In vivo promoter analysis on refeeding response of hepatic sterol regulatory element-binding protein-1c expression

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Abstract

Sterol regulatory element-binding protein (SREBP)-1c is the master regulator of lipogenic gene expression in liver. The mRNA abundance of SREBP-1c is markedly induced when animals are refed after starvation, although the regulatory mechanism is so far unknown. To investigate the mechanism of refeeding response of SREBP-1c gene expression in vivo, we generated a transgenic mouse model that carries 2.2 kb promoter region fused to the luciferase reporter gene. These transgenic mice exhibited refeeding responses of the reporter in liver and adipose tissues with extents essentially identical to those of endogenous SREBP-1c mRNA. The same results were obtained from experiments using adenovirus-mediated SREBP-1c-promoter-luciferase fusion gene transduction to liver. These data demonstrate that the regulation of SREBP-1c gene expression is at the transcription level, and that the 2.2 kb 5′-flanking region is sufficient for this regulation. Moreover, when these transgenic or adenovirus-infected mice were placed on insulin-depleted state by streptozotocin treatment, the reporter expression was upregulated as strongly as in control mice, demonstrating that this regulation is not dominated by serum insulin level. These mice are the first models to provide the mechanistic insight into the transcriptional regulation of SREBP-1c gene in vivo.
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The conversion of carbohydrate into fat is known as de novo lipogenesis, which is catalyzed by series of lipogenic enzymes such as acetyl-CoA carboxylase and fatty acid synthase [1]. The activities of these enzymes are nutritionally regulated, i.e. downregulated when animals are starved and upregulated when they are refed. This regulation of these lipogenic enzymes has two remarkable features. First, their overall enzymatic activities largely depend on the amount of expressed protein that is primarily controlled at the transcriptional level. Second, their rates of transcription are coordinately regulated [2]. Therefore, it has been postulated that these genes share a regulatory sequence in their promoters that interacts with common trans-acting factors. In the liver, the most likely factor conducting this coordinate transcriptional regulation has been revealed to be sterol regulatory element-binding protein (SREBP)-1 [3,4].

SREBPs are transcription factors that belong to the basic-helix–loop–helix-leucine zipper (bHLH-Zip) family

and are considered to be profoundly involved in the transcriptional regulation of cholesterogenic and lipogenic enzymes [5]. The role of SREBP-1 in the regulation of lipogenesis in the liver has been well established by several lines of evidence, especially from transgenic and knockout mouse models [4,6–8]. In these models, hepatic SREBP-1 protein levels dominate the mRNA expression levels for a battery of lipogenic genes. Thus, SREBP-1 is now well established as the key transcription factor for the regulation of lipogenic gene expression and by extension triglyceride storage in liver [9,10].

It has been well known that the mRNA and nuclear protein abundance of SREBP-1c, the main isoform of SREBP-1 in lipogenic organs such as liver and adipose tissue, is physiologically regulated by nutrient availability, i.e. it is downregulated when animals are starved and upregulated when they are refed, thereby adjusting lipogenic gene expression levels to the nutritional conditions [11]. Because the quantity of mRNA and nuclear protein of SREBP-1c goes up and down in parallel depending on dietary condition, the control at the mRNA expression level is considered primary at least in regard to refeeding response. However, the mechanism of this regulation is yet to be clarified, including the point whether this regulation is at the transcription level or not.

It has been reported that elevated concentrations of glucose and insulin mimic the refeeding state in vivo and upregulate SREBP-1 expression in cultured hepatocytes [12,13]. However, these responses induced by high dosage of glucose and insulin are within two to three-fold changes at best, and far weaker than the refeeding response observed in the in vivo liver. Therefore, to elucidate the precise mechanism of SREBP-1 regulation by nutrient availability, it is inevitable for one to study the in vivo mouse model.

