Cholesterol supply and SREBPs modulate transcription of the Niemann-Pick C-1 gene in steroidogenic tissues

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Abstract **We tested whether sterol-regulatory element binding proteins (SREBPs) mediate sterol-regulated transactivation of the Niemann-Pick C-1 (NPC-1) gene. Loading granulosa cells with 22- or 25-hydroxycholesterol decreased NPC-1 mRNA, whereas culturing in cholesterol-depleted medium or inhibition of cholesterol biosynthesis increased NPC-1 promoter activity and NPC-1 mRNA abundance. Cotransfection of SREBP1a, SREBP1c, and SREBP2 and the NPC-1 promoter-luciferase reporter into granulosa cell lines increased the transcriptional activity of porcine, human, and mouse NPC-1 promoters. Deletion analysis of the 5′ flanking region of the pig NPC-1 gene demonstrated significant promoter activity between fragments −934 and −636 bp upstream from the transcription initiation site. Sequence analysis revealed three sterol-regulatory elements (SREs) clustered between −558 and −650 bp. Each site, along with E-box sequences, bound recombinant SREBP in electromobility shift assays. Mutation of all three sites attenuated the SREBP induction of promoter activity. Chromatin immunoprecipitation (ChIP) assays revealed that cholesterol depletion enriched the association of both SREBP and acetylated histone H3 with the NPC-1 promoter fragment containing the three SREs. ChIP analysis confirmed that SREBP's association with SRE and the E-box was enriched in cells cultured in cholesterol-depleted medium. We conclude that NPC-1 is sterol-regulated, achieved by SREBP acting via SRE and the E-box sequences.**—Gévry, N., K. Schoonjans, F. Guay, and B. D. Murphy. **Cholesterol supply and SREBPs modulate transcription of the Niemann-Pick C-1 gene in steroidogenic tissues. *J. Lipid Res.* 2008.** 49: **1024–1033.**

Supplementary key words sterol regulatory element binding protein • gene transcription • cholesterol trafficking • granulosa cells

Intracellular cholesterol is regulated by a negative feedback mechanism that senses sterol concentrations and modifies transcription and, by consequence, the expression of a diverse array of proteins that synthesize, import, and transport cholesterol (1, 2). The key elements in this transcriptional regulation are the sterol-regulatory element binding proteins (SREBPs), members of a family of three membrane-bound transcription factors (3). The SREBP1a and -1c isoforms result from alternate splicing of a single gene, whereas SREBP2, which bears 47% homology to SREBP1a, is derived from a second gene (3). The SREBP cleavage-activating protein (SCAP), which contains a sterol-sensing domain, is the proximate regulator of SREBP-modulated transcription (1). At low intracellular sterol concentrations, SCAP translocates the inactive, membrane-bound form of SREBP to the Golgi, where two distinct proteolytic cleavage steps convert it to its nuclear and transcriptionally active form (4). Cholesterol is believed to act directly on SCAP via its sterol-sensitive domain, inducing conformational changes that interdict its translocation function (5). After activation, the SREBPs translocated to the nucleus dimerize and interact with cognate regulatory sequences, the sterol-regulatory elements (SREs), to transactivate genes (3) in concert with coactivators (6).

The Niemann-Pick C-1 (NPC-1) gene codes for a protein in the network of intracellular cholesterol homeostasis (7). It is a late endosomal protein and is required for the delivery of low density lipoprotein-derived cholesterol to the endoplasmic reticulum (8). Humans and mice bearing spontaneous inactivating mutations of this gene have nearly ubiquitous pathological and fatal intracellular cholesterol accumulation (8). The NPC-1 protein has a sterol-sensing domain that mediates sterol binding (9), and point mutation studies have shown that this domain is required for the transport of cholesterol imported by means of the LDL pathway (10). Early studies of the sequence of the human NPC-1 gene suggested that that it was constitutively expressed rather than under active transcriptional regulation (11). By deletion analysis of the 5′-flanking region of the porcine promoter, it was shown

Manuscript received 28 November 2007 and in revised form 18 January 2008.

Published, JLR Papers in Press, February 13, 2008.
DOI 10.1194/jlr.M700554-JLR200

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that there is a requirement for a segment of 1.2 kb upstream of the translation initiation site for full transcriptional expression (12). The increases in NPC-1 expression in steroidogenic tissues that followed gonadotropin stimulation (13) were shown to result from transcription modulated by the cyclic AMP, protein kinase A pathway (12), indicating active, rather than constitutive, regulation.

The essential role for NPC-1 in cholesterol trafficking and homeostasis argues that, as with most other genes that affect intracellular cholesterol abundance, its expression should be sterol-modulated. Indeed, the transcription of a structurally related plasma membrane protein, the Niemann-Pick C-1-like protein (NPC-1L1), was recently shown to be regulated by intracellular cholesterol via a SREBP system (14). In a recent study, mouse body cholesterol load was manipulated, either by feeding mice high-cholesterol diets or by reducing cholesterol by feeding cholestyramine (15). From subsequent measurement of NPC-1 mRNA and protein in this experimental paradigm, it was concluded that cholesterol entering cells via the coated-pit, endosomal pathway does not regulate NPC-1 expression (15). In contrast, a more recent investigation using human fibroblasts demonstrated that LDL cholesterol does, in fact, downregulate NPC-1 expression via SREBP (16). We undertook parallel experimentation in the porcine model to examine this question. Here, we demonstrate that, in porcine granulosa cells in primary culture, cholesterol depletion increases the expression of NPC-1 and this effect is abolished by exogenous cholesterol. Our results contrast with those in the mouse whole animal model (15) but concur with findings in human fibroblasts. In addition, we show that the promoter regions of the pig, human, and mouse contain conserved consensus SREs and that SREBP associates with SREBP regions of the porcine NPC-1 promoter bearing these SREs. Furthermore, both depletion of cholesterol in the cellular culture medium and transient overexpression of SREBP induce the activity of the NPC-1 promoter. Putative SRE sequences bind recombinant SREBP, and their ablation prevents SREBP induction of transcriptional activity. Our findings provide novel and compelling evidence indicating that NPC-1 is regulated by intracellular cholesterol in steroidogenic cells.

