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Transcriptional regulation of the adipocyte fatty acid synthase gene by agouti: interaction with insulin

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Claycombe, Kate J., Yanxin Wang, Brynn H. Jones, Suyeon Kim, William O. Wilkison, Michael B. Zemel, Joseph Chun, and Naima Moustaid-Moussa. Transcriptional regulation of the adipocyte fatty acid synthase gene by agouti: interaction with insulin. *Physiol Genomics* 3: 157–162, 2000.—Mice carrying dominant mutations at the agouti locus exhibit ectopic expression of agouti gene transcripts, obesity, and type II diabetes through unknown mechanisms. To gain insight into the role of agouti protein in modulating adiposity, we investigated regulation of a key lipogenic gene, fatty acid synthase (FAS) by agouti alone and in combination with insulin. Both agouti and insulin increase FAS activity in 3T3-L1 and in human adipocytes. Agouti and insulin independently and additively increase FAS activity in 3T3-L1 adipocytes. We further investigated the mechanism responsible for the agouti-induced FAS expression in these cells and demonstrated that both insulin (3-fold increase) and agouti (2-fold) increased FAS gene expression at the transcriptional level. Furthermore, insulin and agouti together exerted additive effects (5-fold increase) on FAS gene transcription. Transfection assays of FAS promoter-luciferase fusion gene constructs into 3T3-L1 adipocytes indicated that the agouti response element(s) is (are) located in the −435 to −415 region (−435/−415) of the FAS promoter. Nuclear proteins binding to this novel sequence are adipocyte specific. Thus the agouti response sequences mapped to a region upstream of the insulin-responsive element (which we previously reported to be located at −67/−52), consistent with additive effects of these two factors on FAS gene transcription.

gene transcription; regulatory sequences

AGOUTI, A PARACRINE FACTOR composed of 131 amino acids, is normally secreted within hair follicles during the hair growth period (26, 32). Secreted agouti protein regulates hair pigmentation by competitive antagonism of α-melanocyte stimulating hormone at its receptor (MC1-R), resulting in a switch from eumelanin to phaeomelanin production (16, 26). Promoter mutations at the mouse agouti locus resulting in ectopic expression of the agouti gene cause yellow coat color, marked obesity, hyperinsulinemia, type II diabetes, and development of cancer (15, 29).

Although agouti was the first obesity gene to be cloned (4), the role of agouti in the development of obesity is not fully understood. Both peripheral and central effects of agouti have been implicated in yellow mouse obesity (22). Centrally, agouti has been shown to antagonize the hypothalamic melanocortin receptor (MC4-R), resulting in inhibition of feeding behavior (7). In addition, we have obtained evidence supporting peripheral actions of agouti in the etiology of yellow mouse obesity. Fatty acid synthase (FAS) is expressed at significantly higher levels in the fat tissues of A^{vy}/a mice (viable yellow) compared with lean control and in transgenic mice that ubiquitously overexpress the agouti gene (11, 12). We also have shown that agouti increases FAS expression and lipogenesis via an intracellular Ca^{2+} ($[Ca^{2+}]_i$)-dependent mechanism (11–13). Furthermore, insulin treatment of transgenic mice expressing the agouti gene specifically in adipocytes under the control of the aP2 (adipocyte fatty acid binding protein) promoter (6) causes a significant increase in body weight compared with untreated transgenic mice (23). Consequently, we hypothesized that agouti interacts with insulin to induce obesity by upregulating lipogenesis in adipose tissue.

To gain insight into the specific role of agouti protein in adipocytes and its interaction with insulin, we investigated the effects of these two factors on FAS expression in adipocytes. FAS plays a key role in long-term regulation of lipogenesis and catalyzes de novo synthesis of palmitate from acetyl-CoA and malonyl-CoA; this pathway is highly regulated by nutritional factors as well as hormonal signals (10). Insulin has been shown to induce FAS in several species (2, 5, 10, 20, 24). We have previously demonstrated that this increase in FAS gene expression is mediated via an insulin response element within the proximal rat FAS promoter (18).

In our present study, we report an additive effect of agouti and insulin in inducing FAS expression in 3T3-L1 adipocytes. Furthermore, this induction of FAS

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activity is due to increased FAS gene transcription in 3T3-L1 adipocytes. Finally, we map the agouti response element to a region distinct from the insulin response element, and we demonstrate that specific adipocyte nuclear proteins bind this novel agouti-responsive sequence.

MATERIALS AND METHODS

Expression of agouti cDNA. Full-length agouti cDNA was subcloned into a baculovirus expression vector and expressed in *Trichoplusia ni* cells. Medium was collected 48 h after infection and partially purified as previously described (16).

