The expression of prosaposin is temporally and spatially regulated at transcriptional and post-translational levels. Transgenic mice with various 5′-flanking deletions of the prosaposin promoter fused to luciferase (LUC) reporters were used to define its temporal regulatory region. LUC expression in the transgenic mice carrying constructs with 234 bp (234LUC), 310 bp (310LUC) or 2400 bp (2400LUC) of the 5′-flanking region was analysed in the central nervous system and eye throughout development. For 310LUC and 2400LUC, low-level LUC activity was maintained until embryonal day 18 in brain, eye and spinal cord. The peak level of LUC activity was at birth, with return to a plateau (1/3 of peak) throughout adulthood. Deletion of the region that included the retinoic acid-receptor-related orphan receptor (RORα)-binding site and sequence-specific transcription factor (Sp1) cluster sites (44–310 bp) suppressed the peak of activity. By comparison, the peak level for 234LUC was shifted 2 weeks into neonatal life in the brain, but not in the eye, and no peak of activity was observed in the spinal cord. The endogenous prosaposin mRNA in eye, spinal cord and cerebellum had low-level expression before birth and continued to increase into adulthood. In cerebrum, the endogenous mRNA showed similar expression profile to constructs 310LUC, 2400LUC and 234LUC, with the peak expression at 1 week and a decreased level in adult. In the brain of the newborn, 2400LUC was highly expressed in the trigeminal ganglion and brain stem regions when compared with the generalized expression pattern for endogenous prosaposin mRNA. These results suggest that the modifiers (RORα- and Sp1-binding sites) residing within 310 bp of the 5′-flanking region mediate developmental regulation in the central nervous system and eye. Additional regulatory elements outside the 5′ region of the 2400 bp promoter fragment appear to be essential for the physiological control of the prosaposin locus.

Key words: central nervous system, developmental regulation, luciferase, retinoic acid-receptor-related orphan receptor-binding site, sequence-specific transcription factor (Sp1).

INTRODUCTION

Prosaposin is a multifunctional protein that plays physiological roles intra- and extracellularly. Intracellularly, prosaposin is the precursor of four glycoproteins, saposin A, B, C and D, which function as activators of selected glycosphingolipid (GSL) hydrolases [1–3]. Extracellularly, prosaposin has in vitro lipid-transfer properties as well as neurotrophic and nerve growth-promoting properties ex vivo and in vivo [4–8]. The physiological significance of these latter activities was evaluated in transgenic mice and gross trophic effects of saposin C were not found in the organization of the central nervous system (CNS) [9,10]. Inherited mutations at the prosaposin locus lead to total prosaposin or individual saposin deficiencies, which produce complex GSL storage diseases mimicking deficiencies of the cognate enzymes [11–16]. Targeted disruption of the murine prosaposin gene produces total prosaposin deficiency and a complex GSL phenotype that includes demyelination and extensive storage of GSLs [17].

The expression of prosaposin is temporally and spatially regulated at transcriptional and post-translational levels [18,19]. High-level expression of its mRNA and protein has been shown in neurons of the CNS, including neurons of the cerebral cortex, spinal cord, and Purkinje cells and granular cells in cerebellum. In contrast with postnatal tissues, expression of prosaposin in embryos was at low levels in the CNS [18,20,21]. Posttranslationally, the proteolytic processing of prosaposin also has cell-type specificity, particularly in the CNS [19]. The variation in the proteolytic processing to mature saposins is cell-differentiation-specific for human and mouse neuron-like cell lines in culture [19].

The transcriptional control region of the prosaposin locus has been defined ex vivo for the mouse and human [22,23]. Both promoters are TATA-less with positive and negative regulatory elements in the first 2400 bp, 5′ to the transcription start sites [22,23]. In the proximal promoter region (310 bp, 5′ to transcription start site) of mouse prosaposin gene, retinoic acid-receptor-related orphan receptor (RORα)-binding site (RORE), a sequence-specific transcription factor (Sp1) and an unknown ‘U’ site were shown to modulate prosaposin expression in vitro by site-directed mutagenesis analysis [24]. The importance of these elements for tissue-specific expression in vivo was determined using transgenic mice bearing 5′-flanking deletions of prosaposin gene fused to luciferase (LUC) reporter cDNA. The transgenic analysis showed nearly exclusive expression in CNS with the constructs containing 310 bp of 5′-flanking DNA. This CNS expression was controlled by the RORE and Sp1 cluster. Internal deletion of RORE and the Sp1 cluster from longer constructs with 2400 bp of 5′-flanking DNA significantly diminished the expression in the CNS [25]. The in vivo analyses of the mice deficient in RORα and containing selected prosaposin-promoter deletion transgenes showed that RORα played an important role in the regulation of prosaposin locus [26].
To define the temporal regulatory region(s) of the prosaposin gene in vivo, transgenic mice containing various deletions of the 5'-flanking region were constructed to contain the LUC reporter gene. The LUC activities in CNS tissues throughout development in these transgenic mice were evaluated to delineate important promoter regions of prosaposin for its CNS developmental expression.