EXPERIMENTAL PROCEDURES

Comparative sequence analysis

A comparison was made among the 5′ flanking region of the porcine (12), mouse (17), and human (11) NPC-1 gene promoter regions by combining the conserved sequence analysis algorithm (BLASTZ) with transcription factor binding site analysis (MatInspector; http://trafac.chmcc.org). Sequences with at least 95% homology to the SRE site consensus direct repeat 5′-ATCACCCCAC-3′ were mapped across these three species.

Plasmid constructions

The 1.8 kb NPC-1 gene promoter was cloned into pGL3 basic vector (Promega, Nepean, Ontario, Canada), and various deletion constructs were prepared by PCR with *Eco*RI and *Mlu*I insertions for directed cloning. Potential SREBP response sites and E-box sequences were mutated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Expression vectors for SREBP1a, -1c, and -2 used were as described in previous investigations (18). To compare the responses of the porcine, human, and mouse promoters, 1.9 kb of the human and 2.1 kb of the mouse 5′ flanking regions upstream from the putative transcription start sites were cloned into the pGL3 basic vector.

Cell culture, transient transfections, and promoter activity assays

Primary cultures of porcine granulosa cells were collected as described previously (19) and cultured for 24 h in MEM (Gibco BRL, Burlington, Ontario, Canada) containing 1 mg/l insulin (Sigma, Oakville, Ontario, Canada), 0.1 mM nonessential amino acids (Gibco BRL), 5×10^4 IU/l penicillin (Gibco BRL), 50 μg/l streptomycin (Gibco BRL), 0.5 mg/l fungizone (Gibco BRL), and 10% fetal calf serum (Gibco BRL), then for a further 24 h in serum-free MEM. The porcine granulosa cell line PGC-2 (20) was cultured in MEM with the additives described above and 10% fetal calf serum (Gibco BRL). Y-1 mouse adrenal tumor cells [American Type Culture Collection (ATCC), Manassas, VA] were maintained in Dulbecco's modified Eagle's medium/F12 (Gibco BRL) supplemented with 10% horse serum, 2.5% FBS, and antibiotics. SVG40 human granulosa cells (a gift from Dr. P. Leung, University of British Columbia, Vancouver, British Columbia, Canada) were cultured in Opti-MEM (Gibco BRL) supplemented with 5% FBS and antibiotics. HepG2 cells acquired from the ATCC were cultured in MEM with 6% FBS. MCF-7 cells (ATCC) were maintained in DME H-21 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with 100 nM/well of the deletion constructs of the pig NPC-1 promoter in the vector pGL3 using Effectene reagent (Qiagen) according to the manufacturer's protocol. Cells were cotransfected with the SV40 *Renilla* luciferase control vector pRL.SV40 (Promega) at a ratio of 10:1 pNPC-LUC/pRL.SV40 to normalize results for transfection efficiency. The cotransfection experiments were performed with the transfection of 10 ng of vectors expressing the nuclear (and transcriptionally active) forms of SREBP (18) or an empty expression vector for the correction of total DNA. Some cultures were treated with 1 mM dibutryl cAMP (Sigma) for 24 h as described previously (12). Luciferase activity was detected by the Promega Dual Luciferase Assay System, and chemiluminescence was measured in a Berthold 9501 luminometer. Control transfections included the inclusion of an equal amount of the promoterless pGL3 basic plasmid (Promega). Some cultures were treated with the protease inhibitor calpain [acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN); Sigma] at a dose of 50 μg/ml in DMSO. Control cultures were treated with DMSO in the absence of ALLN.

Cholesterol depletion

Primary cultures were incubated serum-free in MEM to which was added 50 μM cerivastatin (Sigma) and 50 μM mevalonate (Sigma). Cell lines were washed, and medium was replaced with depletion medium containing 10% (v/v) fetal bovine lipoprotein-depleted serum (Gibco BRL) in place of FBS along with the same concentrations of cerivastatin and mevalonate. Control cells were fed with repletion medium composed of the depletion medium with 10 μg/ml 25-OH-cholesterol (Sigma) or 1 μg/ml 22-OH-cholesterol (Sigma) added. Further pools of

cells were incubated with serum-free medium as the depletion medium or serum-free medium to which 2 μg/ml 22-OH- or 25-OH-cholesterol had been added.

Electrophoretic mobility shift assays

The electrophoretic mobility shift assays (EMSAs) were performed as described (12) with some modifications. Double-stranded oligonucleotides corresponding to the NPC-1 promoter region (**Table 1**) were labeled with [α-^{32}P-deoxy]CTP by polynucleotide kinase. Recombinant SREBP was incubated with 1 ng of labeled probe in binding buffer [20% glycerol, 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 mg/ml poly(dI-dC)-(dI-dC)] for 20 min at room temperature. The binding reaction products were loaded on a 6% nondenaturing polyacrylamide gel in 0.25 μl of Tris-buffered EDTA. An oligonucleotide of a demonstrated SRE sequence, SRE 2 from the lipoprotein lipase promoter (18), was included as a positive control (Table 1). EMSA was performed using a double-stranded oligonucleotide representing the principal putative SRE cluster (SRE 2-3; Table 1) to establish that SREBP binding to the radiolabeled probe could be inhibited by an excess of unlabeled probe.

