Transcriptional regulation of the sodium-coupled neutral amino acid transporter (SNAT2) by 17β-estradiol

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The sodium-coupled neutral amino acid transporter 2 (SNAT2) translocates small neutral amino acids into the mammary gland to promote cell proliferation during gestation. It is known that SNAT2 expression increases during pregnancy, and in vitro studies indicate that this transporter is induced by 17β-estradiol. In this study, we elucidated the mechanism by which 17β-estradiol regulates the transcription of SNAT2. In silico analysis revealed the presence of a potential estrogen response element (ERE) in the SNAT2 promoter. Reporter assays showed an increase in SNAT2 promoter activity when cotransfected with estrogen receptor alpha (ER-α) after 17β-estradiol stimulation. Deletion of the ERE reduced estradiol-induced promoter activity by 63%. Additionally, EMSAs and supershift assays showed that ER-α binds to the SNAT2 ERE and that this binding competes with the interaction of ER-α with its consensus ERE. An in vivo ChIP assay demonstrated that the binding of ER-α to the SNAT2 promoter gradually increased in the mammary gland during gestation and that maximal binding occurred at the highest 17β-estradiol serum concentration. Liquid chromatography-elevated energy mass spectrometry and Western blot analysis revealed that the SNAT2 ER-α–ERE complex contained poly(ADP-ribose) polymerase 1, Lupus Ku autoantigen protein p70, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins and that the silencing of each of these proteins nearly abolished 17β-estradiol–stimulated SNAT2 promoter activity. Nuclear levels of GAPDH increased progressively during gestation in the mammary gland, and GAPDH binding was nucleotide-specific for the SNAT2 ERE. Thus, this study provides new insights into how the mammary epithelium adapts to control amino acid uptake through the transcriptional regulation of the SNAT2 transporter via 17β-estradiol.

coactivator | amino acid transport

The mammary gland has a high demand for amino acids during the gestation and lactation periods. It requires the uptake of amino acids for cell proliferation during pregnancy and for milk protein synthesis during lactation. Studies examining arteriovenous differences have demonstrated that glutamine and alanine are efficiently transported into the mammary gland at the start of lactation (1). These amino acids are primarily transported by amino acid transport system A, which is comprised of three subtypes known as sodium-coupled neutral amino acid transporter (SNAT) 1, 2, and 4 (2). SNAT2 is characterized as the classical system A transporter, is ubiquitous in mammalian cells, and plays various roles in different tissues and depending on specific physiological conditions (3–5). SNAT2 also provides efflux substrates for other amino acid transport systems, like the amino acid heteroexchanger system L, facilitating the uptake of branched-chain amino acids, particularly leucine that activates the TOR pathway involved in protein synthesis and cell proliferation (6). Up-regulation of SNAT2 gene expression is mediated by different signal transduction pathways. In the liver, it is activated by glucagon via cAMP/PKA, which phosphorylates the transcription factor CREB, allowing it to bind to the CRE site in the SNAT2 promoter region (7). In addition, system A activity is induced by insulin and glucocorticoids (8–11).

During pregnancy, there is a progressive increase in SNAT2 mRNA levels in the mammary gland. Interestingly, it has been shown that SNAT2 mRNA expression is induced by incubating rat mammary gland explants in the presence of 17β-estradiol, an effect repressed by the addition of the estrogen receptor (ER) inhibitor ICI-182780 (12). Similarly, Bhat and Vadgama (13) found that the activity and expression of SNAT2 are increased in mammary carcinoma cell lines that express ER alpha (ER-α) after 48 h of stimulation with 17β-estradiol.

A previous study in female mice with ER-α knocked out conditionally in the mammary gland demonstrated that pups born and nursed by these dams during lactation exhibited reduced body weight gain compared with pups born and fed by wild-type dams (14), possibly due to a reduction in the uptake of

Significance

During gestation, several metabolic adaptations occur to prepare the mammary gland for lactation, a process essential to sustain the nutritional needs of the newborn. Thus, the capacity of the gland to actively synthesize proteins is dependent on the supply of amino acids regulated by specific amino acid transporters, including the sodium-coupled neutral amino acid transporter 2 (SNAT2). Our findings reveal that 17β-estradiol activates the expression of SNAT2 during gestation via an estrogen response element in the SNAT2 promoter that binds to a specific complex containing poly(ADP-ribose) polymerase 1, Lupus Ku autoantigen protein p70, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins in the presence of estrogen receptor alpha (ER-α). Accordingly, estrogens regulate the expression of SNAT2 via ER-α, utilizing a transcriptional mechanism in which GAPDH, a metabolic enzyme associated with carbohydrate metabolism, is a coactivator.

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substrates for milk production, including the amino acids transported by SNAT2.

