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Construction and Characterization of Novel Expression Vectors for Genetic Adipose Tissue Ablation

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Obesity, one of the most common metabolic diseases in industrial countries is characterized by an increase in the number or size of adipocytes. In an effort to create transgenic mouse models for the study of obesity, we developed a novel technique in which adipose tissue can be ablated genetically at will, at any specific developmental stage and/or physiological condition, by the treatment of ganciclovir. We made a series of adipocyte-specific expression vectors using minimal regulatory regions of brown adipocyte-specific uncoupling protein (UCP-1) gene and adipocyte-specific aP2 gene, and then analyzed their expression characteristics in cultured cell lines. When both constructs pUCP-LacZ and paP2-LacZ were transfected transiently into differentiating 3T3-L1 (pre-white adipocytes) and HIB-1B (pre-brown adipocytes) cell lines *in vitro* and then monitored by X-gal staining of cells, these regulatory regions were sufficient to show proper differentiation stage-specific expression in adipocytes. To confirm that adipocytes expressing HSV-TK controlled by these minimal regulatory elements are sufficient to kill themselves with ganciclovir treatment, pUCP-TK and paP2-TK expression constructs were transfected stably into HIB-1B and 3T3-L1 cells, respectively, and their ganciclovir sensitivities were tested during *in vitro* differentiation of cells. As expected, more than 80% of cells were dead by the 7th day of treatment with ganciclovir, while negative control cells were not affected at all. The data suggest that the constructed vectors are suitable for obtaining novel obese transgenic models based on a conditional genetic tissue ablation method.

Obesity, an excessive accumulation of adipose tissue by an increase in the number and/or size of adipocyte, is one of the most common metabolic diseases in the industrial world, and a major risk factor for serious health problems, such as non-insulin dependent adult-onset diabetes mellitus, hypertension, hyperlipidemias, atherosclerosis. These problems in turn can cause ischaemic heart disease, stroke and premature death (Grundy and Barnett, 1990; Spiegelman et al., 1993; Kuczmarski et al., 1994; Flier, 1995). In most populations, obesity is more common among women than men and is a multifunctional phenotype, which may result from a complex network of genetic and non-genetic factors. Thus the underlying mechanisms behind imbalance in energy intake and energy expenditure that lead to obesity are still controversial.

There have been several animal models in which obesity has been shown to be inherited as a genetic trait (Friedman and Leibel, 1990), and the last several years have seen progress in two obesity-related areas of investigation; the identification of genomic loci at which mutations cause obesity in rodents, and the identification and characterization of transcription factors that regulate the differentiation, pattern of gene expression, and lipid content of fat cells (see Spiegelman and Flier, 1996, for review).

A current model of a homeostatic cycle for the control of energy balance (Spiegelman and Flier, 1996) is shown in Fig. 1. The white adipocyte produces leptin, an *ob* gene product, as a function of adipose energy storage. Leptin acts through receptors in the hypothalamus, where actions are initiated that control food intake and alter autonomic output, to control appetite, thermogenesis (brown adipose tissue activity), and hormone secretion and action via sympathetic nervous system output and neuroendocrine function (including reproduction and thyroid hormone). Although the consequences of the absence of leptin signaling are clear, determining the normal physiological role for leptin signaling requires further study. Furthermore, in the great majority of obese humans and in most rodent models, most obesity is leptin resistant (Maffei et al., 1995). This suggests that the regulatory mechanism of

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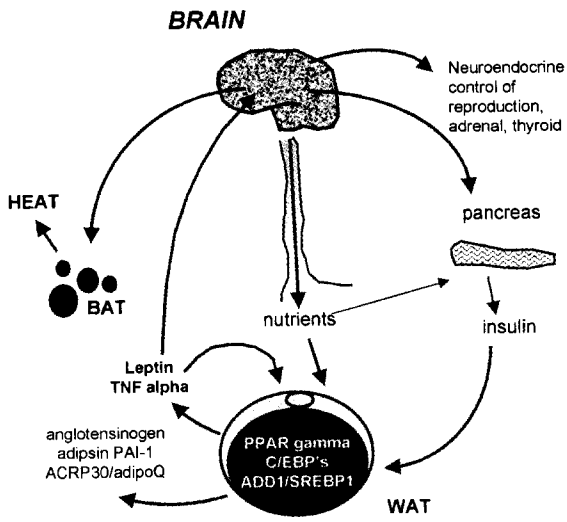

Fig. 1. A current model showing homeostatic cycle for the control of energy balance (Quoted from Spiegelman and Flier, 1996). SNS, sensory nervous system; WAT, white adipose tissue; BAT, brown adipose tissue.

obesity remains to be solved. Because the available genetic models are still limited in number, and because many obese animals exhibit decreased fertility, attributable to both physical constraints and inappropriate gonadotropic secretion stemming from hypothalamic dysfunction (Bray and York, 1979), more genetic obese mouse models are required.

There have been several transgenic mouse models generated by a method of deleting specific cell lineages that entails microinjection into fertilized eggs of a chimeric gene in which lineage-specific regulatory elements is used to drive the expression of a toxin gene product, such as diphtheria toxin A (DT-A) polypeptide (Palmiter et al., 1987; Breitman et al., 1987). These animal models resulted from systematic dysfunction of specific organs. For example, brown adipose tissue deficient transgenic mice, generated by transfection of DT-A regulated by 850 bp 5'-distal UCP-1 gene regulatory region, showed obesity (Lowell et al., 1993). Reduced adiposity was also accomplished by targeted expression of an attenuated DT-A chain to adipose tissue, using 5.4 kb 5' regulatory region of the adipocyte P2 (aP2) gene (Ross et al., 1993). However, even though these DT-A-based genetic tissue ablation models provide new insights into the physiological significance of obesity, they at best provide limited function as the majority of mice show prenatal death or infertility, and survivors often do not have a specific organ from birth. If we can induce tissue ablation under physiological conditions as well as at specific developmental stages, the resulting mouse model will be more versatile for studies of physiology, pathogenesis (e.g., for obese research, what are the adaptations to knockout or overexpression of individual obese-related genes, and how do these potential adaptations interact with genetic background and environmental conditions such as diet and stress?) and for devising eventual therapy.