RNA analysis

RNA was isolated from untreated cells, cholesterol-depleted or cholesterol-replete cells, and cells treated with 1 mM dibutryl cAMP (Sigma). Northern blot analysis of total RNA was performed as described previously (13). In brief, cultured cells were homogenized in 4 M guanidine isothiocyanate (Gibco BRL), 26.5 mM sodium acetate (Sigma), and 0.12 M β-mercaptoethanol (Sigma) and stored at −70°C until analysis. Total RNA was purified with Qiagen Easy Spin columns (Qiagen). Aliquots of 15 μg of total RNA were subjected to electrophoresis on 1% agarose-formaldehyde gels using a 20 mM morpholinopropanesulfonic acid buffer (pH 7.0), transferred overnight to nylon membranes, and cross-linked for 30 s at 150 mJ in an ultraviolet light chamber (GS Gene Linker; Bio-Rad, Richmond, CA). Blots were hybridized with a 1 kb probe from the 5′ region of the porcine NPC-1 open reading frame. All probes were labeled by random priming (Boehringer Mannheim, Laval, Quebec, Canada). Hybridized blots were subjected to phosphorimaging for visualization and quantitative estimatation of the most prominent NPC-1 transcript.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays followed the method of Kuo and Allis (21) with minor modifications. DNA and cell proteins in granulosa cell cultures were cross-linked by the addition of formaldehyde to a final concentration of 1% for 10 min at room temperature. Cells were washed and scraped in ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A), collected by centrifugation for 4 min at 2,500 rpm, and resuspended in 200 μl of ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and protease inhibitors). Cells were disrupted by sonication after incubation for 10 min on ice and then centrifuged (10 min, 13,000 rpm at 4°C). The supernatant was then diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl, and protease inhibitors; Sigma). An aliquot of 2 μl of lysate was used for the purification of total DNA. Each sample was precleared by incubating with 80 μl of salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology, Inc., Lake Placid, NY) for 60 min at 4°C to reduce nonspecific background. One sample (2 ml) was divided into two separate 1 ml parts, and each 1 ml was incubated with 5 μg of antibody and treated overnight at 4°C with agitation. Antibodies used in this experiment were against the biacetylated (lysine-9 and lysine-14) form of histone H3 (Upstate) and anti-SREBP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Control precipitation was performed with rabbit IgG (Upstate). Immunocomplexes were collected with 60 μl of salmon sperm DNA/protein A-agarose for 2 h at 4°C with rotation and washed once with the following buffers in sequence: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl); high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% Nonidet-P40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1); and TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Immunocomplex elution was achieved by adding 250 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$). The DNA-protein cross-linking was reversed by incubation at 65°C for 6 h followed by proteinase K treatment. DNA was recovered by purification with the Qiaquik PCR purification column (Qiagen). In the first trials with PGC-2 cells, a 0.5 kb fragment from the proximal promoter region of NPC-1 was amplified by PCR in total DNA and immunoprecipitated DNA. As a negative control, primers derived from the 3′ region of the porcine NPC-1 open reading frame were used. PCR products were resolved on agarose gels and visualized by means of the Alpha-Imager gel documentation system. A second trial used primary granulosa cells and primer sequences to amplify 0.1 kb regions specific to the putative SRE and E box (the palindromic motif 5′-CANNTG-3′) regions of the promoter. Controls were regions distal and proximal to these regions. Real-time PCR was conducted to quantify the amplicons in the latter trial. The primer sequences for PCR in both trials are listed in **Table 2**.

TABLE 1. DNA sequences used in electromobility shift assays

Probe	Sequence
NPC S1	CAAAATGGGCTGAGCAG
NPC S2	CAGATCACAACACTGTC
NPC S3	ACTGTCAGGCCACTACG
NPC E2	AACATCAGCTGCATCG
LPL S2	TTCTCGTTGGCAGAGATAATCCTCATTACT
NPC S2-3	TCACAACACTGTCAGGCCACTACGTGGCACC

RESULTS

Several lines of evidence indicate that the expression of the NPC-1 gene in vitro is regulated by the concentrations of intracellular sterols in steroidogenic cells. Northern blot analysis from the first experiments revealed a dose-dependent reduction in porcine NPC-1 mRNA abundance in primary cultures of porcine granulosa cells incubated over 24 h with either of 22-OH- or 25-OH-cholesterol (**Fig. 1A**). We used cAMP stimulation as a positive control for the upregulation of NPC-1 expression, as described previously (13). To further address the role of cholesterol in the regulation of NPC-1 expression, granulosa cells were incubated for 24 h in cholesterol-

TABLE 2. Primers used in NPC-1 chromatin immunoprecipitation assays

Region and Primer	Sequence
SRE region	
pNPC1-SRE-fwd	ATCAGCTGCATCGTGGTCCTAACTC
pNPC1-SRE-rev	CCAAGTGTAAGACTCAAAACCCAAGATG
Coding region	
pNPC1-SRE-fwd	AAGGGGAGAAATGAGTTGAAGC
pNPC1-r ev	GAATTCCAGCAGGAGGAGGCCGAA
Promoter distal region	
NPC1-distal-fwd	TGCTAGGCTTTTGGGTACTG
NPC1-distal-rev	ATATTTCTGGGATGGGCAGA
E-box region	
NPC1-E2-fwd	CCAGCCAGTTACGTGGACTT
NPC1-E2-rev	CACGATGCAGCTGATGTTCT
SRE region	
NPC1-SREBP-fwd	GGTGCAGTTCTAGCGCTTTT
NPC1-SREBP-rev	TGGCCTGACAGTGTTGTGAT
Promoter proximal region	
NPC1-prox-fwd	ACGCCTTCCTTCCTGACCT
NPC1-prox-rev	GAGGTTCAGCTGGCAGTCC

SRE, sterol-regulatory element.

depleted medium supplemented with the HMG-CoA reductase inhibitor cerivastatin to reduce endogenous cholesterol synthesis. This depletion of intracellular cholesterol resulted in an increase in the abundance of NPC-1 mRNA (Fig. 1B). Repletion of cholesterol by the addition of either 22-OH- or 25-OH-cholesterol prevented the increases in NPC-1 mRNA in response to cholesterol depletion (Fig. 1B). We then sought to determine whether there was a transcriptional component to this response by transient transfection analysis. Cells from the PGC-2 porcine granulosa line, cultured in a cholesterol-replete medium (10% serum) or in cholesterol-depleted medium as described above, were transfected with the 1.8 kb fragment of the porcine NPC-1 promoter ligated to the luciferase reporter. Cells cultured in the depleted medium displayed a 10-fold increase in promoter activity relative to cells in cultures replete with cholesterol over the 12 h after transient transfection (Fig. 1C). The increase in transcriptional activity in response to incubation in cholesterol-depleted medium was reduced significantly by the addition of either 22-OH- or 25-OH-cholesterol to the culture medium (Fig. 1C).

