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(71) Applicant *(for all designated States except US)*: **BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITYAND AGRICULTURAL AND MECHANICAL COLLEGE** [US/US]; Louisiana State University, 111 System Building, Baton Rouge, LA 70808 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KOZAK, Leslie, P.** [US/US]; 1072 Kimbro Drive, Baton Rouge, LA 70808 (US). **RIM, Jong, S.** [KR/US]; 18605 Perkins Oak Road, Prairieville, 70769-3753 (US).

(74) Agent: **DAVIS, Bonnie, J.**; Taylor, Porter, Brooks & Phillips, P.O. Box 2471, Baton Rouge, LA 70821-2471 (US).

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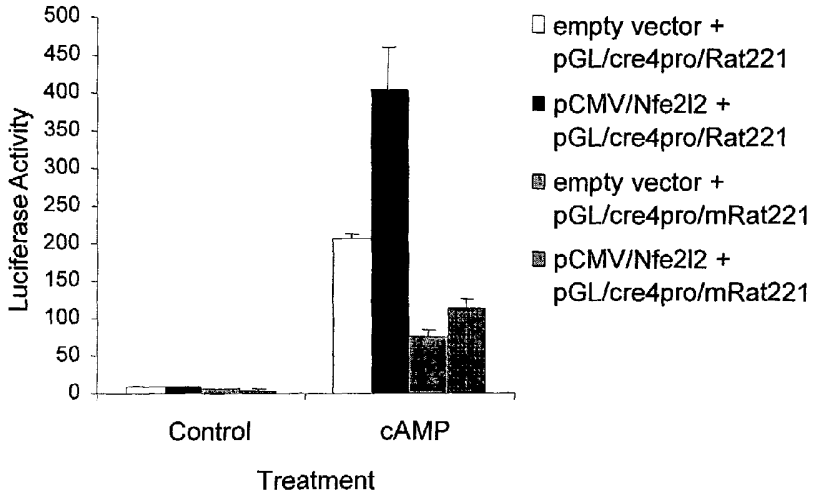
(54) Title: INDUCTION OF BROWN ADIPOCYTES BY TRANSCRIPTION FACTOR NFE2L2



(57) Abstract: New methods have been developed to treat obesity or other human disorders by increasing the degree of brown adipose thermogenesis, by both increasing the growth and differentiation of brown adipose tissue and increasing its activity. A new use for the transcription factor NFE2l2 was developed to increase BAT thermogenesis by increasing the expression of *Ucp1*. For the first time, NFE2l2 was found to interact with the NF-E2 motif and to stimulate the expression of *Ucp1*. By modulating the expression of the *Nfe2l2* gene or the concentration of NFE2l2 protein, changes in brown adipose tissue thermogenesis can be achieved to treat various weight disorders. Additionally, the direct involvement of CREB binding to CRE2 to regulate *Ucp1* expression was demonstrated. Furthermore, transient transfection assays of luciferase reporter constructs and site-directed mutagenesis indicated that CRE2 was involved in transcriptional regulation of the *Ucp*1 through interaction with a phosphorylated CREB. NFE2l2 can be combined with other known transcription factors, e.g., CREB, PGC1, RXR, RAR, and PPARγ, to increase BAT thermogenesis by increasing the expression of *Ucp1*.

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INDUCTION OF BROWN ADIPOCYTES BY TRANSCRIPTION FACTOR NFE2l2

[0001] The benefit of the 24 September 2001 filing date of United States provisional patent application serial number 60/324,400 is claimed under 35 U.S.C. § 119(e) in the United States, and is claimed under applicable treaties and conventions in all countries.

[0002] The development of this invention was partially funded by the Government under grant nos. R01-DK58152-01 from the National Institute of Diabetes and Digestive and Kidney Diseases and RO1-HD08431 from the National Institute of Child Health and Human Development, both divisions of the National Institutes of Health. The Government has certain rights in this invention.

TECHNICAL FIELD

[0003] This invention pertains to a new method to prevent or alleviate mammalian obesity by increasing the effective concentration of the transcription factor, NFE2l2, which will increase the number and activity of brown adipocytes, whose role is to burn fat to produce primarily heat.

BACKGROUND ART

Brown Adipose Tissue

[0004] Mammals possess two forms of adipose tissue -- white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue stores fat to be released upon demand for the nutritional and metabolic needs of the mammal. In contrast, the cells of BAT (brown adipocytes)

function to burn fat to release heat, and thus to help the mammal maintain or attain its body temperature. BAT is characterized by the presence of numerous mitochondria. In muscles and other tissues with numerous mitochondria, the oxidation of fuel (e.g., sugars or fats) generates a proton gradient across the mitochondrial membrane; the energy of this gradient is coupled to the synthesis of adenosine triphosphate (ATP), a universal energy source for the body. This production of ATP occurs with relatively little loss of energy as heat. In BAT mitochondria, the oxidation of fat results in a proton gradient as in muscle and other tissues. However, in contrast to other tissues, the BAT mitochondria have a unique ability to generate a large amount of heat by uncoupling the normal mitochondrial process of oxidation of fat to produce ATP. This uncoupling is due to the presence of a unique protein found only in the membranes of BAT mitochondria, called "mitocondrial uncoupling protein 1" or "UCP1." UCP1 uncouples the usual process of catabolism of fuel to form ATP by serving as a proton channel to decrease the proton concentration gradient across the mitochondrial membrane which normally (in mitochondria of non-BAT tissue) powers the production of ATP. See U.S. Patent No. 5,453,270.

[0005] At various points in the life of a mammal, the growth and differentiation of BAT are important to the mammal's ability to maintain energy balance, body temperature, and prevent obesity; and the expression of UCP1 is essential for this function of BAT. Moreover, an increased level of transcription of the *Ucp1* gene is a critical event leading to elevated BAT activity, namely thermogenesis. Several rodent models of obesity (including leptin and lepin-receptor mutants) have diminished or defective BAT function. See B. Lowell *et al.*, "Development of Obesity in Transgenic Mice after Genetic Ablation of Brown Adipose Tissue," Nature, vol. 366, pp. 740-742 (1993). Additionally, in rodents *Ucp1* expression is increased in response to cold stress and to administration of norepinephrine and other β-adrenergic receptor agonists. Four hours of cold stress increased *Ucp1* mRNA by seven fold in mice. See S. Rehnmark *et al.*, "Alpha- and Beta-adrenergic Induction of the Expression of the Uncoupling Protein Thermogenin in Brown Adipocytes Differentiated in Culture," J. Biol. Chem., vol. 265, p. 16464-16471 (1990); and A. Jacobsson *et al.*, "Mitochondrial Uncoupling Protein from Mouse Brown Fat. Molecular Cloning, Genetic Mapping, and mRNA Expression," J. Biol. Chem., vol. 260, pp. 16250-16254 (1985).

Mitochondrial Uncoupling Protein (UCP1)

[0006] Increased thermogenesis of BAT can be induced by cold exposure and/or a high fat diet in brown adipose tissue (BAT) through the induction of the mitochondrial uncoupling protein (UCP1). See J.A. Levine *et al.*, "Role of Nonexercise Activity Thermogenesis in Resistance to Fat Gain in Humans," Science vol. 283, pp. 212-214 (1999). Although four homologues of UCP have been identified, definitive proof establishing that an uncoupling protein is essential for thermogenesis has been shown only for UCP1. See S. Enerback *et al.*, "Mice Lacking Mitochondrial Uncoupling Protein Are Cold-sensitive but Not Obese," Nature, vol. 387, pp. 90-94 (1997). UCP1 is located in the inner membrane of mitochondria, where it dissipates the mitochondrial membrane potential resulting in the generation of heat instead of ATP. Overexpression of *Ucp1* has been achieved pharmacologically by administration of thermogenic β_3-adrenergic receptor agonists, or genetically by using tissue-specific gene promoters to drive expression of *Ucp1* in transgenic mice, or by the increase in *Ucp1* transcription due to increase protein kinase A (PKA) activity in PKA RIIb knockout mice. See J. Himms-Hagen *et al.*, "Effect of Cl-316,243, a Thermogenic Beta 3-agonist, on Energy Balance and Brown and White Adipose Tissues in Rats," Am. J. Physiol., vol. 266, pp. R1371-1382 (1994); and D.E. Cummings *et al.*, "Genetically Lean Mice Result from Targeted Disruption of the RII Beta Subunit of Protein Kinase A," Nature, vol. 382, pp. 622-626 (1996). Each of these experimental mammals with increased UCP1 showed an increase in brown fat activity and energy expenditure, and a reduction in adiposity. Accordingly, determining mechanisms to increase UCP1 has practical applications to the problem of obesity.

[0007] There are several important aspects of *Ucp1* expression. One is the molecular basis of its unique expression in BAT; and a second is the tightly controlled regulation by the hypothalamus via the sympathetic nervous system in response to cold and possibly diet. See A. Jacobsson *et al.*, 1986. A third aspect is the cellular mechanisms that can increase the number of mitochondria in the adiopocyte; and a fourth is the cellular mechanisms that can increase the formation of new brown adipocytes. These mechanisms may not be mutually exclusive. In the rodent, obesity has been reduced by a high fat diet or by a mutant gene that increases the transcription of *Ucp1* in pre-existing brown adipocytes. This strategy is unlikely to succeed in humans because adult humans have lost the large number of brown adipocytes that were present at birth. It is unknown whether the brown adipocytes of newborn humans undergo apoptotic

death or are converted to white adipocytes. Any effective increase in brown fat-based thermogenesis in adult humans must first increase or reactivate the brown adiopocytes.

[0008] Many investigators of human obesity consider the number of brown adipocytes in the adult human to be too small to be effective as a mechanism for reducing obesity. See M.E. Lean *et al.*, "Brown adipose tissue uncoupling protein content in human infants, children and adults," Clin. Sci., vol. 71, pp. 291-297 (1986). However, little is known about the capacity in humans for brown adipocyte expression, because in the absence of an appropriate stimulus a brown adipocyte will assume the morphology of a white adipocyte. Nonetheless, histological examination of post-mortem human fat depots always reveals the presence of small numbers of brown adipocytes. P. Huttunen *et al.*, "The occurrence of brown adipose tissue in outdoor workers," Eur. J. Appl. Physiol., vol. 46, pp. 339-345 (1981). In addition, chronic exposure to high levels of catecholamines, e.g., secreted by a phaeochromocytoma tumor, in humans leads to a large increase in brown fat depots. M.E. Lean *et al.*, "Brown adipose tissue in patients with phaeochromocytoma," Int. J. Obes., vol. 10, pp. 219-227 (1986). Human adipocytes in culture have also been stimulated to produce UCP1 by treatment with thiazolidinediones, indicating that brown adipocytes are present in adult humans and that the number of brown adipocytes can be increased by β-adrenergic stimulation. See J.E. Digby *et al.*, "Thiazolidinedione exposure increases the expression of uncoupling protein 1 in cultured human preadipocytes," Diabetes, vol. 47, pp. 138-141 (1998). Although brown adipoctyes were once though to be restricted to defined brown fat depots, recently the emergence of brown adipocytes in white fat depots was found in mice, rats, and dogs. The capacity to induce brown fat in white fat depots was found to be under genetic control and displayed a wide genetic variability, which also affected the ability of the mice to respond to drugs that reduce adiposity. See C. Guerra *et al.*, "Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity," J. Clin. Invest., vol. 102, pp. 412-420 (1998). In mice, at least four genes were found that control this genetic variability, three of which conferred high levels of UCP1. The three gene locations were on Chromosomes 2, 3 and 8. See R.A. Koza *et al.*, "Synergistic Gene Interactions Control the Induction of the Mitochondrial Uncoupling Protein (*Ucp1*) Gene in White Fat Tissue," J. Biol. Chem., vol. 275, pp. 34486-34492 (2000).

[0009] The considerable body of information that has accumulated on the molecular basis of *Ucp1* expression in BAT indicates that in mice there is a 200 bp enhancer, located approximately 2.5 kb upstream of the transcription start site for mouse *Ucp1*. This enhancer is known to contain *cis*-acting elements that play a critical role in the regulation of mouse *Ucp1* expression. These *cis*-acting elements include peroxisomal proliferator activator receptor binding motif (PPRE), thyroid hormone regulatory element /retinoic acid regulatory element (TRE/RARE), and cAMP responsive elements (CRE). See U.C. Kozak *et al.*, "An Upstream Enhancer Regulating Brown-fat-specific Expression of the Mitochondrial Uncoupling Protein Gene," Mol. Cell Biol., vol. 14, pp. 59-67 (1994); I.B. Sears *et al.*, "Differentiation-dependent Expression of the Brown Adipocyte Uncoupling Protein Gene: Regulation by Peroxisome Proliferator-activated Receptor Gamma," Mol. Cell Biol., vol. 16, pp. 3410-3419 (1996); A.M. Cassard-Doulcier *et al.*, "In Vitro Interactions Between Nuclear Proteins and Uncoupling Protein Gene Promoter Reveal Several Putative Transactivating Factors Including Ets1, Retinoid X Receptor, Thyroid Hormone Receptor, and a CACCC Box-binding Protein," J. Biol. Chem., vol. 269, pp. 24335-24342 (1994); R. Rabelo *et al.*, "Delineation of Thyroid Hormone-responsive Sequences Within a Critical Enhancer in the Rat Uncoupling Protein Gene," Endocrinology, vol. 136, pp. 1003-1013 (1995); and U.S. Patent Nos. 6,166,192 and 6,033,656. (See also, Table 1 for a listing of abbreviations used in the Specification and the Claims). Recently, it has been shown that synergism between retinoids, isoproterenol and thiazolidinedione regulates human *Ucp1* transcription in an enhancer region located 3.5 kb upstream of the gene. See M. del Mar Gonzalez-Barroso *et al.*, "Transcriptional Activation of the Human Ucp1 Gene in a Rodent Cell Line. Synergism of Retinoids, Isoproterenol, and Thiazolidinedione Is Mediated by a Multipartite Response Element," J. Biol. Chem., vol. 275, pp. 31722-31732 (2000).

