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Obese gene expression at *in vivo* levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes

(adipocytes/differentiation/obesity/adipose/leptin)

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ABSTRACT **3T3-F442A preadipocytes implanted s.c. into athymic mice develop into fat pads that are indistinguishable from normal adipose tissue. Implanted preadipocytes harboring a β-galactosidase transgene gave rise to fat pads in which almost all adipocytes expressed β-galactosidase. This finding proved that the implanted 3T3-F442A preadipocytes, rather than endogenous preadipose cells, gave rise to the newly developed "adipose tissue." 3T3-F442A preadipocytes, when differentiated into adipocytes in cell culture, express the obese gene at an unexpectedly low level, i.e., ≤1% the level in adipose tissue. However, adipose tissue derived from s.c. implanted 3T3-F442A preadipocytes expressed leptin mRNA at a level comparable to that in epididymal adipose tissue. These findings indicate that a factor(s) or condition, present in the tissue context and necessary for maximal obese gene expression, is lacking in cell culture. Furthermore, adipocytes derived from the implanted cells were hormonally responsive in that leptin mRNA levels were up-regulated 3- to 8-fold by glucocorticoid injection into the host animal. Thus, these findings indicate that adipose-specific promoter–reporter constructs, transfected into 3T3-F442A preadipocytes, can be tested in an *in vivo* context during and after development of these cells into adipose tissue. Furthermore, the effect of transgenes on the adipogenic development of the implanted preadipocytes can be assessed. Thus, this approach offers a faster and less costly alternative to the transgenic mouse method for assessing adipose gene function.**

The positional cloning of the obese gene led to the identification of its gene product (1), i.e., leptin, a peptide hormone produced by adipocytes that is involved in the regulation of food intake and energy expenditure. Cloning of the leptin receptor gene and characterization of its RNA transcripts revealed multiple splice variants (2, 3), one of which encodes a receptor isoform that transmits its "signal" via the JAK/STAT system (4). This, along with earlier evidence (5), indicates that the interaction of leptin with receptors of this type located in the hypothalamus triggers a response that leads to appetite suppression and increased energy expenditure (5). These findings provided new insight into how adipose tissue mass is regulated.

The expression of leptin by the adipocyte appears to be subject to both positive and negative control. Insulin (6, 7), glucocorticoid (8, 9), and certain endotoxins and cytokines (10) potently up-regulate expression of the gene whereas cAMP and β-adrenergic agonists down-regulate its expression (11, 12). In *ob/ob* mice, which possess a mutated obese gene, and in *db/db* mice, which possess a mutated leptin receptor gene, the obese gene is markedly overexpressed (6). These findings suggest that the gene is under negative feedback control by the leptin "signal" and that disruption of the leptin signaling pathway increases expression of the obese gene.

Recent studies in this (13) and other laboratories (14–16) have shown that the proximal promoter of the obese gene possesses a C/EBP binding site that mediates transactivation by C/EBPα. This and other evidence (13) indicate that, like many other adipocyte genes (17), the obese gene is transcriptionally activated by C/EBPα during adipocyte differentiation. Although attempts have been made to identify other functional regulatory elements in the obese gene promoter, to our knowledge, none has been reported.

Promoter analysis of the obese gene with differentiated preadipocyte cell lines in culture is complicated by the fact that the level of leptin expression is extremely low [≤1% the level of expression of the endogenous gene (6)]. Although adipocytes in primary culture have proven useful in identifying exogenous agents (e.g., hormones) that affect leptin expression (8, 9), these cells lose their capacity to express leptin when carried in culture and, therefore, are of limited use for promoter analysis. We have sought to develop a methodology by which the obese gene promoter, as well as other adipocyte gene promoters, can be analyzed in a more appropriate adipose tissue context.