SREBP isoforms are factors that regulate the transcription of numerous proteins involved in cholesterol homeostasis (3, 22). To test the implication of SREBP in NPC-1 transcription, we examined the effects of the transfection of plasmids expressing SREBP isoforms on the transcriptional activity of a fragment of the NPC-1 promoter comprising the 1.8 kb upstream of the transcription initiation site. SREBP1a, SREBP1c, and SREBP2 all induced 2- to 5-fold increases in promoter activity in the pig granulosa cell line over the 18 h after transfection (**Fig. 2A**). Treatment of the porcine granulosa cell line PGC-2 with the protease inhibitor calpain (ALLN) along with transfection with SREBP1a tripled the reporter signal relative to SREBP1a transfection alone (Fig. 2B). We investigated the ubiquity of the SREBP response by transfection of SREBP1a along with the NPC-1 promoter-

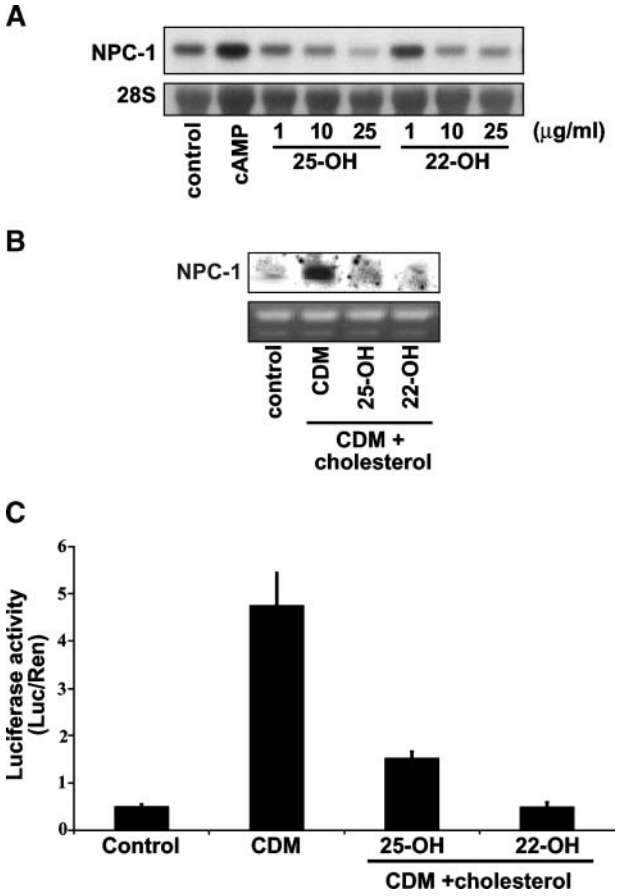

Fig. 1. Sterol regulation of the expression and transcription of the Niemann-Pick C-1 (NPC-1) gene. A: A Northern blot representative of three replicates demonstrating the reduction in NPC-1 mRNA in porcine granulosa cells incubated for 24 h with doses of hydroxylated steroids (22-OH and 25-OH). A dose of 1 mM cAMP was used as a positive control. B: A Northern blot depicting the increase in NPC-1 mRNA in porcine granulosa cells incubated for 24 h in cholesterol-depleted medium (CDM) or cholesterol-replete medium (CDM + 2 μg of 22-OH-cholesterol or an equivalent amount of 25-OH-cholesterol). Results shown are representative of three replicate experiments. C: Promoter activity of the 1.2 kb 5′-flanking region of the NPC-1 gene fused to the luciferase reporter transfected into the spontaneously immortalized PGC-2 porcine granulosa cell line. Cells were depleted of cholesterol for 24 h, and measurement of luciferase activity was effected at 18 h after transient transfection. Bars represent means ± SD of three replicate experiments.

reporter construct into two additional steroidogenic cell lines, the SVG40 human granulosa line and the Y-1 mouse adrenal cortical cell line. The human HepG2 liver cell line, which is not steroidogenic, was also transfected. All lines responded with significant increases in promoter activity, with, for the most part, the greatest responses present in steroidogenic cells (**Table 3**). We then sought to establish whether the response was common to promoters of the NPC-1 orthologs by cotransfection of fragments of the human, mouse, and pig promoters into PGC-2 cells and the MCF-7 human breast cancer line. The results (**Table 4**) demonstrate that the reporter constructs derived from the 5′-flanking regions of all three genes display significant gains in transcriptional activity

