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Regulation of the bovine SCD5 promoter by EGR2 and SREBP1

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ABSTRACT

In rodents, the transcription factors early growth response 2 (EGR2) and sterol regulatory element binding protein 1a (SREBP1a) regulate transcription of the stearoyl-CoA desaturase 2 (SCD2) gene during peripheral nerve myelination, which may be important for synthesis of the lipid component of myelin. Most non-rodent genomes do not contain the SCD2 gene, but rather express SCD5 in brain and nervous tissues. In this paper, we asked whether bovine SCD5 is regulated in a similar manner to rodent SCD2. Expression of EGR2 did not result in an increase in endogenous SCD5 mRNA expression in JEG3 cells, but did result in activation of truncated bovine SCD5 promoter luciferase reporter constructs. Similar results were obtained with expression of the active form of SREBP1a; however, unlike rodent SCD2, there was no synergistic activation of the bovine SCD5 promoter reporters when EGR2 and SREBP1a were co-expressed. Mutation of the putative EGR2 binding site in the SCD5 promoter abolished activation by SREBP1a, suggesting that EGR2 and SREBP1a bind to the same site in the SCD5 promoter. Finally, we have identified a region of the bovine SCD5 promoter between 505 and 305 base pairs upstream of the transcriptional start site that appears to be important for maintaining basal levels of transcription of this gene. While it appears that there are some differences between the regulation of rodent SCD2 and bovine SCD5, the promoters of both genes can be activated by EGR2 and SREBP1a. This is the first report of potential regulators of SCD5 transcription.

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1. Introduction

The transcriptional regulator EGR2 plays a critical role in peripheral nerve myelination. EGR2 expression is induced in Schwann cells at the onset of myelination, and its increase in expression follows a similar developmental time course as several other myelin genes, such as myelin protein zero (MPZ), myelin associated glycoprotein, and periaxin (PRX), as well as the lipid synthetic genes SREBP1 and SCD2 [1]. In murine Schwann cells, microarray and Real-time PCR analysis suggests that these myelin protein genes are actually targets of EGR2 [2,3]. In addition, EGR2 has been shown to regulate the expression of many other genes involved in peripheral myelin formation, including enzymes involved in the formation of critical lipid components of myelin such as oleate and cholesterol.

EGR2-deficient mice have a severe defect in the myelination of peripheral nerves [4]. Studies with EGR2 knockout mice have shown that while Schwann cells are still present, they are blocked at an early stage of differentiation and fail to myelinate peripheral nerves [4]. Further, the mRNA expression of SCD2 was significantly reduced in sciatic nerves from EGR2 knockout mice [3].

SCD2 is a member of the stearoyl-CoA desaturase enzyme family that is highly expressed in the brain and peripheral nerves of rodents. It is responsible for catalyzing the desaturation of saturated fatty acyl-CoA substrates, including palmitoyl-CoA and stearoyl-CoA, at the delta-9 position, resulting in the synthesis of palmitoleoyl-CoA and oleoyl-CoA [5]. Oleate is a major component of myelin, and the oleate synthesized by SCD2 may play an important role in normal brain and myelin development [1].

A rodent SCD2 promoter reporter vector containing two EGR2 sites and an SRE was significantly activated when co-transfected with either an EGR2 expression vector or SREBP1a, 1c, or 2 expression vectors in JEG3 cells. When co-transfected with both EGR2 and SREBP expression vectors, activation of the SCD2 promoter was potentiated [3]. Further, while SREBP1a was able to activate both the SCD1 and SCD2 promoters, EGR2 only activated the SCD2 promoter and there was no potentiation of the SCD1 promoter by SREBP1a and EGR2 expressed together [3].

The gene encoding SCD2 appears to be specifically found in rodent and lagomorph genomes, but is absent from other mammalian genomes [6]. Multiple isoforms of SCD have been found in most species examined to date; however, different species appear to have different subsets of SCD isoforms. Some rodent genomes contain four SCD isoforms (SCD1-4) with different expression profiles. For example, murine SCD1 is expressed predominantly in lipogenic tissues [7], while SCD2 is preferentially expressed in

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brain and neuronal tissues [8]. In contrast, the genomes of many other species, including humans, cows, pigs, sheep, chickens, and more, have been found to contain two SCD isoforms: SCD1 is most highly expressed in lipogenic tissues, while SCD5 is highly expressed in brain and pancreas [6,9–11].