We developed a novel technique in which adipose tissue could ablated genetically at will, at any specific developmental stage and/or at any specific physiological condition by exploiting the herpes simplex virus thymidine kinase (HSV-TK) gene as a toxin gene. An advantage of incorporating the HSV-TK gene to effect cell death is that thymidine kinase is not toxic itself, and therefore offers a conditional killing mechanism for proliferating cells by treatment with ganciclovir. In this study, as a first step toward generating an obese mouse model based on conditional genetic tissue ablation, we constructed brown adipocyte-specific and adipocyte-specific LacZ expression vectors by employing minimal regulatory elements of UCP-1 or aP2 genes. We then tested whether the transgenes show proper developmental stage-specific and adipose cell-specific expression in differentiating preadipose cell lines. After making HSV-TK expression vectors by emplying minimal regulatory elements characterized above, we also tested whether the level of HSV-TK gene expression is sufficient to kill HSV-TK expressing cells by ganciclovir treatment.

Materials and Methods

Oligonucletides and polymerase chain reaction (PCR)

Oligonucleotides used in this study are as follows: **UCPE1**, 5'-AAG CTT GCT GTC ACT CCT CT-3'; **UCPE2**, 5'-TCT AGA GTC TGA GGA AAG GG-3'; **UCPP1**, 5'-CAT GCA GCT CTT TGG AGA CCT GGG-3'; **UCPP2**, 5'-CCT GGC TTG GAG GGC AGA GA-3'; **aP2E1**, 5'-AGC TAT TTC ACC CAG AGA GAA GGG ATT G-3'; **aP2E2**, 5'-AGC TCA ATC CCT TCT CTC TCT GGG TGA AAT-3'; **aP2P1**, 5'-AGT CAA AAC AGG AAC CTT TAA AA-3'; **aP2P2**, 5'-GTA AAC CTT CGA GGA GGA GC-3'; **TK**, 5'-GCC ACC AAG ATC TGC GGC AC-3'.

PCR was performed at standard reaction conditions for 35 cycles (denaturation at 94℃ for 1 min, annealing at 55℃ for 30 sec, and elongation at 72℃ for 2 min and 30 sec) by using 100 ng of genomic DNA and 1 U of Dynazyme (Finnzymes Oy).

Construction of adipocyte-specific expression vectors

The adipose tissue-specific expression vectors were constructed by juxtaposing a reporter gene to the core enhancer and promoter region of the adipose tissue-specific aP2 gene (see Fig. 2). A dimer of double stranded oligonucletides corresponding to ARE6 of the aP2 enhancer and a PCR-amplified promoter fragment (238 bp corresponding a region from - 170 to +67) of the aP2 gene (Graves et al., 1992) were subcloned into a HindIII site of pBluescript II -KS (+) and pCR II vectors, respectively. The HindIII/EcoRI enhancer fragment was moved into a HindIII/EcoRI site of a promoter-containing vector, yielding the pPBS-aP2. The paP2-

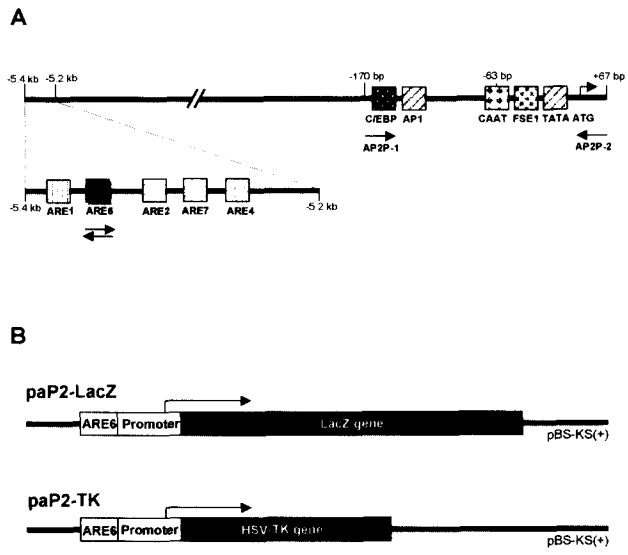

Fig. 2. Construction of adipocyte-specific expression vectors. A, Map of 5'-flanking region of the murine aP2 gene. The *cis*-acting elements in the enhancer and promoter regions of aP2 gene are marked with filled boxes. The coordinates are from the data of Ross et al. (1990) and the transcription start site is indicated by a bent arrow. Horizontal arrows below the map indicate the ds oligonucleotides used for the cloning of ARE6 enhancer sequences and the primers for the PCR cloning of promoter sequences, respectively. B, A schematic representation of the adipocyte-specific expression vectors, paP2-LacZ and paP2-TK. Dimer of ARE6 enhancer sequences and proximal promoter of the aP2 gene are linked to *E. coli* β-galactosidase gene or HSV-TK gene. The transcription start site is indicated by a bent arrow.