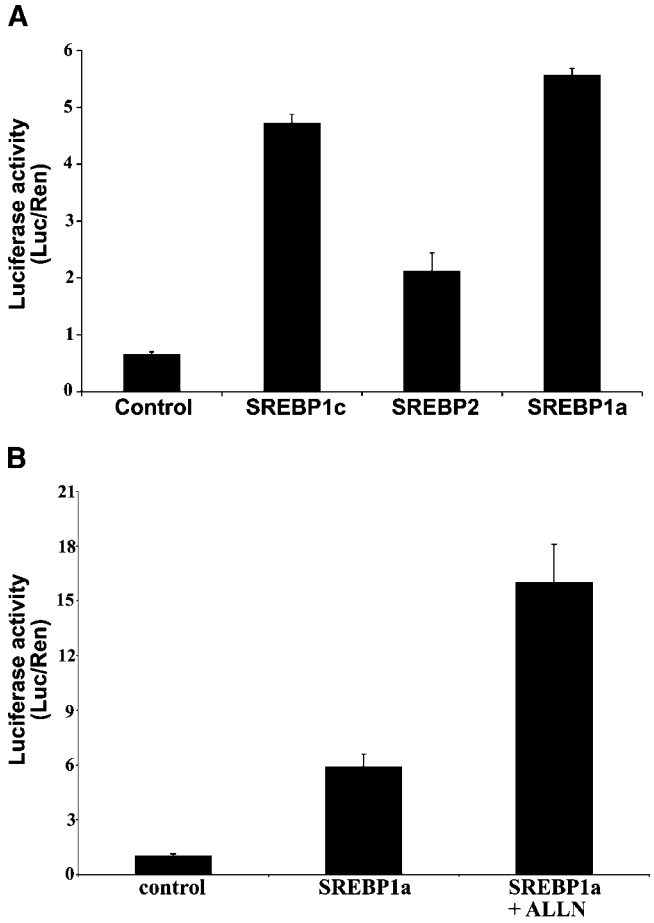

Fig. 2. The sterol-regulatory element binding protein (SREBP) induces NPC-1 transcription. A: The promoter NPC-1 activity was calculated from the luciferase/*Renilla* signal at 18 h after cotransfection of the 1.8 kb NPC-1-luciferase construct and a plasmid constitutively expressing the transcriptionally active isoforms of SREBP into PGC-2 cells. B: Addition of the protease inhibitor calpain [acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN)] at the time of transfection magnified the response of the NPC-1 promoter to the nuclear form of SREBP over 24 h. Control and SREPB1a cultures received the same dose of DMSO. Bars represent means ± SD of three replicate experiments.

when cotransfected with SREBP1a relative to the response in the absence of exogenous transcriptional activator.

The sequences of the pig, human, and mouse NPC-1 promoters were then subjected to in silico analysis to determine the regions with potential to interact with SREBP transcription factors. As can be seen from **Fig. 3A**, three sequences bearing homology with the SRE were identified in the first 1 kb upstream of the translation start site in the pig promoter, three were present in the human 5′ flanking region of similar length, and two were present in the mouse. Deletion fragments were then used to determine the components of the porcine promoter associated with the response to constitutive expression of SREBP induced by the transfected plasmid (Fig. 3B). The minimum promoter sequence required for both the basal and SREBP-induced promoter activity was at least 120 bp. The basal or constitutive signals remained constant, with promoter lengths from 0.2 to 1.2 kb, and the SREBP response did not differ when the promoter construct was derived from fragments of 1.4 to 1.2 kb. Comparison of the luciferase signal revealed a significant decline in promoter activity between fragments at −853 bp and −636 bp (Fig. 3B), suggesting that the putative SREs in this region were involved in the regulation of this gene. The three SREs, designated S1, S2, and S3, were then subjected to EMSA to determine their capacity to bind to recombinant human SREBPs. These results demonstrate that all three sites, along with the E-box sequence, interacted with the transcription factor (**Fig. 4A**). In a separate EMSA trial, we examined the span containing two of the three SRE sites and demonstrated that excess unlabeled probe extinguished SREBP binding to the labeled oligonucleotides (Fig. 4B).

We next produced promoter-reporter constructs with each of the putative SRE sites, the E-box, or all three sites and the E-box mutated. These were then cotransfected into PGC-2 cells with SREBP1a. None of the mutations affected the basal transcriptional activity. Mutation of the most distal site (S1) reduced the signal, whereas mutation of the central site (S2) alone had no effect. (Fig. 4B). Mutation of the proximal and distal (S1 and S3) sites or the E-box both significantly reduced, but did not abolish, the SREBP-induced increase in promoter activity. Reduction to basal levels of activity in the presence of transfected SREBP was achieved by mutation of all three SRE sites along with the E-box (Fig. 4B).

ChIP assays were used to further establish the link between cholesterol deprivation of cells and SREBP in NPC-1 transcription and to further define the regions of the promoter responsive to reduced cholesterol. PGC-2 cells were incubated in the cholesterol-depleted and

TABLE 3. Transient transfection experiments indicate that the porcine NPC-1 promoter reporter construct displays activity in response to SREBP in homologous and heterologous cell lines

Cell Line	Fold Induction
PCG-2	4.7 ± 0.5
SVG40	22.7 ± 3.8
Y1	12.8 ± 0.3
HepG2	2.5 ± 0.2

SREBP, sterol-regulatory element binding protein. The 1.8 kb promoter construct was cotransfected with a plasmid expressing SREBP1a and incubated for 12 h. Values represent fold induction over the control (no SREBP1a) and are means ± SD of three replicate experiments.

TABLE 4. The activity of three mammalian promoter-reporter constructs is augmented by cotransfection of the SREBP1a promoter in two mammalian cell lines, PGC-2 and the human breast cancer line MCF-7

Promoter Sequence	PGC-1	MCF-7
Pig	6.0 ± 0.7	6.8 ± 2.0
Human	2.5 ± 0.3	6.6 ± 0.4
Mouse	1.5 ± 0.1	4.1 ± 0.1

Values represent fold increases relative to the control (no SREBP1a) and are means ± SD of three replicate experiments.