TABLE I: ABBREVIATIONS		
Tissues		
	BAT	brown adipose tissue
	WAT	white adipose tissue
DNA enhancer regions		
	CRE	c-AMP responsive element
	NF-E2 binding motif	DNA binding site for the transcription factors NF-E2, NFE2l1 (NRF1), NFE2l2 (NRF2)
	PPRE	peroxisome proliferator activator receptor responsive element
Proteins		
	ATF-1	activating transcription factor-1 (binds CRE)
	CBP	CREB-binding protein
	CREB	CRE-binding protein
	NF-E2	nuclear factor erythroid 2 (transcription factor)
	NFE2l2	transcription factor that binds NF-E2 binding motif (also known as "NRF2")
	PGC1	$PPAR_{\lambda}$ coactivator 1
	PKA	protein kinase A
	PPARγ	peroxisome proliferator activated receptor
	RAR	retinoic acid receptor
	RXR	retinoic acid X receptor
	UCP1	mitochondrial uncoupling protein 1

[0010] The control of expression of *Ucp1* has been studied with transient expression assays of reporter constructs in brown adiopocytes in tissue culture, suggesting the interaction of the transcription factors PPARγ, RXR, and PGC1 via the PPRE binding site. See U.S. Patent No. 6,166,192; see also Table 1 for definitions of abbreviations. Additional regulatory elements and transcription factors are likely to be involved. There is evidence that induction is initiated by norepinephrine action on G protein-coupled β_1 and β_3 adrenergic receptors. See U.C. Kozak *et al.*, "Adrenergic Regulation of the Mitochondrial Uncoupling Protein Gene in Brown Fat Tumor Cells," Mol. Endocrinol., vol. 6, pp. 763-772 (1992). It is also known that *Pgc1* mRNA levels are increased in BAT in response to cold exposure. See U.S. Patent No. 6,166,192; and P. Puigserver *et al.*, "A Cold-inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis," Cell, vol. 92, pp. 829-839 (1998). Thyroid hormones, retinoids, and thiazolidinediones (TZD) have been reported to increase transcription of the *Ucp1* in rodents, both *in vivo* and *in vitro*. See C. Guerra *et al.*, "Triiodothyronine Induces the Transcription of the Uncoupling Protein Gene and Stabilizes its mRNA in Fetal Rat Brown Adipocyte Primary Cultures," J. Biol. Chem., vol. 271, pp. 2076-2081 (1996); P. Puigserver *et al.*, "In Vitro and in Vivo Induction of Brown Adipocyte Uncoupling Protein (Thermogenin) by Retinoic Acid," Biochem. J., vol. 317, pp. 827-833 (1996) ; and J.E. Digby *et al.*, (1998).

[0011] In mice, transient transfection analyses utilizing primary cell cultures from a SV40 T-antigen- induced brown adipocyte tumor have shown that mutations in two of four half-site CREs in a CAT-reporter construct carrying 3 kb of the 5'-flanking region almost completely abolished expression of *Ucp1*. See U.C. Kozak *et al.*, 1994. These two sites, CRE2 and CRE4, were located in the enhancer region and just 5' of the TATA box region, respectively. Mutations to the other two sites, CRE1 and CRE3, only slightly reduced reporter activity.

[0012] Recently, the human *Ucp1* gene was cloned and evidence describing key elements controlling its transcriptional regulation obtained. See M. del Mar Gonzalez-Barroso *et al.*, 2000. A 350 bp, hormone-sensitive region of the human gene showed significant (60.1%) similarity with the mouse BAT-specific enhancer element. This region in the human gene was able to bind the nuclear factors RARs, RXRs, CREB/ATF, and PPARγ, indicating that transcriptional regulation of the *Ucp1* gene in rodents and humans share mechanisms in common. It would be desirable to identify additional factors which could activate *Ucp1* expression and which could also promote an increase in both the number of brown adipocytes and the amount of BAT thermogenesis.

NF-E2 Binding Proteins

[0013] NF-E2 was first discovered as a binding site in the beta-globin gene locus control region where the hematopoietic specific NF-E2 p45 subunit and the ubiquitously expressed small Maf protein, an important regulator of cell differentiation in various systems, form heterodimers. See P. Moi *et al.*, "Isolation of NF-E2-related Factor 2 (Nrf2), a NF-E2-like Basic Leucine Zipper Transcriptional Activator That Binds to the Tandem NF-E2/AP1 Repeat of the Beta-globin Locus Control Region," Proc. Natl. Acad. Sci. USA, vol. 91, pp. 9926-9930 (1994); V. Blank *et al.*, "The Maf Transcription Factors: Regulators of Differentiation," Trends Biochem. Sci., vol. 22, pp. 437-441 (1997); H. Motohashi *et al.*, "Mesodermal- vs. Neuronal-specific Expression of MafK Is Elicited by Different Promoters," Genes Cells, vol. 1, pp. 223-238 (1996); and K. Igarashi *et al.*, "Regulation of Transcription by Dimerization of Erythroid Factor NF-E2 p45 with Small Maf Proteins," Nature, vol. 367, pp. 568-572 (1994). Transcription factors of the NF-E2 family, which were originally identified as having erythrocyte-specific DNA binding activity, belong to the cap'n'collar (CNC)-type basic region leucine zipper (bZIP) superfamily, which represents a class of transcription factors that bind DNA using a simple, dimeric, alpha-helical recognition motif. Cap'n'collar (CNC) is a homeotic gene involved in the development of the head and neck structure in *Drosophila.* See J. Mohler *et al.*, "Segmentally Restricted, Cephalic Expression of a Leucine Zipper Gene During Drosophila Embryogenesis," Mech. Dev., vol. 34, pp. 3-9 (1991); and J. Mohler *et al.*, "Control of Drosophila Head Segment Identity by the bZIP Homeotic Gene cnc, "Development, vol. 121, pp. 237-247 (1995); and P. Moi *et al.*,1994. The nuclear DNA binding protein NF-E2 regulates expression of globin genes in developing erythroid cells. Two additional members of CNC-bZIP family, NFE211 and NFE212 (also known as NRF1 and NRF2), have been cloned; they are expressed ubiquitously in tissues, but with variable expression among these different tissues. See K. Chan *et al.,* "NRF2, a Member of the NFE2 Family of Transcription Factors, Is Not Essential for Murine Erythropoiesis, Growth, and Development," Proc. Natl. Acad. Sci., vol. 93, pp. 13943-13948 (1996); R. Yu *et al.*, "Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism," J. Biol. Chem., vol. 275, pp. 39907-39913 (2000); and H.-C. Huang *et al.*, "Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2," Proc. Natl. Acad. Sci., vol. 97,

pp. 12475-12480 (2000). NFE2l2 is a member of the CNC (cap'n'collar)-basic region leucine zipper (bZIP) superfamily.

[0014] The transcription factors, NFE2l2 and NFE211 (or NRF2 and NRF1) are not to be confused with the nuclear respiratory factors, NRF-1 and NRF-2 which bind to DNA binding motifs (NRF-1 and NRF-2) in the regulatory regions of nuclear genes that encode proteins destined for the mitochondia. NRF-1 and NRF-2 are known to be different proteins. See J.V. Virbasius *et al.*, "Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: A potenital regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis," Proc. Natl. Acad. Sci., vol. 91, pp. 1309-1313 (1994); and Z. Wu *et al.*, "Mechanisms controlling mitochondrial biogenesis and respiration through the termogenic activator PGC-1," Cell, vol. 98, pp. 115-124 (1999).

[0015] Protein-protein interactions between NFE2l2 and other bZIP proteins including CREB, Fos, and Jun have been reported. See R. Venugopal *et al.*, "Nrf2 and Nrf1 in Association with June Proteins Regulate Antioxidant Response Element-mediated Expression and Coordinated Induction of Genes Encoding Detoxifying Enzymes," Oncogene, vol. 17, pp. 3145-3156 (1998); T. Nguyen *et al.*, "Transcriptional Regulation of the Antioxidant Response Element," J. Biol. Chem., vol. 275, pp. 15466-15473 (2000); and R. Venugopal *et al.*, "Nrf1 and Nrf2 Positively and c-Fos and Fra1 Negatively Regulate the Human Antioxidant Response Element-mediated Expression of NAD(P)H:quinone Oxidoreductase1 Gene," Proc. Natl. Acad. Sci. U S A, vol. 93, pp. 14960-14965 (1996). PPARγ can also directly interact with NFE2l2 via the NF-E2/AP1 binding site (TGCTGATTCAT) of the thromboxane synthesis gene in macrophages. See Y. Ikeda *et al.*, "Suppression of Rat Thromboxane Synthase Gene Transcription by Peroxisome Proliferator-activated Receptor Gamma in Macrophages via an Interaction with NRF2," J. Biol. Chem., vol. 275, pp. 33142-33150 (2000). Also, *in situ* hybridization with sections from 15.5-day-old embryos has shown that the *Nfe2l2* gene is expressed in brown fat. See K. Chan *et al.*, 1996.

[0016] NRf2 (NF-E2-related factor 2) was discovered to be involved in the cellular response to oxidative stress. NRf2 is normally retained in the cytoplasm, and then is liberated in response to oxidative stress to translocate into the nucleus. See H.-C. Huang *et al.*, 2000. NRF2 (or NFE2l2) has been shown to be phosphorylated by protein kinase C which triggers nuclear translocation of Nrf2 and increases the binding to the NF-E2 binding motif. Compounds known to activate protein kinase C are known to increase the concentration and binding of NRF2

in the nucleus, e.g., phorbol esters (e.g., phorbol 12-myristate 13-acetate), *tert*-butylhydroquinone, and β-naphthoflavone.

[0017] NRF2 has not been previously implicated as a transcription factor to affect *Ucp1* expression and increase BAT thermogenesis.

DISCLOSURE OF THE INVENTION

[0018] We have discovered a new method to treat obesity or other human disorders by increasing the degree of brown adipose thermogenesis, by both increasing the growth and differentiation of brown adipose tissue and increasing its activity. We have found a new use for the transcription factor NFE212 to increase BAT thermogenesis by increasing the expression of *Ucp1*. For the first time, NFE212 binding to the NF-E2 motif was found to stimulate the expression of *Ucp1*. By modulating the expression of the *Nfe2l2* gene or the concentration of NFE212 protein, changes in brown adipose tissue thermogenesis can be achieved to treat various weight disorders. Additionally, the direct involvement of CREB binding to CRE2 to regulate *Ucp1* expression was demonstrated. Furthermore, transient transfection assays of luciferase reporter constructs and site-directed mutagenesis indicated that CRE2 was involved in transcriptional regulation of the *Ucp1* through interaction with a phosphorylated CREB. NFE212 can be combined with other known transcription factors, e.g., CREB, PGC1, RXR, RAR, and PPARγ, to increase BAT thermogenesis by increasing the expression of *Ucp1*.

Brief Description of the Drawings

[0019] Fig. 1 illustrates the nucleotide sequence of the 221 bp (4828/5048) of BAT tissue specific region of the mouse *Ucp1* gene (SEQ ID No: 1), with the enhancer elements shown inside boxes and the NF-E2 binding site shown as underlined bold letters.

[0020] Fig. 2A illustrates the binding of half site CRE sequences to nuclear extracts from various tissues of A/J mice exposed to cold (4°C overnight) in an autoradiogram using ^{32}P end-labeled CRE2 from mouse *Ucp1* gene, with arrows indicating the bands representing CREB (dark arrow) and free probes (light arrow).

[0021] Fig. 2B illustrates the competitive binding activity of CRE2 with half-site CREs from mouse *Ucp1* gene from nuclear extracts from BAT of A/J mice which were exposed to cold (4°C overnight), and a palindromic CRE from a somatostatin gene (CRE) in an autoradiogram showing only the CREB bands, with the percent competition of ^{32}P end-labeled CRE2 to CREB

by a CRE sequences from mouse *Ucp1* and somatostatin genes calculated from the radioactivity of the slow migrating bands in the lane without (first lane) and with individual competitors (calculations shown at the bottom).

[0022] Fig. 3A illustrates a Western blot analysis showing the increase in CREB/ATF1 phosphorylation in HIB-1B cells treated with norepinephrine (NE) for 0, 5, 10, 20, 30 and 60 minutes.

[0023] Fig. 3B illustrates the increase in CRE2 binding to nuclear proteins in HIB-1B cells treated with norepinephrine for 0, 10, and 60 min; and compared with intensity of BAT of A/J mouse (cold, overnight) and for competition with cold CRE2. The arrows indicate CREB bands.

[0024] Fig. 4 illustrates transient expression analyses of luciferase reporter constructs to determine the function of individual CREs, (CRE1, CRE2, CRE3 and CRE4, represented by ovals) and of mutations of CRE2 and CRE3 (open ovals with cross marks).