These situations prompted us to generate transgenic mice that carry SREBP-1c-promoter-driven reporter gene cassette to investigate the nutritional regulation of the promoter activities in vivo. Moreover, based on the results from these experiments, we further assessed the promoter activities using adenovirus-mediated gene delivery to the liver, which complemented the data from transgenic mice

A. Transgene construct
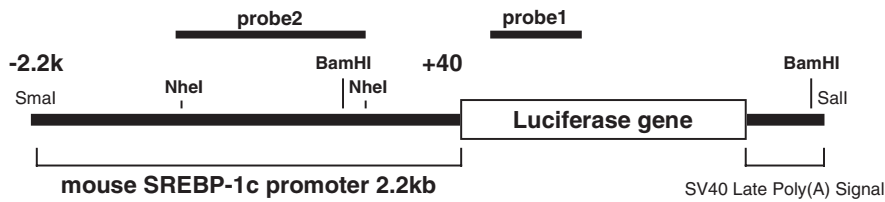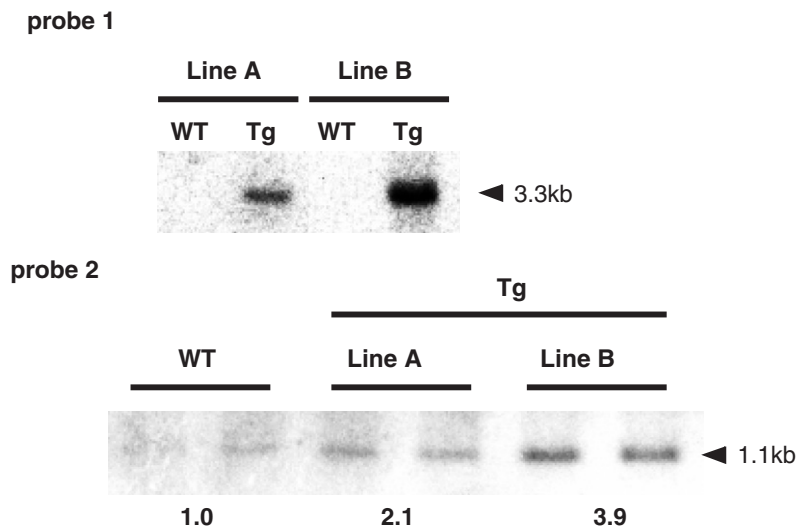

B. Southern blot

Fig. 1. Transgene construct and Southern blot. (A) Construct used to generate −2.2 kb-SREBP-1c-promoter-Luc transgenic mice. The mouse SREBP-1c promoter region spanning −2.2 k to +40 bp was fused to a luciferase reporter gene and SV40 late polyadenylation sequence. (B) Southern blotting of genomic DNA from transgenic mice. Genomic DNA prepared from tail samples of wild-type (WT) controls and two lines (line A and B) of transgenic mice were analyzed by Southern blotting using two distinct probes. For probe 1 (shown in upper panel), a cDNA probe for luciferase gene was used. DNA samples were digested with BamHI. For probe 2 (shown in lower panel), a cDNA probe for mouse SREBP-1c promoter region was used to determine the copy number of transgene. DNA samples were digested with NheI.

by excluding the potential possibility of positional effects in transgenic mouse model.

Materials and methods

Animals and treatment. Seven- to nine-week-old ICR and C57BL/6J male mice were purchased from CLEA. All animals were housed in a temperature-controlled environment with a 12 h-light/dark cycle and free access to standard laboratory diet (MF from Oriental Yeast, composed of 60% carbohydrate, 13% fat, and 27% protein on a caloric basis) and water. For the fasting group, animals were starved 24 h, and for the refeeding group, they were refed at 16 h after a 24 h starvation. To make insulin-depleted states, streptozotocin (100 mg/kg body weight, Sigma) was administered by two intraperitoneal injections with 1-day interval following an overnight fasting period as described previously [14]. Mice were considered diabetic when tail vain blood glucose levels exceeded 350 mg/dl, and euthanized within 8 days from the first injection. All the experiments were repeated more than twice and reproducibility was confirmed.

Production of transgenic mice. pBP1c2600Luc plasmid was constructed into pGL2 basic vector (Promega) as previously described [15]. The SmaI–SalI fragment of this plasmid spanning −2.2 kb to +40 bp of the mouse SREBP-1c promoter fused to luciferase gene was microinjected into BDF1 eggs. Among the 55 offsprings, eight mice had the integrated transgene. Among eight founder mice, two were crossbred to C57BL/6J background more than 5 times, and thus two lines of −2.2 kb-SREBP-1c-promoter-Luc transgenic mice were established.