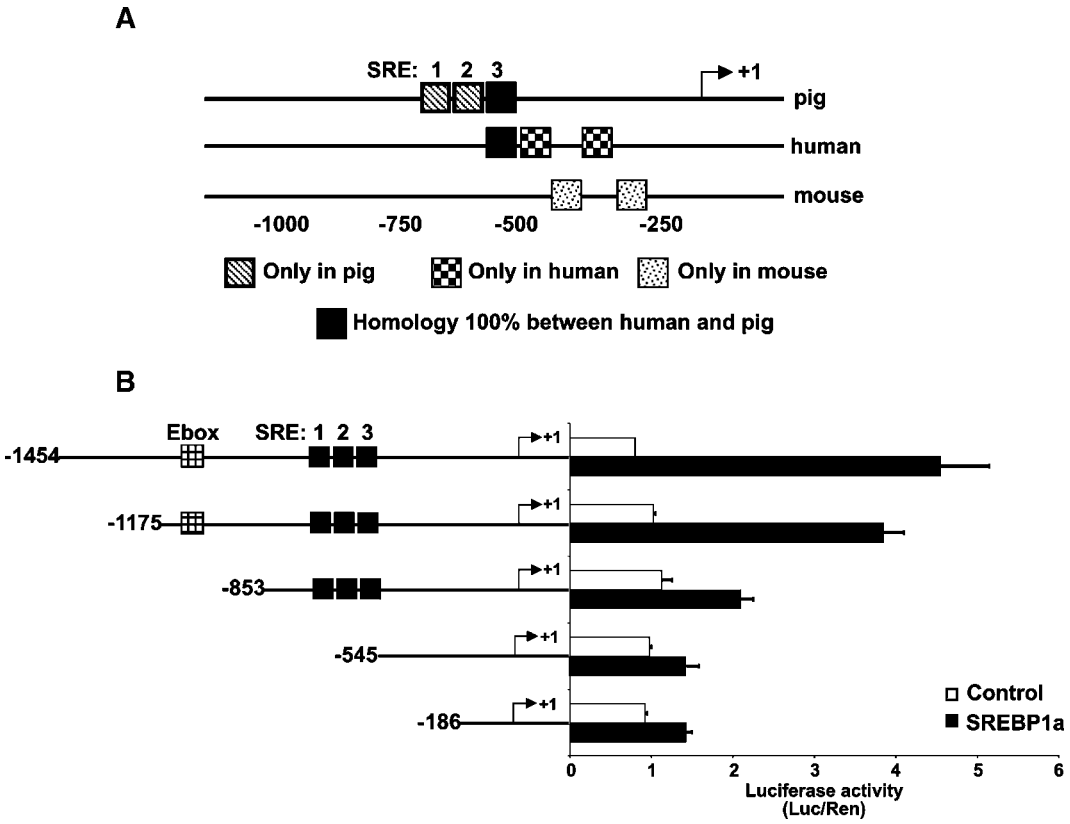

Fig. 3. Deletion and band shift analyses demonstrate that the SREBP signal is mediated by specific elements in the NPC-1 promoter. A: Comparative in silico analysis of the 5′-flanking regions of the human, pig, and mouse genes indicating the presence of orthologous sterol response elements (SREs). B: Deletion analysis indicating that a sequence of constitutive expression requires a minimum of 120 bp upstream of the transcription start site. SREBP1a-induced transcription is affected by promoter deletions that exclude the putative SRE between −835 and −527 bp upstream of the putative transcription start site.

cholesterol-replete media described above. DNA bound to SREBP immunoprecipitates of nuclear extracts was amplified by PCR using primers derived from the region that spanned the three putative SRE sites on the NPC-1 promoter. The results demonstrate increased association of the promoter with SREBP in cells deprived of cholesterol (**Fig. 5A**). PCR amplification of a sequence of similar size from the coding region of the NPC-1 gene indicated that the response was specific to the promoter region. It was shown previously that SREBP activation of two sterol-sensitive genes, those encoding the LDL receptor and HMG-CoA reductase, engenders a promoter-associated increase in histone H3 acetylation (23). To establish the presence of a similar regulation of NPC-1 expression, extracts of primary porcine granulosa cells cultured in cholesterol-replete or -depleted medium were immunoprecipitated with antiserum specific to H3 acetylated on lysine residues 9 and 14. Subsequent PCR amplification of the region of the NPC-1 promoter revealed a marked increase in the association of the promoter with acetylated H3 in cholesterol-deprived cells (Fig. 5B).

We then used porcine cells in primary culture incubated under conditions of depleted or replete cholesterol to clarify the significance of the SRE sites and the E-box in the porcine NPC-1 promoter. This ChIP analysis using SREBP for immunoprecipitation and real-time PCR amplification of defined regions of the promoter demonstrated that both the sequences containing the E-box and the SRE sites were immunoprecipitated by the SREBP antibody, whereas the regions proximal or distal to these sites were not (Fig. 5C). There was 4- to 5-fold greater enrichment of the sequences of the E-box and the cluster of SRE in cells incubated in cholesterol-depleted medium compared to the response in cells in cholesterol-replete medium, indicating that this treatment influenced the association of the transcription factor with both of these elements.

DISCUSSION

Studies of a mouse line bearing spontaneous mutation of the NPC-1 gene have demonstrated its essential role in the trafficking of LDL-imported cholesterol in steroidogenic tissues (24). The absence of the intact protein results in the sequestering of cholesterol in pathologic late endosomal structures and compromises the normal progression of the esterification of LDL-borne cholesterol and its incorporation into membranes (8). Regulation of many of the elements of cholesterol homeostasis, including its de novo synthesis, importation, and efflux, are modulated by cholesterol itself via the