LacZ expression vector was constructed by inserting a 3.4 kb BamHI/BglII fragment of LacZ gene from pCMV-βgal into a BamHI site located at the 3' end of UCP promoter in pBS-aP2. paP2-TK expression vector was constructed by inserting a 2.7 kb BamHI/BglII fragment of promoterless HSV-TK gene from ptk03tk into a BamHI site located at the 3' end of aP2 promoter in pBS-aP2.

The brown adipose tissue-specific expression vectors were constructed by linking a reporter gene to the regulatory regions of the brown adipocyte-specific UCP-1 gene (see Fig. 4). The enhancer (220 bp corresponding to a region from -2,530 to -2,310) and the promoter (340 bp corresponding to a region from -98 to +230) fragments were PCR-amplified from genomic DNA using the UCPE1/ UCPE2 primer set and the UCPP1/UCPP2 primer set, respectively. The resulting PCR products were subcloned into a PCR II vector (TA cloning, Invitrogen). The HindIII/EcoRI fragment (for enhancer) and EcoRI fragment (for promoter) were sequentially subcloned into pBluescript II KS(+) (Stratagene), and then it was named pBS-UCP. pUCP-LacZ expression vector was constructed by inserting a 3.4 kb BamHI/BglII fragment of the LacZ gene from pCMV-βgal into a BamHI site located at the 3' end of UCP promoter in pBS-UCP. pUCP-TK expression vector was constructed by inserting a 2.7 kb BamHI/BglII fragment of the hsv-tk gene from ptk03tk into a BamHI site at the 3' end of UCP promoter in pBS-UCP.

Cell culture and cell differentiation

3T3-L1 preadipocytes were maintained in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum (Hyclone). Differentiation was induced by treating the 2-day post-confluent cell (designated day 0) with DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone), 5 μg/ml insulin, 1 μM dexamethasone (DEX) and 0.5 mM methylisobutylxanthine (MIX) for 48 h as described (Reed and Lane, 1980). The medium was then replaced with DMEM supplemented with 5 μg/ml insulin and 10% FBS. The appearance of cytoplasmic triglyceride droplets as the cells differentiated into adipocytes was visualized by staining with Oil-Red-O (Preece, 1972).

A brown adipose tumor cell line, HIB-1B, can be differentiated into UCP-expressing brown adipocytes. HIB-1B preadipocytes, kindly provided by Dr. Spiegelman in Harvard Medical School, were cultured and differentiated essentially as described (Ross et al., 1992). Briefly, HIB-1B preadipocytes were maintained in DMEM supplemented with 10% calf serum (Hyclone). Differentiation was induced by treating the 2-day post-confluent cell with DMEM supplemented with 10% FBS, 5 μg/ml insulin, 1 μM DEX and 0.5 mM MIX for 48 h. The medium was then replaced with DMEM supplemented with 5 μg/ml insulin, 1 nM T3, and 10% FBS. The appearance of cytoplasmic triglyceride droplets was visualized by staining with Oil-Red-O.

Primary cell culture of brown and white adipocytes were prepared according to Poissonnet et al. (1988). Briefly, interscapular brown adipose tissue and inguinal white fat from a 3-month-old mouse (ICR strain) were dissected. The tissues were thoroughly minced and digested with collagenase. After isolation from the whole tissue, the stromal cells were filtered through a 80 μm-mesh nylon filter. The cells from both tissues were plated separately at a density of 6×10^5 cells per 100 mm dish in a differentiation medium (DMEM supplemented with 10% FBS, 5 μg/ml insulin, 1 μM DEX and 0.5 mM MIX). Once the monolayer reached confluence (4~5 day after plating), the appearance of cytoplasmic triglyceride droplets was visualized by staining with Oil-Red-O.

Cell transfection, selection, and β-galactosidase assay

Differentiation of the 3T3-L1 and HIB-1B preadipocytes was set up in 100 mm dishes, and transient transfection was performed by the modified calcium phosphate coprecipitation method with 30 μg of supercoiled vectors as described (Wigler et al., 1979). Primary cultured adipocytes were transfected at confluence (4~5 day after plating) in the differentiation medium. All transfections were done in duplicate and repeated at least twice. The level of lacZ gene expression was determined by X-gal staining of cells. For β-galactosidase assay, cells were rinsed with phosphate buffered saline