In silico analysis of the SNAT2 promoter region revealed the presence of a putative estrogen response element (ERE) between bases −1,486 and −1,471 upstream of the transcriptional start site, suggesting that the promoter region of the SNAT2 gene is regulated by 17β-estradiol and that this regulation is activated during gestation when the levels of this hormone (15) and amino acid requirements (16) increase, supporting the proliferation of the mammary tissue. The aim of this study was to elucidate the molecular mechanism by which 17β-estradiol regulates the transcription of the SNAT2 gene.

Results

SNAT2 ERE-Containing Promoter Region Is Activated by 17β-Estradiol. To determine whether the putative ERE found in the SNAT2 promoter region was functional, we performed assays using SNAT2 promoter constructs and the luciferase reporter gene. We observed (Fig. 1*A*) that 1×10^{-8} M 17β-estradiol increased luciferase activity approximately twofold when using base pairs −1,872 to +265 of the SNAT2 promoter region. To corroborate that loss of the ERE site between bases −1,486 and −1,471 diminished the effect of the hormone, we used a series of unidirectional 5′ deletion constructs generated from the complete sequence. The 17β-estradiol–induced luciferase activity was reduced by ∼63% and 75% with the constructs encoding base pairs −872 to +265 and −336 to +265, respectively. The remaining activity was not generated by a direct interaction of ER-α with a specific ERE because there are no other putative EREs in the SNAT2 promoter. Deletion of base pairs −1,872 to −192 resulted in the loss of 17β-estradiol–induced activity.

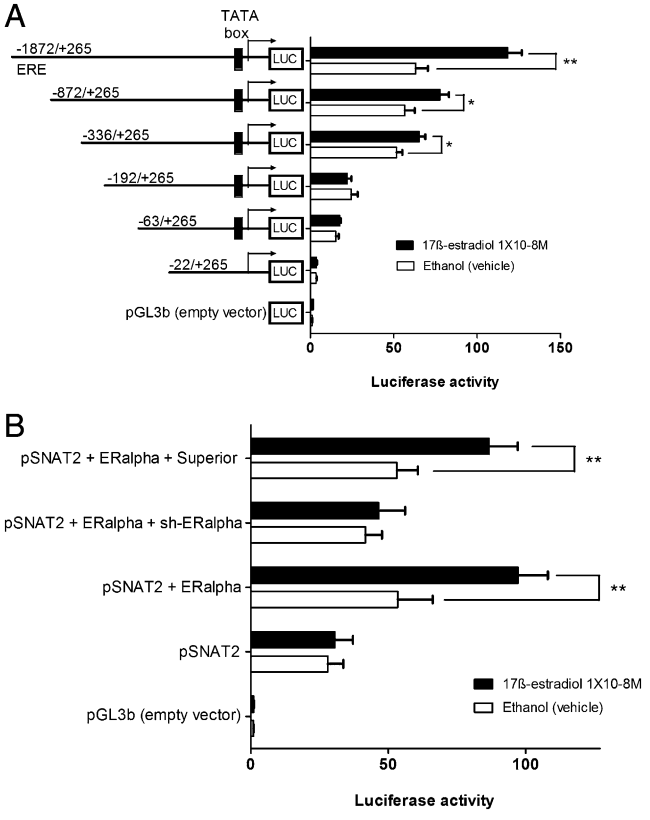
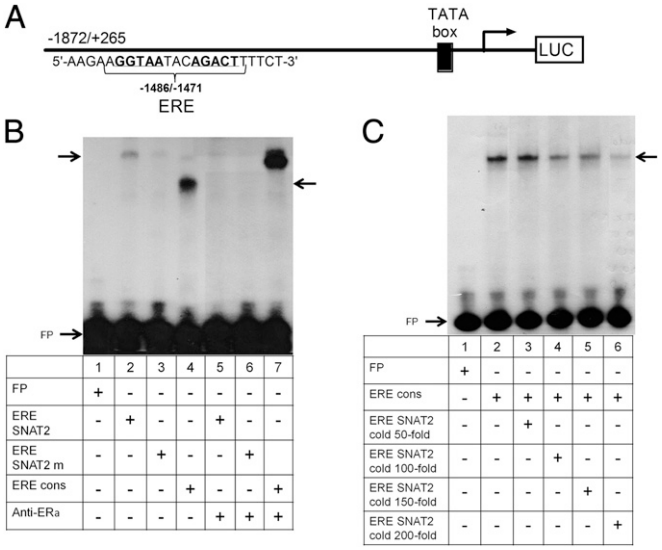


Fig. 1. Functional analysis of the ERE site in the rat SNAT2 promoter. (*A*) Effect of 1×10^{-8} M 17β-estradiol on SNAT2 promoter activity. HeLa cells were cotransfected with each promoter–reporter construct, the ER-α expression vector, and pRL-TK and incubated in the presence of 1×10^{-8} M 17β-estradiol or vehicle (ethanol). Luciferase activities were measured 24 h after transfection. The results are the mean ± SE of three independent experiments per construct. (*B*) Effect of the ER-α-sh plasmid on SNAT2 promoter activity. HeLa cells were cotransfected with the ER-α-sh plasmid or empty vector (Superior), the SNAT2 promoter–reporter construct (pSNAT2), or empty vector (pGL3b), the ER-α expression vector, and pRL-TK and incubated in the presence of 1×10^{-8} M 17β-estradiol or vehicle (ethanol). Luciferase activities were measured 24 h after transfection. The results are the mean ± SD of three independent experiments per construct. $*P < 0.05$; $**P < 0.01$ (significant difference compared with their respective control).

