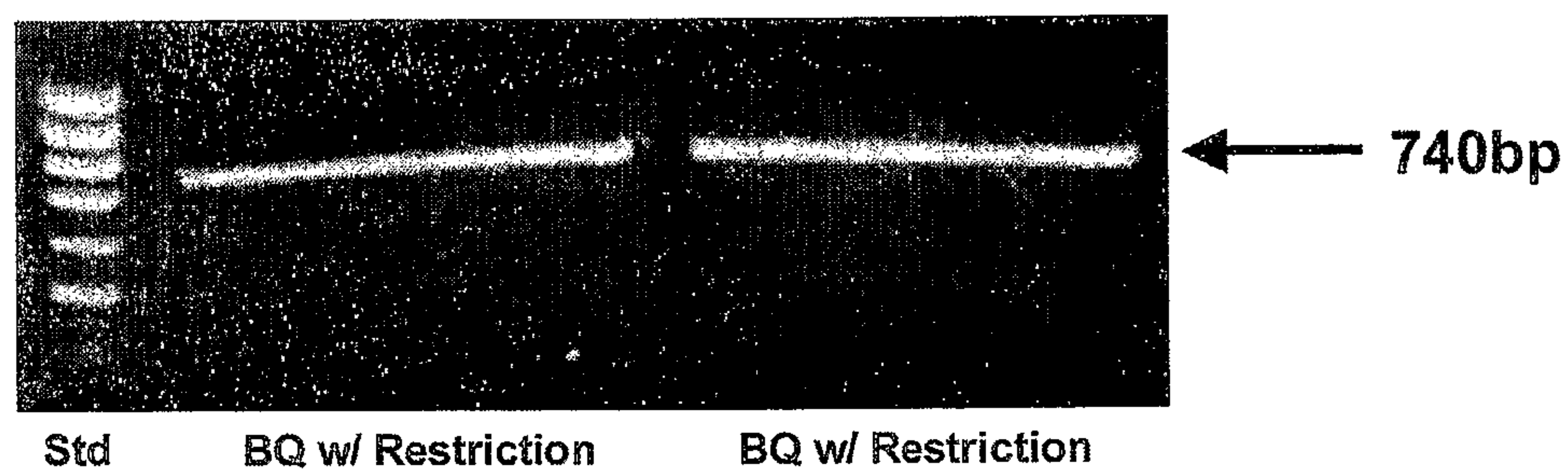


Figure 2

FIG. 3

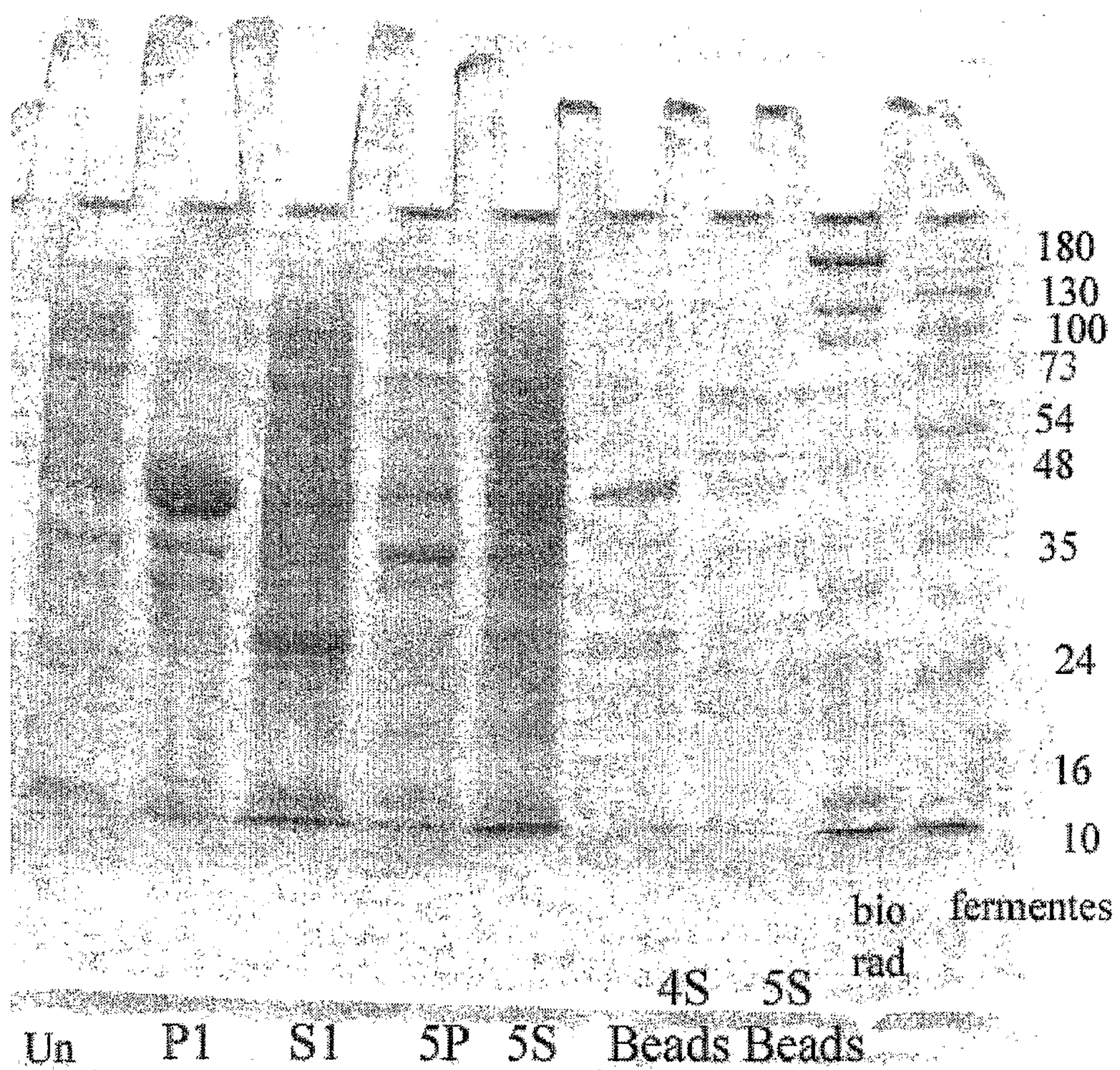
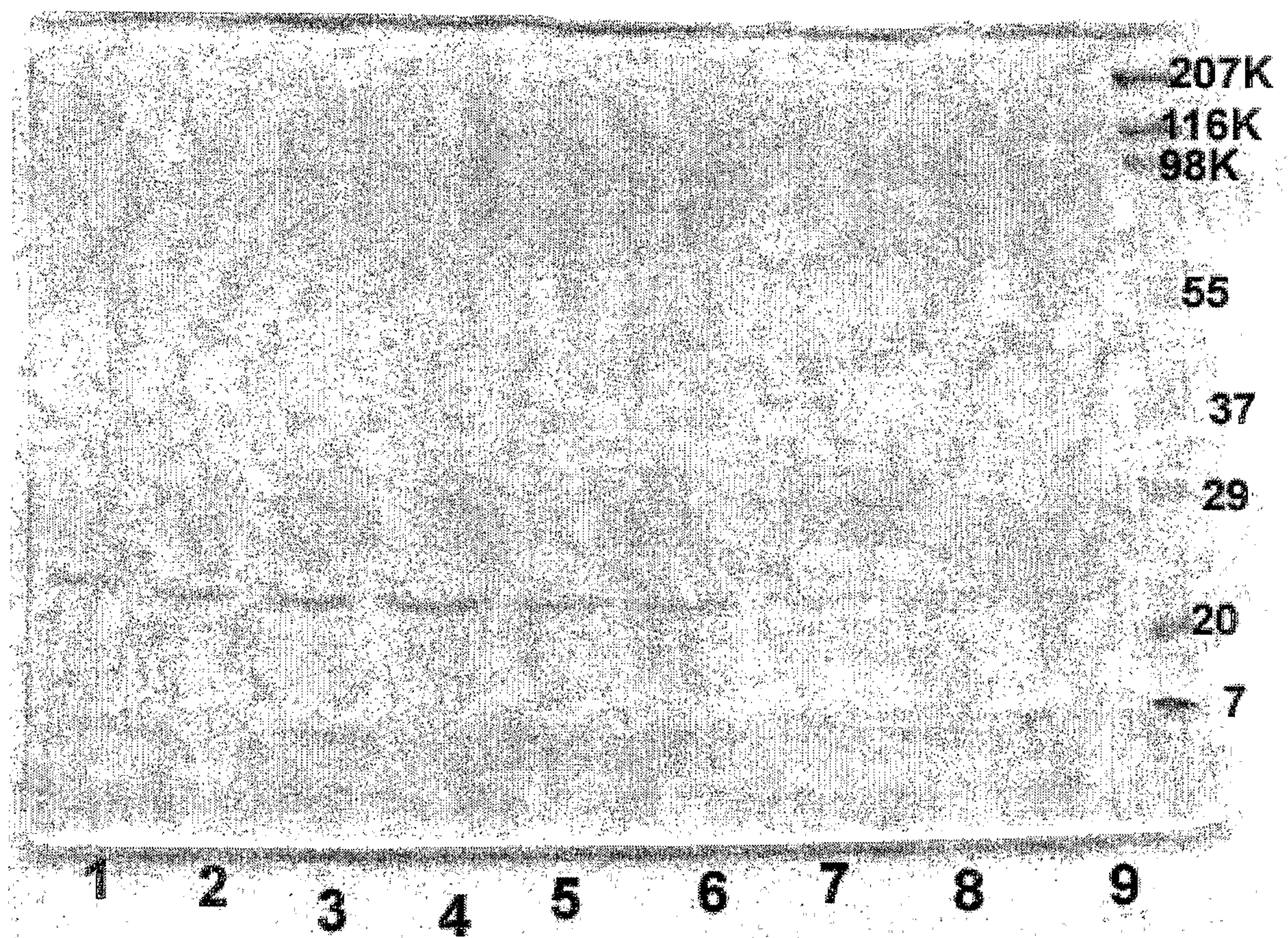