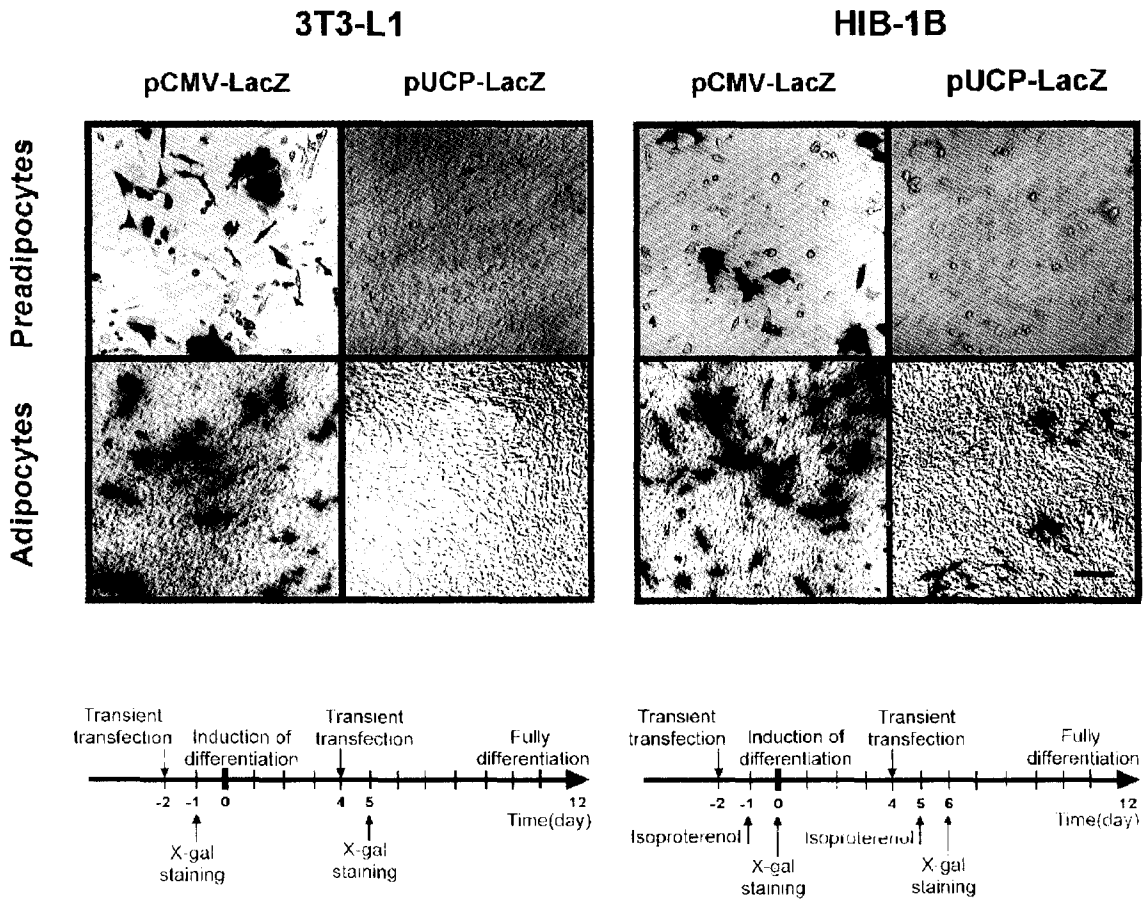

Fig. 3. Adipocyte-specific and differentiation-dependent expression of the paP2-LacZ in cultured cell lines. paP2-LacZ construct (30 ug) is transiently transfected into differentiating 3T3-L1 (A) and HIB-1B (B) cell lines *in vitro* as schematically indicated below the figures, respectively. During the differentiation of HIB-1B cells, isoproterenol (1 uM) is supplemented into medium 24 h after transient transfection of construct. Expression of β -galactosidase gene is monitored by X-gal staining at specific times as indicated. As a positive control, pCMV-LacZ construct is also analyzed, separately. Bar=100 um.

(PBS) and fixed for 2 min with 2% formaldehyde and 0.1% glutaraldehyde. Then they were stained with staining solution containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide.

For stable transfection, 3T3-L1 and HIB-1B preadipocytes were cotransfected at 80% confluence with 20 μg of linearized HSV-TK expression vector (e.g., paP2-TK and pUCP-TK) and 2 μg of linearized pRSV-neo by calcium phosphate coprecipitation method. After selection with 500 μg/ml of G418 for 10 to 14 days, the resistant clones were subsequently isolated and propagated into cell lines in DMEM containing 10% FBS and 250 μg/ml of G418. Chromosomal integrity of HSV-TK gene was monitored by genomic PCR.

Ganciclovir sensitivity assay

Aliquoted cells (at a density of 10^5 cells per well) in 6 wells/group were differentiated in the presence or absence of ganciclovir (20 ug ganciclovir/ml of medium was added starting from -2 day of differentiation initiation), and then the surviving cells at days of -2, 0, 4, 7, and 10 were counted from one well per group per day. The cells were harvested using trypsin and counted in a hemocytometer. Trypan blue exclusion was used to assess the percentage of viable cells. The numbers of surviving cells were expressed as a percentage of the number of cells in untreated cultures.

Results

Adipocytes play a central role in lipid homeostatis and the maintenance of energy balance. These cells store excess energy in the form of triglycerides during periods of nutritional abundance and release it in the form of free fatty acids at time of nutritional deprivation. Furthermore, as a function of adipose energy storage, white adipocytes send signals (leptin) to hypothalamus to coordinate energy balance, thus reducing appetite and burning out extra energy in brown adipocytes. Obesity animal models have been used as a critical genetic tool for the understanding of

pathogenesis and pathophysiology of disease. To create an useful transgenic model not only for the study of obesity but also for the development of diagnosis and treatment of human obese syndrome and related diseases, we made a strategy in which adipose tissue could be ablated genetically at will, at any specific developmental stages and/or physiological condition by treatment of ganciclovir. Here, we have constructed two kinds of adipocyte specific HSV-TK expression vectors by using the minimum regulatory elements of adipocytes-specific genes. Before attempting to generate transgenic mice directly, we have analyzed at first their expression properties in cultured preadipose cell lines as well as in mouse primary cultured adipocytes to know whether they would be sufficient to show proper differentiation stage specific expression in adipocytes as well as to know whether the levels of expression would be sufficient to kill the cells themselves by the treatment of ganciclovir.

Construction and characterization of the adipocyte-specific expression vectors

Vertebrates possess two distinct types of adipose tissue, brown and white. White adipose tissue stores and releases fat according to the nutritional needs of the animal, whereas brown adipose tissue burns fat, releasing the energy as heat. To target the expression of HSV-TK gene to both adipose tissues, we have chosen the regulatory regions of aP2 gene.