Green and Kehinde (18) found that s.c. injection of 3T3-F442A preadipocytes into athymic mice gave rise to fat pads resembling normal adipose tissue. We have exploited this approach and now show that the obese gene of 3T3-F442A preadipocytes differentiated in this *in vivo* context is expressed at a high level (i.e., comparable to that in white adipose tissue) and is responsive to hormonal stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture. 3T3-F442A and 3T3-L1 cells were cultured and differentiated as described (19, 20), respectively. 89CRIP cells stably transfected with pLLZ (89CRIR/nls-lacZ) (21) were from Nicolas Ferry (Rennes, France). Proliferating 3T3-F442A cells were stably transfected by incubation with 20 ml of undiluted 89CRIP/nls-lacZ conditioned medium six times over 5 days. β-Galactosidase was detected by staining fixed cells with 1% 5-bromo-4-chloro-3-indolyl β-D-galactoside. Clones were selected for high levels of β-galactosidase expression and efficient differentiation.

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s.c. Implantation of Preadipocytes, Excision of Fat Pads, and Histology. 3T3-F442A preadipocytes were grown to near confluence, trypsinized, and suspended in bovine calf serum. After centrifugation, cell pellets were resuspended in calf serum or DMEM and injected s.c. (3×10^7 cells per site) at the sternum or back of Crl:NU/NU-nuBR or BALB/cAnNCrl-nuBR athymic mice (Charles River Laboratories) housed in micro-isolator cages. At various times after implantation, mice were anesthetized with inhaled metofane, fat pads derived from the implanted cells and epididymal fat pads were excised, and the mice were killed by cervical dislocation. Small pieces of the fat pads were fixed in neutral-buffered formalin (Baxter Scientific Products, McGaw Park, IL), and the remaining tissue was frozen in liquid nitrogen and stored at −80°C for RNA isolation. When indicated, some mice were treated with dexamethasone [3.7 μg/g (body weight)] by i.p. injection 6 h before death. To assess DNA synthesis *in vivo*, some animals received i.p. injections of BrdUrd [200 μg/g (body weight)] 48 h and 24 h before death.

For light microscopy and immunohistochemical analysis, fat pads derived from implanted preadipocytes and epididymal fat pads were paraffin-embedded after 24 h of fixation in buffered formalin. Four-micrometer paraffin tissue sections were stained with hematoxylin and eosin for histological analysis and selection of tissues for immunohistochemical staining. Immunohistochemical localization of β-galactosidase (Oncogene Science) and BrdUrd (Becton Dickenson) with mouse mAb was performed with a BioTek model 1000–28 automated immunostainer. Antibody reactivity was detected with biotinylated anti-mouse goat Igs and avidin-horseradish peroxidase conjugate (Vector Laboratories). To reduce background staining, sections were blocked with nonimmune mouse F(ab) fragments (Dako) at 4°C before incubation with the primary antisera. 3,3′-Diaminobenzidine was used as the chromogen with hematoxylin counterstain. Omission of the primary antibodies served as the negative controls.

RNA Isolation and Northern Blot Analysis. Total RNA was purified as described (22). Transcript levels were assessed by Northern blot analysis (23) with random-primed, ^{32}P-labeled cDNA probes (24) for leptin, C/EBPα, stearoyl-CoA desaturase (SCD1), 422/aP2, and 28S rRNA. Hybridization signals were quantitated with a Fuji BAS 1500 phosphorimager.

RESULTS

Expression of leptin mRNA by 3T3-L1 and 3T3-F442A adipocytes differentiated in culture is unexpectedly low (≤1% the level found in adipose tissue RNA) (6). After numerous unsuccessful attempts to up-regulate expression of the gene in cell culture, we considered the possibility that expression required factors or conditions present only in an *in vivo* tissue context.