The regulation of the SCD5 gene is currently unknown. The expression of SCD5 in nervous tissues could indicate that it functions similarly to SCD2 in rodent nervous tissues and could be regulated by similar factors.

2. Materials and methods

2.1. Cell culture

The human placental choriocarcinoma cell line JEG3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X antibiotic/antimycotic solution (Sigma, St. Louis, MO).

2.2. Construction of plasmids

Restriction enzymes and T4 DNA ligase used for cloning were purchased from New England Biolabs (Ipswich, MA). *SCD5 promoter reporter vectors*: Portions of the bovine SCD5 promoter were initially amplified by PCR using different upstream primers paired with the same reverse primer (Table 1). PCR products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA), and then subcloned into the pGL4.10[luc2] vector (Promega, Madison, WI) using the SpeI site in pCR4-TOPO and the SmaI site in the SCD5 promoter inserts, and cloning into the NheI and EcoRV sites in the pGL4 vector. Successful cloning was confirmed by sequencing. *SREBP1a expression vector*: A portion of bovine SREBP1a encompassing the sequence coding for the mature peptide was amplified by PCR, cloned into the pCR4-TOPO vector, and then subcloned into the pcDNA3.1 + expression vector (Invitrogen, Carlsbad, CA) using EcoRI. Orientation was confirmed by sequencing. A stop codon was introduced by site-directed mutagenesis in order to produce a mature peptide of 480 amino acids. Site directed mutagenesis was performed using the QuikChange Lightning Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and using mutagenic primers as shown in Table 1. Successful mutagenesis was confirmed by sequencing. *EGR2 expression vector*: The coding sequence of bovine EGR2 was amplified by PCR, cloned into the pCR4-TOPO vector, and then subcloned into the pcDNA3.1+ expression vector (Invitrogen, Carlsbad, CA) using EcoRI. Orientation was confirmed by sequencing.

Table 1
Primers.

Gene	Forward primer	Reverse primer	Prod. size
Real-time PCR primers			
hMPZ	gttccccctgaccactttg	ccccatttcccatttggtttt	173
hPRX	agcaagaaccccacggaag	cctgtgtcctctccccttt	164
hSCD5	gacctgcttgctgatcctgt	agggctgatgtgcttgtcat	237
hACT	ttgcgttacacccttttcttg	caccttcaccgttccagttt	147
hSCD1	acctggcttgctgatgatgt	cgggggctaatgttcttgt	192
Expression vector cloning primers			
bEGR2	ccccaactctttcggactca	gagggaagggcaggaatg	1587
bSREBP1a	ccgagaggaggtgactgc	aggagagacagaggaagacgag	1516
Bovine SCD5 promoter cloning primers			
FL	aacaagacgctcatgtgtgg		
−772F	atacctgggcctgtctcctt		
−644F	taggggaatgagcagacaa		
−505F	agaggagaatttgccacgaa		
−260F	tggccttaacttgtgtgcaa		
−161F	tatttcggggaactggagaa		
+97R	ggggaagaaagacctgcata		
−403F	ggcgctatctgagcacctt		
−305F	ataggagccgggagacaca		
SCD5 promoter mutagenic primers (uppercase shows mutated nucleotides)			
F: ctcccccgcgctcAgcTcAcTgcggaggc tgccc		R:gggcagcctccgcAgTgAgcTgagcgcgggggag	

Abbreviations: h = human, b = bovine.

2.3. Real-time PCR

Cells were trypsinized and seeded (approximately 3×10^5 cells per well) into 6-well plates 24 h prior to transfection. Medium was discarded and replaced with 3 mL per well of fresh growth medium 1 h prior to transfection. Cells were transfected with 3 μg of the indicated expression vector or empty pcDNA3.1+ per well, using jetPEI transfection reagent (PolyPlus Transfection, New York, NY). Unstransfected cells were used as an additional control. After 48 h, cells were harvested in 1 mL RNAzol RT (Molecular Research Center, Inc., Cincinnati, OH) per well, and total RNA was isolated according to the manufacturer's instructions. RNA (2 μg per sample) was reverse transcribed into cDNA using the Omniscript RT kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time PCR reactions were performed using the GoTaq qPCR Master Mix (Promega, Madison, WI) and an Applied Biosystems 7300 Real-time PCR machine (Applied Biosystems, Foster City, CA). Each reaction was performed in duplicate wells. Beta actin (ACT) was used as an endogenous control gene. Untransfected cells were used as the calibrator for making relative comparisons between tissues. Fold change was calculated using the $2^{-\Delta\Delta cT}$ method [12]. Primer pairs are shown in Table 1 and reaction conditions were as follows: 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min.