The lipid binding protein aP2 gene is expressed only in adipose cells (Bernlohr et al., 1985; Zezulak and Green, 1985). Previous studies have shown that the proximal promoter region (168 or 247 bp of the 5'-flanking region) and enhancer region (183 bp fragment at -5.4 kb to -5.2 kb) are sufficient to direct gene expression to the adipose tissue of transgenic mice as well as differentiation-dependent gene expression in cultured adipocytes (Cook et al., 1988; Distel et al., 1987; Graves et al., 1991; Ross et al., 1990). Both the AP1 and C/EBP binding sites are essential for expression from the aP2 proximal promoter (Christy et al., 1989; Herrera et al., 1989). Among several cis- and trans-acting components that contribute to the activity of the aP2 enhancer, ARE6, a binding site of a novel adipocyte differentiation-dependent factor ARF6 (also known as RXRα/PPARγ, Tontonoz et al., 1994, 1995) was demonstrated to be a key regulatory enhancer element (Graves et al., 1992; see also Fig. 2A). Therefore, we exploited these minimum regulatory elements (ARE6 region of the enhancer and proximal promoter) of the aP2 gene for the construction of adipocyte-specific expression vectors (Fig. 2B). ARE6, multimerized two copies in tandem, was linked to a minimal aP2 promoter to drive the expression of reporter genes, lacZ or HSV-TK (see Materials and Methods), and then resulting vectors were named paP2-LacZ and paP2-TK, respectively.

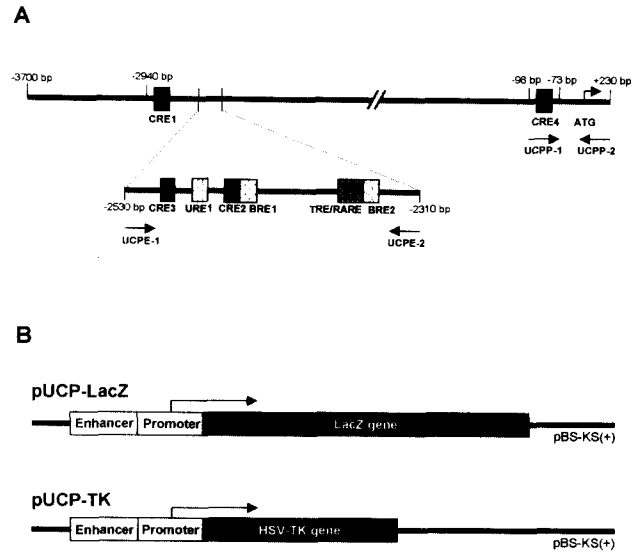

Fig. 4. Construction of brown adipocyte-specific expression vectors. A, Map of 5'-flanking region of the murine UCP-1 gene. The region of the UCP-1 gene from -3.7 kb to +230 bp is shown. The coordinates are from the data of Kozak et al. (1994) and the transcription start site is indicated by a bent arrow. The 220 bp enhancer region is enlarged, where the *cis*-acting elements are marked with filled boxes. Horizontal arrows below the map indicate the positions of oligonucleotide primers used for the PCR cloning of minimal enhancer sequences and promoter sequences, respectively. B, A schematic representation of the brown adipocyte-specific expression vectors, pUCP-LacZ and pUCP-TK. PCR-amplified enhancer sequences and proximal promoter sequences of the UCP-1 gene are linked to *E. coli* β-galactosidase gene or HSV-TK gene. The transcription start site is indicated by a bent arrow.

At first, we attempted to determine whether the exploited regulatory regions were sufficient to drive the transgene to adipocyte-specific expression. paP2-LacZ construct was transiently transfected into the differentiating preadipocyte cell lines, 3T3-L1 and HIB-1B, *in vitro*. The 3T3-L1 preadipocyte cell line, originally derived from mouse fibroblasts (Green and Kehinde, 1974), could be induced to differentiate by exposure of a confluent population of cells to Dex, MIX, and insulin. The differentiation is marked by a major change in cell morphology that includes the accumulation of large lipid droplets and the induction of a program of adipocyte-specific genes (Wu et al., 1995). HIB-1B cell line, originally derived from a hibernoma generated in a transgenic mouse through expression of the SV40 T antigen under the control of the aP2 5'-flanking region (Ross et al., 1992), readily differentiates into brown adipocytes that express the UCP-1 gene when treated with several natural or synthetic β-adrenergic agonists or when treated with insulin, DEX, MIX, and isoprotenenol (Ross et al., 1992).

As shown in Fig. 3, in the transient transfection of paP2-LacZ, the X-gal positive cells appeared in differentiated but not in undifferentiated preadipocytes. The proper differentiation of cells was also confirmed by cell morphology as well as by Oil Red O staining (data not shown). On the contrary, when the positive control vector (pCMV-LacZ) was transfected, X-gal positive cells emerged in both undifferentiated and differenti-