Generation of recombinant adenovirus. The SmaI–BglII fragment of pBP1c2600bp-Luc containing −2.2 kb to +40 bp of the mouse SREBP-1c promoter was subcloned into the pGL3 basic vector (Promega) at SmaI and BglII sites. Then the SREBP-1c promoter-luciferase gene cassette was cut out of the vector by NotI and SalI, and after both ends were blunted, it was subcloned into the pENTR4 vector (Invitrogen) at the EcoRI site (blunted). Then, −2.2 kb-SREBP-1c-promoter-Luc adenoviral plasmid was generated by homologous recombination between the subcloned pENTR4 plasmid and pAd promoterless vector using Clonase recombinase (Gateway system, Invitrogen). Similarly, SV40-promoter-Luc control adenoviral plasmid was generated from pGL3 promoter vector (Promega). After the transfection into 293A cells, adenoviruses were collected by CsCl gradient centrifugation. Following titer determination by limiting dilution, adenoviruses were injected intravenously into ICR male mice at the dose of 6.0×10^6 P.F.U./body unless otherwise indicated.

Northern blotting. Total RNA from liver and subcutaneous fat pad was isolated with Trizol Reagent (Invitrogen), and 7.5 μg RNA equally pooled among each group was analyzed. cDNA probes were cloned previously [4,16,17]. Blots were exposed to imaging plate for BAS2000 BIO IMAGING ANALYZER (Fuji Photo Film).

Luciferase assays. Tissue samples were homogenized in Reporter Lysis Buffer (Promega) and after centrifugation, luciferase activity in the supernatant was measured by standard kits (Promega) on a luminometer.

Adenoviral DNA isolation and quantification. The pellets obtained in the process described above were digested in SNET buffer (1% SDS, 400 mM NaCl, 5 mM EDTA, and 20 mM Tris–HCl at pH 8.0) containing proteinase K (0.01 mg/ml). After DNA extraction with phenol/chloroform and RNase treatment, the amount of adenovirus DNA was determined with Sybr-Green Dye (ABgene)-based real-time PCR with primers 5′-GTCCGGTTATGTAAACAATCC-3′ and 5′-ATGAAGAAGTGT TCGTCTTCG-3′.

Results and discussion

Generation of transgenic mice carrying SREBP-1c promoter-luciferase fusion gene

To examine the transcriptional regulation of SREBP-1c gene *in vivo*, we have generated transgenic mice carrying the luciferase reporter gene driven by the 2.2 kb SREBP-1c 5′-flanking sequence that we have studied *in vitro* previ-

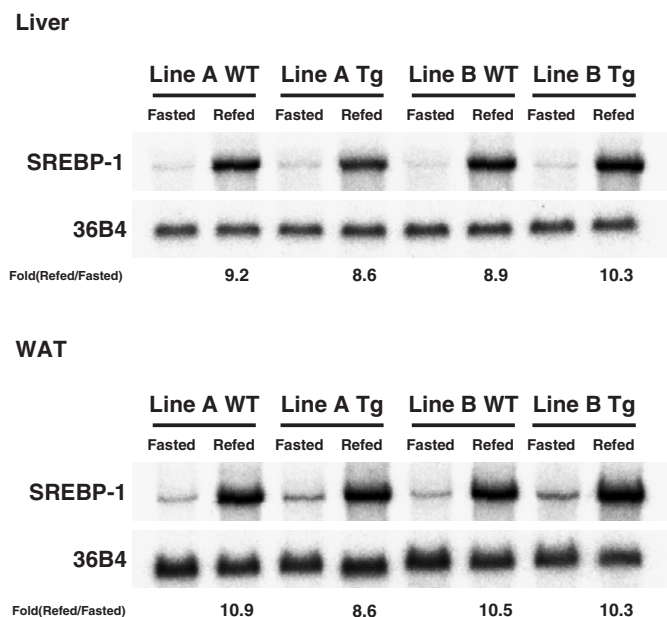## A. Luciferase activities
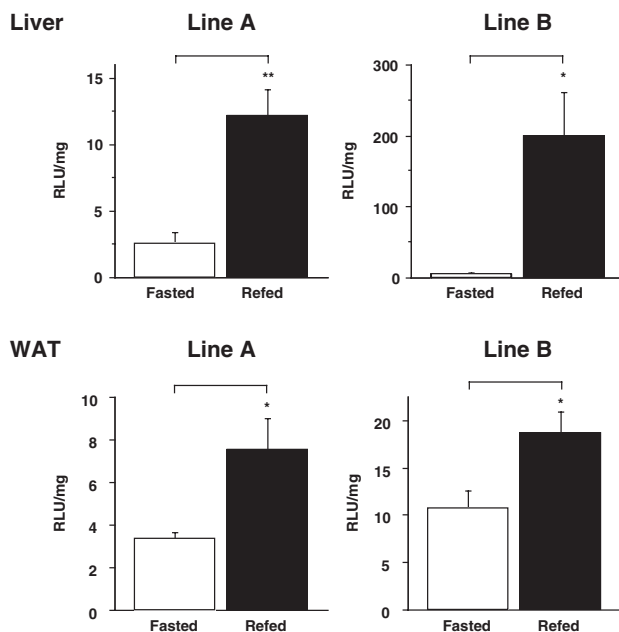
B. Intrinsic gene expression