Cell culture. 3T3-L1 cells were grown and differentiated as previously described (11, 21). Briefly, cells were grown to confluence in standard medium (DMEM supplemented with 10% FBS). At confluence, cells were induced to differentiate by addition of dexamethasone (250 nM) and isobutylmethylxanthine (0.5 mM) to standard medium for 2 days. Cells were maintained for 3 additional days in standard medium, then cultured overnight in serum-free medium (containing 1% BSA) prior to treatment with agouti protein and/or insulin as indicated in the legends to Figs. 1–6. HepG2 cells were maintained in DMEM supplemented with 10% FBS, and subconfluent cells were used for nuclei preparation.

Human adipocyte cell culture. Human adipose tissues were obtained from abdominal fat of patients undergoing elective liposuction or abdominoplasty. These tissues were processed as previously described (5, 20). Briefly, adipose tissue was minced into small fragments, digested with collagenase (GIBCO BRL; Life Technologies, Bethesda, MD), and filtered (350 μm mesh). The floating adipocyte fraction was then maintained in DMEM medium supplemented with 1% FBS, and cells were treated as described in the legend to Fig. 2.

FAS activity. FAS activity was measured spectrophotometrically in crude cytosolic extracts of 3T3-L1 or human adipocytes by measuring the oxidation rate of NADPH at 340 nm/min (19). FAS activity was normalized per milligram protein or per microgram DNA, assayed as we previously described (5, 11).

Northern Blot analysis. Differentiated cells were maintained overnight in serum-free medium prior to hormone treatment (insulin and/or agouti), as indicated in the legends to Fig. 1–6. Total RNA was isolated by the CsCl method (11, 21). RNA was subjected to agarose gel electrophoresis, and Northern blot analysis was performed using FAS and actin cDNAs as probes. Autoradiograms were analyzed by densitometric scanning.

Nuclear run-on assay. Nuclei were isolated from control, agouti and/or insulin-treated 3T3-L1 adipocytes as previously described (21). Nuclei were then labeled with [^{32}P]UTP, and labeled RNA were purified and hybridized to rat FAS cDNA (cloned in Dr. Porter's lab and kindly provided by Dr. A. G. Goodridge, University of Iowa, Iowa City, IA) and lipoprotein lipase (LPL) cDNA, which was used as a control (kindly provided by Dr. S. Fried, Rutgers University, NJ).

FAS-luciferase fusion gene construct. A fragment of the FAS 5′-flanking region spanning −2100 to +67 was generated by PCR using rat genomic DNA and subcloned into the pGL-basic vector (Promega, Madison, WI). Various deletions of this fragment were subsequently generated using *Exo* III mung bean nuclease deletion kit (Stratagene, La Jolla, CA) (18) or by PCR using previously published FAS promoter sequences (1, 3). For fusion constructs containing sequences −450 to −395 or −435 to −415, these oligonucleotides were first synthesized, annealed, and then subcloned into pGL2 SV promoter-luciferase plasmid.

Transient transfections. 3T3-L1 adipocytes were transfected with each of the above fusion gene constructs using the calcium phosphate-DNA coprecipitation method (kit purchased from GIBCO BRL) as we previously described (18). Transfected cells were maintained overnight in serum-free medium before treating with agouti and/or insulin for up to 48 h, as indicated in the legend to Fig. 5. SV-β-galactosidase fusion construct (Promega) was cotransfected with each of the fusion constructs to normalize for the transfection efficiency. pGL2 control, which contains the SV40 promoter linked to the luciferase gene, was used as a control to determine specificity of agouti effect on FAS promoter. Agouti did not affect luciferase activity when driven by the SV promoter alone (pGL2 SV promoter).

Luciferase and β-galactosidase assays. The cells were lysed in 100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol. The cytosolic extracts of lysed cells were used for luciferase activity. Luciferase and β-galactosidase activities were measured utilizing a luminometer (Berthold, Nashua, NH) and a dual-luminescence assay kit (Tropix, Bedford, MA).

Gel-mobility shift assays. Nuclei were isolated as described above from 3T3-L1 and HepG2 cells and used to prepare nuclear extracts as previously described (18, 20). HeLa nuclear extracts were purchased from Promega. Two complementary single strands of 20-bp oligonucleotides containing FAS promoter sequences spanning −435/−415 region or mutations within this region (oligonucleotides M3 and M5, described in Fig. 6*B*) were synthesized, then annealed by incubating equal molar concentrations in 1 M Tris·HCl (pH 7.5), 5 M NaCl, and 0.5 M EDTA at 65°C for 10 min followed by cooling to room temperature. The annealed oligonucleotides were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Gel shift binding assays were performed at room temperature in 2 μl of binding buffer [5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris·HCl, pH 7.5, and 0.25 μg/μl poly(dIdC) in 20% glycerol] for 30 min. Each reaction contained 50,000 cpm (1.75 pmol) of oligonucleotides and 2 μg of nuclear extracts. Unlabeled competitors were added to reaction mixtures containing nuclear extract and binding buffer for 20 min at room temperature prior to addition of labeled oligonucleotides. Electrophoresis of DNA-protein complexes was conducted using a 3% nondenaturing polyacrylamide gel in 50 mM Tris·HCl, 45 mM boric acid, and 0.5 mM EDTA (pH 8.3). The gels were dried and exposed to X-ray film at −80°C.