FIG. 4



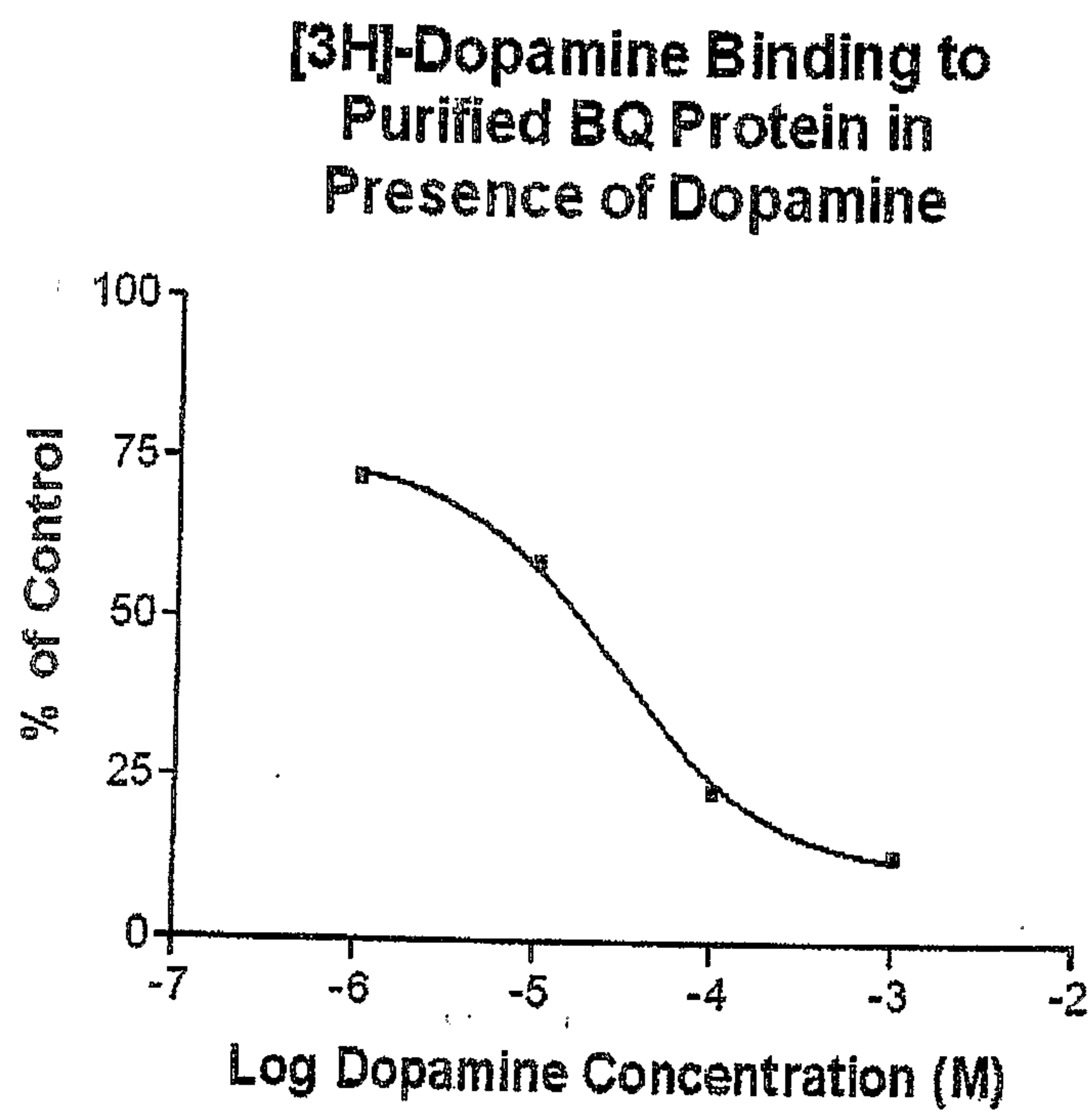


FIG 5

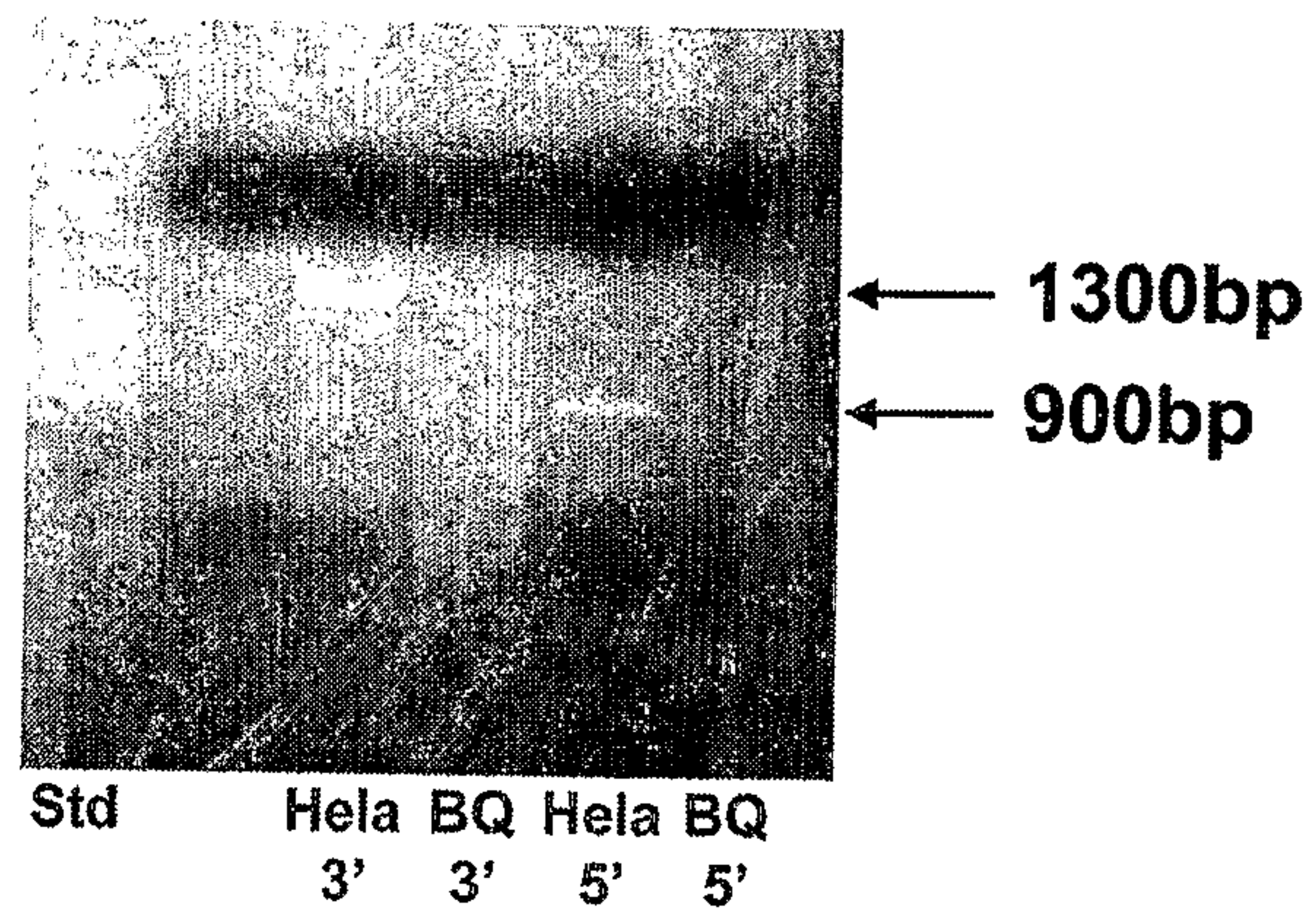


Figure 6

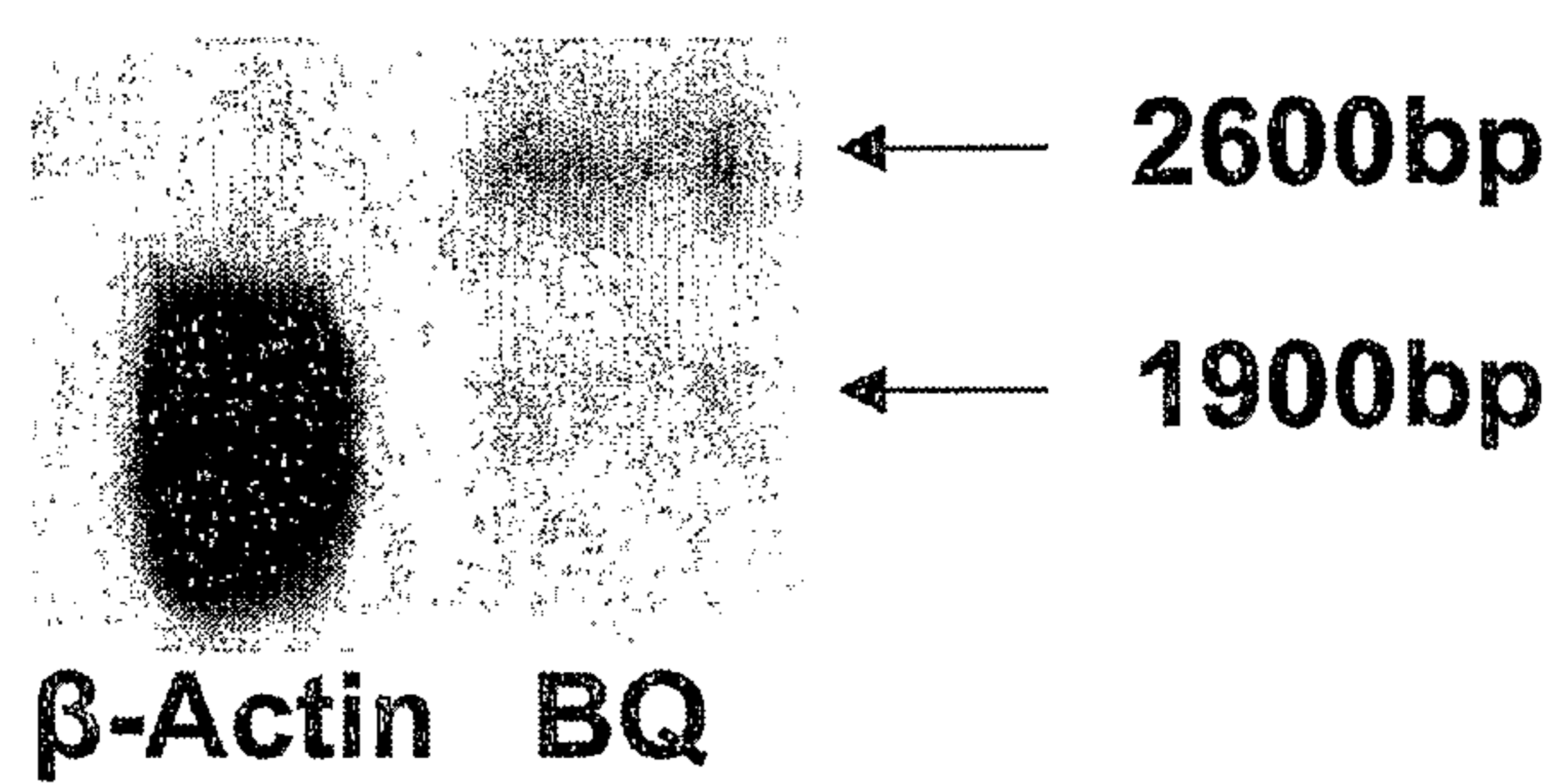


Figure 7

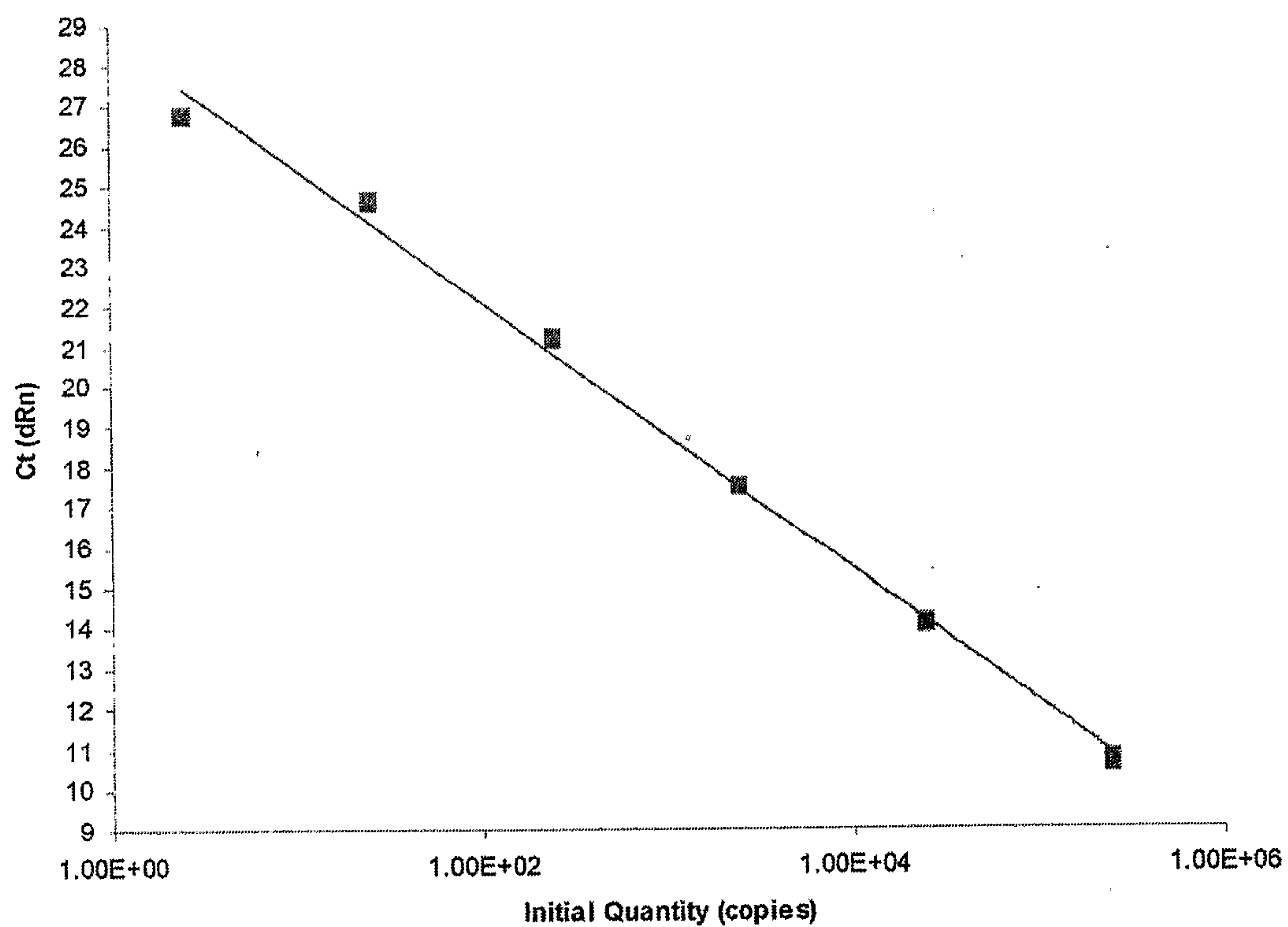


Figure 8