2.4. Luciferase assays

Cells were trypsinized and seeded (approximately 1×10^5 cells per well) into 24-well plates 24 h prior to transfection. Medium was discarded and replaced with 1 mL per well of fresh growth medium 1 h prior to transfection. For experiments examining basal transcription of the SCD5 promoter, cells were co-transfected with 1 μg of the indicated pGL4 reporter construct and 30 ng of renilla luciferase control vector (hRLuc-TK, Promega, Madison, WI) per well, using jetPEI transfection reagent. For experiments examining transcription factor effects on the SCD5 promoter, cells were co-transfected with 1 μg of the indicated expression vector, 0.5 μg of the indicated pGL4 reporter construct, and 30 ng of renilla luciferase control vector (hRLuc-TK, Promega, Madison, WI) per well, using jetPEI transfection reagent (PolyPlus Transfection, New York, NY). After 48 h, cells were washed with PBS, lysed, and assayed for firefly and renilla luciferase activity using the Dual Luciferase Reporter Assay (Promega, Madison, WI) and a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA). Firefly luciferase values were normalized to renilla luciferase values for each sample. Untransfected cells and cells transfected with empty (promoterless) pGL4 were included with each experiment as negative controls (data not shown).

2.5. Statistics

Statistical analysis of data was completed using the Mixed procedure of SAS (SAS 9.2; SAS Institute, Inc., Cary, NC). Real-time PCR data were analyzed using ΔcT values (cT gene of interest – cT β actin). The model for Real-time PCR data analysis was analyzed as a one-way analysis of variance and included treatment as the model term. When a significant effect of treatment was detected,

means were separated using Tukey's adjustment for pairwise comparisons. Luciferase data from promoter constructs in the presence or absence of a transcription factor expression vector were analyzed as a two-way analysis of variance and the model included vector, promoter fragment, and the interaction of vector and promoter fragment. Means comparisons within promoter fragment were accomplished using the Slice function of the Mixed procedure. Two-way analysis of variance was also used to analyze the additive effects of transcription factor expression vectors on two SCD5 promoter fragments. The model included the main effects of expression vector and promoter fragment and the interaction of the main effects. Data from examining the SREBP1 DNA binding site using mutated promoter fragments were analyzed by two-way analysis of variance. The model included the main effects of SREBP1a expression vector and promoter fragments and the interaction of the two main effects. Means separation was completed using Tukey's adjustment for pair-wise comparisons. Truncated promoter data were analyzed as a one-way analysis of variance and means were separated using Tukey's adjustment for pair-wise comparisons.

3. Results and discussion

3.1. Effect of EGR2 expression on the SCD5 promoter

It has been shown that the rodent SCD2 promoter is activated by the transcription factor EGR2 when transfected into JEG3 cells [3]. Whereas SCD2 is highly expressed in the brain and nervous tissues of rodents, many non-rodent species lack the SCD2 gene, but express SCD5 in these tissues. We used AliBaba2, a program for predicting binding sites of transcription factor binding sites in DNA sequences (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), to analyze the bovine SCD5 promoter. We found a potential EGR2 binding site at position −64, leading us to hypothesize that bovine SCD5 might be regulated by EGR2 in a similar manner to rodent SCD2. JEG3 cells were transfected with an expression vector containing the coding region of bovine EGR2, pcDNA3.1 + EGR2, and Real-time PCR was used to assess effects on the mRNA expression levels of SCD5, as well as SCD1 as a negative control, and two genes involved in myelination that have been previously described to be upregulated by EGR2, MPZ and PRX. In JEG3 cells, we found that overexpression of EGR2 resulted in a significant upregulation of PRX mRNA (Fig. 1a). However, the mRNA of SCD5, SCD1 and MPZ were not increased compared to cells transfected with empty vector. In murine Schwann cells, EGR2 expression was shown to induce mRNA expression of both MPZ and PRX [2], and stimulation of cultured rat Schwann cells with forskolin and neuregulin resulted in induction of EGR2 mRNA expression, as well as SCD2 expression [3]. EGR2 has also been shown to increase protein expression of PRX and MPZ when expressed in Swiss 3T3 cells. It is possible that the regulation of these myelin genes is different in JEG3 cells, or that the bovine EGR2 expressed in our experiments may not bind with high efficiency to the native human promoters in JEG3 cells.