Statistical analysis. One-way ANOVA was used (SAS, Cary, NC) for statistical analysis. All data are expressed as means ± SE. All tests were conducted using a 95% confidence interval.

RESULTS

Effects of agouti and insulin on FAS activity and mRNA levels. We have previously shown that insulin induces FAS expression (5, 18, 20). In addition, we have reported that agouti significantly increases FAS activity and triglyceride content in 3T3-L1 adipocytes (11). However, mechanisms of agouti regulation of FAS or its interaction with insulin are not known. In both 3T3-L1 (Fig. 1) and primary human adipocytes (Fig. 2), agouti and insulin each increased FAS enzyme activity by approximately threefold compared with control cells. Combined agouti and insulin treatment caused an approximately sixfold increase in FAS activity in 3T3-L1 adipocytes, demonstrating additive effects (Fig.

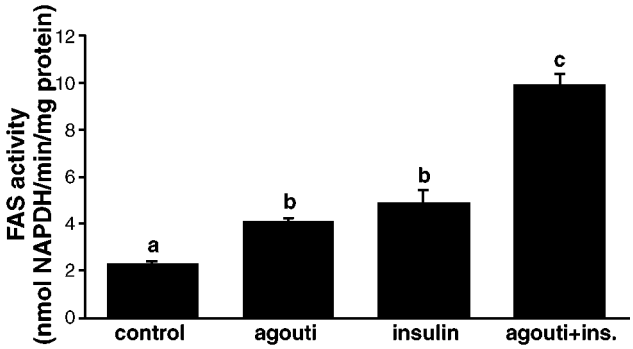

Fig. 1. Effect of agouti and insulin on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were maintained overnight in serum-free media. Cells were then treated with agouti (100 nM) and/or insulin (100 nM) for 48 h, and FAS enzyme activity was assayed as described in MATERIALS AND METHODS. Data are means ± SE. $^{a,b,c}P < 0.01$, groups with different symbols are statistically different from each other. Each treatment represents an average from 4 separate dishes, and this is representative of 3 independent culture experiments.

1). These additive effects were not however, observed in human adipocytes (Fig. 2). These data demonstrate that *1*) consistent with our previous report, agouti, like insulin, directly regulates lipogenesis by inducing FAS activity and *2*) agouti and insulin independently and additively increase FAS activity. Similar effects of agouti and insulin were observed on FAS mRNA content (Fig. 3). Insulin (10 or 100 nM) and agouti (10 or 100 nM) increased FAS mRNA content in 3T3-L1 adipocytes by approximately fourfold, whereas the combination of both hormones increased FAS mRNA content by sevenfold. This demonstrates that agouti, like insulin, regulates FAS gene expression at the pretranslational level.

Effects of agouti on FAS gene transcription. Changes in mRNA content normally correlate with parallel changes in the amount of transcribed message and/or stability of the message. We therefore first tested whether increased FAS mRNA by agouti was due to increased FAS gene transcription. Results from the nuclear run-on assays using nuclei isolated from agouti- and/or insulin-treated 3T3-L1 adipocytes demonstrated that both insulin (6-fold increase) and agouti (3-fold) increased FAS gene expression at the transcriptional level (Fig. 4). Agouti-induced FAS gene transcription was specific since transcription rate of