SNAT2 Promoter Activation by 17β-Estradiol Is ER-α–Dependent. To determine whether the SNAT2 promoter activation mediated by 17β-estradiol is ER-α–dependent, we used an ER-α–sh plasmid to knock down the ER-α protein. As shown in Fig. 1*B*, ER-α knockdown prevented SNAT2 promoter activation by 17β-estradiol. There was no reduction in SNAT2 promoter activity induced by 17β-estradiol in control cells transfected with an empty plasmid. These results corroborate the importance of ER-α regulation of the SNAT2 promoter in response to 17β-estradiol and suggest that the remaining activity observed in the promoter constructs containing base pairs −872 to +265 and −336 to +265 could be ER-α–dependent.

ER-α Binds to the ERE in the SNAT2 Promoter in Vitro. Because it has been established that ER-α activated by 17β-estradiol binds to the ERE sites of target genes, the next step was to corroborate whether ER-α could bind to the ERE site found in the SNAT2 promoter region. As shown in Fig. 2*A*, lane 2, electrophoretic mobility-shift assay (EMSA) analysis of the SNAT2 ERE site using extracts from HeLa cells transfected with ER-α indicated binding with a retarded band. Mutation of the SNAT2 ERE site (Table S1) abolished the formation of this complex (Fig. 2*A*, lane 3), suggesting that this site binds to ER-α. As a positive control, we used the ERE consensus sequence, which produced a very intense retarded band (Fig. 2*A*, lane 4). Interestingly, the ERE consensus band traveled further than the SNAT2 ERE complex in the gel, suggesting that different cofactors might bind to each complex. To demonstrate whether ER-α was bound to the SNAT2 ERE sequence, we used an anti–ER-α antibody. Fig. 2*A*, lane 5, demonstrates that preincubation with the antibody abolished the formation of the complex, possibly by blocking the ER-α DNA-binding site. The formation of a super-shifted band was observed (Fig. 2*A*, lane 7) with the consensus ERE sequence, indicating that the proteins involved in the formation of the complex might vary between the SNAT2 ERE and the ERE consensus sequence. In fact, liquid chromatography-elevated energy mass spectrometry ($LC\text{-}MS^E$) analysis confirmed the difference between both complexes (see below). The SNAT2 ERE sequence competed with the ERE consensus sequence for ER-α binding because the addition of a 200-fold molar excess of unlabeled (cold) SNAT2 ERE sequence nearly obliterated binding to the ERE consensus sequence (Fig. 2*C*).

ERα Binds to the SNAT2 ERE Site in the Rat SNAT2 Promoter in Vivo. The functionality of the SNAT2 ERE site was also demonstrated in vivo. The increase of 17β-estradiol levels during gestation favored the binding of ER-α to the SNAT2 ERE site in the mammary gland as assessed by chromatin immunoprecipitation (ChIP) assays, demonstrating an amplification product on days 5, 14, and 20 of gestation (Fig. 3 *A* and *B*). Quantitative PCR was performed to quantify the binding of ER-α to the SNAT2 ERE site in the rat SNAT2 promoter. There was a progressive increase in binding during the gestation period, which corresponded to the increase in serum estradiol levels in the rat (Fig. 3 *B* and *C*, respectively). Virgin control rats in the diestrus period have very low levels of the hormone, and no ER-α binding was observed. A high level of hormone is needed to activate SNAT2



	1	2	3	4	5	6	7
FP	+	-	-	-	-	-	-
ERE SNAT2	-	+	-	-	+	-	-
ERE SNAT2 m	-	-	+	-	-	+	
ERE cons	-	-	-	+	-	-	+
Anti-ERa	-	-	-	-	+	+	+

	1	2	3	4	5	6
FP	+	-	-	-	-	-
ERE cons	-	+	+	+	+	+
ERE SNAT2 cold 50-fold	-	-	+	-	-	-
ERE SNAT2 cold 100-fold	-	-	-	+	-	-
ERE SNAT2 cold 150-fold	-	-	-	-	+	-
ERE SNAT2 cold 200-fold	-	-	-	-	-	+