To further assess potential regulation of the bovine SCD5 promoter by EGR2, we constructed a panel of bovine SCD5 promoter luciferase reporters (Fig. 1b) ranging in length from 1400 base pairs upstream of the transcriptional start site, down to 161 base pairs upstream of the transcriptional start site. To determine whether expression of EGR2 could activate any of these promoter constructs, JEG3 cells were co-transfected with either empty vector or EGR2 expression vector, and the panel of SCD5 promoter reporter vectors. There was no effect of EGR2 on the four longest promoter reporters. However, EGR2 did significantly increase luciferase activity for the two shortest promoter fragments, −260 and −161 (Fig. 1c). The lack of activity with the longer promoter

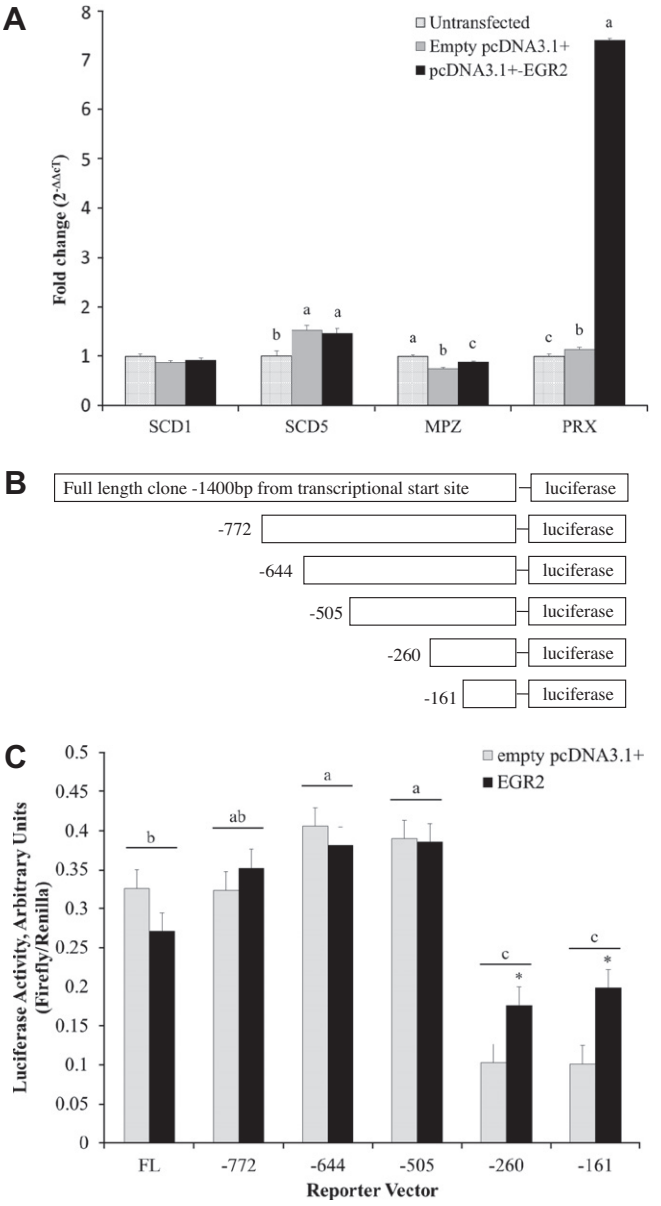

Fig. 1. Effect of EGR2 expression on the SCD5 promoter. (A) Quantitative Real-time PCR using RNA from JEG3 cells untransfected or transfected for 48 h with empty pcDNA3.1 + vector or pcDNA3.1 + EGR2 expression vector. Results are presented as means of 4 experiments ($n = 4$) + S.E.M. $^{a-c}$Means lacking a common letter within each transcript are different ($P < 0.05$). (B) Diagram showing panel of SCD5 promoter luciferase reporter vectors. (C) Cells were co-transfected with or without pcDNA3.1 + EGR2 expression vector in combination with promoter constructs as indicated, and a renilla luciferase reference vector for 48 h. Firefly luciferase activity was measured and normalized to renilla luciferase activity for each sample. Results are presented as means of 4 experiments ($n = 4$) + S.E.M. $^{a-c}$A significant main effect of reporter vector was observed and means lacking a common letter are different ($P < 0.05$). An interaction of EGR2 expression vector and reporter vector was observed and an asterisk (*) denotes a difference ($P < 0.05$) of EGR2 transfection compared to empty vector within reporter vector.