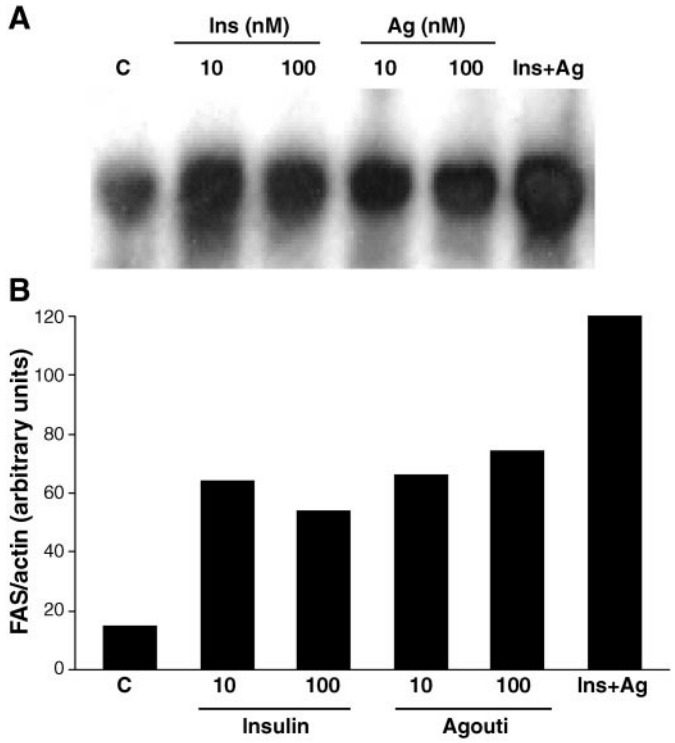

Fig. 3. Effect of agouti and insulin on FAS mRNA content in 3T3-L1 adipocytes. Total RNA was isolated from cells treated with or without insulin (Ins, 10 and 100 nM), agouti (Ag, 10 and 100 nM), or insulin plus agouti (100 nM each) for 24 h. Northern blot analysis was performed as described in MATERIALS AND METHODS using FAS and actin as probes. A representative autoradiogram is shown (*A*), and data averaging two experiments were analyzed by densitometry scanning (*B*).

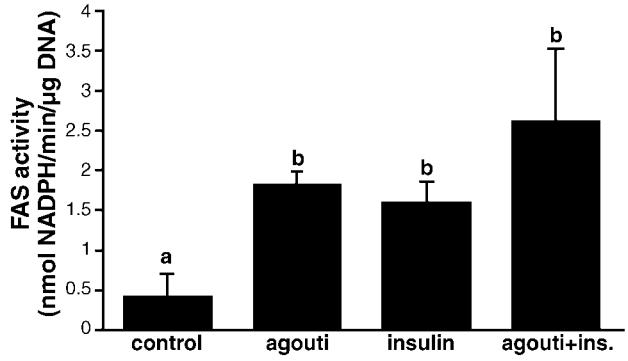

Fig. 2. Effect of agouti and insulin on FAS activity in human adipocytes. Primary human adipocytes were cultured in 1% FBS-supplemented media and treated with agouti (100 nM) and/or insulin (100 nM) for 48 h. FAS enzyme activity was then was assayed as described in MATERIALS AND METHODS. Data are means ± SE. $^{a,b}P < 0.05$; groups with different characters are statistically different from each other. Each treatment represents an average from 6 separate flasks. This experiment was repeated in 5 other patients, which provided comparable results.

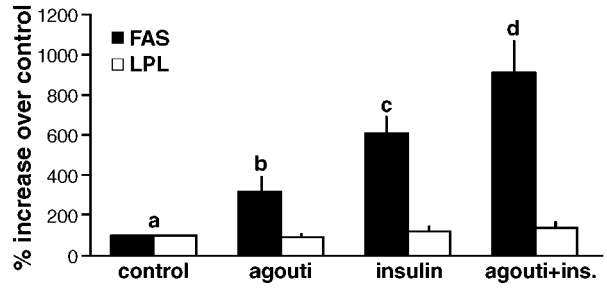

Fig. 4. Effect of agouti and insulin on FAS gene transcriptional rate in 3T3-L1 adipocytes. Nuclei were isolated from 3T3-L1 adipocytes that had been treated with agouti (50 nM) and/or insulin (20 nM) for 24 h. Nuclei were then labeled with [^{32}P]UTP and hybridized to FAS or lipoprotein lipase (LPL) cDNA, which were immobilized on nylon membrane, and radioactivity was counted as arbitrary units. Results were obtained from an average of 3 separate assays in which triplicate samples were prepared. Data are means ±SE. $^{a,b,c,d}P < 0.05$, groups with different letters are statistically different from each other.

LPL (a key gene in triglyceride accumulation in adipocytes) was not changed by agouti treatment. Furthermore, a combination of these two hormones showed additive effects (9-fold increase) on FAS gene transcription rate (Fig. 4). These changes in FAS gene transcription by agouti or insulin are consistent with those observed for FAS enzyme activity and mRNA levels (Figs. 1–3). Collectively, results from these studies demonstrate that *1*) agouti, like insulin, regulates FAS gene expression primarily at the transcriptional level and *2*) insulin and agouti act via independent transcriptional elements/ factors to regulate the FAS promoter.