s.c. Implanted 3T3-F442A Cells Give Rise to Adipose-Like Tissue. Green and Kehinde (18) have reported that s.c. injection of 3T3-F442A preadipocytes into athymic mice gave rise to fat pads indistinguishable from normal adipose tissue. These investigators did not prove, however, that the "new" fat pads were derived from the implanted cells rather than preadipose cells of tissue origin. To determine whether 3T3 preadipocytes can give rise to adipose tissue adipocytes, 3T3-F442A and 3T3-L1 preadipocytes were grown to near confluence, harvested by trypsinization, and resuspended in a small volume of medium, and 3×10^7 cells were injected s.c. at the sternum of athymic mice. The swelling observed initially at the site of injection disappeared over the next 2 days. Within a week, a flat thickened pad began to develop at the site of implantation, and this pad further enlarged over the next 2–3 weeks (Fig. 1*A*). However, pads were not observed in the mice that had been implanted with 3T3-L1 preadipocytes. Dissection of the mice 3–4 weeks after s.c. injection revealed well defined tissue resembling fat pads developed at the site of implantation of 3T3-F442A but not 3T3-L1 cells. Consistent with the findings of Green and Kehinde (18), the newly developed fat pads exhibited histological features of adipose tissue, i.e., epididymal fat pads isolated from the same mouse (Figs. 1*B* and 2).

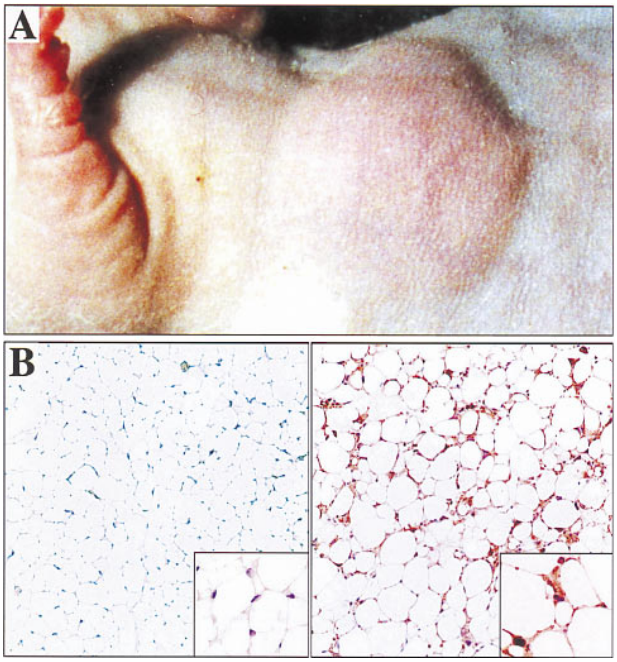

FIG. 1. Development of fat pads from s.c. implanted 3T3-F442A preadipocytes. 3T3-F442A preadipocytes (3×10^7 cells) harboring a β-galactosidase expression vector were harvested at near confluence and injected s.c. at the sternum of outbred Crl athymic mice. (*A*) A typical fat pad that developed at the site of injection 4 weeks after implantation. (*B*) Fat pads that developed at the site of implantation (*Right*) and epididymal fat pads (*Left*) were excised, fixed in buffered formalin, and immmunohistochemically stained for β-galactosidase with eosin as a counterstain.

To verify that the fat pads were in fact derived from the s.c. implanted preadipocytes rather than by recruitment of endogenous preadipose cells, 3T3-F442A cells were tagged by retroviral transduction with the *nls-lacZ* gene encoding β-galactosidase containing a nuclear localization signal (*nls*). Successive infections of 3T3-F442A cells with medium from 89CRIP cells stably transfected with pLLZ (21) gave rise to a population in which ≈15% of the cells expressed detectable levels of nuclear β-galactosidase. β-Galactosidase-positive clones that retained the capacity to differentiate into adipocytes in culture were selected and propagated for implantation. Approximately 3×10^7 preadipocytes expressing the *nls-lacZ* gene were implanted s.c. at the sternum of athymic (outbred Crl) mice. After 4 weeks, epididymal fat pads and adipose-like tissue that developed at the site of implantation were excised, fixed, and subjected to immunohistochemical staining for β-galactosidase. Adipocytes in the fat pads derived from 3T3-F442A preadipocytes exhibited substantial staining for β-galactosidase, particularly in the nuclei but also in the cytoplasm, whereas adipocytes of epididymal fat pads did not (Fig. 1*B*). These findings prove that fat pads that developed at the sites of implantation were derived from 3T3-F442A preadipocytes. It should be noted that both nuclear and cytoplasmic staining were also observed in 3T3-F442A adipocytes differentiated in cell culture (results not shown).