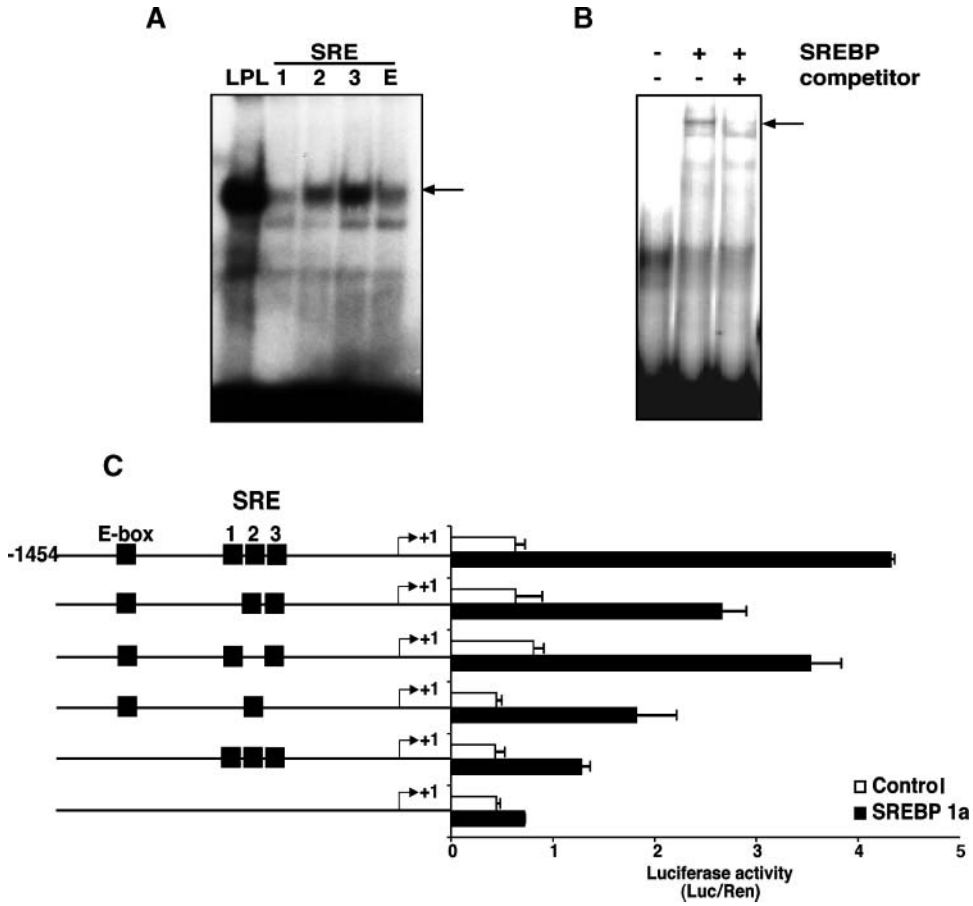

Fig. 4. Putative SREs bind recombinant SREBP, and their mutation interferes with SREBP-induced NPC-1 transcription. A: Electrophoretic mobility shift assay (EMSA) indicating the binding of recombinant SREBP to the three putative SREs (Table 2) in the region of SREBP responsiveness defined by deletion analysis. The positive control for binding was confirmed sequences in the lipoprotein lipase promoter. B: Excess unlabeled oligonucleotide displaces SREBP binding. EMSA was performed with an oligonucleotide encompassing the principal SRE binding region in the presence or absence of excess probe. The arrow indicates the migration of the probe/SREBP complex on the gel. C: Effects of the mutation of individual SRE and E-box consensus sites on the response of the porcine NPC-1 promoter-luciferase construct to SREBP1a cotransfection in PGC-2 cells. Abrogation of the response required mutation of all three sites and the E-box.

SREBP transcriptional mechanism (25, 26). It is reasonable, therefore, to assume that NPC-1 is regulated by the same mechanism. Nonetheless, early investigations of the structure of the human NPC-1 promoter (11, 27) suggested that the gene is constitutively expressed. Furthermore, in a recent study of cholesterol dynamics in an in vivo model in which mice were fed a cholesterol-supplemented diet, it was concluded that NPC-1 expression is independent of LDL-imported cholesterol and therefore constitutive (15). The ensemble of data we present here demonstrates that intracellular cholesterol and the SREPB transcriptional pathway modulate, at least in part, the expression of NPC-1 in steroidogenic cells. This view, along with results indicating the SREBP regulation of NPC-1 expression in human fibroblasts (16), contrast with the constitutive expression scenario.

Our analysis of noncoding sequences of the NPC-1 gene across species (28) indicated the potential for sterol-mediated SREBP regulation. Comparison of the 5′-flanking region of the NPC-1 gene between three species of mammals revealed conserved SRE motifs and E-box sequences, both theoretically capable of responding to SREBP. Furthermore, the authenticity of these SRE sequences identified in the pig promoter between 0.6 and 0.9 kb upstream of the translation initiation site was verified in the EMSA analysis using recombinant SREBP as bait, and ChIP assays demonstrated that the portions of the porcine promoter that contained the E-box and the three regulatory SRE sites could be immunoprecipitated by SREBP antibodies. Together, these results provide sequence-based evidence that SREBP-responsive elements are present in the NPC-1 promoter. The 5′-flanking structurally related human gene that has been shown to be regulated by the concentration of cholesterol, NPC-1L1, contains two SRE sites (14). Multiple SREs have been shown to be present in the porcine promoter LDL, a gene known to be regulated by cholesterol availability via SREBP (29).

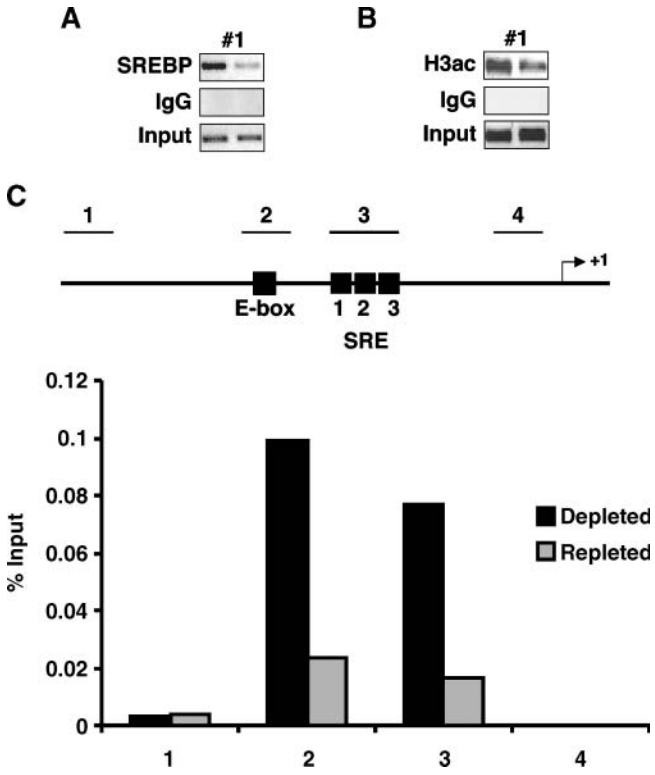

Fig. 5. Chromatin immunoprecipitation (ChIP) analysis of the NPC-1 gene indicates an association of SREBP and acetylated histone H3 with the region of the promoter implicated in cholesterol regulation. A: SREBP ChIP demonstrating that cholesterol depletion of primary porcine granulosa cells increases the association of the region of the NPC-1 promoter from −835 to −527 with SREBP. B: Amplification of the same promoter region in samples immunoprecipitated with antiserum against acetylated histone H3 likewise shows that the association of the promoter with the covalently modified histone is increased in response to cholesterol depletion. C: ChIP analysis of the 5′-flanking region of the porcine NPC-1 gene demonstrating the binding of SREBP to regions containing the three putative SRE sites (3) and the E-box (2). Regions 1 and 4 contained no consensus sequences for SREBP binding and thus were not amplified in immunoprecipitates. This figure further demonstrates the effects of the reduction in cholesterol substrate on the association of SREBP with the cognate regions of the NPC-1 promoter. In each case, amplifications are representative of three replicate experiments.