Fig. 2. Refeeding response of transgenic mice. (A) Transgene expression as assessed by luciferase activities of liver and white adipose tissue (WAT) from two lines of transgenic mice. Transgenic mice were either fasted for 24 h or refed for 16 h after a 24 h fast before euthanized. Each group consists of 4–7 male mice. Results are means ± SE. *$P < 0.05$ and **$P < 0.01$, respectively. (B) Intrinsic gene expression as assessed by Northern blot analyses of wild type (WT) control and transgenic (Tg) mice. The quantification results were obtained with BAS2000 system and normalized to the signal generated from 36B4 mRNA (used as a loading control), and the fold-changes between refed *vs.* fasted states are shown.

ously [15,18]. The genomic structure of SREBP-1 gene has been previously clarified in detail [19], and the SREBP-1c promoter is located at 10 kb downstream of the SREBP-1a promoter. Because refeeding manipulation selectively upregulates SREBP-1c rather than SREBP-1a [20], the responsible element was expected to be within the SREBP-1c 5′-flanking region. Since expression of reporter genes in transgenic animals might be affected by the location of genomic integration, we analyzed two independent founder lines, designated as line A and B. From Southern blotting analyses of genomic DNA (Fig. 1B), line A and B mice were estimated to harbor 2 and 6 copies of transgene per allele, respectively.

Transcriptional activation of the SREBP-1c promoter-driven transgene by refeeding

For these lines of transgenic mice, the expression of reporter gene in liver and white adipose tissue was assessed by luciferase activity. As shown in Fig. 2, the luciferase activities in the livers of both lines were markedly upregulated by refeeding manipulation, which paralleled the responses of endogenous SREBP-1c expression. This result means that the SREBP-1c expression is regulated at the transcription level in this situation, rather than through the variable sta-

bility of mRNA, which is reported to be involved in the regulation by polyunsaturated fatty acids [21].

SREBP-1c induction by refeeding without insulin

We have previously reported that the refeeding response of SREBP-1c does not require insulin [14]. To examine the role of insulin in this transcriptional upregulation of the SREBP-1c by refeeding, we placed the transgenic mice on insulin-depleted state with streptozotocin treatment. As shown in Fig. 3, increase in plasma insulin by refeeding was essentially abolished, but the SREBP-1c promoter activities as assessed by luciferase reporter expression were vigorously induced in the liver accompanied by elevation in blood glucose levels. This result indicates that insulin is not inevitable for the upregulation of SREBP-1c gene transcription by refeeding, consistent with our previous report [14].
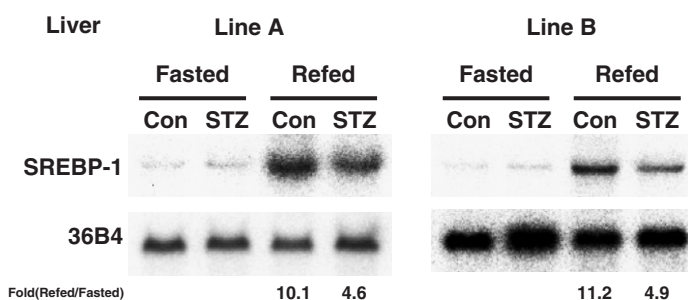
Adenovirus-mediated transduction of SREBP-1c promoter-luciferase fusion gene into the mouse liver and assessment of refeeding response