The kinetics of adipose development from 3T3-F442A preadipocytes s.c. implanted into athymic mice were followed for a period of 10 weeks. Within 1 week after implantation, only a few cells had begun to differentiate as evidenced by the

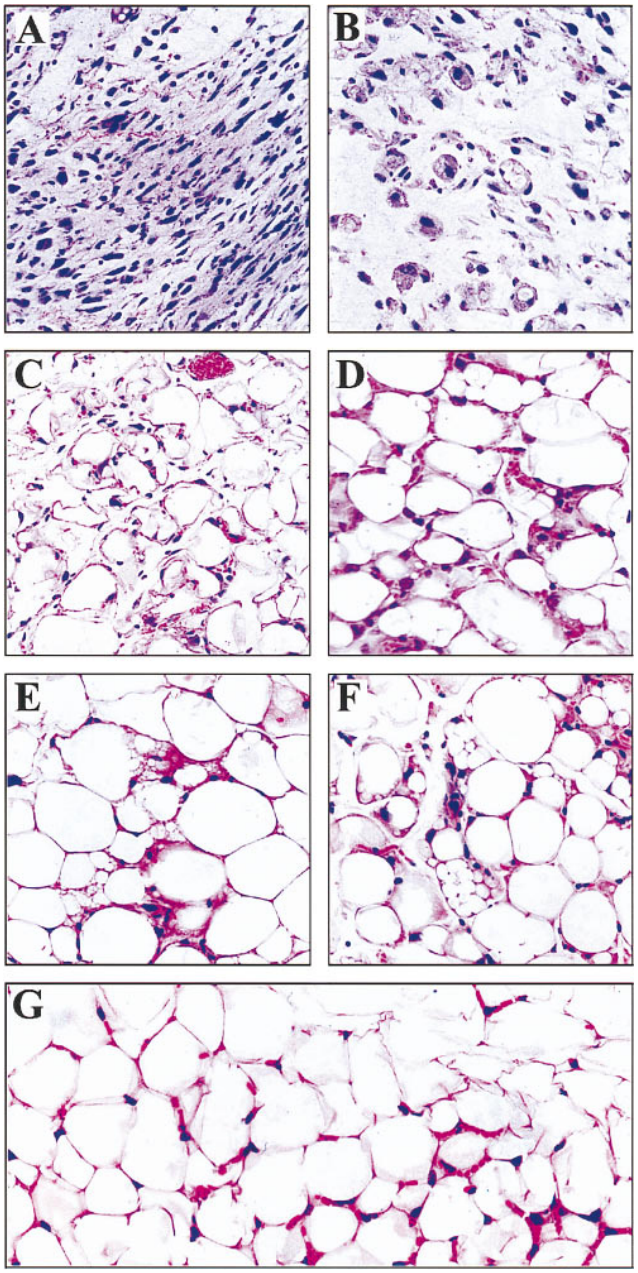

FIG. 2. Time course of adipose development from s.c. implanted 3T3-F442A preadipocytes. Outbred Crl athymic mice were injected s.c. with 3×10^7 3T3-F442A preadipocytes. Fat pads that developed from the implanted preadipocytes were excised, fixed, sectioned, and stained with hematoxylin and eosin at 1 (*A*), 2 (*B*), 3 (*C*), 5 (*D*), 8 (*E*), and 10 weeks (*F*) after implantation. (*G*) Epididymal adipose tissue from a mouse harboring implanted 3T3-F442A preadipocytes for 6 weeks was excised and treated in an identical manner.

accumulation of cytoplasmic triglyceride droplets (Fig. 2*A*). A few necrotic cells were also observed. Two weeks after implantation, neovascularization was evident, and many cells had accumulated cytoplasmic triglyceride in cytoplasmic vesicles (Fig. 2*B*). By 3 weeks, most cells were unilocular and vascularization of the tissue was more extensive (Fig. 2*C*). Cytoplasmic triglyceride-containing vesicles reached maximal size at ≈6 weeks, at which time innervation of the tissue was evident. After 6, 8, and 10 weeks, tissue at the site of implantation was virtually indistinguishable from adipose tissue of epididymal fat pads (compare Fig. 2 *D–F* with *G*). The only differences were that the nuclei of adipocytes derived from 3T3-F442A cells appeared somewhat larger than those in epididymal fat pads and that a small fraction of the implanted cells was multilocular. By 15 weeks after implantation, it appeared that the lipid content of the implanted cells had decreased slightly.