Agouti response element(s) in FAS promoter. To further investigate the mechanisms of transcriptional regulation of the FAS gene by agouti, we searched for an agouti-responsive region(s) within the FAS promoter. Using transfection assays, we initially tested a promoter region containing 500 bp of the FAS 5′-flanking region. We have previously mapped the insulin response sequence to −67/−52 region within the FAS proximal promoter (18). We first transfected 3T3-L1 adipocytes with FAS-promoter luciferase fusion gene constructs (−500 to +67 and −300 to +67), both of which contain the insulin-responsive region, and the effects of agouti on luciferase activity were measured. Agouti significantly increased luciferase activity (Fig. 5*A*) in 3T3-L1 cells transfected with the −500 to +67 regions of the FAS promoter compared with cells transfected with −300 to +67 of the FAS promoter or pGL2 control, which were not responsive to agouti. To further narrow down the agouti-responsive region, we generated smaller fragments within the region spanning from −500 to −300. Agouti increased reporter gene activity in cells transfected with luciferase reporter constructs containing −450/−395 or −435/−415 sequences of the FAS promoter, ligated into pGL2 SV promoter-luciferase plasmid to levels comparable to those observed in cells transfected with −500/+67 fragment (Fig. 5*A*). The highest inductions of luciferase activity (~3-fold) were achieved with cells transfected with the −435/−415 sequence (Fig. 5*A*). The SV promoter (pGL2 control) or other FAS luciferase fusion constructs spanning the −500/−300 region were not responsive to agouti (not shown). These results indicate that *1*) agouti response element, AgRE, is located in the −435 to −415 region upstream of the FAS transcription start site and *2*) agouti-responsive region is distinct from the insulin-responsive region (15) (Fig. 5*B*).

Binding of nuclear factors to the putative FAS agouti response sequences. After we identified a 20-bp region of the FAS promoter that contains the agouti response element(s), we used gel shift assays to determine whether nuclear factors present in 3T3-L1 adipocytes indeed bind to this region of the FAS promoter. As indicated in Fig. 6*A*, nuclear extracts from 3T3-L1 adipocytes contained proteins that altered the electrophoretic mobility of oligonucleotides specific for the AgRE. This major DNA-protein complex was competed away by the same unlabeled oligonucleotide. Very low binding was observed with nuclear extracts from pre-

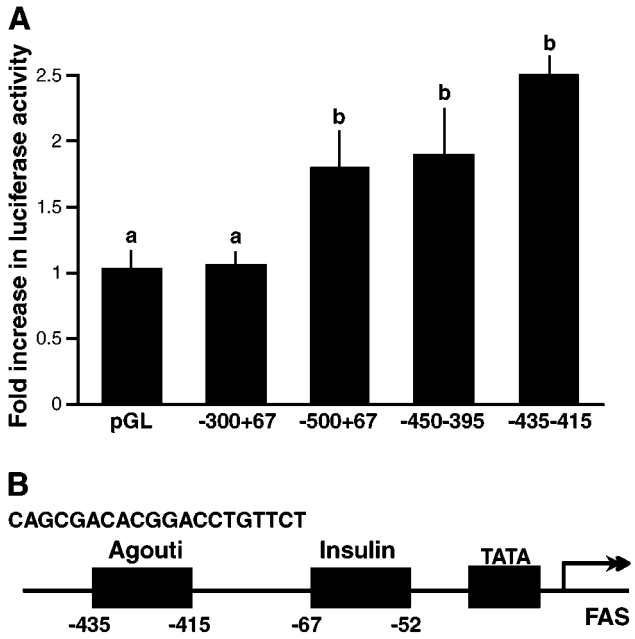

Fig. 5. Identification of agouti-responsive region in FAS promoter in transfections assays. *A*: 3T3-L1 adipocytes were transfected with FAS-luciferase fusion gene constructs as described in MATERIALS AND METHODS. pGL is the control plasmid containing SV promoter and luciferase and no FAS sequences. Transfected cells (6 dishes for each treatment) were maintained overnight in serum-free media prior to treatment of agouti (100 nM) and/or insulin (100 nM) for 48 h. Cells were then harvested and subjected to luciferase activity, which was normalized to β-galactosidase activity. Data are means ± SE. $^{a,b}P <$ 0.05; groups with different characters are statistically different from each other. This experiment was repeated three times. *B*: agouti and insulin response elements in the FAS promoter.

adipocytes and HepG2 cells, and no binding was detected with nuclear extracts from HeLa cells. These results suggest that the transcription factor(s) that binds to the AgRE in the FAS promoter is adipocyte differentiation dependent.

To further define the binding specificity of AgRE to adipocyte nuclear proteins, we synthesized two mutants (M3, 5′-AAGAGACACGGACCTATTAT-3′; and M5, 5′-CAACATGTCGGACCTGTTCT-3′). As shown in Fig. 6*B*, unlabeled M3 competitor did not displace AgRE (5′-CAGCGACACGGACCTGTTCT-3′) binding to adipocyte nuclear extracts. However, M5 competed away the labeled AgRE. These results suggest that the sequence CGGACCTGTTCT is critical for binding of AgRE to adipocyte nuclear proteins.