[0025] Fig. 5A illustrates the binding of NF-E2 sequences to nuclear extracts from HIB-1B cells, in an autoradiogram using ^{32}P end-labeled NF-E2 (0.1 pmoles) from mouse *Ucp1* gene, and using nuclear extracts prepared from HIB-1B cells with (+) or without (-) norepinephine, with the bands representing NFE212 indicated with an arrow on the right.

[0026] Fig. 5B illustrates the binding of NF-E2 sequences to nuclear extracts from BAT of A/J mouse (cold, overnight) in an autoradiogram showing only the NFE212 bands using ^{32}P end-labeled NF-E2 (0.1 pmoles) with an antibody or a competitor.

[0027] Fig. 5C illustrates the increase in binding activity of NF-E2 sequence in BAT of A/J mouse under cold stress in an autoradiogram showing only the NFE212 bands (indicated by arrow) using ^{32}P end-labeled NF-E2 probe.

[0028] Fig. 6A illustrates the nucleotide sequences for the combined probe of NFCRE containing both NF-E2 and CRE2 binding sites (SEQ ID NO: 2), with the enhancer elements shown with underlined bold letters (NF-E2) or a box (CRE2), and with the nucleotide sequences for cold probes for NF-E2 (SEQ ID NO: 3) and CRE2 (SEQ ID NO: 4).

[0029] Fig. 6B illustrates the binding activity of NFCRE, NF-E2 and CRE2 with nuclear extracts from HIB-1B cells (exposed to norephinephrine for 60 min), using a ^{32}P end-labeled NFCRE (SEQ ID NO: 2) , NF-E2 (SEQ ID NO: 3), and CRE2 (SEQ ID NO: 4) probes.

[0030] Fig. 7A illustrates the100 bp (-3762/-3662) of nucleotides sequences from the human 350 bp enhancer (SEQ ID NO. 5) and corresponding mouse (SEQ ID NO. 16) and rat (SEQ ID NO. 17) enhancer sequences, with the half-sites for ATF/CREB, a putative NF-E2 binding site, and PPRE labeled and shown within boxes, and with the similar bases among the three species shown in bold letters.

[0031] Fig. 7B illustrates the binding of human NF-E2 sequences to nuclear extracts from BAT of A/J mouse kept in RT or cold (4°C) for 7 days, using a ^{32}P end-labeled NF-E2 probe with competitors as indicated. Only the NFE2l2 bands are shown (arrow).

[0032] Fig. 8A illustrates the effect of NFE2l2 overexpression on the transcriptional activity of the luciferase reporter constructs containing mouse *Ucp1* NF-E2 using the luciferase reporter construct pGL3/3.1kb, that was cotransfected with expression vector which is empty (pCMV/tag1, open box), contains cDNA for *Nfe2l2* correct (closed box), or contains cDNA for *Nfe2l2* with reversed orientation (hatched box) into HIB-1B cells, incubated in medium with either cAMP, norepinephrine (NE), or troglitazone (Trog) for an additional 16 hrs.

[0033] Fig. 8B illustrates the effect of NFE2l2 overexpression on the transcriptional activity of the luciferase reporter constructs containing mouse *Ucp1* NF-E2 using the luciferase reporter construct pGL3/CRE4pro/220, that was cotransfected with expression vector which is empty (pCMV/tag1, open box), contains cDNA for *Nfe2l2* correct (closed box), or contains cDNA for *Nfe2l2* with reversed orientation (hatched box) into HIB-1B cells, incubated in medium with either cAMP, norepinephrine (NE), or troglitazone (Trog) for an additional 16 hrs.

[0034] Fig. 9 illustrates the effect of NFE2l2 overexpression on the transcriptional activity of the luciferase reporter constructs containing rat *Ucp1* NF-E2 using the luciferase reporter constructs (pGL/cre4pro/Rat221 and pGL/cre4pro/mRat221), that were cotransfected with expression vector which is empty (pCMV/tag1, Stratagen) or contains cDNA for *Nfe2l2* into HIB-1B cells, incubated in medium with cAMP for an additional 16 hrs.

[0035] Fig. 10A illustrates the binding of NF-E2 sequences to nuclear extracts from BAT of B6 mice of different ages (from 19 days of gestation to 4 months after birth) in an autoradiogram showing only the NFE2l2 bands using ^{32}P end-labeled NF-E2 probe (0.1 *pmoles*) with an antibody or a competitor, after separation on 6% non-denaturing acrylamide gel.

[0036] Fig. 10B illustrates a Western blot analysis for NFE212 from nuclear extracts from BAT of B6 mice (ages from 19 days of gestation to 3 months after birth), using a specific antibody for NFE212 and after separation on a 10% SDS-polyacrylamine gel.

[0037] Fig. 10C illustrates the binding of NF-E2 sequences to nuclear extracts from BAT of B6 mice at room temperature or a 4 C for 1 day or 7 days in an autoradiogram showing only the NFE212 bands using ^{32}P end-labeled NF-E2 (0.1 *pmoles*) with an antibody or a competitor, after separation on 6% non-denaturing acrylamide gel.

MODES FOR CARRYING OUT THE INVENTION

Example 1

Materials and Methods

Cell culture and transfection:

[0038] HIB-1B cells, obtained from the Dana-Farber Cancer Insitute (Boston, Massachusetts), were maintained in Dulbecco's modified Eagle's medium (DMEM, 4,500 mg/L D-glucose, 584 mg/L L-glutamine, and 15 mg/L phenol red, GIBCO, Invitrogen Corp., Carlbad, California) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 0.1 mM non-essential amino acids. HIB-1B is an immortalized brown adipose cell line from hybridoma tissue that expresses *Ucp1* in response to retinoic acid and β-adrenergic agonists such as norepinephrine and isoproterenol. Medium was changed every two days. Reporter constructs were transiently transfected into HIB-1B cells using Lipofectamine Plus reagent (GIBCO) according to the manufacturer's protocol. The day before transfection, $2x10^5$ cells were seeded into a 24- well cluster dish (Corning Inc., Acton, Massachusetts). Briefly, 0.5 μg of reporter construct was transfected with 50 ng of pRL/SV40 (Promega Corp., Madison, Wisconsin), a plasmid containing *Renilla* luciferase gene under control of SV40 promoter, in a mixture of PLUS and Lipofectamine reagent. For the co-expression experiment, each 0.3 μg of reporter construct and expression vector was transfected with 50 ng of pRL/SV40. Transfected cells were cultured in medium in the presence or absence of 1 mM of norepinephrine (Sigma) or 0.5 mM 8-Br-cAMP (Calbiochem-Novabiochem Corp., San Diego, California) for 16 hrs. Cell extracts were prepared, and the activity of both *Photinus* and *Renilla* luciferase was determined using the dual-luciferase reporter assay system (Promega). For each construct, the activity of the *Photinus* luciferase was divided by the activity of the *Renilla* luciferase to correct for transfection efficiency. Under each treatment, the corrected activity was again divided by activity from the

empty vector, pGL3/basic (Promega), to estimate the degree of increase for each construct. The degree of increase for the over-expression experiment was obtained by dividing the corrected activity by the empty vector (pCMV/tag). Each experiment was performed in duplicate dishes.

Subcloning of 5'-flanking region of the mouse* Ucp1 *gene and reporter constructs:

[0039] The 3.1 kb of 5'-flanking region containing the four cAMP responsive elements (CRE) and the 220 bp BAT specific enhancer of the mouse *Ucp1* gene were obtained by PCR amplification. The 3.85 kb *Bgl*I fragment in pGEM, which was previously used in our characterization of *Ucp1* (note that nucleotide positions correspond to those in the *Ucp1* gene as described in GenBank U63418), was used as a template with forward and reverse primers, 5'-ggggtaCCGTGCACACTGCCAAATCATCTC (SEQ ID NO: 6) (4379/4355, a new *Kpn*I site is underlined) and 5'-gggagCTCCTGCAGAGCCACCTGGGCTAGG (SEQ ID NO: 7) (7514/7538, a new *Sac*I site is underlined), respectively, and subcloned into pGL3/basic using the *Kpn*I and *Sac*I restriction enzyme sites. See Kozak *et al.*, 1994. To obtain the *Ucp1* promoter with or without CRE4, forward primers 5'-ggggatccGAG**TGACG**CGCGGCTGGG (SEQ ID NO: 8) (nucleotide sequences for CRE4 are shown as bold and a new *BamH*I site is underlined, 7261/7278) or 5'-ggggatcCGGCTGGGAGGCTTGCGCA (SEQ ID NO: 9) (a new *BamH*I site is underlined, 7271//7289) and reverse primer 5'-gggaagcttGGGCTAGGTAGTGCCAG (SEQ ID NO: 10) (a new *Hind*III site is underlined, 7504/7520) were used for PCR amplification and subcloned into pGL3/basic using *Bgl*II and *Hind*III restriction enzyme sites. For the 220 bp of BAT specific enhancer region, the 3.85 kb *Bgl*I fragment was PCR amplified using primers 5'-ggggagCTCCTCTACAGCGTCACAGAGG (SEQ ID NO: 11) (*Sac*I site is underlined, 4841/4862) and 5'-gggctcgagAGTCTGAGGAAAGGGTTGA (SEQ ID NO: 12) (a new *Xho*I site is underlined, 5025/5045) and subcloned into luciferase reporter construct containing the *Ucp1* promoter. The structure of each fragment was verified by DNA sequencing. *Nfe2l2* cDNA was kindly provided by Dr. Paul Ney (St. Jude Children's Research Hospital, Memphis, Tennessee). A *Nfe2l2* expression vector was made by cloning a *Not*I fragment into pCMV/tag1 (Stratagene, La Jolla, California).

***Site-directed mutagenesis for CRE2 and CRE3*:**

[0040] CRE2 and CRE3 sequences in the 220 bp of BAT-specific-enhancer region were mutated using PCR and subcloned into the luciferase reporter plasmid, pGL3/basic. For CRE3 the forward primer was 5'-ggggagCTCCTCTACAG***Ctga***ACAGAGG (SEQ ID NO: 13) (CRE3 shown in *bold* with lowercase *italic* letters which represent mutations; a new *Sac*I site is

underlined, 4841/4862) and the reverse primer was 5'-gggctcgagAGTCTGAGGAAAGGGTTGA (SEQ ID NO: 12) (a new *Xho*I site is underlined, 5025/5045). To mutate CRE2, two pairs of primers were required in separate amplifications. The first pair was 5'-ggggagCTCCTCTACAGCGTCACAGAGG (SEQ ID NO: 11) (forward primer, a new *Sac*I site is *underlined*, 4841/4862) and 5'-AGTGGAAAGG**T*tca*G**ACTAGTTCAG (SEQ ID NO: 14) (reverse primer, CRE2 is shown in *bold* with lowercase *italic* letters representing mutations, 4883/4907). The second pair was 5'-CTGAACTAGT**C*tga*A**CCTTTCCACT (SEQ ID NO: 15) (forward primer, CRE2 is shown in *bold* with lowercase *italic* letters representing mutations, 4883/4907) and 5'-gggctcgagAGTCTGAGGAAAGGGTTGA (SEQ ID NO: 12) (reverse primer, a new *Xho*I site is underlined, 5025/5045). To generate the 220 bp of enhancer region with mutations in CRE2, aliquots (1 ul of each 50 ul PCR reactions) of the two PCR products were mixed and subjected to PCR amplification using primer pairs for intact 220 bp BAT specific enhancer region. The resulting mutations were confirmed by sequencing. To mutate both CRE2 and CRE3, the 220 bp fragment, which contains the mutation in CRE2, was subjected to PCR amplification using primer pairs described above to generate the CRE3 mutation: 5'-ggggagCTCCTCTACAG**C*tga*A**CAGAGG (SEQ ID NO: 13) (CRE3 shown in bold with lowercase *italic* letters which represent mutated sites; a new *Sac*I site is underlined, 4841/4862) and 5'-gggctcgagAGTCTGAGGAAAGGGTTGA (SEQ ID NO: 12) (a new *Xho*I site is *underlined*, 5025/5045). After the mutations were verified by sequencing, the DNA fragments containing the mutated sites in CRE2 and/or CRE3 were subcloned into luciferase reporter plasmid containing *Ucp1* promoter with CRE4.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA):

[0041] Nuclear extracts from various tissues of A/J mice (The Jackson Laboratory, Bar Harbor, Maine) and HIB-1B cells were prepared as described in J.D. Dignam *et al.*, Nucleic Acids Res., vol. 11, pp. 1475-1489 (1983), except that phosphatase inhibitors cocktail 1 and 2 (Sigma, St. Louis, Missouri) were added. The protein concentration was determined by the Lowry method using BSA as a standard as described in O.H. Lowry *et al.*, J. Biol. Chem., vol. 239, pp. 18-30 (1964). To prepare probes for EMSA, single-stranded oligonucleotides were synthesized and purified (Operon Technologies, Inc., Alameda, California). 200 pmol each of the complementary oligonucleotides were annealed in 100 ul containing 100 mM NaCl to obtain a double-stranded probe. Five μg of nuclear extract were incubated initially for 10 min at room

temperature in 29 µl containing 20 mM HEPES (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1.5 µg of poly(dA-dT), and 5 mM $MgCl_2$. The mixture was then incubated for an additional 20 min after adding ^{32}P-labeled probe ($4X10^5$ cpm/ul) with or without an unlabeled competitor or antibody for the supershift analysis. The antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). The reaction was electrophoresed on a 6% polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, California) in 0.5X TBE buffer. The gel was then dried and exposed to a PhosphorImage screen. The radioactivity was visualized and quantified using PhosphorImager and ImageQuant software from Molecular Dynamics (Sunnyvale, California).