An attempt was made to accelerate *in vivo* adipose development from 3T3-F442A cells. Thus, 3T3-F442A cells were induced to differentiate in cell culture for 2 or 7 days before implantation into athymic mice (see *Experimental Procedures*). Cells that had undergone differentiation in culture for 7 days before implantation failed to give rise to fat pads (results not shown). Cells that had been differentiated for 2 days in culture before implantation developed into adipocyte-like cells within 4 weeks; however, this tissue had abnormal morphology in that the intercellular spaces appeared to contain large amounts of extracellular matrix (results not shown). Thus, it appears that the optimal stage for implantation is before induction of differentiation before preadipocytes achieve confluence. Conceivably, preadipocytes must be actively dividing at the time of implantation to undergo normal adipogenesis.

To ascertain whether preadipocytes undergo cell division after implantaion, mice were injected with BrdUrd 48 and 24 h before death at 1, 2, or 3 weeks after implantation of 3T3-F442A cells. Sections of the fat pads derived from the implanted preadipocytes, from epididymal fat pads (negative control), and from skin (positive control) were fixed for immunohistochemical detection of BrdUrd incorporation into DNA. Although the skin showed significant DNA synthesis as measured by BrdUrd staining, no incorporation was detected at any time point in sections of the fat pads derived from the implanted cells (results not shown). Thus, if the implanted cells proliferated *in vivo*, this must have occurred within the first 5 days after the implantation.

Expression of Leptin mRNA During Adipose Conversion of s.c. Implanted 3T3-F442A Preadipocytes. To determine whether expression of the obese gene (and representative adipocyte genes) by 3T3-F442A adipocytes in a tissue context differs from that in cell culture, mRNA levels of implanted 3T3-F442A cells were compared at various times after implantation with those of epididymal fat pads and 3T3-F442A adipocytes in culture. As observed (5), the level of leptin message in 3T3-F442A adipocytes differentiated in cell culture was below the limit of detection by Northern blot analysis (Fig. 3). However, leptin mRNA in fat pads derived from implanted 3T3-F442A cells was readily detected 2 weeks after implantation and by 6 weeks had reached a maximal level, ≈15% of that of epididymal adipose tissue from the same animal (Fig. 3*B*). Consistent with the role of C/EBPα as a transcriptional activator of many adipocyte genes (17), including the obese gene (13), expression of C/EBPα mRNA precedes expression of the leptin message (Fig. 3). The kinetics of expression of the 422/aP2 and stearoyl–CoA desaturase 1 (*SCD1*) genes, both of which are transcriptionally activated by C/EBPα, were similar to that of the obese gene (Fig. 3).

The first appearance of leptin mRNA after implantation of 3T3-F442A cells correlates well with adipose development as assessed by light microscopy. Thus, expression of leptin mRNA (Fig. 3; week 2) and the appearance of adipocyte morphology (Fig. 2*B*) first became evident during the second week after implantation of the preadipocytes. Leptin mRNA levels reached a maximum around week 6, at which time almost all implanted cells had developed into unilocular adipocytes.