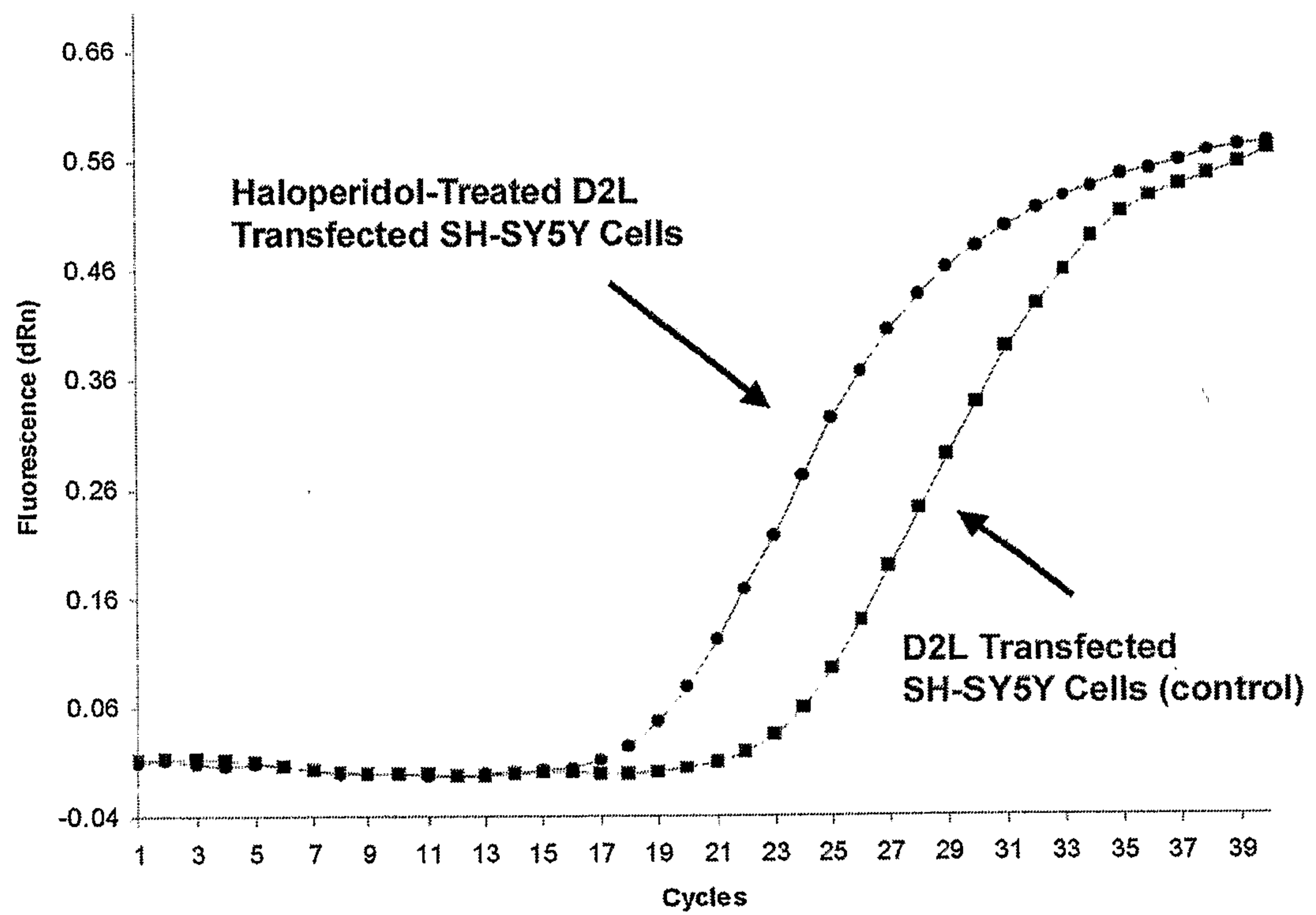


Figure 9

10/21

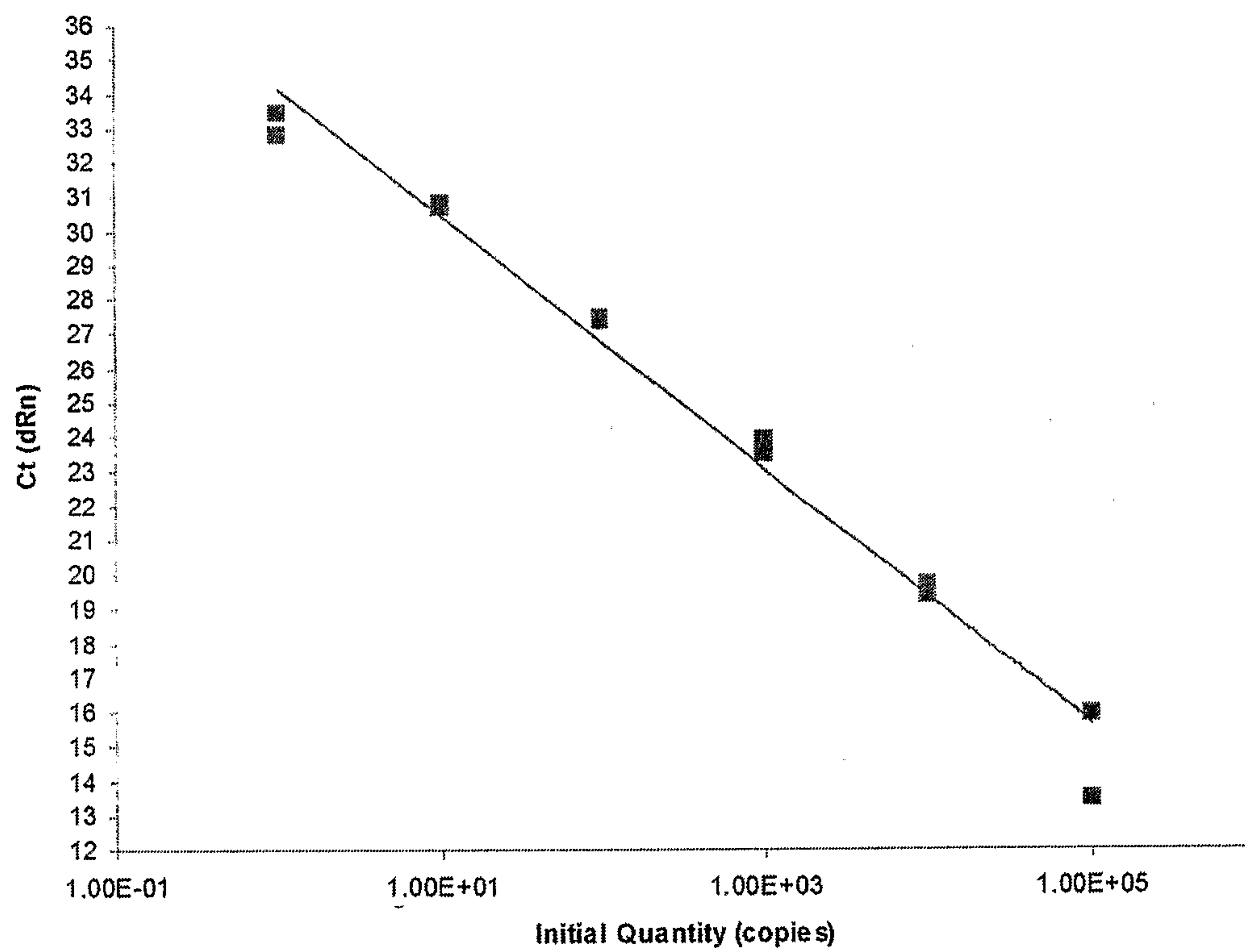


Figure 10

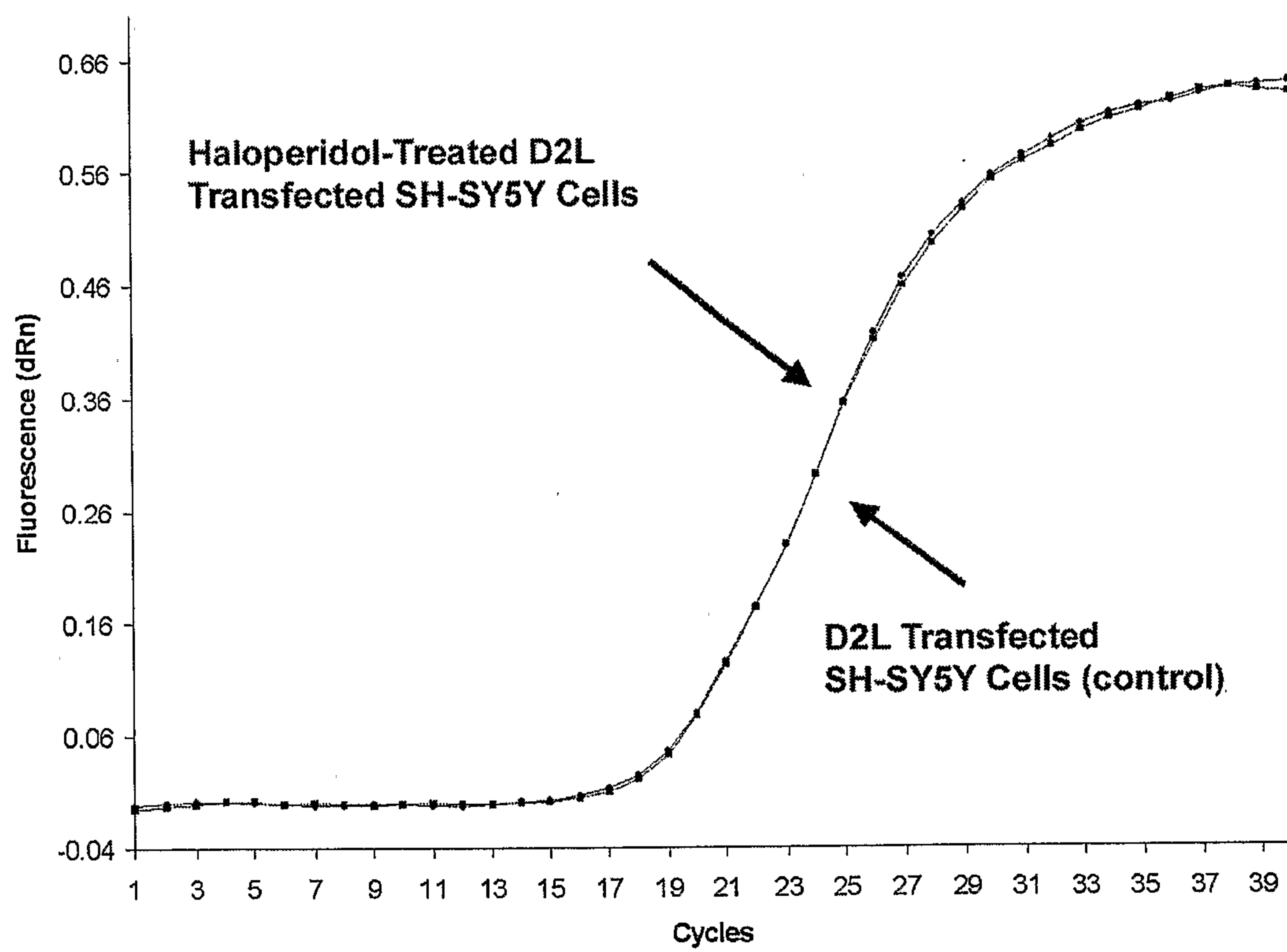


Figure 11

FIG. 12

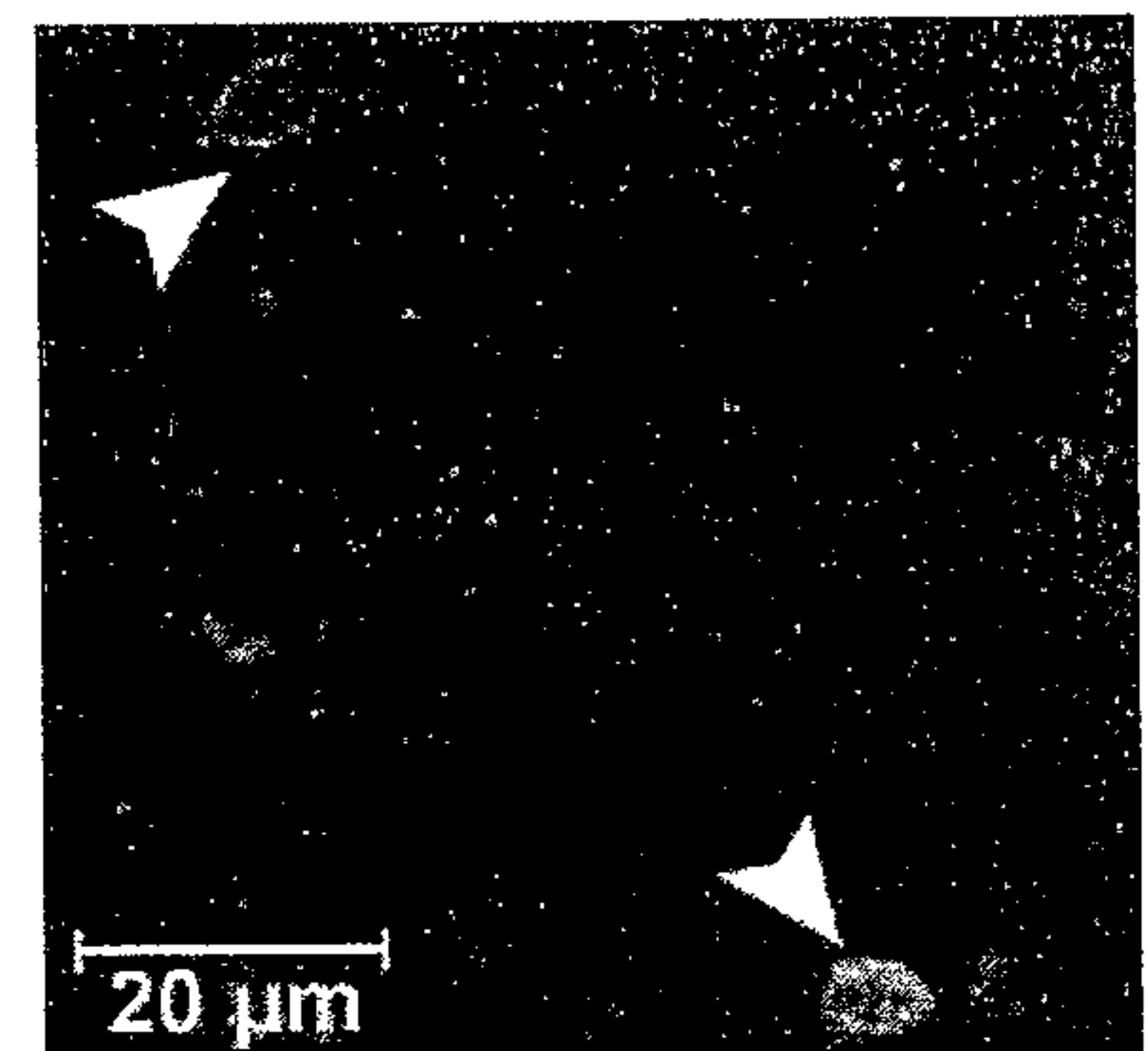