***Western blot analysis*:**

[0042] Western blot analyses were performed with minor modifications as described in U.K. Laemmli, Nature, vol. 227, pp. 680-685 (1970); and H. Towbin *et al.*, Proc. Natl. Acad. Sci. USA, vol. 76, pp. 4350-4354 (1979). Cell lysates from HIB-1B cells were prepared by adding SDS sample buffer containing 62.5 mM Tris-Cl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromphenol blue with 1% v/v phosphatase inhibitor cocktail 1 and 2. All chemicals were from Sigma unless otherwise indicated. After cell lysates were separated on 8% SDS-polyacrylamide gel, protein was transferred onto a nitrocellulose membrane (Millipore Corp., Bedford, Massachusetts). The blots were then incubated with rabbit antibody against CREB (1:1,000 dilution, Santa Cruz Biotechnology), or phospho-CREB (Ser133, 1:1,000 dilution, New England Biolabs, Beverly, Massachusetts), overnight at 4°C with gentle agitation, followed by incubation with donkey anti-rabbit IgG as a secondary antibody (horseradish peroxide conjugated, Amersham Pharmacia Biotech, Piscataway, New Jersey). Bands were visualized by using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and exposed to X-Omat film (Eastman Kodak Co., Rochester, New York).

Example 2

Identification of CRE sequences for the binding of CREB

[0043] Four potential CRE sites are located in the 5' flanking region of *Ucp1* (Table 2). All four CREs have half-site consensus sequences (CGTCA). Evidence that these half-sites are involved in the regulation of *Ucp1* is limited to loss of CAT reporter activity in transient expression assays in a BAT cell line. From this prior analysis CRE2 and CRE4 appeared to be essential; mutations to CRE1 showed no loss of expression and mutations to CRE3 only slightly

reduced expression. See Kozak *et al.*, 1994. This experiment was designed to establish the function of CRE2 located in the upstream enhancer. A CRE2 probe for EMSA was made with 5 bp of half-site CRE2 (CGTCA) flanked by 14 bp of 5' and 3'-flanking sequences as shown in Fig. 1. Fig. 1 shows the nucleotide sequence of the 221 bp (4828/5048) of BAT specific region of the mouse *Ucp1* gene, with the enhancer elements shown within boxes and the NF-E2 binding site underlined and in bold letters.

Table 2. Synthetic double-stranded CRE sequences used for electomobility shift assay. Each DNA containing half-site CRE motif (CGTCA) from mouse *Ucp1* or palindromic sequences from somatostatin gene was annealed as described under "Example 1." Consensus sequences for CRE are underlined (lowercase letters represent mutations).

Name	Location	Sequences	SEQ ID NO:
CRE1	4419/4437	TTATAGTGCCGTCACTAAC AATATCACGGCAGTGATTG	SEQ ID NO: 18
CRE2	4884/4902	TGAACTAGTCGTCACCTTT ACTTGATCAGCAGTGGAAA	SEQ ID NO: 19
CRE3	4843/4861	CCTCTACAGCGTCACAGAG GGAGATGTCGCAGTGTCTC	SEQ ID NO: 20
CRE4	7258/7276	TGGGAGTGACGCGCGGCTG ACCCTCACTGCGCGCCGAC	SEQ ID NO: 21
CRE (Somatostatin)		TTGGCTGACGTCAGAGAGA AACCGACTGCAGTCTCTCT	SEQ ID NO: 22
m1CRE2		AACTAGTCtgaACCTTT TTGATCAGactTGGAAA	SEQ ID NO: 23
m2CRE2		AACTAtgaGTCACCTTT TTGATactCAGTGGAAA	SEQ ID NO: 24

[0044] Fig. 2A is an autoradiogram of an EMSA using ^{32}P end-labeled CRE2 (0.1 pmoles) from mouse *Ucp1* gene. Each lane (except for liver which was $1/10^{th}$ of the reaction) was loaded with a binding reaction containing 5 μg of nuclear extracts from A/J mice exposed to cold (4°C, overnight) with 2 pmoles of cold probe or antibody (1 μl) as indicated on 6% non-denaturing acrylamide gel. Slowly migrating bands representing CREB (dark arrow) and free probes (light arrow) are indicated on the right. However, probes prepared from the region just downstream of the CRE2 motif failed to form a similar retarded band (*data not shown*). The

complex from liver was ~ 10-times stronger than that of other fat tissues (loading for liver was $1/10^{th}$ of the reaction; the second retarded band in liver is non-specific and can be seen with other probes, data not shown). Nuclear extracts, prepared from brown adipose tissue (BAT), retroperitoneal fat tissue (RP), inguinal fat tissue (IG), and liver of A/J mice kept in the cold (4°C) overnight, showed a major retarded band that was eliminated by competition with a 20-fold excess of cold CRE2 (specific shifted bands are shown with the dark arrow in Fig. 2A).

[0045] To further characterize the binding sites of CRE2, competitive binding assays were performed with the same mutation, GTC to TGA, in two contiguous locations in the sequence. For the m1CRE2 probe, the mutation occurs in the middle of half-site CRE motif, while the m2CRE2 mutation only overlaps the first C in CRE motif (Table 2). In competitive EMSA, the m1CRE2, but not the m2CRE2 mutant oligonucleotide, lost the ability to compete with the labeled CRE2 probe (Fig. 2A). This indicates that the half-site CRE motif, but not the flanking 5' region, is active in binding the specific factor(s).

[0046] To identify the nuclear factor(s) which binds to CRE2, specific antibodies against Fos, Jun, CBP, or CREB/ATF1 were applied in an EMSA reaction. Because of the sequence similarity of CRE and AP-1 binding sites for the *Jun/Fos* heterodimer (palidromic CRE, TGACGTCA; palindromic AP-1, TGA$^{C}/_{G}$TCA; half-site CRE sequences are shown underlined) and the known interaction between CREB and CBEB binding protein (CBP), the antibodies were tested in a super shift assay. The data (Fig. 2A) demonstrated both that the factors that bind to CRE2 are part of the CREB/ATF1 family, and that CREB/ATF1 does not interact with either *jun* and *fos* or CBP.

[0047] To quantify binding of the four half-site CREs to CREB/ATF1, the ability of each CRE to compete with the CRE2 probe that binds to CREB/ATF1 as described in Table 2 was measured. Fig. 2B shows an autoradiogram of an EMSA showing only the CREB bands. Each lane was loaded with a binding reaction containing 0.1 pmoles of ^{32}P end-labeled CRE2, 5 μg of nuclear extracts from BAT of A/J mice which were exposed to cold (4°C, overnight), and 0.4 pmoles of cold competitors as indicated on the top of Fig.2B. Percent competition of ^{32}P end-labeled CRE2 to CREB by a CRE sequences from mouse *Ucp1* and somatostatin (named CRE) gene was calculated (shown at the bottom) from the radioactivity of the slow migrating bands in the lane without (first lane) and in the lane with the individual competitors. Most of the labeled CRE2 probe complexed with proteins in nuclear extracts (as illustrated in Fig.2A) disappeared with a 40-fold excess (4 pmoles) of cold probe (data not shown). Under these conditions,

palindromic CRE from somatostatin gene competes better than CRE2 itself (percent competition of 58.1% versus 29.1% in Fig. 2B) as expected. As shown in Fig. 2B, all the half-site CREs showed competition to CRE2 binding. This competition data together with the interference on probe binding upon addition of anti-CREB antibody indicates that CRE2 is a high affinity binding site that is enhanced by binding with CREB/ATF. The EMSA evidence for CRE2-CREB interactions corroborated the expression data to lead to the conclusion that CRE2 interacts with CREB to directly regulate *Ucp1* expression.

[0048] To test for a nuclear complex involving CBP or other CREB partners, a supershift assay was performed using nuclear extract from BAT of cold exposed A/J mouse (Fig.2A). Other than the strong interference in band formation found with CREB antibodies, there was no evidence that antibodies against CBP, c-Jun or c-Fos interfered with the interaction of the CRE2 probe with nuclear proteins in EMSA. Consistent with the lack of effects of these antibodies, a yeast two-hybrid screening for cDNA ($4X10^7$ transformants were screened) from BAT of A/J mouse with CREB as a bait, failed to detect positive clones (Data not shown). This is conclusive evidence that CRE2 is a major site for the transcriptional activation of *Ucp1* expression by direct interaction with homodimers of CREB.

Example 3

Changes in CREB/ATF1 phosphorylation and binding to CRE2 in response to norepinephrine

[0049] To assay for changes in CREB/ATF1 binding to CRE2 in response to norepinephrine, HIB-1B cells were treated with fresh medium (control) or medium containing 1 mM norepinephrine (NE) for 0, 5, 10, 20, 30 and 60 minutes. Cell lysates were prepared and analyzed by a Western blot with phospho-CREB (Ser133) specific antibody. The arrows on the right indicate the location of phosphorylated CREB (pCREB) and ATF1 (pATF1) with molecular weights of 43 KDa and 35 KDa, respectively. The treatment of HIB-1B cells with 1 mM norepinephrine significantly increased phosphorylation of both CREB and ATF1 over a 60 min time course, whereas only a modest increase occurred with a change of culture medium (Fig. 3A).

[0050] CRE2 binding to CREB/ATF1 factors from HIB-1B cells was confirmed and quantified with EMSA using nuclear extracts from HIB-1B cells treated with 1 mM norepinephrine (NE). In Fig. 3B, nuclear extracts were isolated from HIB-1B cells treated with 1 mM of NE for 0, 10, and 60 min. Each lane was loaded with a binding reaction containing 5 μg of nuclear extracts and 0.1 pmoles of ^{32}P end-labeled CRE2 as indicated on the *top*. A lane for nuclear extracts from BAT of A/J mouse (cold, overnight) was added to compare intensity (lane BAT). Cold probe (2 pmoles) was added in the reaction for the competition (lane CRE2). Only the CREB bands are shown with arrows in Fig. 3B. The major thick band migrated to the same position on the gel as the single band from nuclear extract of cold exposed BAT of A/J mice (right lane). Treatment of HIB-1B cells with norepinephrine (1 mM) increased the intensity of the four retarded bands 20.6% and 24.2% after 10 and 60 min, respectively (from mean of 2 experiments). As shown in Fig. 3B, nuclear extracts from HIB-1B cells showed at least four retarded bands that were specifically removed with an excess of cold CRE2 (shown with arrows at right). These results indicate that norepinephrine (NE) induces phosphorylation and binding of CREB/ATF1 proteins to CRE2.

[0051] As shown in Fig. 3A, norepinephrine treatment in HIB-1B cell dramatically increased the phosphorylation of both CREB and ATF1 within 5 min, followed by an increased binding of dimerized CREB or CREB/ATF1 heterodimers to ^{32}P end-labeled CRE2 probe in EMSA (Fig. 3B). Similarly, the EMSA data showing an increase in complex formation with nuclear extracts from cells incubated with 1 uM NE indicated that phosphorylation of CREB and ATF1 increase their binding affinity to CRE in *Ucp1* gene.

Example 4

Functional Characterization of CRE1-4

[0052] To further characterize the functionality of CREs, a transient transfection assay was performed using luciferase reporter constructs and site-directed mutagenesis. The same site-directed mutations were introduced into CRE2 and CRE3 in the 220 bp of BAT-specific enhancer region as present in the probes used in the competitive EMSA (Table 2), since changes from GTC to TGA (m1CRE2 probe in Fig. 2A) eliminated the capacity of the oligonucleotide to compete with CRE2 probe.

[0053] Luciferase reporter constructs (named in the *left*) were generated by subcloning the various fragments from 5' flanking region of mouse *Ucp1* gene into pGL3/basic vector (Promega). DNA fragments from mouse *Ucp1* gene are shown in Fig. 4 as thick lines with the position of individual CREs indicated as *ovals*. Mutations of CRE2 and/or CRE3 by mutating key nucleotide residues as described under Example 1 are indicated with open ovals with *X* marks. Each construct was transfected into HIB-1B cells with pRL/SV40 vector (Promega), and the cells were cultured under the medium containing 1 uM of norepinephrine (NE) or 0.5 mM of 8-Bro-cAMP (cAMP) another 16 hrs. Luciferase activity was measured from cell lysates using Dual-Luciferase assay system (Promega), and a fold increase of luciferase activity by NE or cAMP was calculated. Data is presented as the means and standard deviations of fold increase from three experiments. The restriction map shown at the top indicates the position of the restriction enymes --*Hind*III (H) ; *Xba*I (X); and *Bgl*I (B).

[0054] The promoter without CRE4 (pGL3/pro) had low basal promoter activity (Fig. 4). Addition of CRE4 (pGL3/CRE4pro) to the promoter construct showed about a 3-fold increase in luciferase activity in response to NE and cAMP. This level of transient expression was similar to that of the promoter construct containing 220 bp of BAT-specific enhancer region, but without CRE4 (pGL3/pro/220). Importantly, the 220 bp of BAT-specific enhancer region together with CRE4 (pGL3/CRE4pro/220) showed a level of expression activity similar to the 3.1 kb of 5'-flanking region of *Ucp1* (pGL3/3.1 kb). This data suggests that CRE4 cooperates with the 220 bp BAT-specific enhancer region in determining the response to NE and cAMP. When each of CRE2 or CRE3 independently or together in pGL3/CRE4pro/220 were mutated to evaluate the contribution of CRE2 and CRE3 to the enhancer activity, the expression was diminished in assays with the mutant constructs, but it was less dramatic than had been previously observed with a more differentiated BAT cell line. See U.C. Kozak, *et al.*, Mol. Cell Biol., vol. 14, pp. 59-67 (1994).