Adipose tissue from different fat depots differs with respect to leptin mRNA content (7, 25), so we considered the possibilty that the site of implantation of 3T3-F442A preadipocytes might affect fat pad development and the level of leptin expression. To test this possibility, 3T3-F442A cells were implanted s.c. at the sternum and at the back of four athymic mice. No differences in the rate of development or character of fat pads were evident at these different sites; moreover, no

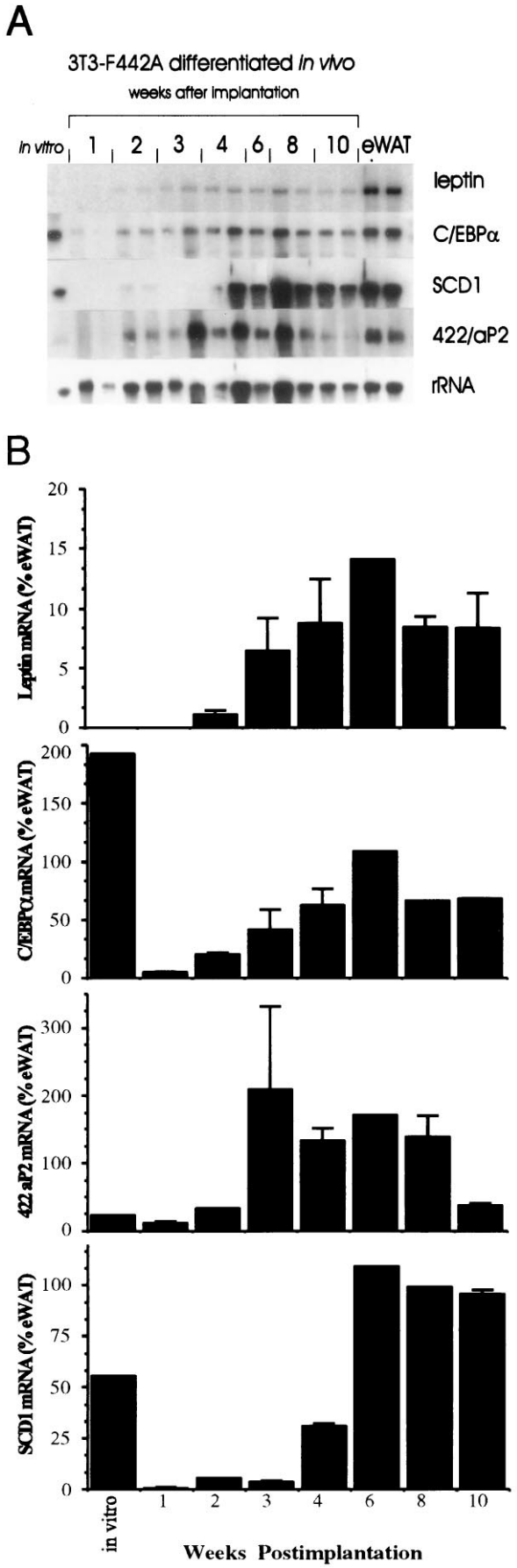

FIG. 3. Expression of leptin mRNA and other adipose-specific mRNAs during adipose development from s.c. implanted 3T3-F442A preadipocytes. The level of expression leptin mRNA and mRNAs encoding other adipocyte markers was determined by Northern blot analysis of total RNA isolated from fat pads derived from the implanted preadipocytes described in Fig. 2. (*A*) Northern blots of RNA isolated from adipose tissue derived from implanted 3T3-F442A preadipocytes, from epididymal white adipose tissue (eWAT), and from *in vitro* differentiated 3T3-F442A adipocytes. Approximately 10 μg of total RNA was loaded per lane. The blot was hybridized to a ^{32}P-labeled probe for leptin and then stripped and reused for hybridization to probes for C/EBPα, SCD1, 422/aP2, and 28S rRNA. (*B*) Plots of the results in *A* showing the relative levels of each transcript expressed as percentage of the level of transcript in RNA from epididymal adipose tissue. Hybridization signals on Northern blots (shown in *A*) were quantified using a phosphorimager and were normalized to the corresponding signals from the rRNA. Transcript levels are expressed as percentage of the mean transcript level of eWAT. Results are represented as the mean ± range (n = 2).

difference in the level of leptin mRNA was observed in fat pads that developed at these two sites (results not shown).