Fig. 2. Binding of ER-α to the ERE in the SNAT2 promoter in vitro. Double-stranded DNA oligonucleotides containing the SNAT2 ERE site (ERE SNAT2), the SNAT2 ERE mutated site (ERE SNAT2 m), or the ERE consensus sequence (ERE cons) were incubated with HeLa nuclear extracts from cells previously transfected with ER-α. The DNA sequences of the oligonucleotides used are indicated in Table S1. (*A*) Localization of the ERE sequence in the SNAT2 promoter region between the bases −1,486 and −1,471 upstream of the transcriptional start site. (*B*) EMSA analysis of HeLa nuclear extracts previously transfected with ER-α using labeled oligonucleotides containing the wild-type SNAT2 ERE site (lane 2), the SNAT2 ERE mutated site (lane 3), or the ERE consensus site (lane 4). The supershift assay was performed by preincubating 1 μg of anti–ER-α antibody with the HeLa nuclear extracts from cells previously transfected with ER-α. Probes for the labeled SNAT2 ERE site (lane 5), the SNAT2 ERE mutated site (lane 6), or the consensus ERE sequence (lane 7) were added. (*C*) Competitive assays using the labeled ERE consensus sequence and an excess of unlabeled SNAT2 ERE sequence (SNAT2 ERE cold) at 50-, 100-, 150-, and 200-fold molar excess are shown in lanes 3, 4, 5, and 6, respectively. FP, free probe.

transcription, and therefore SNAT2 is up-regulated exclusively during the gestation period and possibly during the proestrus phase of the female rat. Additionally, the use of different concentrations of 17β-estradiol (from 1×10^{-6} to 1×10^{-11} M) demonstrated that the SNAT2 promoter requires a higher concentration of hormone to induce transcription compared with the consensus ERE sequence (Fig. 3*D*). For instance, whereas 10^{-11} and 10^{-10} M 17β-estradiol robustly activated the consensus ERE construct, the SNAT2 ERE required higher hormone concentrations to be activated to the same degree.

PARP1, Ku70, and GAPDH Bind to the SNAT2 Promoter ERE Sequence with ER-α. We used a technique based on a desthiobiotin–triethylene glycol (TEG) oligo and streptavidin beads to precipitate the ER-α–ERE complex and identify the other proteins bound to the SNAT2 promoter region following activation by 17β-estradiol. For this assay, double-stranded DNA oligonucleotides containing the SNAT2 ERE site (ERE SNAT2 oligo) and the ERE consensus sequence (ERE cons oligo) were incubated with nuclear extracts from HeLa cells transfected with ER-α. HeLa nuclear extracts from untransfected cells were used as negative controls for each oligo. The ER-α–ERE complex was precipitated with streptavidin beads and eluted with 5 mM biotin (Fig. 4*A*). LC-MS^{E} (Table S2) and Western blot analysis (Fig. 4*B*) revealed the presence of poly(ADP-ribose) polymerase 1 (PARP1), Lupus Ku autoantigen protein p86 (Ku80), Ku70, and topoisomerase IIβ with ER-α on the ERE consensus oligo and PARP1, Ku70, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as well as topoisomerase I and IIβ with ER-α on the SNAT2 ERE oligo. Although PARP1 bound equally well to both ERE sequences, the intensity of some of the bands bound to the SNAT2 ERE oligo was lower than to the ERE consensus oligo, particularly Ku70 and topoisomerase IIβ, possibly because ERα has a lower affinity for the SNAT2 ERE than the consensus ERE. Interestingly, topoisomerase I and GAPDH bind exclusively to the SNAT2 ERE site. Binding of these proteins was dependent on ER-α binding because most of them did not precipitate in the absence of ER-α (Fig. 4*B*).

The activity of the luciferase reporter plasmid encoding the consensus ERE sequence in cells cotransfected with ER-α and stimulated with 17β-estradiol decreased significantly when cotransfected with PARP1 and Ku70 sh-plasmids (Fig. 4*C*). Interestingly, the luciferase reporter plasmid encoding the SNAT2 ERE sequence and the ER-α expression vector showed a significant decrease in activity in response to 17β-estradiol when GAPDH, PARP1, or Ku70 was knocked down (Fig. 4*D*).