[0055] An important conclusion that emerged from this analysis is the requirement for interactions between elements in the distal enhancer with CRE4 in the proximal promoter to confer high levels of expression. Overall, the results confirmed a major role for CRE2 in the enhancer activity.

Example 5

NFE2l2 binds NF-E2 binding sites in the Upstream Enhancer of Mouse Ucp1

[0056] A consensus NF-E2 binding motif, ACTAGTCGT, has been identified that partially overlaps the CRE-2 half-site in mice and is located 6 bp downstream of the peroxisomal proliferator activator receptor binding motif (PPRE)(Fig 1). A probe containing 10 bp of the NF-E2 binding motif with 3 bp of nonspecific flanking sequence (CCC) (SEQ ID NO: 2) was synthesized and incubated with nuclear extracts from HIB-1B cells (Fig 5A). Fig. 5A shows an autoradiogram of an EMSA using ^{32}P end-labeled NF-E2 (0.1 pmoles) from mouse *Ucp1* gene. Nuclear extracts were prepared from HIB-1B cells with (+) or without (-) NE (1 uM, 60 min). Each lane was loaded with a binding reaction containing 5 μg of nuclear extracts with 2 pmoles of cold probe or antibody (1 μl) as indicated in the figure on 6% non-denaturing acrylamide gel. Slowly migrating bands representing NFE2l2 are indicated with an arrow on the right. Nuclear extracts from HIB-1B cells (extracted as described in Example 1) interacted with probes to the NF-E2 binding site (SEQ ID NO: 3) from mouse *Ucp1* gene to generate shifted bands that were eliminated in a competition assay with a 20-fold excess of cold probe (Fig 5A). Nuclear extracts from the HIB-1B cells treated with 1 mM norepinephrine for 30 minute increased the intensity of the complex as did nuclear extracts prepared from brown adipose tissue of cold exposure mice (Fig. 5C). Fig. 5C illustrates that the binding activity of NF-E2 sequence is increased by cold exposure in BAT of A/J mouse. Nuclear extracts were isolated from BAT of A/J mouse from kept in RT or cold (4°C) for 7 days. 5 μg of nuclear extracts were incubated with ^{32}P end-labeled NF-E2 probe (0.1 pmoles), and then separated on 6% non-denaturing acrylamide gel. Only the NFE2l2 bands are shown with an arrow.

[0057] CRE2 probes (SEQ ID NO: 4) that interact with CREB showed a band shift with a different mobility than the NF-E2 probe (Fig. 6B, below). When the ability of antibody against members of NF-E2 binding factors, including NF-E2 p45, NFE2l1 and NFE2l2, to interfere with the band shift was assessed, only the antibody from NFE2l2 interfered with the shifted bands (Fig. 5A). In order to confirm the binding activity of the NFE2l2 from brown adipose tissue with NF-E2 binding sites, the same amount (5 μg) of nuclear extracts from BAT of A/J mouse were incubated with the NF-E2 probe. The effects of cold NF-E2 and antibody to NFE2l2 on the bands in the EMSA were very similar to that observed with extracts from HIB1B cells (Fig. 5B)

[0058] Transcription factors of the NF-E2 family, which were originally identified as an erythrocyte-specific DNA binding activity, belong to the cap'n'collar (CNC)-type basic leucine zipper (bZIP) as described in P. Moi *et al.*, Proc. Natl. Acad. Sci. USA, vol. 91, pp. 9926-9930 (1994). Protein-protein interactions between NFE212 and other bZIP proteins including, CREB, Fos, and Jun have been reported. PPARg can also directly interact with NFE212 via the NF-E2/AP1 binding site (TGCTGATTCAT) of the thromboxane synthesis gene in macrophages. Given the putative role for these transcription factors in *Ucp1* regulation, possible interactions between these transcription factors and NF-E2 in the *Ucp1* enhancer were evaluated by determining whether antibodies to these transcription factors interfere with the EMSA of the NF-E2 probe. Fig. 5B shows an autoradiogram illustrating the binding of NF-E2 sequences to nuclear extracts from BAT of A/J mouse (cold, overnight). Each lane was loaded with a binding reaction of ^{32}P end-labeled NF-E2 (0.1 pmoles) incubated with 5 μg of nuclear extracts from BAT of A/J mouse (cold, overnight) with 2 pmoles of cold probe or antibody (1 ul) as indicated on 6% non-denaturing acrylamide gel. Only the NFE212 bands are shown. Only antibodies to NFE212 interfered with the complexes between NF-E2 probes and nuclear factors from BAT (Fig. 5B).

[0059] These experiments demonstrate that the transcription factor NFE212 induced the expression of *Ucp1* in mice. The gene for NFE212 in mice is located on Chromosome 2. See M.P. Marcias *et al.*, J. Leukoc. Biol., vol. 67, pp. 567-76 (2000); and F. Yehiely *et al.*, Neurobiol. Dis., vol. 3, pp. 339-55 (1997); and the Mouse Genomics Informatics website of The Jackson Laboratory, Bar Harbor, Maine (http://www.informatics.jax.org). Chromosome 2 in mice is the known site of a locus that increases the capacity to induce brown adipocytes in white adipose tissue, and to induce the expression of *Ucp1*. See, C. Guerra *et al.*, 2000.

Example 6

Competition between NFE2l2 and CREB

[0060] An overlap of the binding motif of NF-E2 with the half site CRE2 in mice suggested that competition for binding may exist between NFE212 and CREB. To test this, a 19 bp oligonucleotide probe, NFCRE, (SEQ ID NO: 2) was designed which covered both NF-E2 and CRE2, for a gel shift and super shift assay. Fig. 6A shows the nucleotide sequences for NFCRE containing both NF-E2 and CRE2 binding sites, with the enhancer elements shown with either underlined bold letters (NF-E2) or box (CRE2). The nucleotide sequences for cold probes

for NF-E2 (SEQ ID NO: 3) and for CRE (SEQ ID NO: 4) are shown with underlined bold letters and the 3 bp of flanking sequences.

[0061] Fig. 6B illustrates the binding activity of NFCRE, NF-E2 and CRE2 with nuclear extracts from HIB-1B cells. Each lane was loaded with a binding reaction containing 5 μg of nuclear extracts (HIB-1B cells, 1μM NE for 60 min for the treatment) with different concentrations of cold probe (2 pmole, 0.2 pmole) as indicated on 6% non-denaturing acrylamide gel. Slowly migrating bands representing CREB and NFE2l2 complex with ^{32}P end-labeled probe are shown. The band shifts with the NFCRE probe were very similar to the pattern observed for CRE2, whereas the band shift with the NF-E2 probe migated slightly faster. The NF-E2 band that should have been formed with the NFCRE probe was not detected. Both cold CRE2 and NFCRE was able to compete with the NFCRE probe. However, NF-E2 could not compete away the band shifts with either CRE2 or NFCRE probe, but could with the NF-E2 probe. These findings indicated that CREB binds to CRE2 with a high affinity, and this CREB-CRE2 binding interfered in a competitive manner with the binding of NFE2l2 to the NF-E2 motif. However, since this overlap does not exist in the human gene (see below in Example 7), the competition between CREB and NFE2l2 probably does not occur. This suggests that in humans NFE2l2 may be more efficacious.

Example 7

Human Ucp1 gene contains NF-E2 binding sites

[0062] Recently, the human *Ucp1* gene was cloned and evidence describing key elements controlling its transcriptional regulation obtained. See M. del Mar Gonzalez-Barroso *et al.*, 2000. A 350 bp hormone-sensitive region of the human gene showed significant similarity with the mouse (60.1%) and rat (62.5%) BAT-specific enhancer element. This region in the human gene was able to bind the nuclear factors, RARs, RXRs, CREB/ATF, and PPARγ indicating that transcriptional regulation of the *Ucp1* gene between rodents and human have mechanisms in common. A comparison of 100 bp (-3762/-3662 of human) of the human, rat, and mouse *Ucp1* gene is shown in Fig. 7A. Half-sites for ATF/CREB (CRE2 and CRE3), a putative NF-E2 binding site, and PPRE are shown within boxes. Bold letters represent bases which matched between the three species. A sequence similarity search indicates that NF-E2 binding site (TGCTGYCNCT) in the mouse, human and rat is located in a comparable location. (Fig. 7A) However, unlike the mouse gene, neither the rat nor the human gene contain the downstream NF-

E2 binding site that overlaps with CRE2 in the mouse. Using electromobility shift and supershift assays, the binding of NFE2l2 was identical for the NF-E2 binding sites for human and rodents. (Data not shown).

[0063] Binding activity of the putative human NF-E2 binding site was assayed with nuclear extracts from BAT of A/J mouse. Fig. 7B illustrates the binding of human NF-E2 sequences to nuclear extracts from BAT of A/J mouse. Nuclear extracts were isolated from BAT of A/J mouse kept in RT or cold (4°C) for 7 days. 5 μg of nuclear extracts were incubated with ^{32}P end-labeled NF-E2 probe (0.1 pmoles) corresponding to the human *Ucp1* gene, and separated on 6% non-denaturing acrylamide gel. 2 pmoles of cold probe or antibody for NFE2l2 (1 µl) were added for competition and for the super shift assay, respectively. Only the NFE2l2 bands are shown (arrow). Nuclear extracts from cold exposed mouse (7 days at 4°C) showed an increase in binding activity (Fig. 7B) and a similar binding activity as the mouse NF-E2 probe (Fig. 5c). Mouse NF-E2 binding sites (mNF-E2) showed comparable competition with the human NF-E2 binding site (hNF-E2). Antibody against NFE2l2 (mouse, rat and human reactive) interacted effectively with the complex in the super shift assay. These results indicate that NFE2l2 regulates *Ucp1* expression in humans.

Example 8

Effects of Overexpression of NFE2l2 on Mouse Ucp1 Expression

[0064] Since the binding activity of NFE2l2 to the corresponding NF-E2 sequences increased in response to cold exposure (Fig. 5C) and norepinephrine treatment (Fig. 5a), the effect of *Nfe2l2* overexpression on reporter constructs containing the 5' regulatory region of *Ucp1* was examined. Previously, it has been reported that 3.1 kb of 5' flanking region (4380/7538) of the mouse *Ucp*1 gene which contains 4 DNase I hypersensitive sites revealed strong CAT activity by adding norepinephrine to the cultures. See Kozak *et al.*, 1994. To test the function of NFE2l2 on *Ucp1* gene promoter and transcription, a luciferase reporter construct regulated by the 3.1 kb of mouse *Ucp1* gene was co-expressed with CMV-controlled *Nfe2l2* expression vector in HIB-1B cells. Fig. 8 shows the results of luciferase reporter constructs (pGL3/3.1kb, *left* and pGL3/CRE4pro/220, *right*) that were cotransfected with expression vector which is empty (pCMV/tag1, open box), containing cDNA for *Nfe2l2* correct (closed box), or containing cDNA with reversed orientation (hatched box) into HIB-1B cells. Cells were cultured in medium with or without 0.5 mM of 8-Br-cAMP (cAMP), 1 uM of norepinephrine (NE) or 1uM troglitazone

(Trog) for an additional 16 hrs. Luciferase activity was measured in cell lysates using the Dual-Luciferase assay system (Promega), and activity (fold increase) was obtained by dividing corrected activity (induction after treatment) by the activity from empty vector (pCMV/tag1). Data is presented as the mean and standard deviation of fold increase from two independent experiments.

[0065] As shown in Figure 8, overexpression of *Nfe2l2* increased the luciferase activity of the 3.1 kb of *Ucp1* reporter construct by 3.9-fold (0.5 mM 8-Br-cAMP, 16 hrs) and 2.8-fold (1mM norepinephrine, 16 hrs) relative to the empty vector (pCMV/tag1). However, cells with 1 mM of troglitazone, a PPARγ ligand, showed no effect indicating that activation by NFE2l2 is dependent on β-adrenergic receptor activation and does not involve the upstream PPARγ regulatory site. Additionally, in Fig. 4 mutations to the first and second T and G nucleotides in NF-E2 which overlap with CRE2 decreased the luciferase activity with norepinephrine or cAMP treatment in the same cells. To focus on the NF-E2 binding site of mouse *Ucp1* gene, a similar experiment was performed with the truncated luciferase reporter construct (pGL3/CRE4/220), which was used in characterization of CRE2, as described in Figure 4. As expected from previous experiments, slightly higher activities were observed with pGL3/CRE4/220 luciferase reporter (Fig. 8, *right*). These results indicate that NFE2l2 has a role in regulating mouse *Ucp1* gene expression by interactions with a NF-E2 binding site that overlaps the CRE2 site in the enhancer region.

[0066] An EMSA probe designed from the sequence in the mouse *Ucp1* enhancer was found to form specific bands; however, supershift assays with antibodies showed that the proteins binding to the probe were not against the NF -E2 p45 subunit, but rather against NFE2l2, another member of the NF-E2 family. The binding of NFE2l2 probes was increased in brown fat cells isolated from following treatment with cold or norepinephrine; and coexpression studies of *Nfe2l2* vectors with the *Ucp1* enhancer constructs indicated that transcription of *Ucp1* is mediated by NFE2l2.

[0067] Thus, binding activity of NFE2l2 was increased by norepinephrine treatment (*in vivo*) and cold exposure (*in vitro*). Furthermore, *Nfe2l2* overexpression induced *Ucp1* promoter activity only with norepinephrine and a cAMP analog in HIB-1B cell. That no induction occurred with the PPARγ ligand, troglitazone, suggested that NFE2l2 activation is mediated by protein kinase A signaling pathway, but is independent of PPARγ. This activation does not depend on increased production of *Nfe2l2* mRNA.