We further took another strategy for in vivo assay of SREBP-1c promoter activity using an adenovirus vector for introduction of the reporter gene into the liver. We gen-
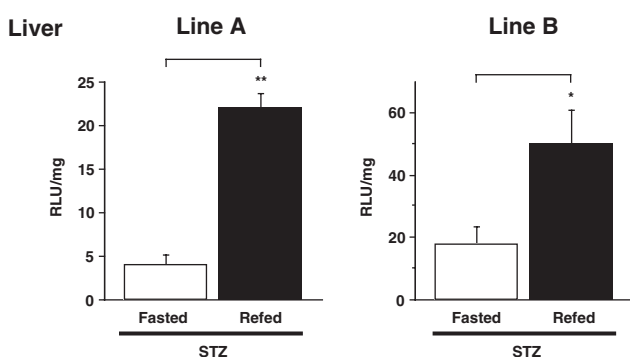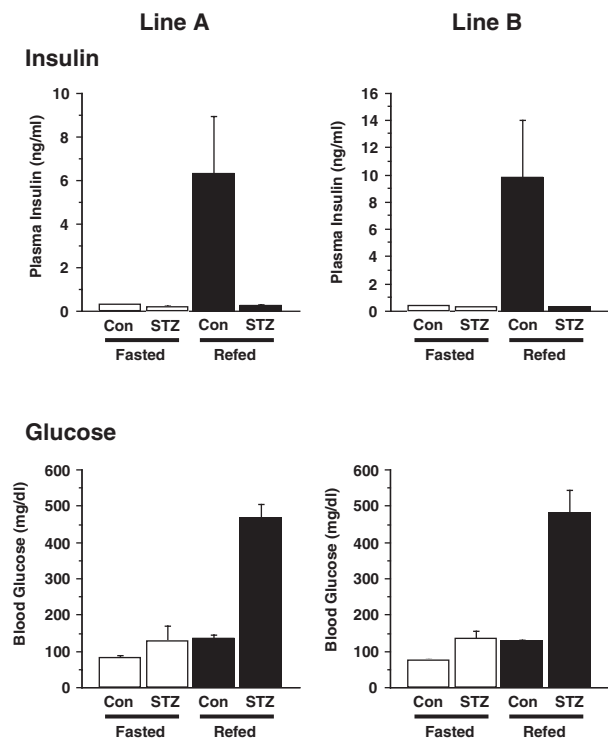

Fig. 3. Refeeding response of streptozotocin-treated transgenic mice. (A) Transgene expression in streptozotocin-treated mouse liver as assessed by luciferase activity. At two days after second administration of streptozotocin, transgenic mice were either fasted for 24 h or refed at 16 h after a 24 h starvation, and euthanized to obtain liver samples. Results are means ± SE of 4–6 male mice. *$P < 0.05$ and **$P < 0.01$, respectively. (B) Northern blot analyses visualizing endogenous SREBP-1 mRNA expression pattern in streptozotocin-treated mouse liver. (C) Blood insulin and glucose levels of streptozotocin-treated transgenic mice.

erated an adenovirus harboring the 2.2 kb promoter region of SREBP-1c fused to the luciferase reporter gene, and validated that intravenously administered adenoviruses are uniformly introduced into the mouse liver as shown in Fig. 4A. Next we examined the refeeding responses of these adenovirus-infected mice expressing SREBP-1c-promoter-luciferase fusion gene in the liver. At the initial estimation, the absolute luciferase activities observed varied among individual mice in a wide range despite the same number of adenovirus particles for injection. As shown in Fig. 4A, it was indicated that the variation among different portions of one liver was negligibly small, thus most of the variation in measured luciferase activities was considered to originate from inter-mouse variation in viral transduction efficiency. Two approaches were taken to cancel this dispersion: (1) the amount of transduced adenoviral DNA was quantified by real-time PCR technique on each