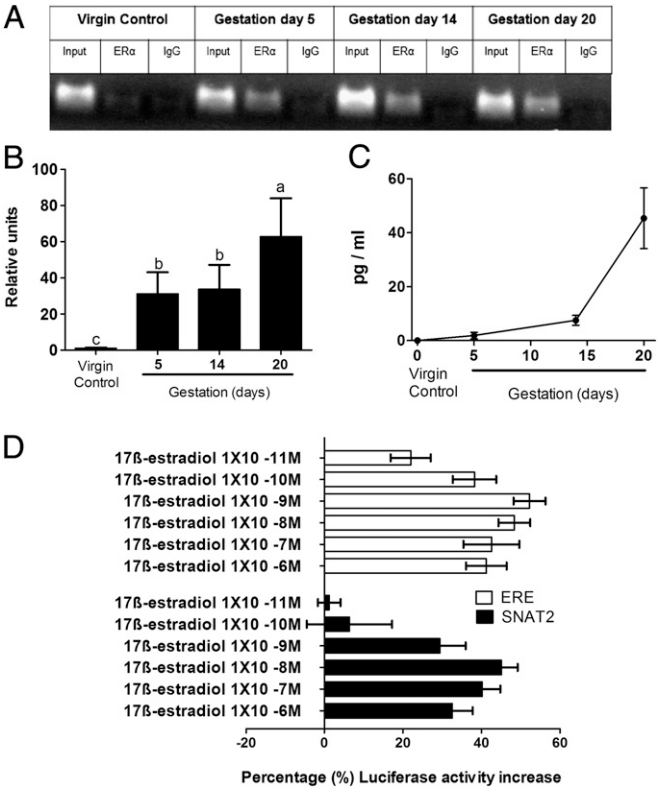


Fig. 3. ER-α binds to the SNAT2 ERE site in the rat SNAT2 promoter in vivo. (*A*) ChIP showing ER-α binding. ChIP assays were conducted on mammary glands from virgin control rats and 5-, 14-, or 20-d pregnant rats. Soluble chromatin was immunoprecipitated with 4 μg of anti–ER-α antibody or negative control IgG and subjected to PCR with a specific primer to the SNAT2 ERE site (Table S1). (*B*) Real-time PCR analysis of amplified products corresponding to ER-α binding to the SNAT2 ERE. This graph is representative of three independent experiments ($n = 5$ rats per group). (*C*) Serum 17β-estradiol levels in virgin control rats and 5-, 14-, and 20-d pregnant rats ($n = 5$ rats per group). (*D*) Effect of different concentrations of 17β-estradiol (1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, 1×10^{-10}, and 1×10^{-11} M) on the ERE consensus promoter–reporter construct and the SNAT2 promoter reporter construct activity. HeLa cells were cotransfected with each promoter–reporter construct, the ER-α expression vector, and pRL-TK and incubated in the presence of 17β-estradiol or vehicle (ethanol). Luciferase activities were measured 24 h after transfection. The results are the mean ± SD of three independent experiments per construct and are expressed as percentage (%) of luciferase activity increase compared with the vehicle (ethanol). Different letters differ: a > b > c. $P < 0.05$.