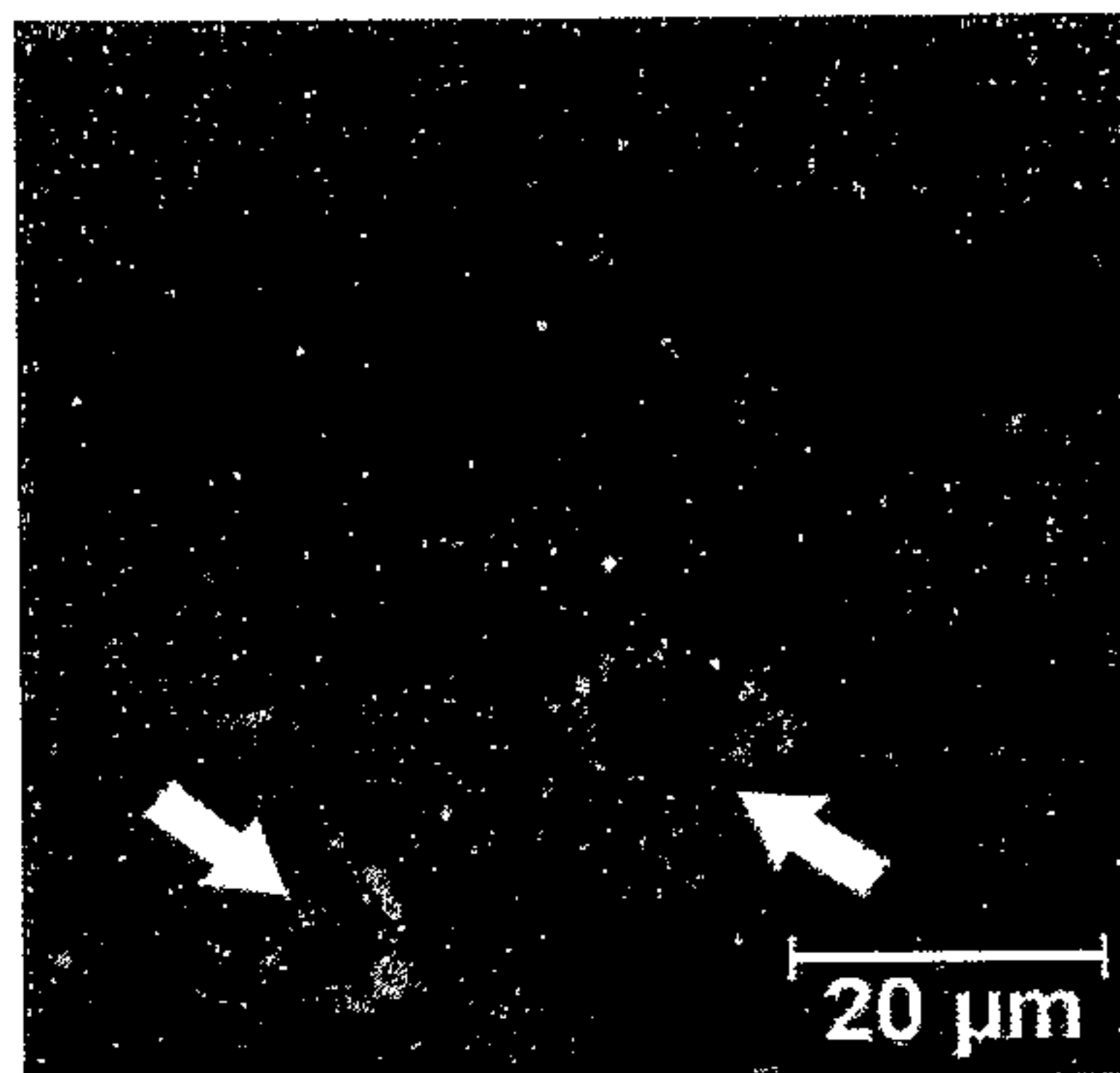
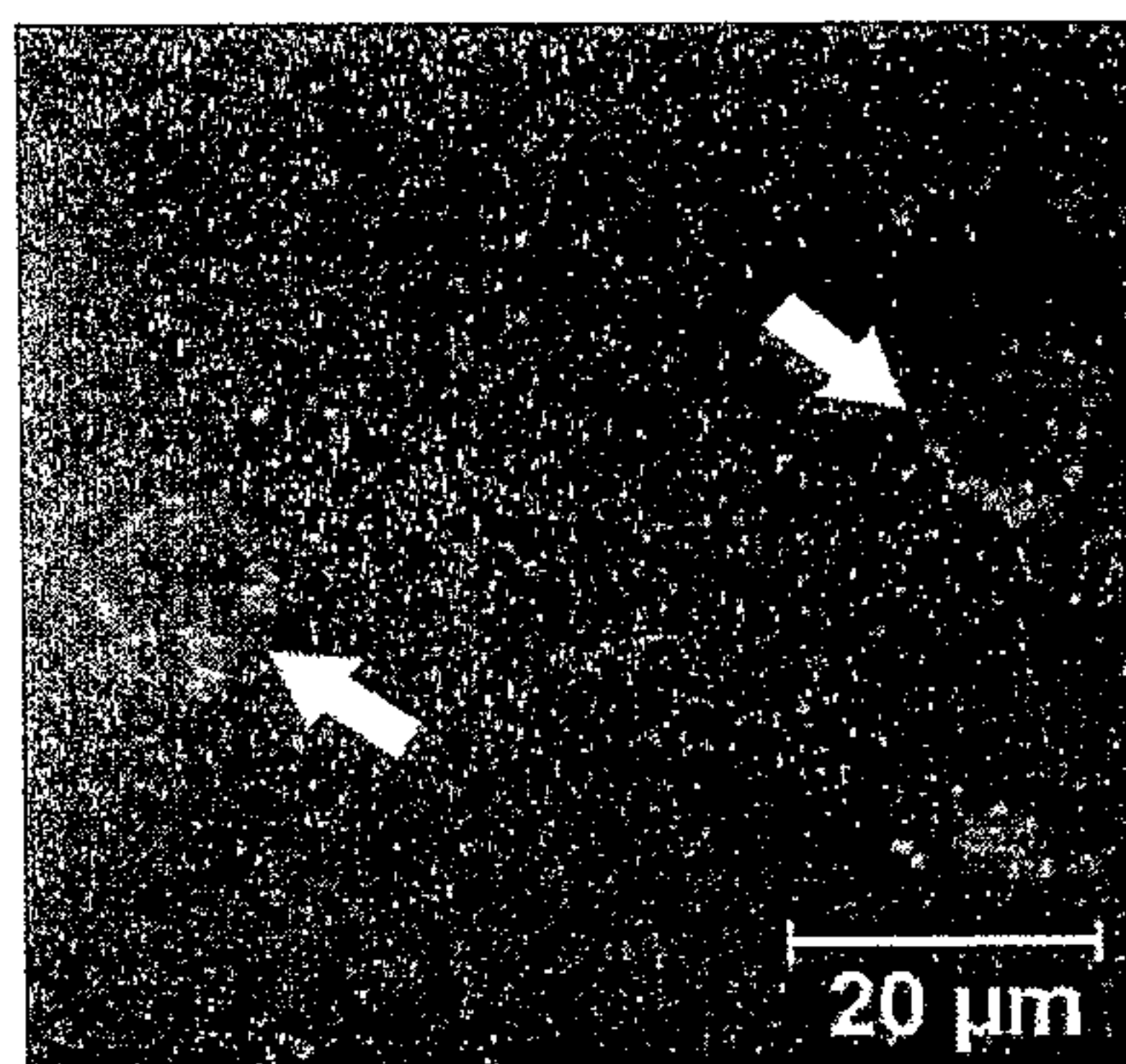


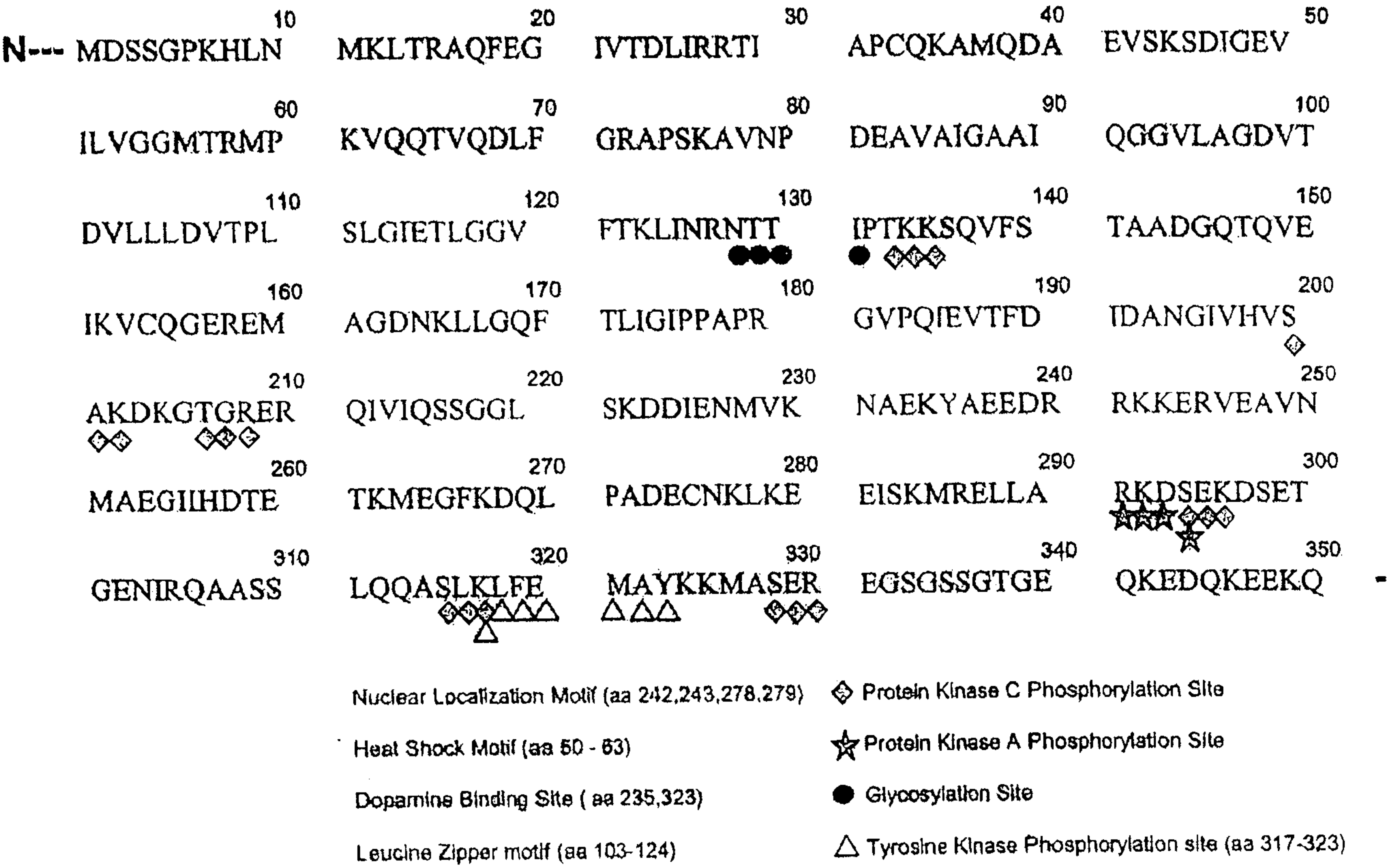
Figure 13**A** SEQ ID NO: 8

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 GVFTKLINRNTTIPTKKSQVFSTAADGQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPPAP
 RGVPQIEVTFDIDANGIVHVSADKDKGTGRERQIVIQSSGGLSKDDIENMVKNAEKYAEEDR
 RKKERVEAVNMAEGIIHDTETKMEGFKDQLPADECNKLKEEISKMRELLARKDSEKDSETG
 ENIRQAASSLQQASLKLFFEMAYKKMASEREGSGSSGTGEQKEDQKEEKQ-350