mouse and luciferase activity was adjusted based on this quantification (Fig. 4B). (2) Liver samples on fasted and refed states were taken from the same mouse by biopsy under brief anesthesia and data of fasting and refeeding conditions from one mouse are analyzed consecutively (Fig. 4C). Both approaches produced essentially the same results, and refeeding responses of 20- to 30-fold increase in luciferase activities were consistently observed in a dose-independent way, which paralleled the increases in the endogenous SREBP-1c mRNA (Fig. 4E and F). In contrast, when SV40-promoter-Luc adenovirus was used as a negative control instead of −2.2 kb-SREBP-1c-promoter-Luc adenovirus, no refeeding responses were observed (Fig. 4D).

These results are in complete accordance with those from transgenic mouse model described above. Moreover, the adenoviral approach can avoid the potential involve-
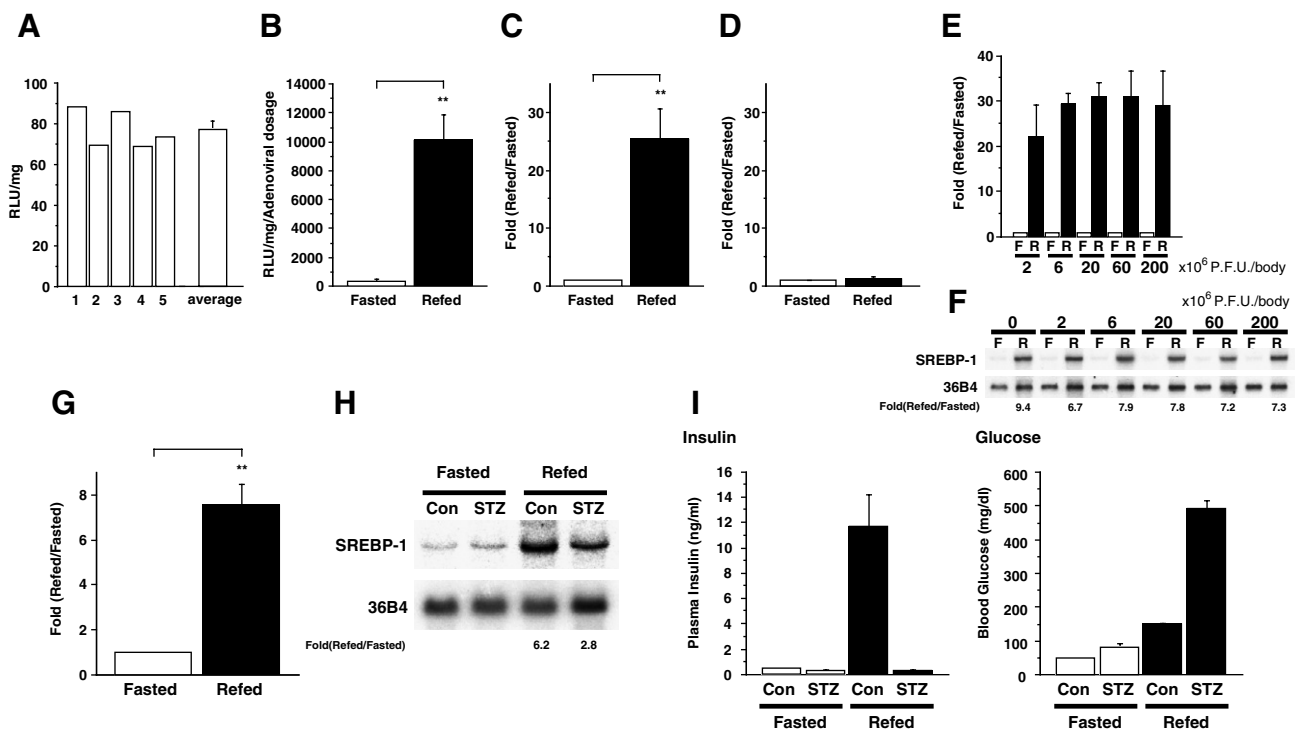