Example 9

Effects of Overexpression of NFE2l2 on Rat Ucp1 Expression

[0068] To further test the function of NFE2l2 on the *Ucp1* enhancer, luciferase reporter constructs containing the rat *Ucp1* enhancer with or without mutations to the NF-E2 site (pGL3/cre4pro/mRat221 and pGL3/cre4pro/Rat221) were co-expressed with a CMV-controlled *Nfe2l2* expression vector in HIB-1B cells, similar to that described above for the mouse *Ucp1* enhancer. (Example 8). Luciferase reporter constructs (pGL/cre4pro/Rat221 and pGL/cre4pro/mRat221) were cotransfected with an expression vector which is either empty (pCMV/tag1, Stratagen) or contains cDNA for *Nfe2l2* into HIB-1B cells. Cells were cultured in the medium with or without 0.5 mM of 8-Br-cAMP (cAMP) for an additional 16 hrs. Luciferase activity was measured in cell lysates using the Dual-Luciferase assay system (Promega). Data is presented in Fig. 9 as the mean and standard deviation from two experiments. The luciferase reporter assay demonstrated that the NF-E2 binding site in the rat *Ucp1* promoter is responsible for its transcriptional activation in response to cAMP or norepinephrine treatment with sequence specific manner (*data for norepinephrine not shown*). As shown in Fig. 9, overexpression of *Nfe2l2* increased *Ucp1* promoter activity but only with the wild-type NF-E2 element (pGL3/cre4pro/Rat221). These results further indicate that in mammals NFE2l2 participates in the BAT development as well as transcriptional activation of *Ucp1* in response to cold exposure or β-adrenergic stimulation.

Example 10

Analysis of mice deficient in NFE2l2 due to a targeted mutation of the gene.

[0069] Mice are currently being bred to be deficient in NFE2l2 due to a target mutation of the gene. These mice will be analyzed for brown adipocyte expression in both brown and white fat depots following exposure to the cold. Mice that are homozygous for the *Nfe2l2* target mutation will be exposed to the cold for periods of time that vary from 6 hr to 3 weeks. Adipose tissue from several depots will be removed and analyzed by the expression of *Ucp1* mRNA and for the increase in brown adipocytes by immunohistology. It is predicted that the mutation to *Nfe2l2* will reduce, but not totally eliminate, the induction of *Ucp1* and brown adipocyte formation.

Example 11

Analysis of mice with overexpression of NFE2l2

[0070] Transgenic mice in which *Nfe2l2* is over-expressed in adipocytes have been generated by driving expression of *Nfe2l2* with the aP2 promoter. This promoter has been used extensively for fat specific over-expression of many genes. Mice carrying the transgene were exposed to cold (4°C) and were analyzed for the expression of *Ucp1* in white fat depots. A prelimary experiment indicated that some transgenic mice showed higher expression of *Ucp1* than control non-transgenic, cold-exposed mice, while other transgenic mice did not. These mice will also be analyzed for the number of brown adipocytes. They will also be fed a high fat diet to stimulate increased obesity.

[0071] We predict that the increase in the expression of *Nfe2l2* will increase *Ucp1* expression and brown adipocyte differentiation and prevent obesity even with a diet high in fat.

Example 12

Interactions between loci on different chromosomes to achieve optimal expression of Ucp1.

[0072] Since the difference in *Ucp1* gene expression depends on variation in *Nfe2l2* on Chromosome 2, as well as genes on Chromosomes 3, 8 and 19, it is possible that effects due to NFE2l2 may require specific interactions with alleles on these other chromosomes. To provide the proper genetic environment to detect the effects of a specific gene, including *Nfe2l2*, congenic mice have been constructed in which the alternative allele for each genetic locus associated with brown fat induction has been placed on the C57BL/6J background by 10 backcross generations. These congenic strains will be analyzed separately as well as in various combinations to identify the interactions between loci on different chromosomes to achieve optimal expression of *Ucp1*. Accordingly, the knowledge gained from the study of these special congenic lines will enable the identification of the genetic environment that will optimize the effect of NFE2l2 on brown fat differentiation.

Example 13

NFE2l2 As a Transcription Factor in BAT Development and Differentiation.

[0073] Brown adipose tissue (BAT) develops during the perinatal period. Breeding pairs of C57BL16J mice were purchased from the Jackson Laboratory. The breeding colon was then

expanded to produce sufficient numbers of progeny to analyze during development. Numerous transcription factors, including peroxisome proliferator-activated receptor (PPAR), CCAAT enhancer-binding proteins (C/EBPs) and cAMP responsive element binding protein (CREB), are involved in the BAT development. Nuclear extracts from BAT isolated from C57BL16J mice ranging in age from 19 days of gestation to 4 months after birth were isolated. The binding of NF-E2 sequences to nuclear extracts from BAT was assayed by loading each lane with a binding reaction containing 5 ug of nuclear extract from C57BL16J mice. Only the NFE212 bands are shown in Fig. 10A. When we compared binding activity of adipogenic transcription factors during BAT development, NFE212 revealed the same pattern of binding activity. (Data for other factors not shown). As shown in Figure 2A, binding activity of NFE212 onto its binding sites which derived from mouse *Ucp1* promoter region reached a maximum at 19-day fetus, but maintained a high level of binding until 1-month-old age when BAT is actively developing.

[0074] An Western blot analysis was conducted to measure protein level of NFE212 in the nucleus of C57BL16J mice of various ages from 19 days of gestation to 3 months after birth. Nuclear extracts (10 μg) from BAT of C57BL16J mice (ages as stated) were separated on a 10% SDS-polyacrylamide gel, and a blot was applied with a specific antibody for NFE212. Only the NFE212 bands are shown. The amount of protein in the nucleus matched the binding activity in terms of highest level at 19 days of gestation and then decrease overtime. (Fig. 10B)

[0075] It has been known that chronic stimulation of β-adrenergic receptors by cold exposure results in increased BAT development. To identify changes in NFE212 during cold adaptation, binding activity of NFE212 from BAT of cold exposed mice (1day and 7 day at 4°C) was measured by same technique. Nuclear extracts were isolated from BAT (as described in Example 1) of B6 mice kept at room temperature (approximately 20°C) or in the cold (4°C) for 1 and 7 days. 5 ug of nuclear extracts were incubated with ^{32}P end-labeled NF-E2 probe (0.1 *pmoles*), and separated on 6% non-denaturing acrylamide gel. The results are shown in Fig. 10C. These results indicate that binding activity of NFE212 increased gradually after cold exposure, a response consistent with other known transcription factors. These results clearly indicate that NFE212 is a transcription factor that plays an important role in both BAT development and differentiation.

[0076] The term "therapeutically effective amount" as used herein refers to an amount of either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene sufficient to increase the expression of *Ucp1* and increase brown adipose tissue thermogenesis. The term "therapeutically effective amount" therefore includes, for example, an amount of either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene sufficient to increase brown adipose tissue thermogenesis to decrease obesity, preferably to reduce by at least 10%, and more preferably to reduce by at least 30%, the degree of obesity. The dosage ranges for the administration of either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene are those that produce the desired effect. Generally, the dosage will vary with the age, weight, condition, and sex of the patient. A person of ordinary skill in the art, given the teachings of the present specification, may readily determine suitable dosage ranges. The dosage can be adjusted by the individual physician in the event of any contraindications. In any event, the effectiveness of treatment can be determined by monitoring the weight of the patient by methods well known to those in the field. Moreover, either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene can be applied in pharmaceutically acceptable carriers known in the art.

[0077] Either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene may be administered to a patient by any suitable means, including parenteral, subcutaneous, intrapulmonary, topically, and intranasal administration. Parenteral infusions include intramuscular, intravenous, intraarterial, or intraperitoneal administration. Either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene may also be administered transdermally, for example in the form of a slow-release subcutaneous implant, or orally in the form of capsules, powders, or granules.

[0078] Pharmaceutically acceptable carrier preparations for parenteral administration include sterile, aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. The active therapeutic ingredient may be mixed with excipients that are pharmaceutically acceptable and are compatible with the active ingredient. Suitable excipients include water, saline, dextrose, glycerol and ethanol, or combinations thereof.

Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases, and the like.

[0079] The form may vary depending upon the route of administration. For example, compositions for injection may be provided in the form of an ampule, each containing a unit dose amount, or in the form of a container containing multiple doses.

[0080] Either NFE2l2 or a compound that will increase the expression of the *Nfe2l2* gene may be formulated into therapeutic compositions as pharmaceutically acceptable salts. These salts include the acid addition salts formed with inorganic acids such as, for example, hydrochloric or phosphoric acid, or organic acids such as acetic, oxalic, or tartaric acid, and the like. Salts also include those formed from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and organic bases such as isopropylamine, trimethylamine, histidine, procaine and the like.

[0081] Controlled delivery may be achieved by admixing the active ingredient with appropriate macromolecules, for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, prolamine sulfate, or lactide/glycolide copolymers. The rate of release of either NFE2l2 or a compound that will increase the expression of the *Nfe2l2* gene may be controlled by altering the concentration of the macromolecule.

[0082] Another method for controlling the duration of action comprises incorporating either NFE2l2 or a compound that will increase the expression of the *Nfe2l2* gene into particles of a polymeric substance such as a polyester, peptide, hydrogel, polylactide/glycolide copolymer, or ethylenevinylacetate copolymers. Alternatively, either NFE2l2 or a compound that will increase the expression of the *Nfe2l2* gene may be encapsulated in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

[0083] The present invention provides a method of treating or ameliorating a disease that can be helped by increasing *Ucp1* expression, such as obesity, comprising administering to the patient, a therapeutically effective amount of either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene. The term "ameliorate" refers to a decrease or lessening of the symptoms of the disorder being treated. The symptoms that may be ameliorated include significant weight loss, and improved insulin senstivity. In addition, the use of either NFE212 or a compound that will increase the expression of the *Nfe2l2* gene may be combined with the administration of another compound that are known to increase *Ucp1* expression, for example, a compound that is known to increase the activity or expression of other transcription factors, e.g., PKA, CREB, PGC1, RAR, RXR, PPARγ, ATF-1, and CBP. Examples of compounds known to increase the expression of *Ucp1* by one or more of these transcription factors, include norepinephrine, β-adrenergic receptor agonists, thiazolidinediones, isoproterenol, thyroid hormone, and retinoids.

[0084] Compounds that are known to increase the level or the activity of protein kinase C can be used to increase the concentration and the binding of NFE212 to its DNA binding motif in the nucleus. These compounds include, but not limited to,phorbol esters (e.g., phorbol 12-myristate 13-acetate), *tert*-butylhydroquinone, and β-naphthoflavone.

[0085] The complete disclosures of all references cited in this specification are hereby incorporated by reference. Also, incorporated by reference is the following reference, which is not prior art to this application: J.S. Rim and L.P. Kozak, "Overlapping regulatory motifs for CREB and NFE212 transcription factors in the upstream enhancer of the mitochondrial uncoupling protein 1 gene," Journal of Biological Chemistry, vol. 277, pp. 34589-34600 (2002). In the event of an otherwise irreconcilable conflict, however, the present specification shall control.

SEQUENCE LISTING

<110> Board of Supervisors of Louisiana State University and Agricultural and Mechanical College

Kozak, Leslie P.

Rim, Jong S.

<120> Induction of Brown Adipocytes by Transcription Factor NFE2L2

<130> 00P01W Kozak

<140> PCT/US02/_____

<141> 2002-09-24

<150> US 60/324,400

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What is claimed:

1. A method of ameliorating or preventing, in a mammals, the symptoms of a disease treatable by increasing *Ucp1* expression, said method comprising administering to the mammal a therapeutically effective amount of a compound that causes an increase in the concentration of NFE212 protein.

2. A method of Claim 1, wherein the disease is a weight disorder.

3. A method of Claim 2, wherein the weight disorder can be ameliorated or prevented with an increase in brown adipose tissue thermogenesis.

4. A method of Claim 1, wherein the adminstered compound causes a change in *Nfe212* gene expression.

5. A method of Claim 2, wherein the weight disorder is obesity.

6. A method of Claim 1, wherein the administered compound is NFE212.

7. A method of Claim 1, further comprising an initial treatment of the mammal with an amount of norepinephrine sufficient to increase the number of brown adiopocytes.

8. A method of Claim 1, further comprising administering to the mammal other compounds that increase *Ucp1* expression, selected from the group consisting of norepinephrine, β-adrenergic receptor agonists, thiazolidinediones, isoproterenol, thyroid hormone, and retinoids.

9. A method of ameliorating or preventing, in a mammals, the symptoms of a disease treatable by increasing *Ucp1* expression, said method comprising administering to the mammal a therapeutically effective amount of a compound that increases the concentration of NFE2l2 protein in the nucleus.

10. A method of Claim 9, wherein the disease is a weight disorder.

11. A method of Claim 10, wherein the weight disorder can be ameliorated or prevented with an increase in brown adipose tissue thermogenesis.

12. A method of Claim 10, wherein the weight disorder is obesity.

13. A method of Claim 9, wherein the administered compound causes an increase in the concentration or activity of protein kinase C.

14. A method of Claim 13, wherein the administered compound is selected from the group consisting of ,phorbol esters, *tert*-butylhydroquinone, and β-naphthoflavone.