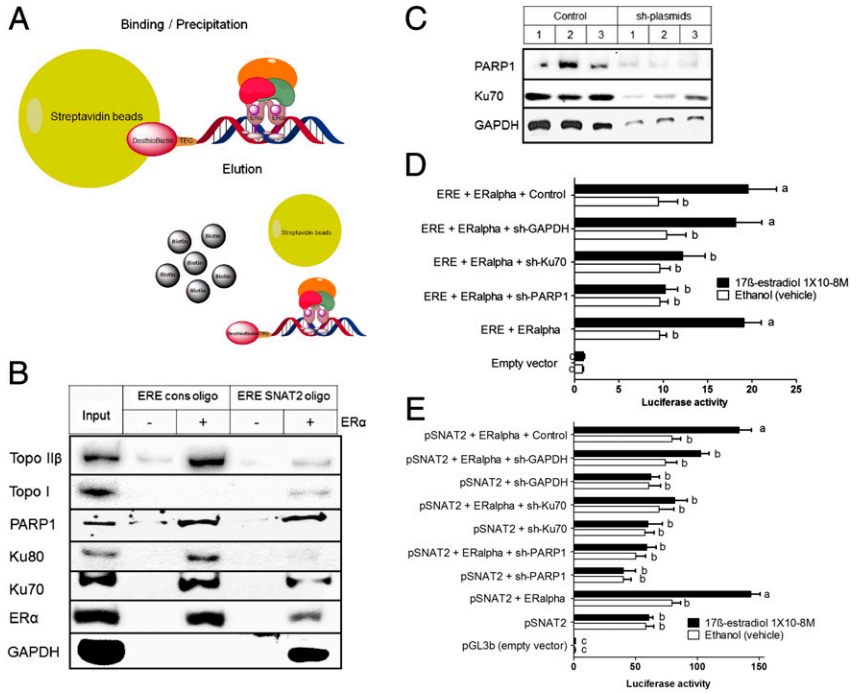


Fig. 4. ER-α–ERE complex purification. (*A*) ER-α–ERE consensus and ER-α–SNAT2 ERE complexes were precipitated by using desthiobiotin–TEG oligos for the ERE consensus and the SNAT2 ERE sites (Table S1). The desthiobiotin–TEG oligos (2 μg) were incubated with HeLa nuclear extracts or HeLa nuclear extracts from cells previously transfected with ER-α, poly(dI-dC) (2 μg) and 1×10^{-8} M 17β-estradiol at 4 °C overnight in a rotation device. The volume was adjusted to 400 μL with binding buffer [50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 μg/μL poly(dI-dC), 20% glycerol]. Each sample was incubated with streptavidin magnetic beads for 2 h at 4 °C and washed three times with the same binding buffer in a rotation device. After the washes, the precipitated oligo-complex was eluted with 5 mM biotin and run on an SDS/PAGE for Western blot analysis. (*B*) Occupancy of Topo IIβ, Topo I, PARP1, Ku80, Ku70, ER-α, and GAPDH on the ERE consensus and SNAT2 ERE sequences. HeLa nuclear extracts without ER-α transfection were used as negative controls for each oligo. (*C*) Representative immunoblot of the knockdown sh-plasmids (PARP1, Ku70, and GAPDH) efficiency. (*D*) Effect of the PARP1, Ku70 and GAPDH sh-plasmids on ERE consensus activity. (*E*) Effect of the PARP1, Ku70, and GAPDH sh-plasmids on SNAT2 promoter activity. HeLa cells were cotransfected with PARP1, Ku70, or GAPDH sh-plasmids, with or without the ER-α expression vector, each promoter–reporter construct, and pRL-TK and incubated in the presence of 1×10^{-8}M 17β-estradiol or vehicle (ethanol). Luciferase activities were measured 24 h after transfection. The results are the mean ± SD of three independent experiments per construct. Different letters differ: a > b > c. $P < 0.05$.

GAPDH Accumulates in the Nucleus, Binds in Vivo to the SNAT2 Promoter of Mammary Epithelium During Gestation, and Is Specific for SNAT2 ERE Activation. GAPDH is a metabolic enzyme involved in glycolysis, gluconeogenesis, and glyceroneogenesis. Intriguingly, most of the amino acids transported by SNAT2 are considered gluconeogenic substrates. Feeding dams a high-protein diet (30%) that contains an excess of gluconeogenic amino acids significantly increased the expression of cytoplasmic GAPDH in the rat mammary gland during gestation on day 20 compared with those fed an adequate-protein (20%) diet (Fig. 5 *A* and *C*). In addition, there was a progressive increase in the nuclear abundance of GAPDH during gestation in rat mammary tissue, reaching a peak on day 20 of gestation as shown by Western blot and immunohistochemical analysis (Fig. 5 *B* and *D*–*G*). Moreover, ChIP assays demonstrated that GAPDH binds in vivo to the SNAT2 ERE in rat mammary gland during gestation, especially on days 14 and 20, whereas virgin control rats lack this binding (Fig. 5*H*). All these data show that GAPDH is translocated to the nucleus during gestation to actively maintain SNAT2 gene expression. To determine the specificity of GAPDH binding to the SNAT2 ERE, we performed a precipitation assay using single-nucleotide mutations and flanking regions similar to the SNAT2 consensus sequence. Our results clearly demonstrated that GAPDH binding to the SNAT2 ERE is exclusively dependent on the palindromic sequence 5′-AGGT**A**ATACAGACTT-3′; 5′-AGGT**C**ATACAGACTT-3′ does not bind. Likewise, GAPDH binds to the 5′ flanking region of the palindromic sequence 5′-**AAGA** AGGTAA TAC AGACTT TTCT-3′ but not to 5′-**TATC** AGGTAA TAC AGACTT TTCT-3′ (bold type letters indicate the nucleotide or nucleotides that were changed in the sequence) (Fig. 5*I*).

Discussion

During gestation and lactation, a series of complex mechanisms function to provide food to the newborn. To activate these mechanisms, the mammary gland initiates a specific program to prepare the tissue to be a secretory organ for feeding the newborn. Therefore, several genes are turned on for the proliferation and differentiation of the mammary epithelium during the gestation period for the synthesis of nutrients to fulfill the requirements of the newborn (17–21).

To trigger the mechanisms of gene expression in the mammary gland during gestation that regulate these processes, the hormonal milieu during this stage (20) controls the transcription of several genes. In particular, 17β-estradiol has been demonstrated to be a powerful activator of genes involved in the cell differentiation and proliferation (22–25) of the lactation process. The effects of 17β-estradiol in the mammary gland take place via ER-α and -β (26, 27).

There are little data examining whether 17β-estradiol is involved in the mechanisms associated with the supply of nutrients for the mammary gland, and less is known about the molecular mechanisms. Amino acids are essential nutrients for protein synthesis, and they are incorporated into cells by specific transporters. It is known that the activities of some of these transporters in the mammary gland are stimulated by estrogens (28–30). In this study, we demonstrated that 17β-estradiol activates the expression of the SNAT2 amino acid transporter via the ERE present in the SNAT2 promoter region and requires the formation of a specific complex containing PARP1, Ku70, and GAPDH proteins.