Fig. 4. Adenovirus-mediated transduction of −2.2 kb-SREBP-1c-promoter-Luc into liver and assessment of refeeding response. (A) Validation of homogenous transfer of reporter gene into various portions of liver. Variation within a mouse liver was estimated by sampling 5 pieces of liver from distinct lobes after three days of −2.2 kb-SREBP-1c-promoter-Luc adenovirus injection (6.0×10^6 P.F.U./body). (B) Estimation of refeeding response of luciferase activity adjusted by real-time PCR. Three days after the administration of −2.2 kb-SREBP-1c-promoter-Luc adenovirus, luciferase activities were measured and at the same time, the amount of transduced adenoviral DNA was quantified by real-time PCR technique on each mouse of fasting and refeeding group and luciferase activities per transduced adenoviral DNA were calculated based on this quantification. Results are means ± SE of 4–5 mice. $^{**}P < 0.01$. (C) Pairwise estimation of refeeding response using biopsy method. −2.2 kb-SREBP-1c-promoter-Luc adenovirus-administered mice were fasted for 24 h and then liver samples were taken by biopsy under inhalation anesthesia. After refed for 16 h, mice were euthanized to obtain refed liver samples. Luciferase activities were determined and fold changes of luciferase activities were calculated by dividing data on a refed state by those of the same mouse on a fasted state. (D) Negative control for data shown in (C). SV40-promoter-Luc control adenovirus was used instead of −2.2 kb-SREBP-1c-promoter-Luc adenovirus. (E) Dose independence of refeeding response. Indicated amounts of −2.2 kb-SREBP-1c-promoter-Luc adenoviruses were injected and refeeding responses were assessed by pairwise method. F, fasted; R, refed. Results are means ± SE of three mice. (F) Northern blot analysis indicating that endogenous SREBP-1 expression is not affected by adenovirus administration. (G) Refeeding response of luciferase reporter activity in adenovirus-infected and streptozotocin-treated mouse liver. At two days after second administration of streptozotocin, −2.2 kb-SREBP-1c-promoter-Luc adenovirus (6.0×10^6 P.F.U./body) was intravenously injected into ICR male mice. Three days thereafter, mice were fasted for 24 h and then liver biopsies were performed under inhalation anesthesia. After refed for 16 h, mice were euthanized to obtain liver sample. Luciferase activities were measured and refeeding responses were determined pairwise. (H) Refeeding responses of endogenous SREBP-1 mRNA expression in streptozotocin-treated mice as assessed by Northern blot. (I) Blood insulin and glucose levels of the control and streptozotocin-administered mice.

ment of positional effects that might influence transgenic mouse data. It is intriguing that whether the promoter-reporter fusion gene is inserted in the genome (*i.e.* in the case of transgenic mouse) or not (*i.e.* in the case of adenovirus-mediated gene transfer) does not affect the regulation of the SREBP-1c promoter activity.

Evaluation of insulin involvement based on adenovirus-mediated reporter gene transfer technique

The involvement of insulin was also evaluated using this adenovirus-mediated introduction of SREBP-1c-promoter-luciferase fusion gene into the liver. As shown in Fig. 4G, streptozotocin-treated diabetic mice exhibited strong refeeding responses of luciferase reporter activities in a similar manner to the responses of intrinsic SREBP-1c mRNA, fully consistent with the data from transgenic mice.

Regarding the role of insulin in the upregulation of lipogenesis, many previous reports indicate that insulin increases the expression of SREBP-1c and thereby upregulates lipogenesis [22–24]. However, our present methods clearly support that insulin signaling pathway does not directly control SREBP-1c expression. This suggests that the insulin response *in vitro* might not be identical to the refeeding response *in vivo*, thus previous knowledge on *in vitro* promoter analyses of SREBP-1c regulation by insulin might not fully hold true of *in vivo* regulation. For better understanding of the *in vivo* physiology, further studies of *in vivo* promoter analyses based on our reporter gene transfer technique utilizing adenovirus vector are currently ongoing.

In summary, we generated two *in vivo* systems to estimate the SREBP-1c promoter activity. It was demonstrated that the fasting-refeeding regulation of SREBP-1c expression in the liver is exerted at the transcription level, and that 2.2 kb of the 5′-flanking sequence is sufficient for this regulation. Our adenoviral strategy provides a feasible approach to identify the promoter regions responsible for refeeding response of SREBP-1c expression, which is currently under going.

Acknowledgments

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