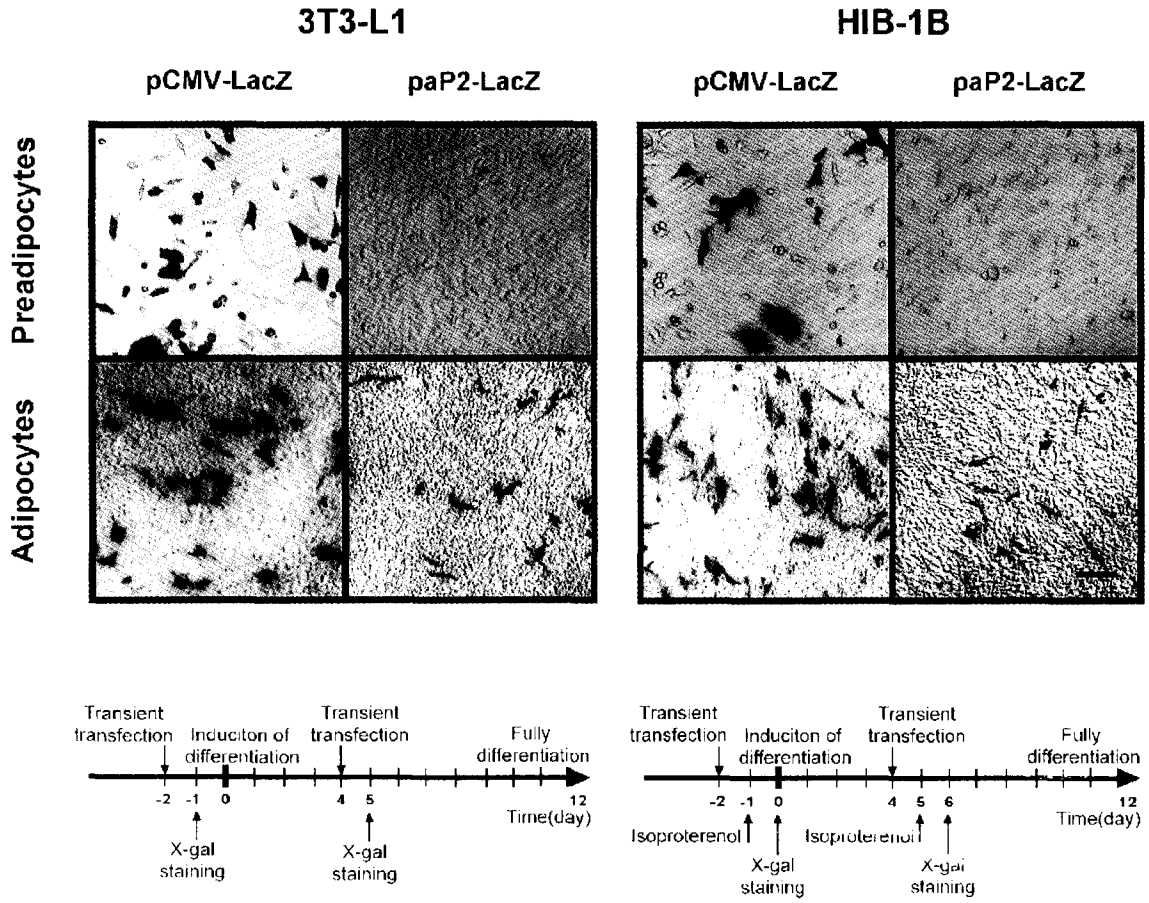

Fig. 5. Brown adipocyte-specific and differentiation-dependent expression of the pUCP-LacZ in cultured cell lines. pUCP-LacZ construct (30 ug) is transiently transfected into differentiating 3T3-L1 (A) and HIB-1B (B) cell lines *in vitro* as schematically indicated below the figures, respectively. During the differentiation of HIB-1B cells, isoproterenol (1 uM) is supplemented into medium 24 h after transient transfection of construct. Expression of β -galactosidase is monitored by X-gal staining at specific times as indicated. As a positive control, pCMV-LacZ construct is also analyzed, separately. Bar=100 um.

ated preadipocytes in both cell lines. Similar results were also obtained in the transient transfections into primary cultured brown and white adipocytes as well as into non-adipose cell lines (e.g., K562, MEL, and E25B2); primary cultured adipocytes were X-gal positive while all of non-adipose cell lines were X-gal negative (data not shown). Therefore, the minimal regulatory regions of the aP2 gene exploited in this study are confirmed to be sufficient to differentiation-dependent adipocyte-specific expression of marker genes.

Construction and characterization of the brown adipose tissue-specific expression vectors

To target the expression of HSV-TK gene to brown adipose tissue, we have chosen the regulatory regions of UCP-1 gene. UCP-1, a brown adipocyte-specific mitochondrial proton carrier, uncouples respiration from oxidative phosphorylation by collapsing the proton gradient established from fatty acid oxidation without concomitant ATP synthesis (Nicholls and Locke, 1984). UCP-1 expression is tightly regulated, primarily by sympathetic nervous systems, in response to physiological signals such as cold exposure and excess caloric intake (Girardier and Seydoux, 1986). Transgenic mice studies have shown that a region from -2.8 to -1.0 kb of the murine UCP-1 gene is required for brown adipocyte-specific expression (Boyer and Kozak, 1991). Subsequent analysis identified a potent 220 bp enhancer at -2.5 to -2.3 kb of the UCP-1 gene (Fig. 4A), which contains a functional cAMP response element (CRE2) and two novel brown adipocyte regulatory factor PPARγ responsive elements (BRE1 and BRE2) (Cassard-Doulcier et al., 1993; Kozak et al., 1994; Sears et al., 1996). For our construction of brown adipocyte- specific expression vectors (see Material and Methods), 220 bp minimal enhancer element was linked to minimal UCP-1 promoter to drive the expression of reporter genes, lacZ and HSV-TK, and then resulting vectors were named individually to pUCP-LacZ and UCP-TK (Fig. 4B).

To see whether the exploited regulatory regions of UCP-1 gene are sufficient to drive the expression of

reporter gene in a manner of brown adipose tissue-specific and differentiation-dependent, pUCP-LacZ constructs were transiently transfected into differentiating preadipocyte cell lines, HIB-1B and 3T3-L1, *in vitro*. To control the transfection efficiency and the expression of β-galactosidase, pCMV-βgal was also transfected into cells independently. As shown in Fig. 5, to the transient transfection of pUCP-LacZ, the X-gal positive cells appeared only in the differentiated HIB-1B cells; β-galactosidase activity was not seen in 3T3-L1 cells at all. Similar results were obtained in the transient transfection of pUCP-LacZ into primary cultured white adipocytes and brown adipocytes of mice (data not shown). Thus, the regulatory regions of the UCP-1 gene exploited in this study are confirmed to be sufficient to show differentiation stage specific and brown adipocytes-specific expression.