15. A method of Claim 14, wherein the phorbel ester is phorbol 12-myristate 13-acetate.

16. A method of Claim 9, further comprising an initial treatment of the mammal with an amount of norepinephrine sufficient to increase the number of brown adiopocytes.

17. A method of Claim 9, further comprising administering to the mammal other compounds that increase *Ucp1* expression, selected from the group consisting of norepinephrine, β-adrenergic receptor agonists, thiazolidinediones, isoproterenol, thyroid hormone, and retinoids

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BRE-2
XbaI

Fig. 1

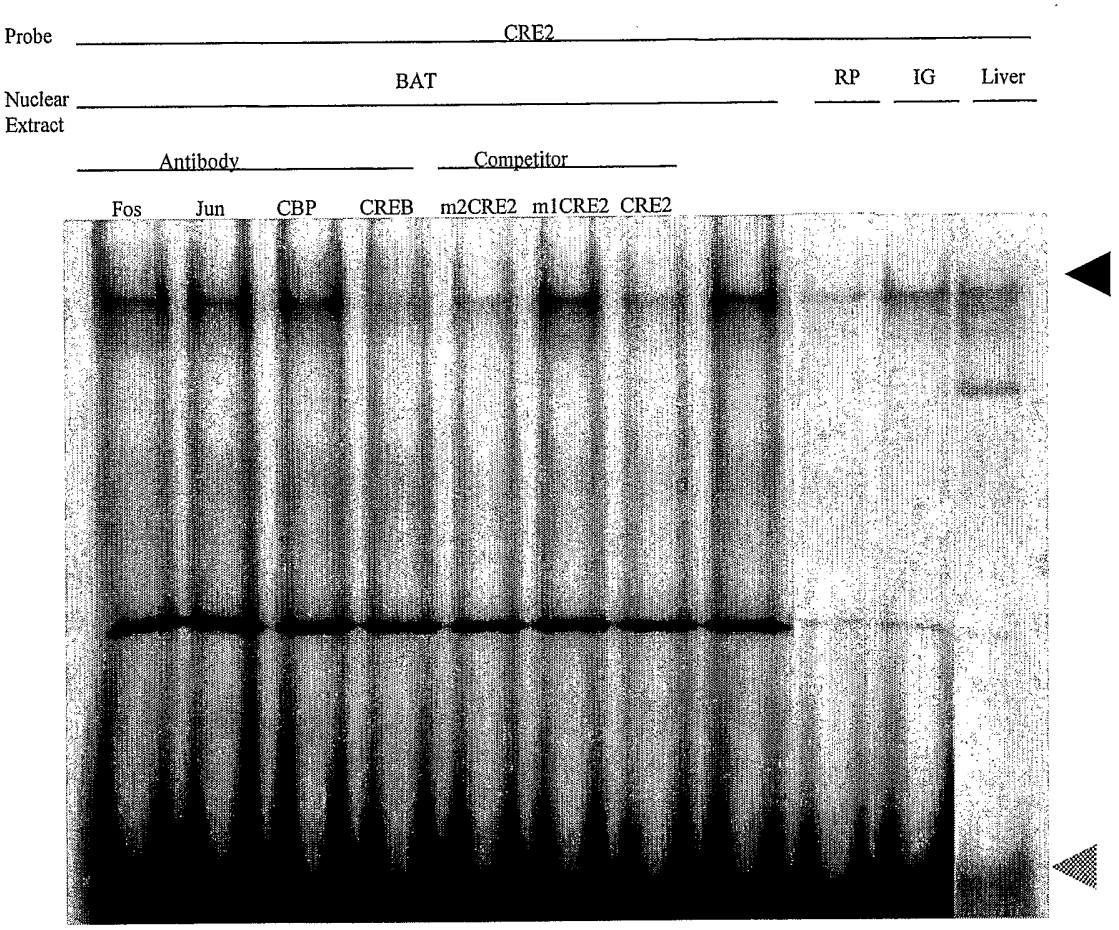
Probe
CRE2
Nuclear Extract
BAT
RP
IG
Liver
Antibody
Competitor
Fos
Jun
CBP
CREB
m2CRE2
m1CRE2
CRE2

Fig. 2A

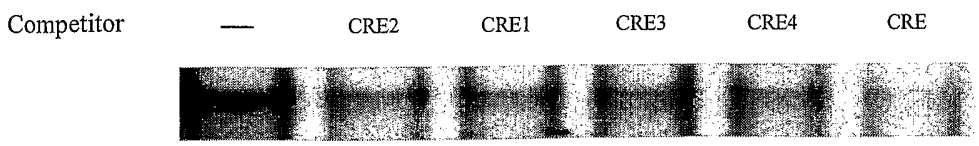
Competitor
—
CRE2
CRE1
CRE3
CRE4
CRE
% competition
0
29.1
7.4
12.6
26.5
58.1

Fig. 2B

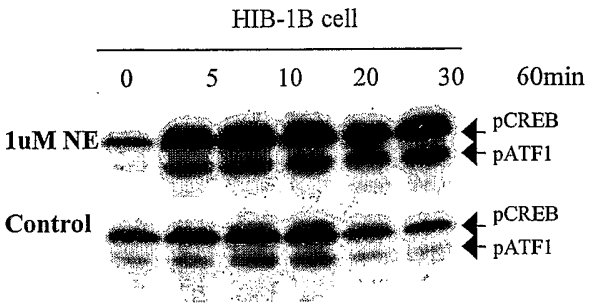
HIB-1B cell
0
5
10
20
30
60min
1uM NE
pCREB
pATF1
Control
pCREB
pATF1

Fig. 3A

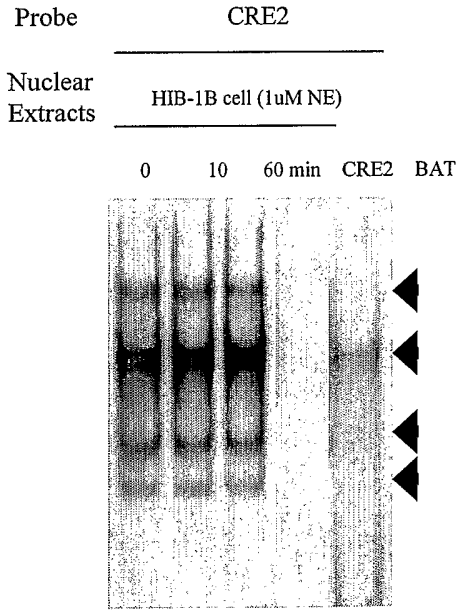
Probe
CRE2
Nuclear Extracts
HIB-1B cell (1uM NE)
0
10
60 min
CRE2
BAT

Fig. 3B

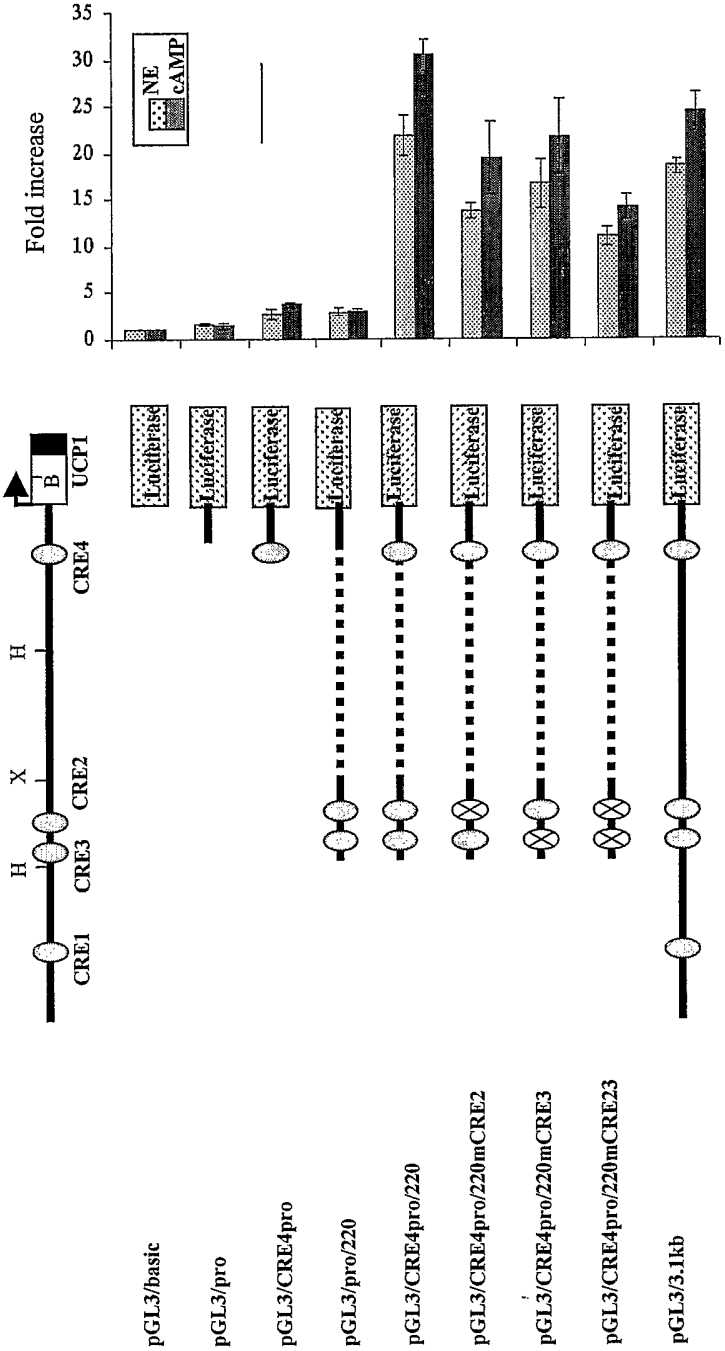
Fold increase
35
30
25
20
15
10
5
0
NE
cAMP
Luciferase
B
UCP1
CRE4
H
X
CRE2
H
CRE3
CRE1
pGL3/basic
pGL3/pro
pGL3/CRE4pro
pGL3/pro/220
pGL3/CRE4pro/220
pGL3/CRE4pro/220mCRE2
pGL3/CRE4pro/220mCRE3
pGL3/CRE4pro/220mCRE23
pGL3/3.1kb

Fig. 4

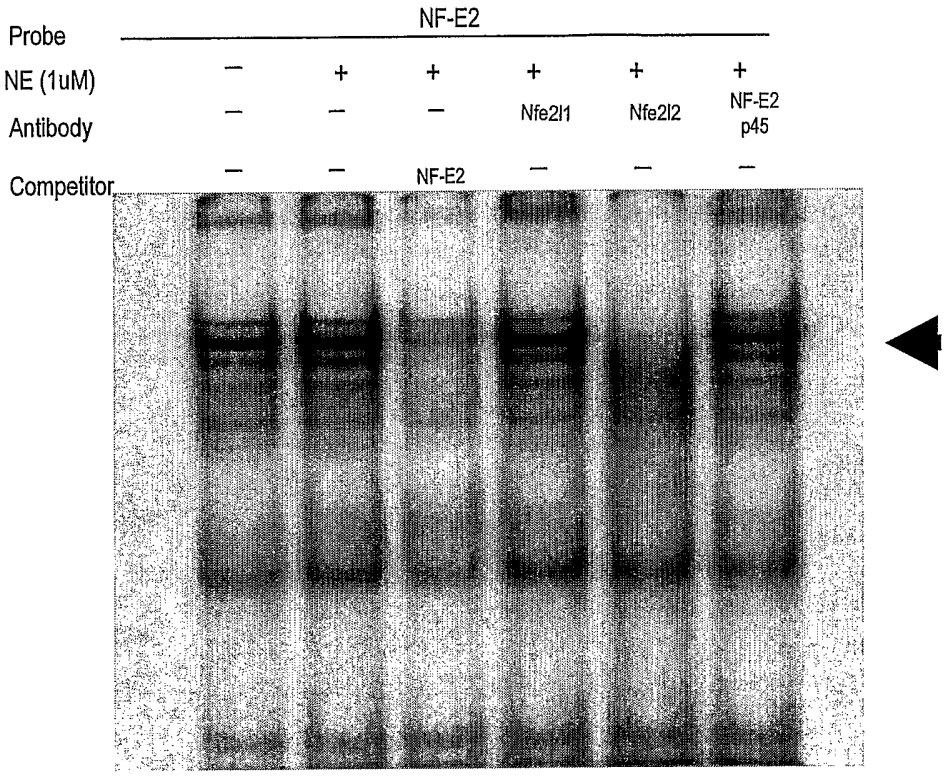

Fig. 5A

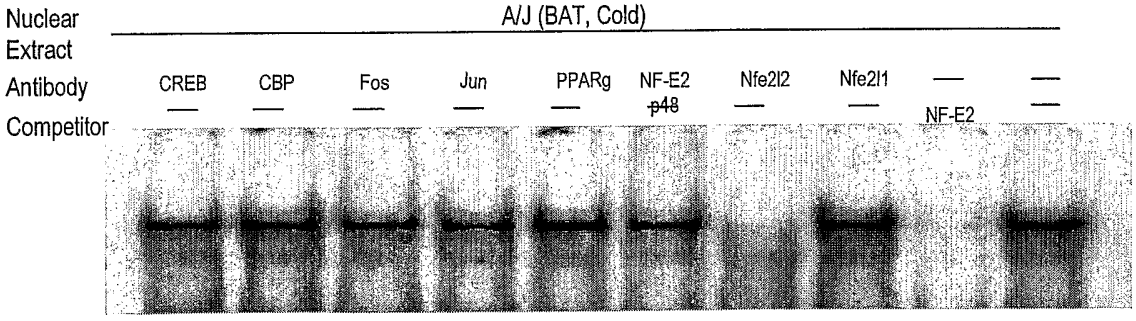

Fig. 5B

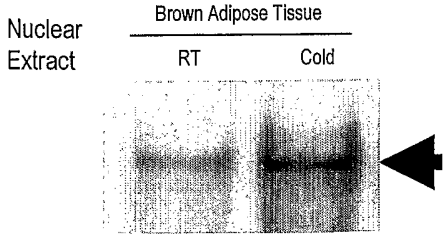

Fig. 5C

Fig. 6A

NFCRE tgaactagtcgtcacctttt
NF-E2 CRE2
NF-E2 gggactagtcgtcgggg
CRE2 gggctagtcgtcaccc