Cotransfection of a plasmid expressing all three isoforms of SREBP provided functional confirmation of SREBP regulation of the porcine NPC-1 gene. Each isoform increased the activation of an NPC-1 promoter-reporter sequence by severalfold in the porcine granulosa cell line, indicating the capacity of the promoter to respond to SREBP. Comparative analysis of 1 kb pig, mouse, and human promoter-reporter constructs demonstrated activity in two cell lines in response to cotransfection with the transcriptionally active form of SREBP. This form of SREBP induced significant upregulation in transcriptional activity of the pig NPC-1 promoter-reporter in diverse cell lines. The strongest responses were seen in steroidogenic cells, suggesting cell-specific SREBP modulation. This compares with early analysis of the human NPC-1 promoter demonstrating that basal transcriptional activity is only modestly cell-specific (27). It has been shown that the nuclear form of SREBP has a relatively brief persistence in cells and that its disappearance can be attenuated by both ALLN (30) and proteosome-specific inhibitors (31). ALLN is an inhibitor of neutral cysteine proteases, inhibiting the degradation of the transcriptionally active form of SREBP (30). Our current demonstration that treatment with ALLN magnified the response to SREBP, presumably by preventing the proteolysis of mature SREBP, strengthens the case for a role of this transcription factor in NPC-1 regulation.

Individual SRE consensus elements regulate SREBP induction of promoter activity in other sterol-sensitive genes, including lipoprotein lipase (18) and the LDL receptor (29). There appears to be functional redundancy among the individual SRE sites of the NPC-1 promoter, as activity persisted after ablation of either one or two of these sites. It is noteworthy that the most proximal SRE was the sequence that is conserved between the human and pig promoters and that displayed the strongest binding to recombinant SREBP in the gel-shift assay. Elimination of the three putative SRE sites and the proximal E-box virtually eliminated SREBP induction of NPC-1 transcriptional activity. It has long been known that SREBP interacts with E-box motifs (32), and there is extensive precedent for a combined interaction of SRE and E-box motifs in the positive (18) and negative (25) regulation of cholesterol- and SREBP-modulated genes. We conclude that a combination of SRE and E-box sequences regulates NPC-1 gene expression.

A compelling argument for the sterol regulation of the NPC-1 gene comes from our cholesterol depletion/repletion studies. Incubation of primary granulosa cells in cholesterol-depleted medium resulted in a significant increase in NPC-1 transcript abundance, whereas sterol treatment resulted in dose- and time-dependent reductions in the expression of this gene. This result was recapitulated in transient transfection of the construct composed of the NPC-1 5′-flanking region fused to the luciferase reporter; in that situation, cholesterol depletion resulted in a 10-fold increase in promoter activity, attenuated completely by the addition of hydroxycholesterols. Furthermore, the ChIP assay demonstrated an increased association of SREBP with the NPC-1 promoter, specifically with the E-box and SRE sites, in primary granulosa cells cultured in cholesterol-depleted relative to cholesterol-replete medium. These responses place NPC-1 in the class with a number of sterol-regulated genes, including HMG CoA-reductase (23), the LDL receptor (2), and lipoprotein lipase (18). The conclusion that cholesterol flowing through the coated-pit pathway did not alter NPC-1 expression (13) was based on a different model, the mouse subjected to chronic overfeeding of cholesterol. In the hamster (33) and mouse (34) liver, this treatment reduced the abundance of the nuclear form of SREBP. Thus, the contradictory conclusions are difficult to reconcile. The fact that our models were primarily steroidogenic cells that require cholesterol substrate for hormone synthesis, whereas whole ani-

mal studies (15) were focused on the liver, may explain the differences. We demonstrate consistent and robust transcriptional activation of NPC-1 by the nuclear form of SREBP in ovarian and adrenal cells. The weakest response to the nuclear form of SREBP was found in the human liver cell line HepG2, indicating that NPC-1 may be regulated in a cell-specific manner. We previously presented clear evidence that NPC-1 is actively rather that constitutively regulated (17, 35). There is evidence for regulation of transcription of cholesterol trafficking genes, including the LDL receptor, that is independent and complementary to SREBP mechanisms (36). This may be the functional mechanism of cell specificity.

SREBPs alone are not generally believed to be strong activators of transcription, and they depend on coregulators to produce high-level expression of target genes. The most common interactions are with the Sp1 transcription factors; indeed, mutation of any of the three Sp1 sites flanking the SRE in the porcine LDL receptor promoter abrogated the response to stimulatory ligands (29). Examination of the pig promoters revealed two Sp1 sites on the proximal upstream side of SRE3 and a NF-Y response element <20 bp distal to the putative SRE3. These sites provide the potential for interaction with Sp1 and appropriate coregulation of the responses to the abundance of cholesterol.

A study of HMG-CoA reductase and LDL receptor transcription in the cholesterol depletion paradigm demonstrated that SREBP activation was accompanied by an increased association of both promoters with acetylated histone H3 (23). In the present investigation, the association of the NPC-1 promoter with acetylated H3 was augmented in cells depleted of cholesterol, further supporting the concept of nutrient regulation of the NPC-1 gene.

In summary, we investigated the regulation of NPC-1 using a battery of techniques in a variety of cell models. We conclude that this gene is not constitutively expressed; rather, it is modulated by cholesterol supply via the intracellular cholesterol feedback pathway by means of SREBP.

This study was supported by Grant MOP-117373 to B.D.M. from the Canadian Institutes of Health Research. F.G. was the recipient of a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada. The authors thank Mira Dobias and Vickie Roussel for invaluable technical assistance.

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