The Obese Gene of Implanted 3T3-F442A Adipocytes Is Responsive to Glucocorticoid. Glucocorticoids are known to increase leptin mRNA levels both *in vivo* (8, 9) and in isolated primary adipocytes (9, 11). The finding that this stimulation is, in large part, independent of *de novo* protein synthesis (9) suggests that glucocorticoids may directly activate transcription of the leptin gene. Glucocorticoid does not, however, significantly up-regulate leptin mRNA in 3T3-L1 adipocytes (O.A.M., unpublished results, and ref. 26) or 3T3-F442A adipocytes (S.M., unpublished results) in cell culture although the hormone does activate expression of other genes in these cell lines (27). To determine whether 3T3-F442A preadipocytes implanted into athymic mice become responsive to glucocorticoid, Crl athymic mice were injected i.p. with dexamethasone 6 weeks after implantation. Six hours after glucocorticoid treatment, RNA was isolated from fat pads and subjected to Northern blot analysis for leptin mRNA. As shown in Fig. 4, glucocorticoid injection caused dramatic and comparable increases (6- and 8-fold, respectively) of leptin mRNA in epididymal fat pads and in fat pads derived from implanted 3T3-F442A cells.

Athymic mouse lines can differ in biological responsiveness, so we compared the outbred Crl line used in the experiments described above with the inbred BALB/c athymic line for the ability to support adipose development of, and obese gene expression in, s.c. implanted 3T3-F442A cells. Although fat pads that developed from implanted 3T3-F442A cells were more well defined in inbred BALB/c mice than in outbred Crl mice, they were histologically indistinguishable. Northern blot analysis revealed that implanted fat pads in BALB/c mice expressed leptin mRNA at a level that equaled that of epididymal white adipose tissue (Fig. 4). Both the implanted and epididymal adipose tissue in the BALB/c mice were responsive to dexamethasone (≈3-fold induction; Fig. 4), consistent with observations with Crl mice.

DISCUSSION

To determine how transcription of the obese gene is regulated, it will be necessary to have an appropriate manipulable system(s) with which to carry out obese gene promoter analysis. The obese gene is expressed only by adipocytes (1), so the choices are limited to isolated adipocytes or to established preadipocyte cell lines, e.g., the 3T3-L1 and 3T3-F442A lines, that can be induced to differentiate into adipocytes in culture. Although the expression of leptin by isolated adipocytes is responsive to certain physiological agents, such as cAMP elevating agents and glucocorticoids (8, 9, 11), these cells are not well suited for promoter analysis because they are difficult to transfect and fail to maintain their differentiated charac-