Our results agree with previous findings that the topoisomerase IIβ/PARP-1 complex mediates the transient formation of a DNA break that is required for 17β-estradiol–stimulated gene activation. PARP-1 uses histone H1 (known to be a transcriptional repressor) as a substrate for poly(ADP ribosyl)ation. Histone H1 is replaced by a high mobility group B 1/2 protein that acts as an activator upon 17β-estradiol stimulation. In this process, topoisomerase IIβ, PARP-1, DNA-PK, Ku80, and Ku70 are recruited to the ERE in the nucleosome after 17β-estradiol treatment (31). Another recent study has demonstrated that PARP1 interacts with ER-α, promoting poly(ADP ribosyl)ation and transactivation of the receptor, indicating that PARP1 is a crucial regulator of ER-dependent gene transcription (32).

Intriguingly, we demonstrated that GAPDH is part of the SNAT2 ER-α–ERE complex. In addition, we observed that its binding to the SNAT2 ERE depends on specific nucleotides present in the palindromic sequence and flanking region of the

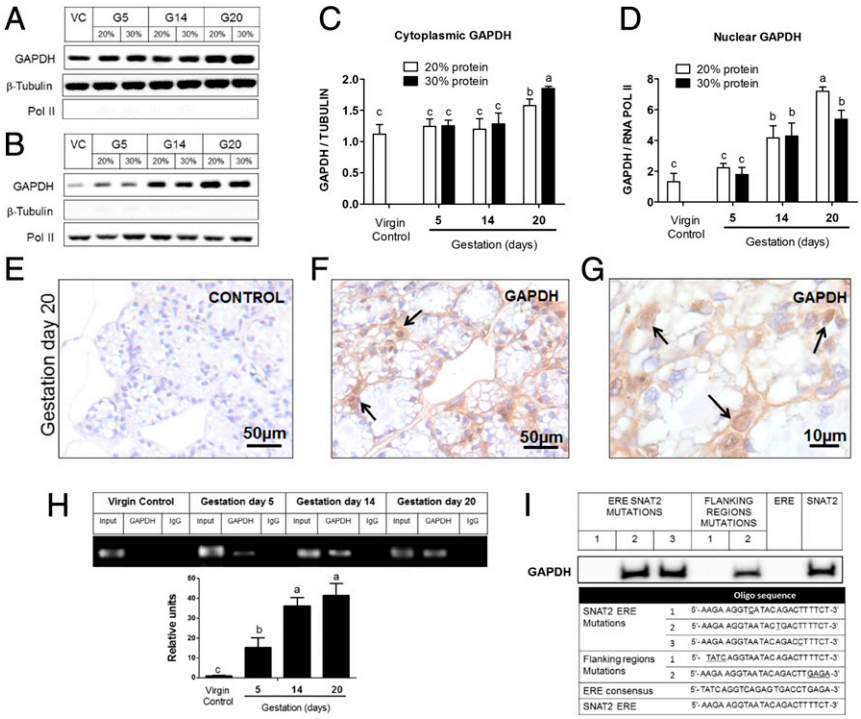


Fig. 5. The expression of GAPDH in rat mammary glands. (*A* and *B*) A representative immunoblot of GAPDH, β-tubulin, and RNA polymerase II (Pol II) in cytoplasmic extract (*A*) and GAPDH, β-tubulin, and Pol II in the nuclear extract of mammary glands from rats fed 20% or 30% dietary protein (*B*). (*C* and *D*) Western blot densitometric analysis of cytoplasmic GAPDH/tubulin (*C*) and nuclear GAPDH/RNA polymerase II of the mammary glands from rats fed 20% or 30% dietary protein (*D*). The results are the mean ± SD of three different blots. (*E*) GAPDH immunohistochemistry in rat mammary gland during gestation on day 20. (*F* and *G*) Negative control (*F*) and GAPDH (*G*). The arrows indicate the presence of GAPDH in the nucleus of mammary epithelium cells. [Scale bars: 50 μm (*E* and *F*); 10 μm (*G*).] (*H*) ChIP showing GAPDH binding to the SNAT2 ERE. ChIP assays were conducted on mammary glands from virgin control rats and 5-, 14-, and 20-d pregnant rats. Soluble chromatin was immunoprecipitated with 4 μg of anti-GAPDH antibody or negative control IgG and subjected to PCR with a specific primer to the SNAT2 ERE site (Table S1). Results shown are representative of three independent experiments ($n = 5$ rats per group). Different letters differ: a > b > c. $P < 0.05$. (*I*) A representative immunoblot of GAPDH precipitation in SNAT2 ERE and SNAT2 ERE mutated sequences using biotinylated oligos incubated with ER-α transfected nuclear extracts from HeLa cells.