Collectively, these data indicate that the AgRE we identified in the FAS promoter is the candidate site for agouti-mediated FAS transcription. Importantly, nuclear proteins from both murine 3T3-L1 adipocytes as well as isolated human adipocytes were able to bind to this region (data not shown). It is also worth noting that, as described for other DNA-binding proteins, agouti treatment did not modify the binding capacity or mobility of this band (data not shown).

DISCUSSION

Several studies have demonstrated that insulin is a lipogenic hormone that regulates transcription of lipo-

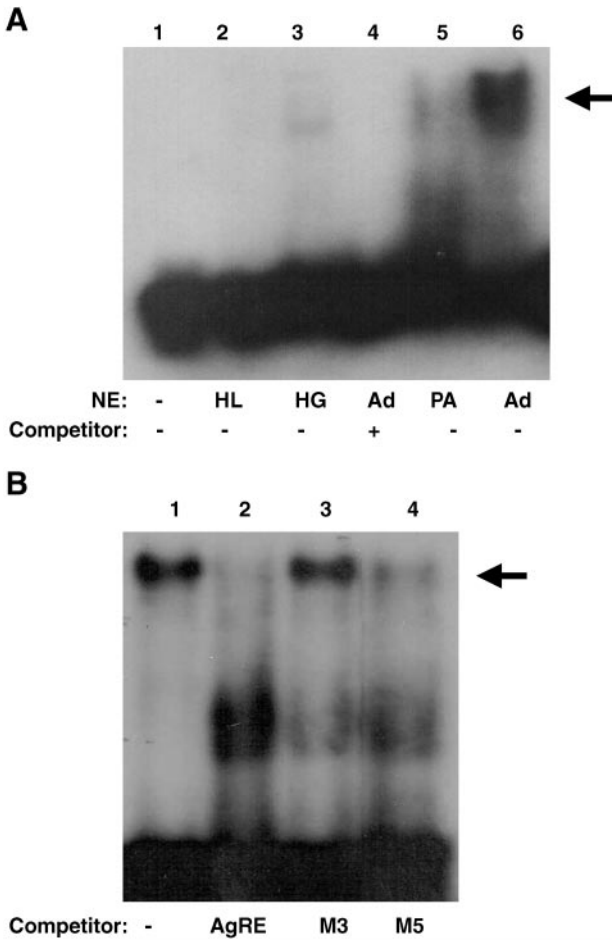

Fig. 6. Binding of 3T3-L1 adipocyte nuclear proteins to the agouti response element in gel mobility shift assay. *A*: nuclear extracts from adipocytes (Ad, *lanes 4* and *6*), preadipocytes (PA, *lane 5*), HeLa cells (HL, *lane 2*), or HepG2 cells (HG, *lane 3*) were incubated with the labeled agouti response element (AgRE, −435/−415 region of the FAS promoter). Excess AgRE competitor was added in *lane 4*, and no extracts were added to *lane 1*. Gel shift assays were performed as described in MATERIALS AND METHODS, and a representative autoradiogram is shown. Arrow indicates specific AgRE binding to adipocyte nuclear extracts. *B*: adipocyte nuclear extracts were incubated with labeled AgRE 5′-CAGCGACACGGACCTGTTCT-3′ in the absence (*lane 1*) or the presence of AgRE (*lane 2*) or mutations within AgRE, named M3 (5′-AAGAGACACGGACCTATTAT-3′) (*lane 3*) and M5 (5′- CAACATGTCGGACCTGTTCT-3′) (*lane 4*), where underlined bases were mutated. Gel shift assays were performed as described in MATERIALS AND METHODS, and a representative autoradiogram is shown.

genic genes including the FAS gene (5, 9, 17, 18, 27, 30). Insulin acts either directly or in conjunction with a glucose metabolite to regulate the FAS gene transcription (8, 9). In all of our studies, insulin effect was tested in the presence of glucose and thus may reflect an insulin/glucose regulation of the FAS gene. Hepatic transcription factors that mediate insulin responsiveness have been identified including upstream stimulatory factor I (USF1) and Sp1, which bind to −71 to −50 and −57 to −35 regions of the FAS promoter, respectively (9, 27). Adipocyte determination differentiation-dependent factor (ADD-1, or sterol regulatory element binding protein, SREBP-1) is another type of transcription factor, which mediates its effect on adipocyte energy homeostasis and adipocyte genes including FAS and leptin through the insulin response element (14).