fragments is consistent with the lack of SCD5 mRNA induction, and may indicate the presence of an upstream repressor which may lead to additional regulation of SCD5 in this cell type. It was also noted that the basal promoter activity was significantly less for the two shortest fragments compared to the longer promoter constructs, suggesting that an element located between the positions −505 and −260 might be important for maintaining a basal level of transcription of SCD5.

3.2. Effect of SREBP1a expression on the SCD5 promoter

SREBP has been shown to play a role in peripheral nerve myelination. Mice with a Schwann cell-specific deletion of the SREBP cleavage activation protein (SCAP) lack expression of all SREBP isoforms in these cells, resulting in congenital hypomyelination and abnormal gait [13]. Other studies have shown that SREBP1a increases the activity of the rodent SCD2 promoter in JEG3 cells [3]. Therefore, we examined the effect of SREBP1a expression on the SCD5 mRNA levels by transfecting JEG3 cells with an expression vector containing the coding region for the mature peptide of bovine SREBP1a. Overexpression of SREBP1a in JEG3 cells did not increase the mRNA levels of SCD1, SCD5, or the two myelin genes MPZ and PRX, compared to cells transfected with empty vector (Fig. 2a).

The effect of SREBP1a on the bovine SCD5 promoter was examined using the panel of luciferase reporters shown in Fig. 2b. Overexpression of SREBP1a did lead to a significant increase in SCD5 promoter activity for all constructs except for the full length (1400 base pair) construct (Fig. 2b). The lack of effect on the full length construct is consistent with the lack of increase in SCD5