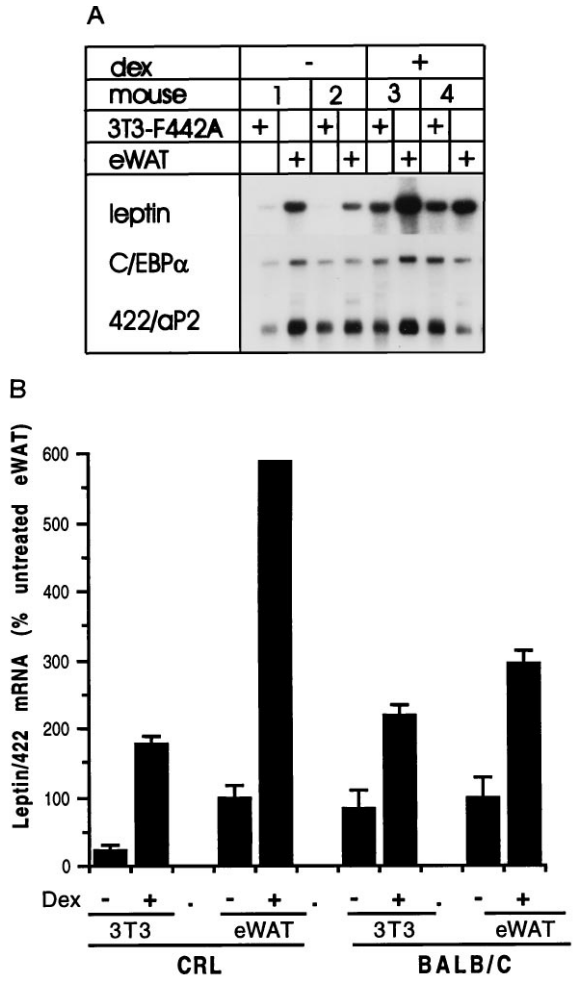

FIG. 4. Hormonal responsiveness of the obese gene in adipose tissue derived from s.c. implanted 3T3-F442A preadipocytes and from epididymal fat pads. (*A*) Six weeks after the implantation of 3T3-F442A preadipocytes, four outbred Crl:NU/NU-nuBR athymic mice were injected i.p., two with dexamethasone [3.7 μg/g (body weight)] and two with vehicle. Similarly, four inbred BALB/cAnNCrl-nuBR athymic mice were injected with dexamethasone or vehicle. Six hours later, RNA was isolated from epididymal fat pads and fat pads derived from implanted 3T3-F442A preadipocytes and used for Northern blotting. Blots were analyzed as indicated in Fig. 3. (*B*) Plots showing the levels of expression of leptin mRNA quantitated from the results in *A* and from the the Northern blot of the RNA from the BALB/c mice (not shown). Transcript levels were normalized to the level of the adipose-specific 422/aP2 transcript and are expressed as percentage of the mean level in eWAT of untreated mice of the same strain.

teristics in cell culture for a sufficient period of time. On the other hand, use of established preadipocyte/adipocyte cell lines has other drawbacks. For example, previous investigations (6) have shown that 3T3 adipocytes in cell culture express leptin mRNA at unexpectedly low levels (i.e., ≤1% the level in adipose tissue) and that expression of the obese gene is not responsive to many physiological agents in culture (ref. 26 and O.A.M., unpublished results).

The approach described in this paper offers an alternative with advantages over promoter analysis in cell culture, perhaps the most important of which is that the analysis is conducted in an *in vivo* tissue context. Our results show that 3T3-F442A preadipocytes, when implanted s.c. into athymic mice, give rise to fat pads (Figs. 1*A* and 2 *B–F*) that express the leptin message at levels approximating *in vivo* levels (Figs. 3 and 4) and that expression of the message is responsive to hormone injected into the host (Fig. 4). Also important is the fact that a transgene, e.g., the β-galactosidase gene, transfected into the preadipocytes before implantation is expressed by the fat pads derived from these cells (Fig. 1*B*). Thus, it should be possible to test adipocyte-specific promoter–reporter gene constructs transfected into 3T3-F442A preadipocytes before, during, and after their development into adipose tissue at the site of implantation. In experiments not reported herein, we have shown that transfected obese gene promoter–luciferase constructs are expressed by fat pads derived from implanted preadipocytes (T.M.L., unpublished results). This approach should be applicable to the study of any adipocyte gene promoter and has advantages over the transgenic mouse method because it is faster and less costly and because a large number of constructs can be tested simultaneously. Furthermore, the effect of transgenes on the adipogenic development of the implanted cells can be assessed.

The present study addressed the question of why the 3T3-L1 and 3T3-F442A adipocyte lines in culture fail to express leptin mRNA at a level comparable to that in adipose tissue. At the outset, two reasons for this low level of expression appeared possible: (*i*) that these established cell lines, which are aneuploid, might lack a heritable factor required for the high level of expression of the gene that occurs in adipose tissue and (*ii*) that a factor (or condition) present in the tissue context might be lacking in the cell culture medium. Our first approach to test the latter possibility was to supplement the cell culture medium with various known physiological effectors, e.g., hormones, growth factors, etc., known to affect adipocyte function and lipid metabolism. These experiments were unsuccessful (6). Our success in obtaining an *in vivo* level of obese gene expression with the preadipocyte implantation approach, however, shows that the 3T3-F442A preadipocytes, when implanted into a tissue context, have the capacity to develop into leptin-expressing adipocytes. Thus, it can be concluded that a factor(s), such as a cytokine or extracellular matrix, or a condition, e.g., neighboring cell–cell contact, is necessary for adipose tissue level expression of the obese gene. Further studies will be necessary to identify this factor(s) or condition.

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