The levels of HSV-TK gene expression are sufficient to kill HSV-TK expressing cells themselves to the treatment of ganciclovir

Before attempting to make transgenic mice, we also tested whether the level of HSV-TK gene expression driven by aP2 gene regulatory regions used for the construction of paP2-LacZ vector was sufficient to kill HSV-TK expressing 3T3-L1 cells with ganciclovir treatment. A linearized paP2-TK construct was cotransfected into 3T3-L1 preadipocytes with the pRSV-neo vector and then 10 G418-resistant clones of cells were isolated. After testing the genomic integrity of this construct by genomic PCR, 5 positive clones (3T3/paP2-TK) were identified (data not shown). One of these positive clones was analyzed for ganciclovir sensitivity during differentiation *in vitro*. To control the experiment, both parental 3T3-L1 and stable 3T3-L1 cell lines containing ptgHygCMVTK, in which the HSV-TK gene was constitutively expressed by CMV promoter/enhancer, were used as negative and positive controls, respectively. As shown in Fig. 6A, with treatment of ganciclovir, 3T3-L1/paP2-TK cells began to die at day 0 of differentiation and showed about 90% cell death rate on the 10th day. In contrast, more than 90% of parental 3T3-L1 cells survived during terminal differentiation of cells, while the positive control cell line containing the ptgHygCMVTK vector showed much more sensitivity, such that more than 90% of the cells died on the 4th day of differentiation. Therefore, the profile of cell survival from ganciclovir treatment not only confirms the differentiation-dependent expression of HSV-TK gene, but also suggests that the level of HSV-TK gene expression is sufficient to kill the TK expressing cells themselves.

The level of HSV-TK gene expression driven by UCP-1 gene regulatory regions used for the construction of pUCP-LacZ vector was also found to be sufficient to kill HSV-TK expressing HIB-1B cells themselves to the treatment of ganciclovir (Fig. 6B). Similar to the

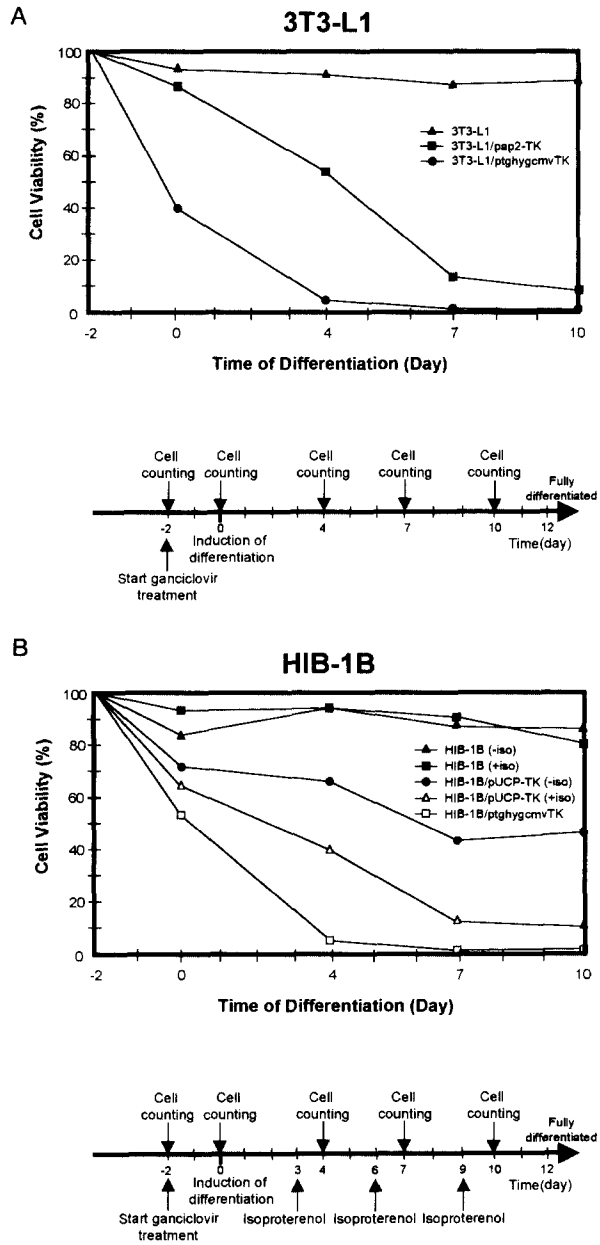

Fig. 6. Ganciclovir sensitivity of the stable 3T3-L1/paP2-TK (A) and HIB-1B/pUCP-TK cell lines (B) during adipogenesis *in vitro*. As shown schematically at the bottom of figure, ganciclovir (20 ug/ml) is supplemented to the culture medium from - 2 days of differentiation, and then the surviving cells are harvested and count at - 2, 0, 4, 7, and 10th day of differentiation. The differentiation of HIB-1B and HIB-1B/pUCP-TK cell lines is induced in the presence (+iso) or absence (-iso) of 1 uM isoproterenol. The numbers of surviving cells are expressed as a percentage of the number of cells in untreated cultures. To control the experiments, both parental cells as well as ptgHygCMVTK transfected cells are also used as negative and positive controls, respectively.