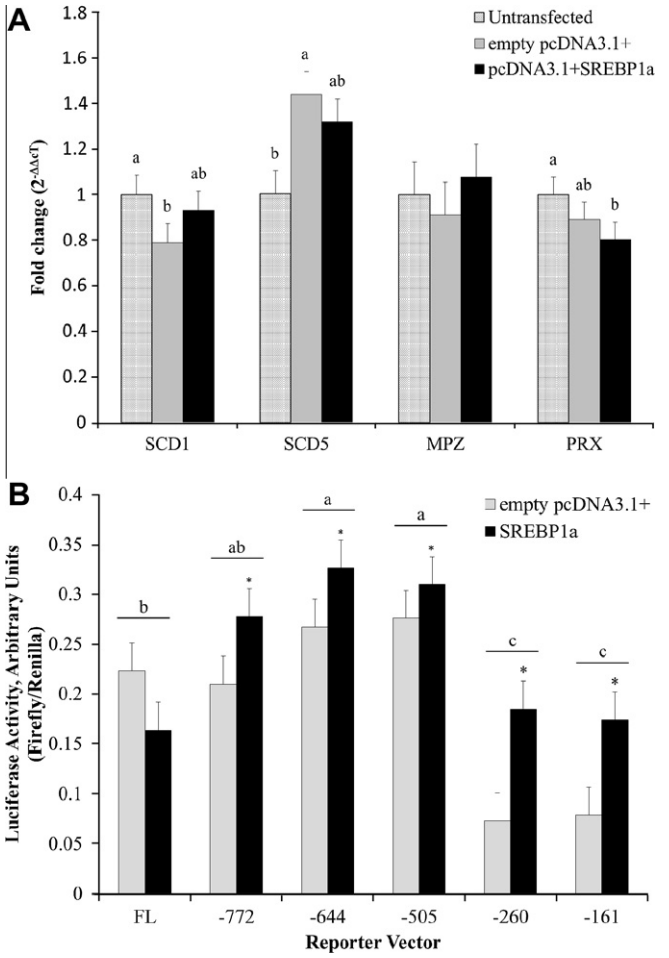

Fig. 2. Effect of SREBP1a expression on the SCD5 promoter. (A) Quantitative Real-time PCR using RNA from JEG3 cells untransfected or transfected for 48 h with empty pcDNA3.1 + vector or pcDNA3.1 + SREBP1a expression vector. Results are presented as means of 4 experiments ($n = 4$) + S.E.M. [a,b]Means lacking a common letter within each transcript are different ($P < 0.05$). (B) Cells were co-transfected with or without pcDNA3.1 + SREBP1a expression vector in combination with promoter constructs as indicated, and a renilla luciferase reference vector for 48 h. Firefly luciferase activity was measured and normalized to renilla luciferase activity for each sample. Results are presented as means of 4 experiments ($n = 4$) + S.E.M. [a–c]A significant main effect of reporter vector was observed and means lacking a common letter are different ($P < 0.05$). An interaction of SREBP1a expression vector and reporter vector was observed and an asterisk (*) denotes a difference ($P < 0.05$) of SREBP1a transfection compared to empty vector within reporter vector.