B SEQ ID NO: 7

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 atttgaagg
 61 attgtcactg atctaatacag aaggactatc gctccatgcc aaaaagctat
 gcaagatgca
 121 gaagtcagca agagtgcacat aggagaagtg attcttgtgg gtggcatgac
 taggatgccc
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C



D

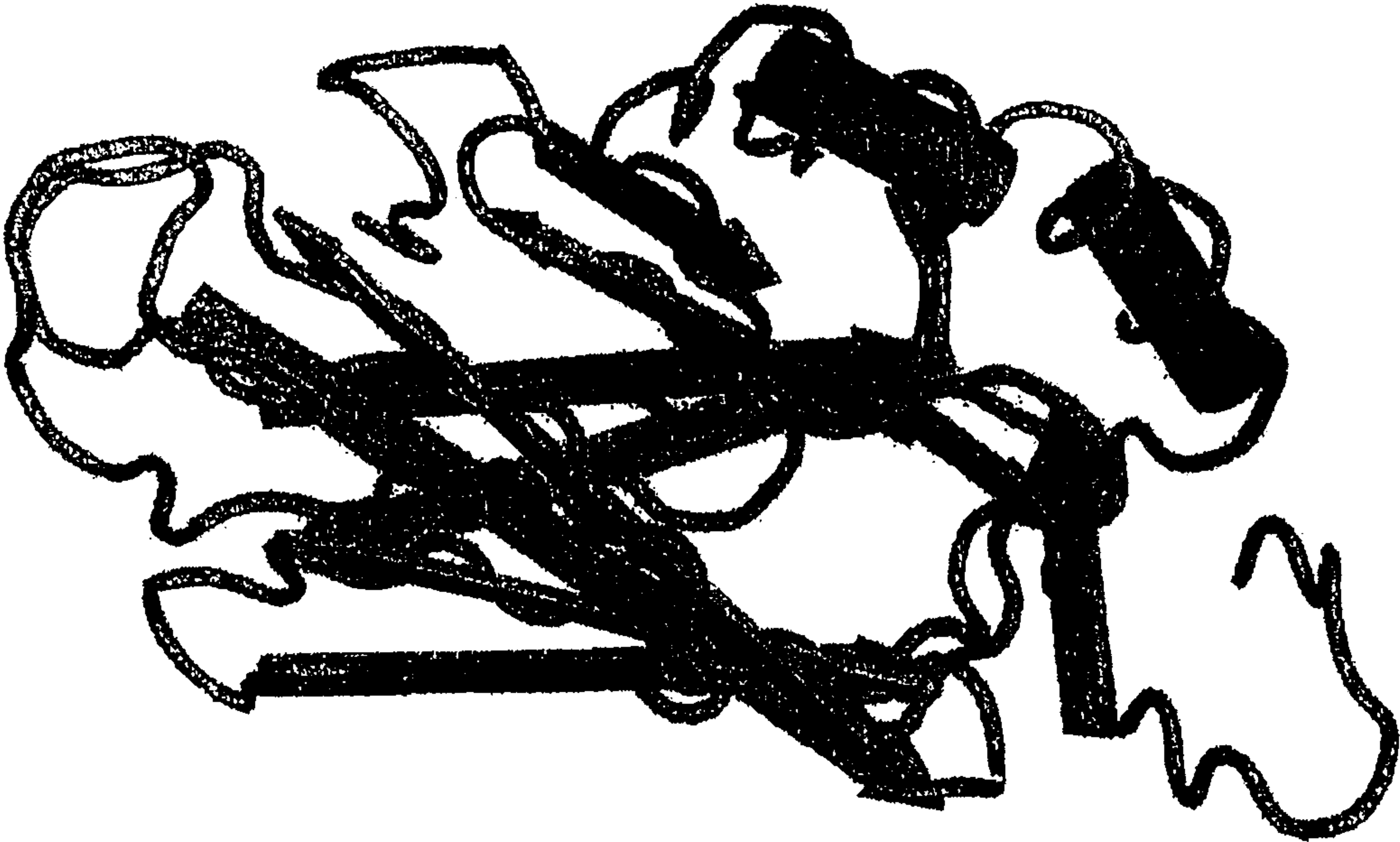


Figure 13 (cont'd)

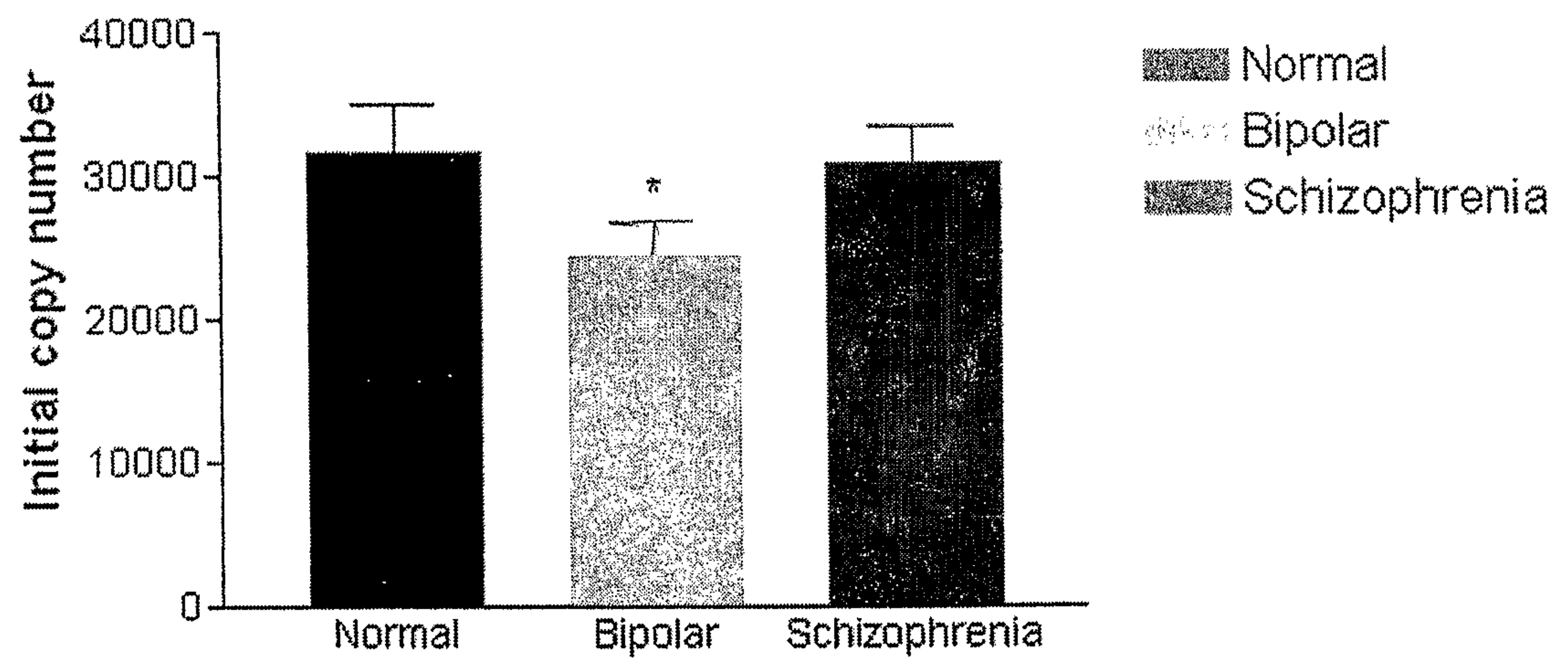


Figure 14.