Four different regions at −500 of FAS promoter bind specific nuclear factors, including Sp1 and NF-Y (25), and transcriptional induction of FAS by NF-Y and Sp1 interaction (cooperate DNA binding) has been suggested (25). Currently, transcription factors mediating agouti regulation of gene transcription are not known. To our knowledge, this is the first report of agouti regulation of gene transcription and of identification of agouti response sequences in any gene.

We demonstrated that agouti, like insulin, increases FAS activity in murine and human adipocytes. In 3T3-L1 adipocytes, this increase was mediated at the transcriptional level, and agouti and insulin together showed additive effects on FAS expression, suggesting that these two proteins act through independent transcription factors and DNA elements within the FAS promoter. Indeed, we identified an agouti-responsive sequence within a FAS promoter region (−435 to −415), which is distinct from insulin-responsive sequences we previously reported (18). Using mutations within the AgRE, we defined a minimal sequence at −426 (CGGACCTGTTCT) that is critical for binding of AgRE to adipocyte nuclear proteins. The agouti response sequence reported here is novel sequence that binds more likely to a novel *trans*-acting factor, since this sequence does not share homology with any other published regulatory sequences. Future studies will use further mutational analysis to fully characterize this agouti response element. Interestingly, we have shown that yellow obese mice carrying agouti mutation exhibit increased $[Ca^{2+}]_i$ levels (33) and that agouti increases $[Ca^{2+}]_i$ in several cell types, including adipocytes (12). Consistent with a Ca^{2+}-linked agouti transduction pathway, we have also shown that agouti induces adipocyte FAS activity, both in vivo and in vitro via a Ca^{2+} -dependent mechanism (11, 13). Therefore, further studies will aim to determine whether this agouti response sequence serves also as a Ca^{2+} response element in the FAS gene.

Agouti is normally expressed in human adipose tissue (28). Furthermore, we have recently shown that agouti is expressed in human pancreas, where it serves as a potent insulin secretagogue (31). Thus these data indicate that the coexpression of agouti and hyperinsulinemia, often found in concert, may be attributable in part to combined actions of agouti in adipose cells and in the pancreas. Our studies demonstrate that, in addition to the effect of agouti (or agouti related protein, AGRP) in the brain to increase food intake, agouti also regulates adipocyte metabolism, and thus this protein may act via central as well as peripheral mechanisms to regulate adiposity (22).