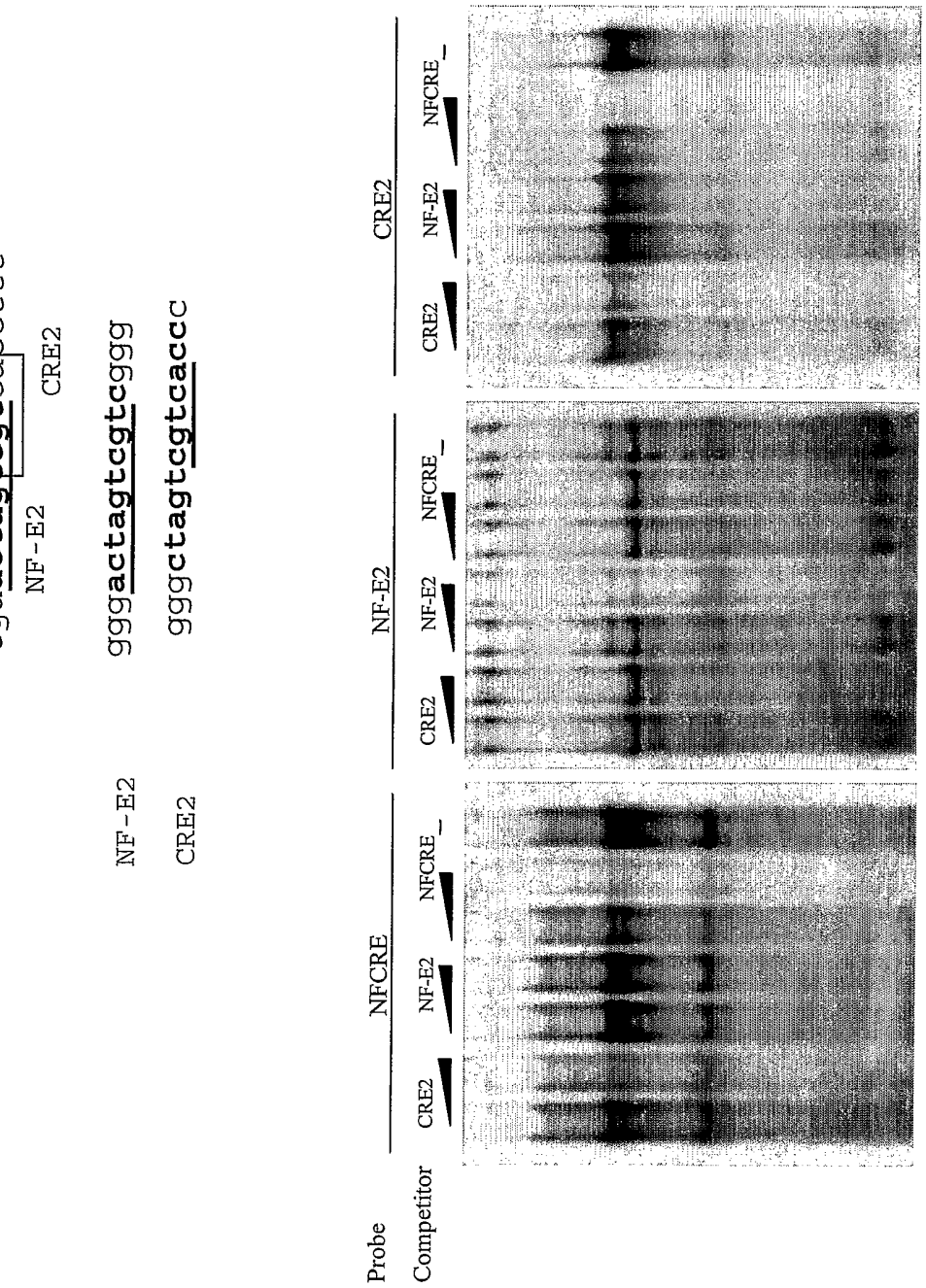

Fig. 6B

```
Human   -3762  agaacttgct gccactcctt tgctacgtca taaagggtca gttgcccttg
Mouse   -2531  gaagcttgct gtcactcctc tacagcgtca cagagggtca gtcacccttg
Rat     -2517  gaacctgct gcctctcctt tgcgacgtca cagtgggtca gtcacccttg
                       NF-E2              CRE3                PPRE

Human          ctcatactga cctattcttt acctctctgc ttcttctttg tgccagaaga  -3662
Mouse          accacactga actagtcgtc acctttccac -tcttc---c tgccagaa--  -2431
Rat            atcacactgc accagtcttc acctttccac -gcttc---c tgccagagca  -2417
                       NF-E2   CRE2
```

Fig. 7A

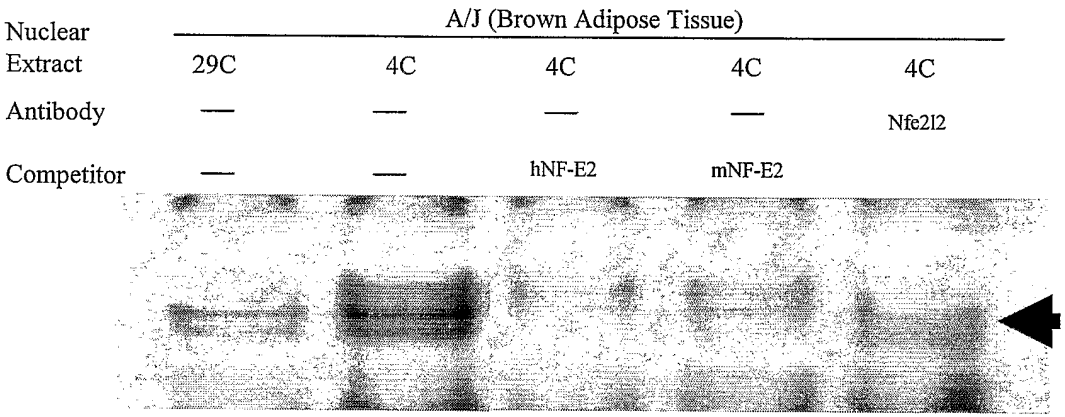

Fig. 7B

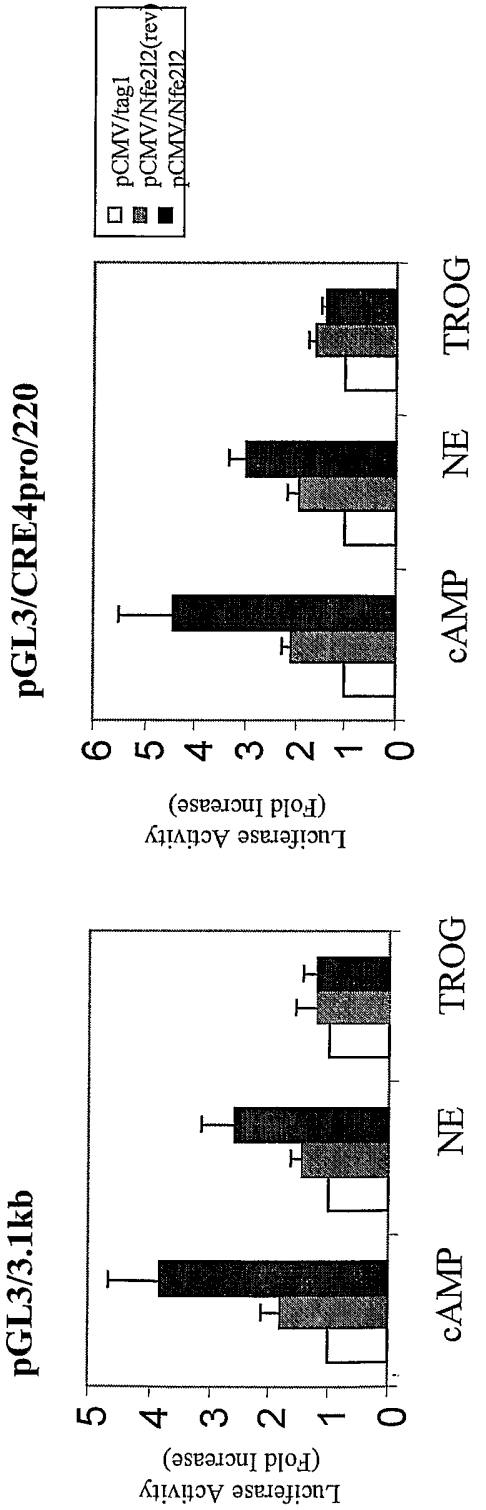
pGL3/3.1kb
Luciferase Activity
(Fold Increase)
0
1
2
3
4
5
cAMP
NE
TROG

Fig. 8A

pGL3/CRE4pro/220
Luciferase Activity
(Fold Increase)
0
1
2
3
4
5
6
cAMP
NE
TROG
pCMV/tag1
pCMV/Nfe212(rev)
pCMV/Nfe212

Fig. 8B

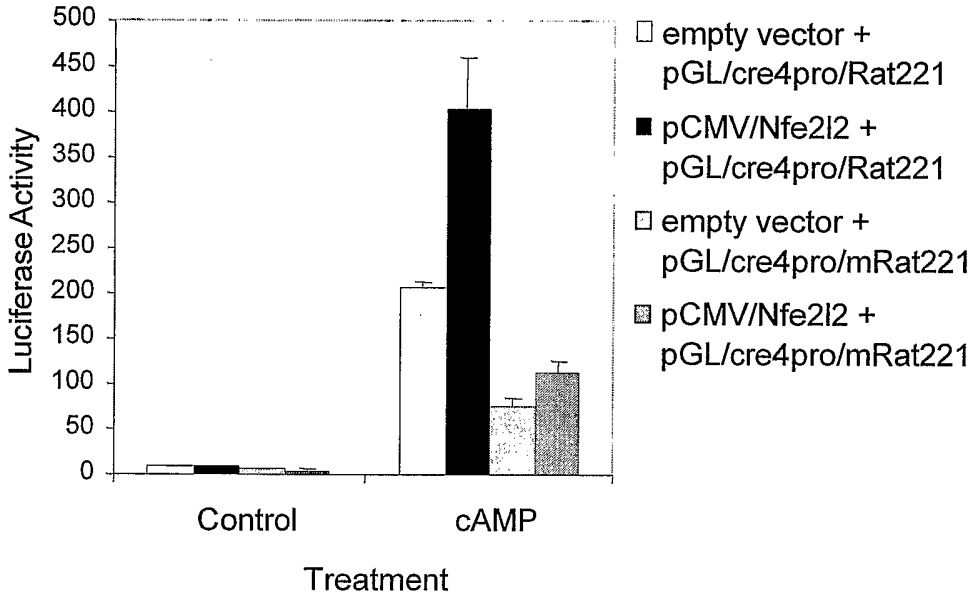
500
450
400
350
300
250
200
150
100
50
0
Luciferase Activity
Control
cAMP
Treatment
empty vector + pGL/cre4pro/Rat221
pCMV/Nfe2l2 + pGL/cre4pro/Rat221
empty vector + pGL/cre4pro/mRat221
pCMV/Nfe2l2 + pGL/cre4pro/mRat221

Fig. 9

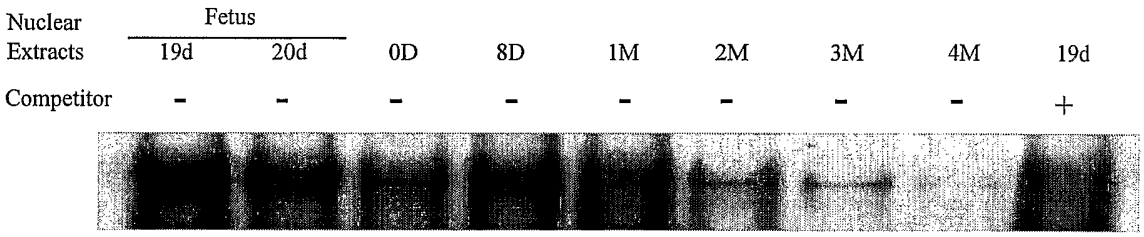

Fig. 10A

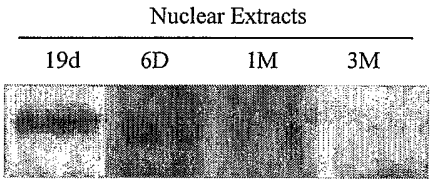

Fig. 10B

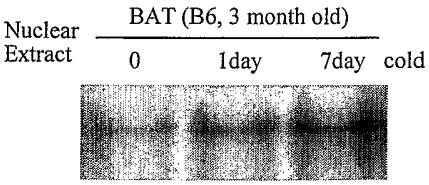

Fig. 10C