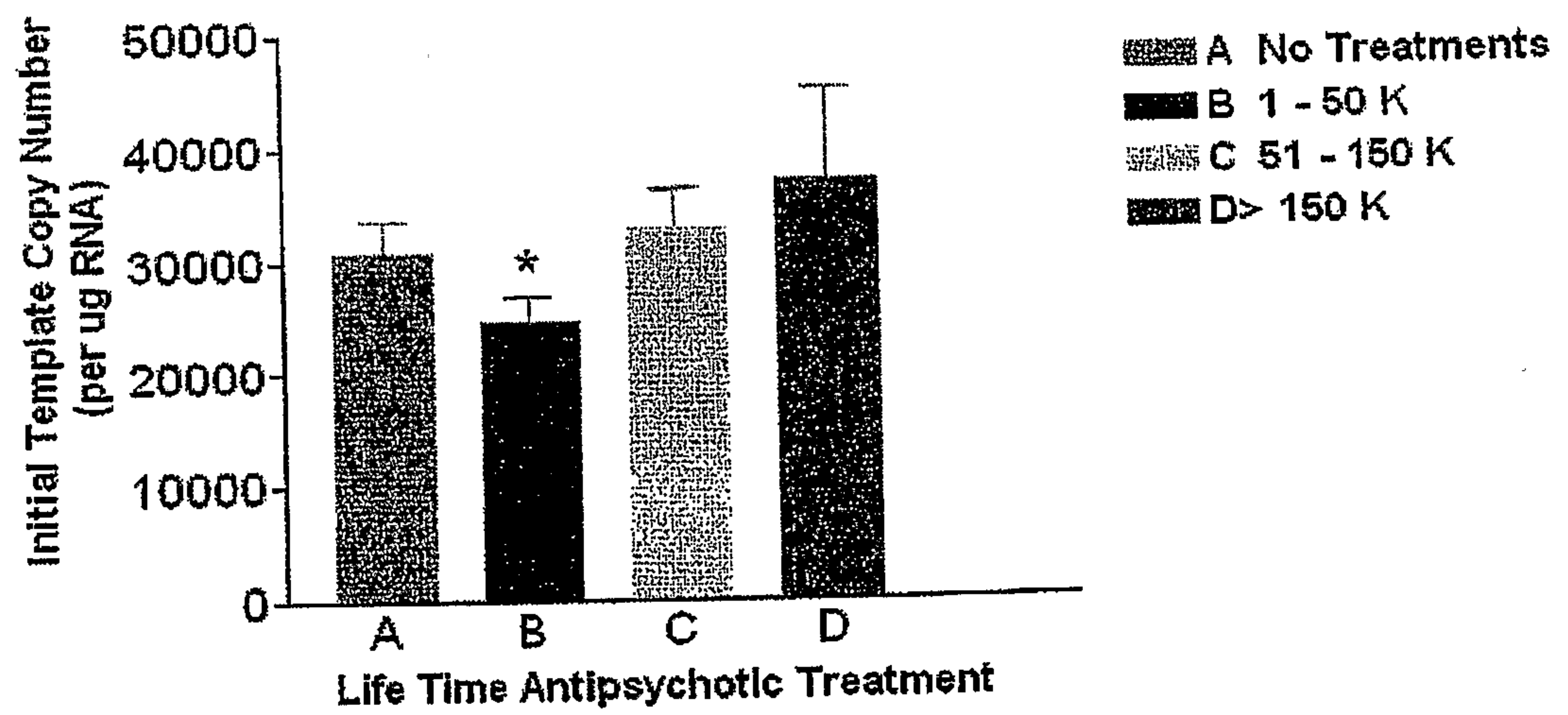


FIG 15

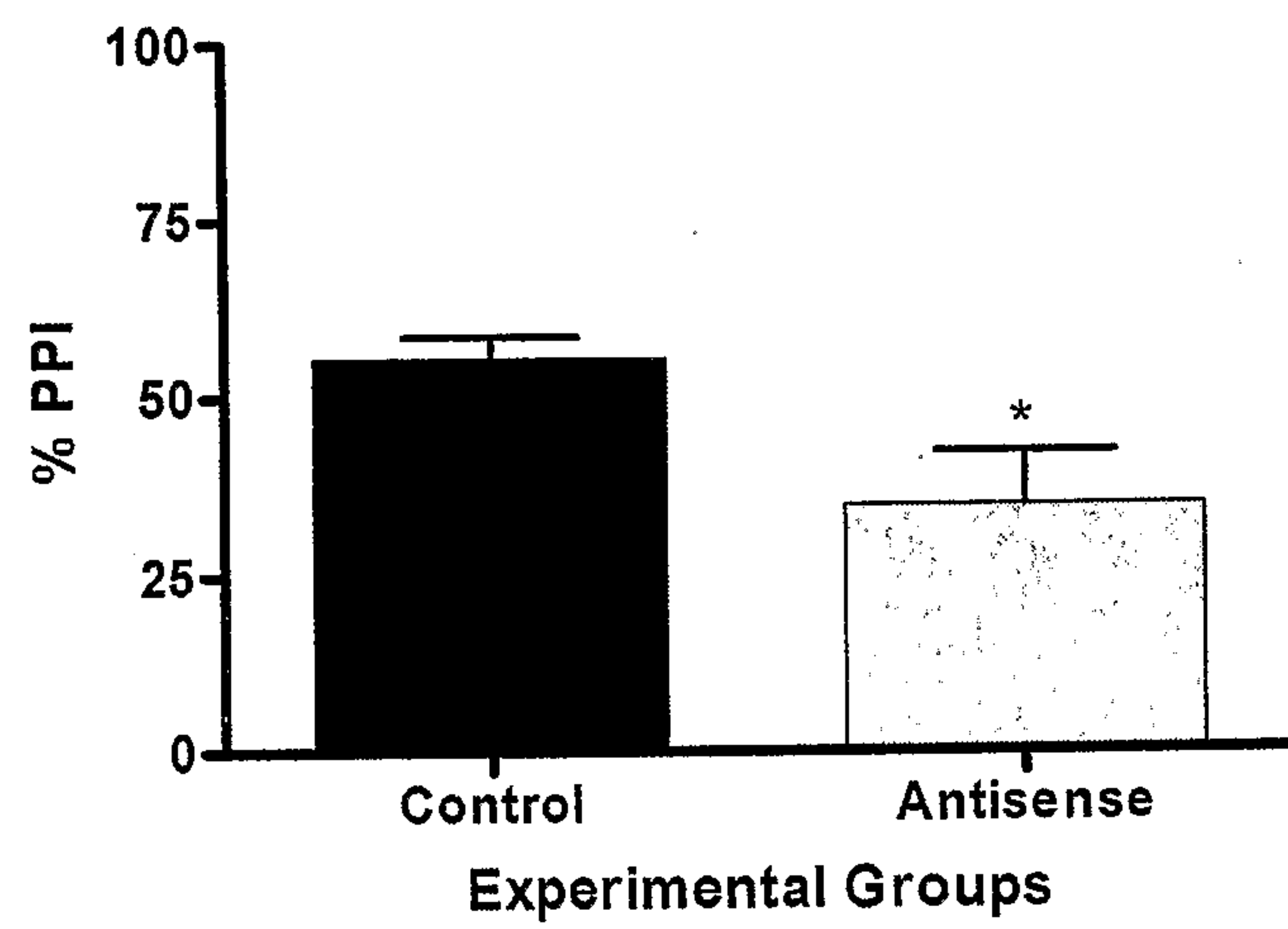


Figure 16

FIG. 17

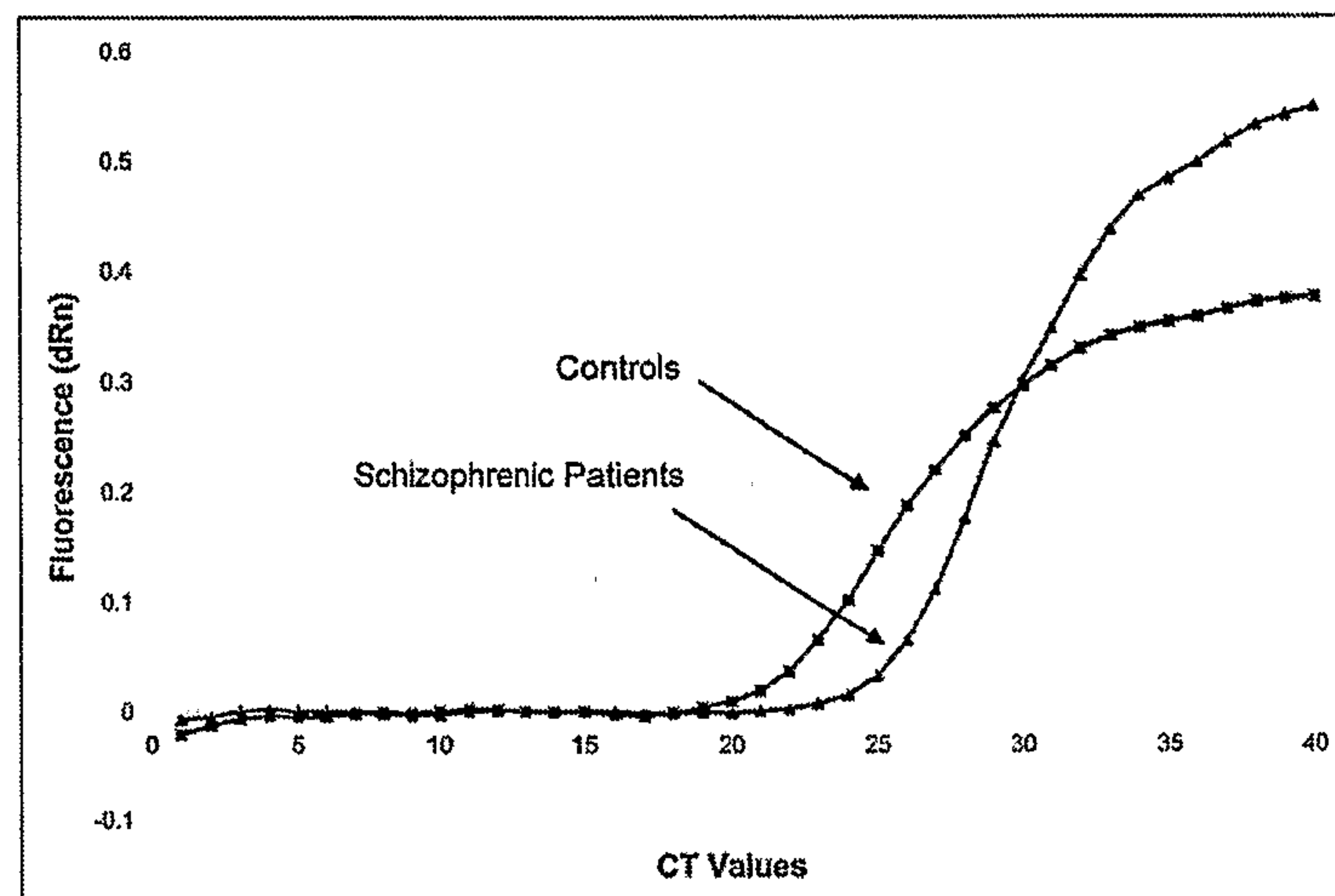


FIG. 18

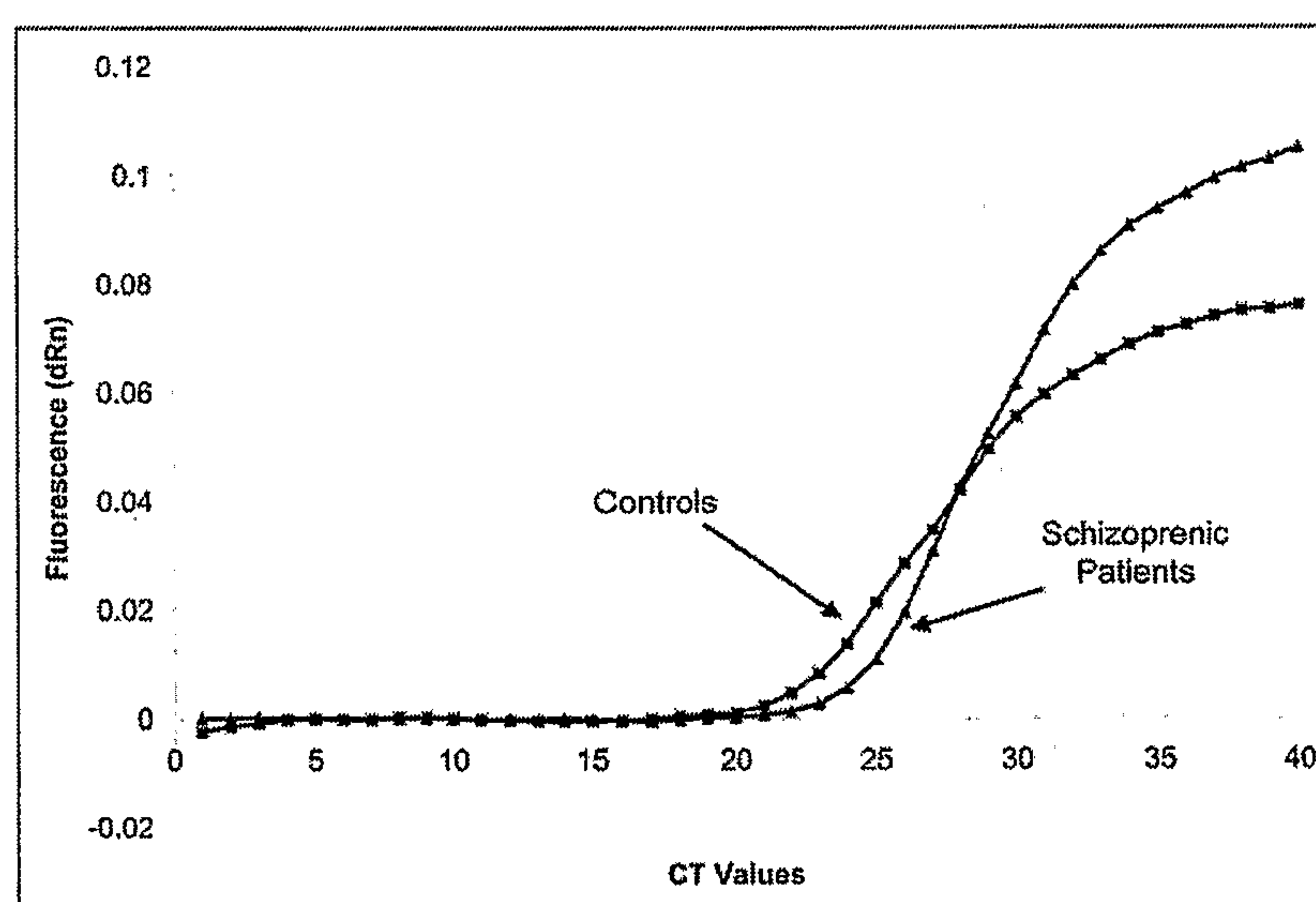
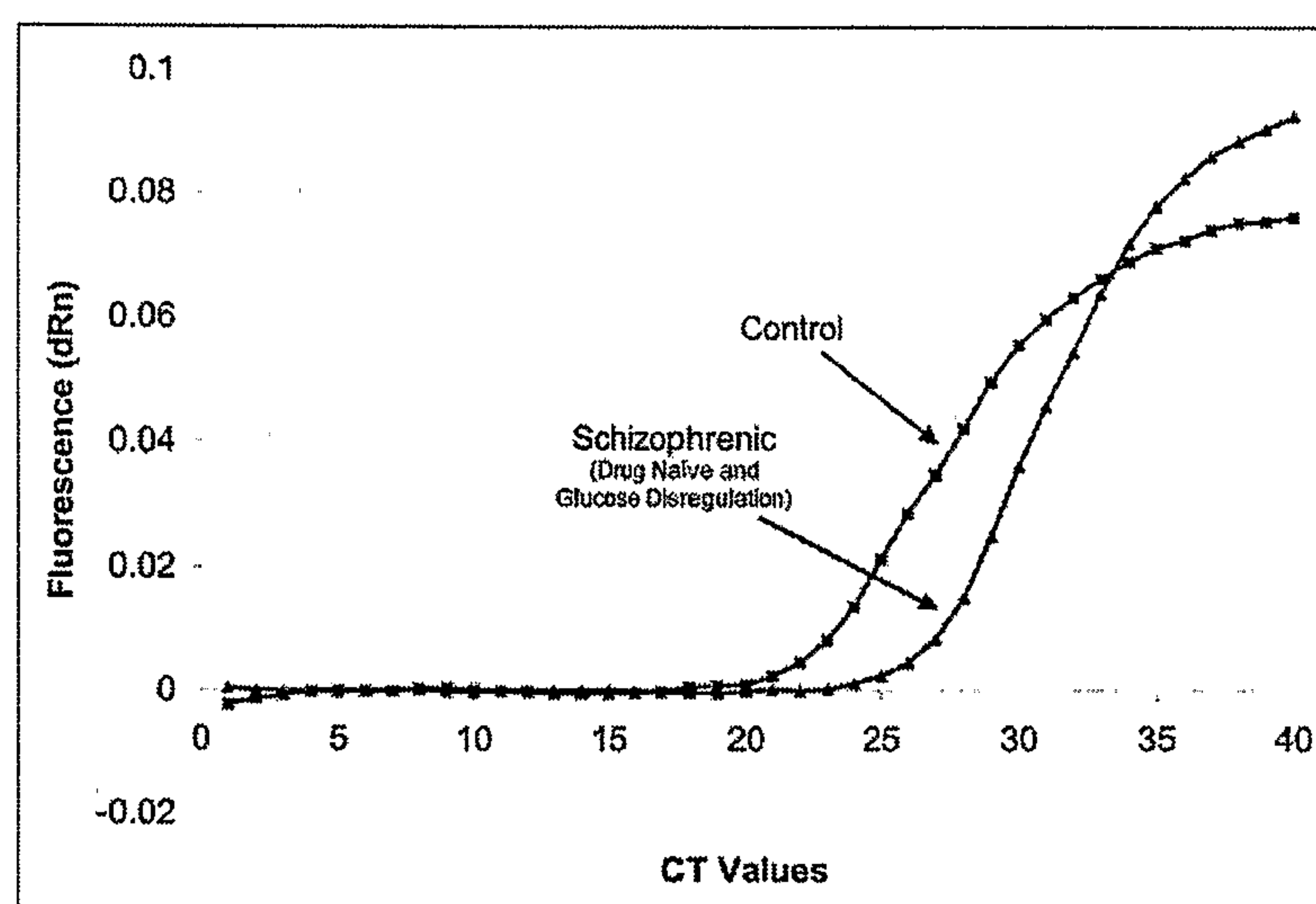