analysis of paP2-TK in 3T3-L1 cells, a linearized pUCP-TK construct was cotransfected into HIB-1B preadipocytes with pRSV-neo vector, and then 7 G418-resistant clones of cells were isolated. After testing the genomic integrity of this construct by genomic PCR, 2 positive clones were identified (data not shown). One positive

clone was further analyzed for its ganciclovir sensitivity in the presence or absence of 1 uM isoproterenol, an inducing agent of UCP-1 gene expression, during differentiation. Both parental HIB-1B and stable HIB-1B cell lines containing the ptgHyg CMVTK construct, were used as negative and positive controls, respectively. As shown in Fig. 6B, in the presence of ganciclovir and isoproterenol, differentiating HIB-1B/pUCP-TK cells showed about 90% cell death rate that corresponded to the value in between parental cells and HIB-1B/ptgHygCMVTK cells; while more than 80% of parental cells survived from the treatment of ganciclovir during differentiation, no cells containing ptgHygCMVTK were survived at 4th day of differentiation. In the absence of isoproterenol, the death rate of HIB-1B/pUCP-TK cells was alleviated up to 40%. Thus, the regulatory region of UCP-1 gene in our construct is not only sufficient to show brown-adipocyte-specific and differentiation-dependent expression of HSV-TK gene, but also suggests that the level of HSV-TK gene expression is sufficient to kill the TK expressing cells themselves.

Discussion

In this study, to examine the feasibility of adipocyte-specific TK expression vectors for the construction of novel transgenic mice models for the study of obesity, we constructed two different kinds of β-galactosidase expression vectors (paP2-LacZ and pUCP-LacZ) and two different kinds of TK expression vectors (paP2-TK and pUCP-TK), and then we characterized their expression profiles in two different preadipocyte cell lines (3T3-L1 and HIB-1B) before and after induction of differentiation *in vitro*.

First, we constructed two kinds of expression vectors by using minimal enhancer and proximal promoter region of aP2 and UCP-1 genes, respectively, to drive *E. coli* LacZ genes (Figs. 2 and 4). When the tissue-specificity and differentiation-dependency were tested by transient transfection of these constructs into differentiating preadipocytes *in vitro*, both constructs showed adipocyte-specific and differentiation-dependent expressions similar to the genes in natural chromosomal context (e.g., β-galactosidase activity in differentiated 3T3-L1 and HIB-1B cells to the paP2-LacZ, and in differentiated HIB-1B cells to the pUCP-LacZ) (Figs. 3 and 5). Therefore, the minimal regulatory regions used for the construction of expression vectors are sufficient to show tissue-specific and differentiation-dependent expression of marker genes. Because the functional binding sites of C/EBP-α and PPARγ are present in the paP2-LacZ construct, the adipocyte-specific and differentiation-dependent expression of LacZ genes in transient transfection of paP2-LacZ corresponds to previous findings that C/EBP-α and PPARγ are sufficient for the induction of adipocyte differentiation of NIH-3T3 fibroblast cells (Yeh et al., 1995). However, the precise delineation of the regulatory element of UCP-1 gene in pUCP-LacZ expressed in brown adipocytes is not yet known, even though 220 bp enhancer elements used in our vector construction contain the PPARγ- RXRα responsive element which is responsible for differentiation-dependent adipocyte-specific expression (Sears et al., 1996).

We also demonstrated that the level of HSV-TK expression driven by these regulatory regions was strong enough to kill the cells themselves with the treatment of ganciclovir (Fig. 6). Even though the cells did not kill themselves completely with the treatment of ganciclovir during the course of induced differentiation of adipose cells *in vitro*, HSV-TK/ganciclovir system has been confirmed to show the bystanding cytocidal effect in which non-expressing cells in proximity to HSV-TK expressing cells are killed with the expressing cells in the presence of ganciclovir (Vrionis et al., 1995). The cytotoxic effect of the HSV-TK/ganciclovir system has been successfully exploited in several tumor models, in some cases with complete regression and long-term animal survival (Borrelli et al., 1988; Caruso et al., 1993; Culver et al., 1992; Ram et al., 1993; Takamiya et al., 1993; Vrionis et al.,1995).

Even though we did not check the copy number of transgenes in our stable cell lines containing paP2-TK or pUCP-2 constructs, the copy number of transgenes might be within a range of several copies based on the intensity of genomic PCR product fragments of HSV-TK gene in the Et-Br stained gel compared to endogenous mouse HPRT gene (data not shown). Whatever the copy number of transgenes is, it may not cause any serious problem for the construction of transgenic obese mice models to induce conditional adipose tissue ablation, as long as the transgene shows the adipocyte-specific and development-dependent expression. In any case, we must choose appropriate transgenic lines from the litters because the level of transgene expression is also affected by the position of chromosomal integration, unless we use a tissue-specific locus control region (Kim et al., 1992; Epner et al., 1992).

Therefore, the data suggest that the constructed HSV-TK expression vectors regulated by minimal regulatory regions of adipocyte-specific genes are useful for the generation of transgenic obese models, where all adipose tissue or only brown adipose tissue can be conditionally ablated by the supplementation of ganciclovir in foods or by injection of ganciclovir into peritoneal cavity at any development stage and/or at any physiological condition of the mouse. Transgenic mice containing paP2-TK or pUCP-TK will be normal unless they intake ganciclovir. These obese models will provide valuable clues for the complicated physiology of obesity, for the contrivance of eventual therapy for the obese and for the exploration of the relationship between obesity and other important clinical correlates, such as hypertension, atherosclerosis, diabetes, infertility and hyperlipidemia. Further usefulness of

these mice models will emerge when they produce offspring by mating with other available obese or other relevant mice models.

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