ERE found in the SNAT2 promoter region. This sequence differs from the consensus by three nucleotides and is unique among other EREs present in several genes, including metabolic genes that also have imperfect palindromic sequences with at least one base pair change (33). Differences between the ERE sequences and their promoter localizations modulate ER transcriptional activity, recruiting different patterns of coactivators (34). Additionally, it has been demonstrated that upstream EREs recruit histone acetyltransferase and/or SWI/SNF complexes, whereas only proximal promoter sequences may be able to recruit mediator complexes (35). Interestingly, in our study of the SNAT2 ERE, we observed that change of the nucleotide "A" to "C" in the 5′ palindromic sequence and of the 5′ flanking region, to make it resemble that of the consensus ERE, resulted in the loss of GAPDH recruitment to the complex. Thus, transcriptional activation of the SNAT2 gene in the mammary gland to fulfill the elevated demand of amino acids occurs during gestation, a physiological condition with elevated levels of 17β-estradiol to overcome the lower affinity of ER-α to the SNAT2 ERE, which specifically binds the metabolic enzyme GAPDH as a coactivator.

GAPDH was recently found to be a cofactor of transcriptional complexes (36, 37). Zheng Lei et al. demonstrated that p38/GAPDH is an essential Oct-1 CoActivator in S phase specific for histone-2B transcription and that it is modulated by NAD+/NADH ratio (38). Several other studies have described the participation of GAPDH in different transcriptional complexes (39–41). However, none of them involves the recruitment of this protein to an ERE–ER-α complex, perhaps indicating a crosstalk with metabolic events as suggested in the present study. Interestingly, we observed an increase in the nuclear abundance of GAPDH, particularly between days 15 and 20 of gestation, and at least cytoplasmic GAPDH concentration depended on the amount of dietary protein consumed.

The expression pattern of other amino acid transporters during gestation is different from SNAT2 (42). The data obtained in this work imply that the regulatory effect of estrogens on the expression of the SNAT2 gene is a unique feature in the transport of amino acids into the mammary gland during this period. It has been postulated that the inhibition of system A by a systemic infusion of an amino acid analog that cannot be metabolized, 2-(methylamino) isobutyric acid, during gestation resulted in a decrease in fetal weight (43). This previous evidence in addititon to the results obtained in this study suggest that SNAT2 plays a significant role in the supply of amino acids, an important function in the mammary epithelium for the differentiation and proliferation of these cells to prepare the gland for lactation.

Materials and Methods

Animals. Female Wistar rats were obtained from the animal research facility at the Instituto Nacional de Ciencias Médicas y Nutrición. The animals were housed and fed as reported (16). For experimental procedure details, see *SI Materials and Methods*.

Cell Culture, Transient Transfection, and Luciferase Reporter Assays. HeLa cells were transfected by using the PolyFect transfection reagent according to the manufacturer's manual. Details for the cell-growing conditions and transfection assays are given in *SI Materials and Methods*.

Preparation of Nuclear Extracts and EMSA. Nuclear extracts from HeLa cells and rat mammary gland tissue were prepared with the Pierce nuclear extract kit according to the manufacturer's instructions. DNA mobility-shift assays were performed by using the gel shift assay system according to the manufacturer's instructions. For experimental details, see *SI Materials and Methods*.

ChIP Assay. Frozen rat mammary glands from virgin control and pregnant rats on days 5, 14, and 20 were used for ChIP assays. Fragmented chromatin from each sample was immunoprecipitated with anti–ER-α or anti-GAPDH antibodies. Details of the technique are given in *SI Materials and Methods*.

Complex Precipitation and Protein Identification by LC/MS^E. ER-α–ERE consensus and ER-α–SNAT2 ERE complexes were precipitated by using desthiobiotin–TEG oligos for the ERE consensus and SNAT2 ERE sites (Table S1). The precipitated proteins were digested with trypsin and prepared for identification by LC/MS^E or Western blot analysis. Details of the techniques can be found in *SI Materials and Methods*.

Western Blot Analysis. Proteins from the complex purification assay or proteins extracted from rat mammary gland using a described method (16) were separated by electrophoresis, transferred onto a poly(vinylidene difluoride)

membrane (Amersham GE Healthcare), and blotted with specific antibodies for protein detection. Details are given in *SI Materials and Methods*.

Immunohistochemistry. For the immunohistochemistry experiments, rat mammary gland was obtained from gestation day 20. Tissue sections 4 mm thick, embedded in paraffin, were mounted on polarized slides for immunohistochemistry detection (details are in *SI Materials and Methods*). The slides were analyzed by using the Leica QWin image-analyzer system on a Leica DMLS microscope.

Statistical Analysis. The results obtained in this study are presented as the means ± SD. The data were evaluated with the Student *t* test and were considered significant at $P < 0.05$. The data were analyzed with Prism for Mac OS X software (Version 5).

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