FIG. 19



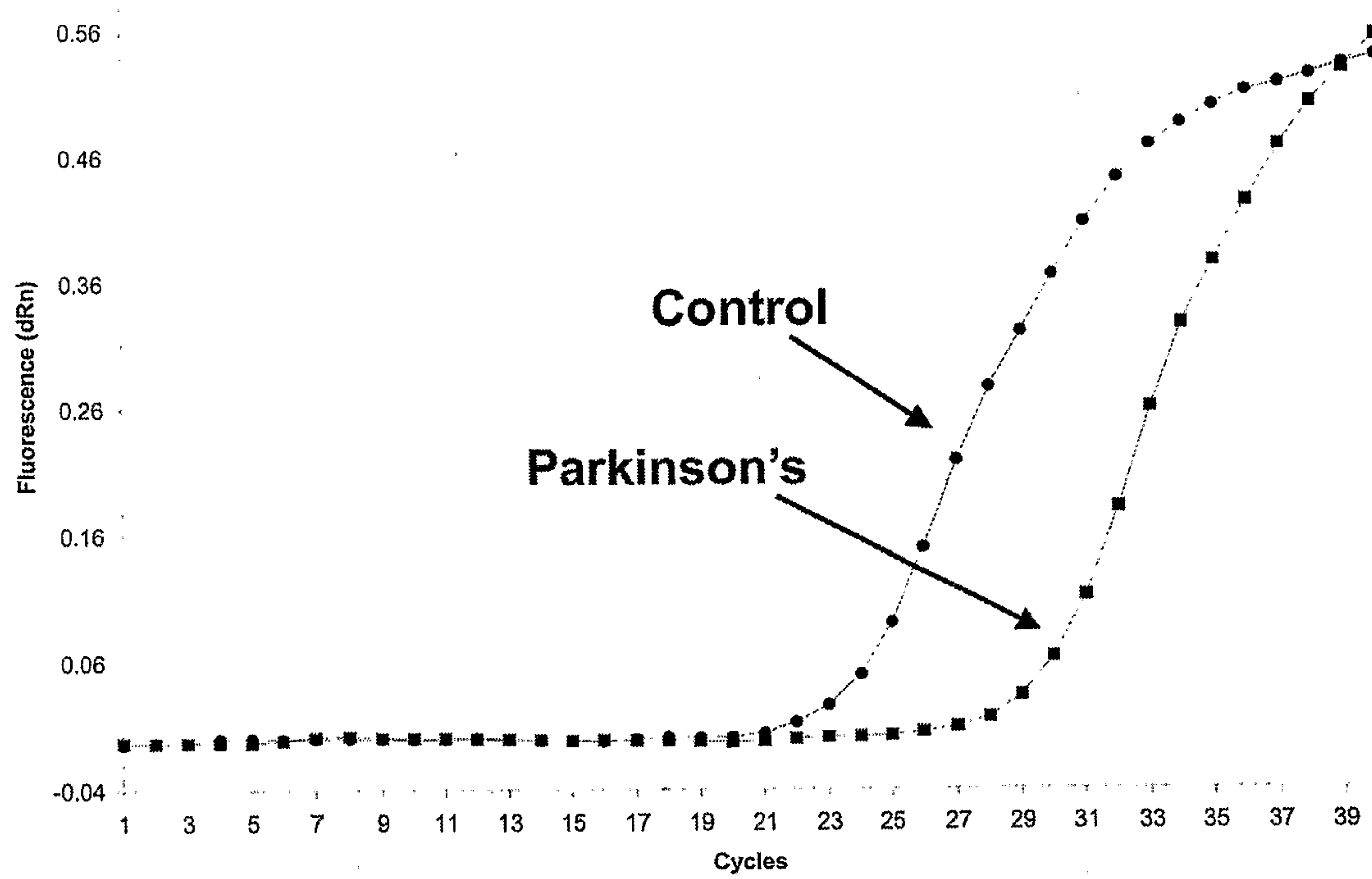


Figure 20

Fluorescence (dRn)

0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1

0 5 10 15 20 25 30 35 40

CT Values

Controls

Schizophrenic Patients