REFERENCES

1. **Amy CM, Williams-Ahlf B, Naggert J, and Smith S.** Molecular cloning of the mammalian fatty acid synthase gene, and identification of the promoter region. *Biochem J* 271: 675–679, 1990.
2. **Assimacopoulos JF, Brichard S, Rencurel F, Cusin I, and Jeanrenaud B.** In vivo effects of hyperinsulemia on lipogenic enzymes, and glucose transporter expression in rat liver and adipose tissues. *Metabolism* 44: 228–233, 1995.
3. **Beck KF, Schreglmann R, Stathopulos I, Klein H, Hoch J, and Schweizer M.** The fatty acid synthase (FAS) gene, and its promoter in *Rattus norvegicus*. *DNA Seq* 2: 359–386, 1992.
4. **Bultman SJ, Michaud EJ, and Woychik RP.** Molecular characterization of the mouse agouti locus. *Cell* 71: 1195–1205, 1992.
5. **Claycombe KJ, Jones BH, Standridge M, Chun J, Taylor JW, and Moustaid-Moussa N.** Insulin induces fatty acid synthase gene transcription in human adipocytes. *Am J Physiol Regulatory Integrative Comp Physiol* 274: R1253–R1259, 1998.
6. **Cristy RJ, Yang VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman AD, Nakabeppu Y, Kelly TJ, and Lane MD.** Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev* 3: 1323–1335, 1989.
7. **Fan W, Boston BA, Kesterson RA, Hurby VJ, and Cone RD.** Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 385: 165–168, 1997.
8. **Ferre P.** Regulation of gene expression by glucose. *Proc Nutr Soc* 58: 621–623, 1999.
9. **Fukuda H, Iritani N, Sugimoto T, and Ikeda H.** Transcriptional regulation of fatty acid synthase gene by insulin/glucose, polyunsaturated fatty acid and leptin in hepatocytes and adipocytes in normal and genetically obese rats. *Eur J Biochem* 260: 505–511, 1999.
10. **Hillgartner FB, Salati LM, and Goodridge AG.** Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. *Physiol Rev* 75: 47–76, 1995.
11. **Jones BH, Kim JH, Zemel MB, Woychik RP, Michaud ED, Wilkison WO, and Moustaid N.** Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. *Am J Physiol Endocrinol Metab* 270: E192–E196, 1996.
12. **Kim JH, Kiefer LL, Woychik RP, Wilkison WO, Truesdale A, Ittoop O, Willard D, Nichols J, and Zemel MB.** Agouti regulation of intracellular calcium: role of melanocortin receptors. *Am J Physiol Endocrinol Metab* 272: E379–E384, 1997.
13. **Kim JH, Mynatt RL, Moore JW, Woychik RP, Moustaid N, and Zemel MB.** The effects of calcium channel blockade on agouti-induced obesity. *FASEB J* 10: 1646–1652, 1996.
14. **Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, and Spiegelman BM.** Nutritional, and insulin regulation of fatty acid synthase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101: 1–9, 1998.
15. **Klebig ML, Wilkinson JE, Geisler JG, and Woychik RP.** Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes, and yellow fur. *Proc Natl Acad Sci USA* 92: 4728–4732, 1995.
16. **Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, and Cone RD.** Agouti protein is an antagonist of the melanocyte stimulating hormone receptor. *Nature* 371: 799–802, 1994.
17. **Moon YS, Latasa MJ, Kim KH, Wang D, and Sul HS.** Two 5′-regions are required for nutritional, and insulin regulation of the fatty-acid synthase promoter in transgenic mice. *J Biol Chem* 275: 10121–1012, 2000.
18. **Moustaid N, Beyer RS, and Sul HS.** Identification of an insulin response element in the fatty acid synthase promoter. *J Biol Chem* 269: 5629–5634, 1994.
19. **Moustaid N, Hainque B, and Quignard-Boulange A.** Dexamethasone regulation of terminal differentiation in 3T3-F442A preadipocyte cell line. *Cytotechnology* 1: 285–293, 1988.
20. **Moustaid N, Jones BH, and Taylor JW.** Insulin increases lipogenic enzyme activity in human adipocytes in primary culture. *J Nutr* 126: 865–870, 1996.
21. **Moustaid N and Sul HS.** Regulation of expression of the fatty acid synthase gene in 3T3-L1 cells by differentiation, and triiodothyronine. *J Biol Chem* 266: 18550–18554, 1991.
22. **Moustaid-Moussa N and Claycombe K.** Mechanisms of agouti-induced obesity. *Obes Res* 7: 506–514, 1999.
23. **Mynatt RL, Miltenberger RJ, Klebig ML, Zemel MB, Wilkinson JE, Wilkison WO, and Woychik RP.** Combined effects of insulin treatment and adipose tissue-specific agouti expression on the development of obesity. *Proc Natl Acad Sci USA* 94: 919–922, 1997.
24. **Paulauskis JD and Sul HS.** Hormonal regulation of mouse fatty acid synthase in liver. *J Biol Chem* 264: 154–167, 1989.
25. **Roder K, Wolf SS, Beck KF, and Schweizer M.** Cooperative binding of NF-Y, and Sp1 at the DNase I hypersensitive site, fatty acid synthase insulin-responsive element 1, located at −500 in the fatty acid synthase promoter. *J Biol Chem* 272: 21616–21624, 1997.
26. **Silvers WK.** An experimental approach to action of genes by the agouti locus in mouse. *J Exp Zool* 137: 189–196, 1958.
27. **Wang D and Sul HS.** Upstream stimulatory factors bind to insulin response sequence of the fatty acid synthase promoter. *J Biol Chem* 270: 28716–28722, 1995.
28. **Wilson BD, Ollmann MM, Kang L, Stoffel M, and Bell GL.** Structure, and function of ASP, the human homologue of the mouse agouti gene. *Hum Mol Genet* 4: 223–230, 1995.
29. **Wolf GL, Roberts DL, and Gealraith DB.** Prenatal determination of obesity, tumor susceptibility, and coat color pattern in viable yellow (A^{vy}/a) mice. *J Hered* 77: 151–158, 1986.
30. **Wolf SS, Hofer G, Beck KF, and Schweizer M.** Insulin-responsive regions of the rat fatty acid synthase gene promoter. *Biochem Biophys Res Commun* 203: 943–950, 1994.
31. **Xue B, Wilkison WO, Mynatt RL, Moustaid N, and Zemel MB.** The agouti gene product stimulates pancreatic β-cell Ca^{2+} signaling, and insulin release (Abstract). *FASEB J* 11: 320, 1997.
32. **Yen TT, Gill AM, Frigeri LG, Barsh G, and Wolff GL.** Obesity, diabetes and neoplasia in yellow A^{vy}/− mice: ectopic expression of the agouti gene. *FASEB J* 8: 479–488, 1994.
33. **Zemel MB, Kim JH, Woychik RP, Michaud EJ, Kadwell SH, Patel IR, and Wilkison WO.** Agouti regulation of intracellular free calcium: role in the insulin resistance of viable yellow mice. *Proc Natl Acad Sci USA* 92: 4733–4737, 1995.