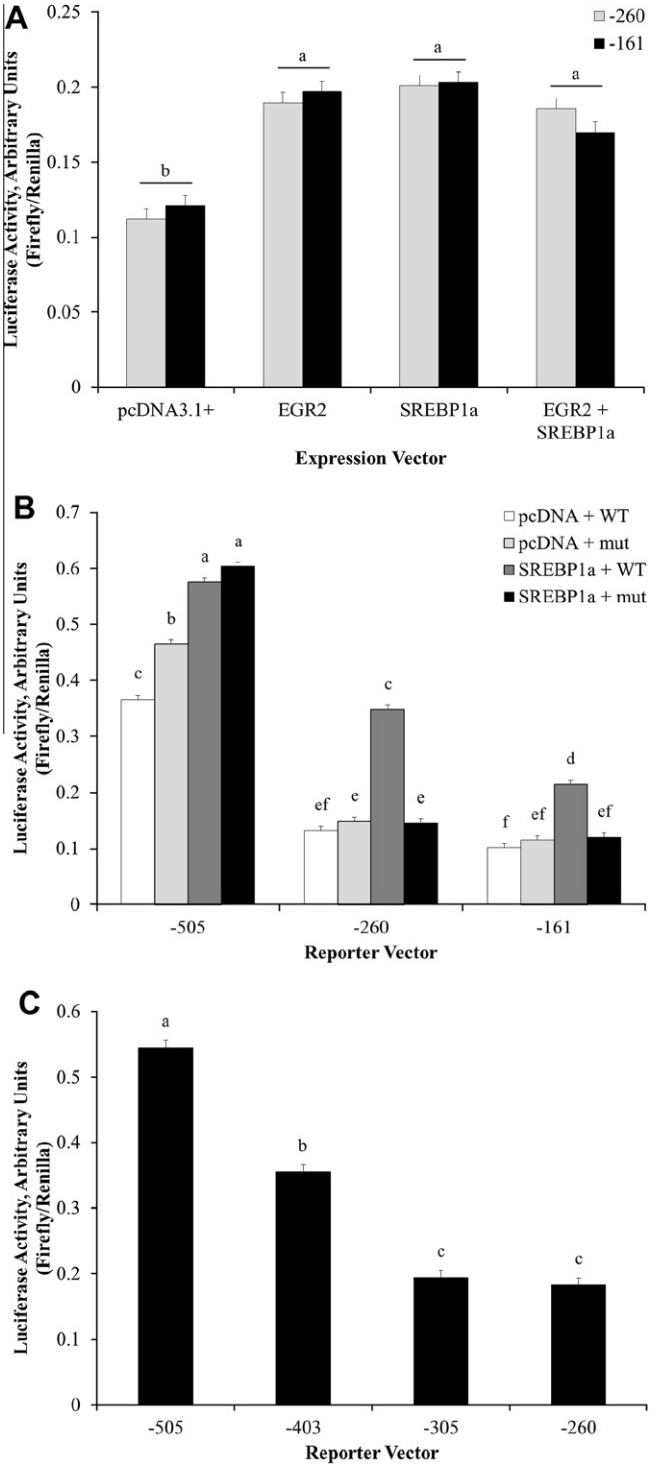

Fig. 3. (A) JEG3 cells were transfected for 48 h with either the 1232F or 1331F reporter vectors, and co-transfected with either empty pcDNA3.1 + vector, pcDNA3.1 + EGR2, pcDNA3.1 + SREBP1a, or with both pcDNA3.1 + EGR2 and pcDNA3.1 + SREBP1a. [a,b]Means lacking a common letter are different ($P < 0.05$). (B) JEG3 cells were transfected for 48 h with either the wild-type or mutant versions of the 987F, 1232F or 1331F reporter vectors, and co-transfected with either empty pcDNA3.1 + vector or pcDNA3.1 + SREBP1a expression vector. [a–f]Means lacking a common letter are different ($P < 0.05$). (C) JEG3 cells transfected for 48 h with SCD5 promoter reporter vectors as indicated. [a–c]Means lacking a common letter are different ($P < 0.05$).

mRNA in SREBP1a transfected JEG3 cells. It is possible that there are additional negative regulation sites found in the full length promoter. As with Fig. 1c, the basal level of SCD5 promoter activity dropped significantly between −505 and −260.

3.3. Effect of co-expression of EGR2 and SREBP1a on the SCD5 promoter

Previous studies have found that when co-transfected with both EGR2 and SREBP1a or SREBP1c expression vectors, the activation of the rodent SCD2 promoter was potentiated in JEG3 cells [3]. To determine whether EGR2 and SREBP1a can synergistically activate the bovine SCD5 promoter, JEG3 cells were co-transfected with either the −260 or −161 reporter vector, and the EGR2 expression vector, the SREBP1a expression vector, or both expression vectors together. While both EGR2 and SREBP1a expression increased luciferase activity compared to empty pcDNA3.1+, there was no synergistic activation of either SCD5 promoter construct when both transcription factors were expressed together (Fig. 3a).

Since both EGR2 and SERBP1a expression individually activated the −260 and −161 SCD5 promoter constructs but dual expression results in no additional increase in activation, we hypothesized that these two transcription factors might be binding to the same site. In fact, a different promoter analysis algorithm, MATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) identified the EGR2 site as a potential SREBP binding site as well. To determine if this was indeed the site mediating the SREBP1a effect on the SCD5 promoter, we used site-directed mutagenesis to alter the DNA sequence at this site in the −505, −260 and −161 SCD5 promoter reporter constructs. JEG3 cells were then transfected with either the wild-type or mutant promoter reporters along with either empty vector or SREBP1a expression vector. Similar to Fig. 2b, expression of SREBP1a increased the activity of all three wild-type reporters (Fig. 3b). However, no increase in activity was seen when SREBP1a was expressed with the mutated −260 and −161 promoters, indicating that the SREBP effect is mediated by the EGR2 site in these constructs. Interestingly, the mutation in the −505 promoter construct had no effect compared to the wild-type −505 promoter construct. One possible explanation would be the presence of an additional SREBP binding site found in this longer promoter piece.

3.4. Basal transcription activity of the bovine SCD5 promoter

Finally, to further explore the region of the bovine SCD5 promoter important for maintaining basal transcription, two additional promoter reporters were developed to examine the region between −505 and −260. JEG3 cells transfected with these reporters showed that basal promoter activity dropped to an intermediate level with the −403 reporter, while the activity of the −305 reporter was no different than that of the −260 reporter. These results seem to indicate that at least two important elements related to maintenance of basal transcription are found with these fragments.

In this paper, we sought answers to the question of whether the transcription of SCD5 is regulated in a similar manner to that of SCD2 in rodents. From these experiments, it appears that there are some differences in the transcriptional regulation of these genes. Expression of bovine EGR2 or bovine SREBP1a in JEG3 cells was not able to increase the mRNA expression of native SCD5 in these cells, or upregulate the activity of a full-length SCD5 promoter reporter construct. This may be due to additional negative regulation of SCD5 in the JEG3 cells, or repressor sites in the full length promoter. Bovine EGR2 and bovine SREBP1a expression were each able to activate shorter bovine SCD5 promoter reporter constructs in a similar manner as seen with rodent SCD2; however, there was no synergism when the two transcription factors were expressed together. Instead, it appears that EGR2 and SREBP1a may bind to the same DNA site in the bovine SCD5 promoter. Further work is needed to determine the contribution of each transcription factor to SCD5 promoter activation in myelin producing cells. This is the first report of potential regulators of SCD5 transcription. Further, we have identified a region of the bovine SCD5 promoter important for maintaining basal transcription of this gene in JEG3 cells.

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