MATERIALS AND METHODS

Materials

LUC assay system and pGL2B LUC reporter vectors were obtained from Promega (Madison, WI, U.S.A.) and TRIzol reagent and SuperScript First-Strand Synthesis System for reverse transcriptase (RT)-PCR were from Invitrogen (Carlsband, CA, U.S.A.). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.), Geneclean kit was from Midwest Scientific Laboratory (Valley Park, MO, U.S.A.), Bradford reagent system was from Bio-Rad Laboratories (Hercules, CA, U.S.A.), and 2 × SYBR Green PCR master mix was from Applied Biosystems (Foster City, CA, U.S.A.).

Transgene constructs

The firefly LUC reporter gene vector pGL2B was used for transgenic constructs. Based on the 5'-flanking sequence of mouse prosaposin gene, a series of PCR primers containing restriction-enzyme sites on the 5'-ends were designed for making deletion constructs as described previously [23]. After restriction digestion, the PCR products were cloned into the HindIII and XhoI sites of pGL2B upstream to LUC gene. Four deletion fragments of the proximal promoter of prosaposin gene were used and were termed 234LUC, 310LUC and 2400LUC (Figure 1). The internal deletion construct 2400D LUC was generated by cloning the PCR fragment containing nucleotide sequences from 2400 to 311 bp into the 5' upstream region of 43LUC [25]. 2400D LUC did not contain the RORE and Sp1 cluster regions (Figure 1).

Generation of transgenic mouse lines

The generation of transgenic mouse lines was described previously [25,27]. For developmental studies, the pups were collected from pregnant wild-type females after breeding to transgenic hemizygous males. The pups at indicated gestational stages were timed by specifying the female copulation plug as 0.5 day of gestation. For genotyping of prenatal transgenic pups, placental genomic DNA was harvested from wild-type pregnant females after killing them at the desired developmental age. For genotyping postnatal transgenic pups, DNA was isolated from tail biopsies. One representative line for each construct was used in the present study: 234LUC, line 2.1 (copy number of transgenes = 5); 310LUC, line 1.1 (copy number = 3); 2400LUC, line 4.2–2 (copy number = 4); and 2400D LUC, line 19.4 (copy number = 3). The tissues were collected from mouse embryos (E) at 15 and 18 days, and postnatal mice from newborns to adulthood (> 6 weeks).

Genotyping of transgenic mice by PCR

The genomic DNA was extracted from the tail clips of postnatal mice or placenta of embryo of prenatal mice. The oligonucleotide primers specific for LUC cDNA (upstream primer 5'-CACAG-AATCGTCGTATGCAGTG-3' and downstream primer 5'-ACTTGACTGCCGACGTATCCTCA-3') were used to generate a 1.3 kb fragment by PCR. Transgene fragments were amplified using Taq polymerase for 30 cycles as follows: 94 °C, 5 min; 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min and extended at 72 °C for 7 min. The solutions contained 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTP and 10 pmol of each primer. The PCR product was electrophoresed for identifying the mice-genotype-specific fragments.

Determination of LUC activity and protein concentration

Tissues were harvested from the transgenic pups between E15 to 6 weeks of postnatal age and included cerebrum, cerebellum, spinal cord, kidney, liver, lung, spleen, eye, heart, thymus, testes

Figure 1  Schematic representation of the mouse prosaposin promoter region and transgene constructs

Mouse prosaposin promoter 5' DNA fragment fused with the LUC cDNA. The numbers in the name indicate the size (bp) of fragments as determined from the transcription start site. The segregation of promoter function was demonstrated by transgenic analyses [25]. The binding sites for transcription factors were identified by in vitro analyses [24]. 2400ALUC indicates that the region from 44 to 310 bp of the 2400LUC was deleted. Bm-2, PDU (Pit-1, Oct-1 and Oct-2, unc-86) domain transcription factor 2; Oct-1 and Oct-6, octamer-binding factors; AP-1, activator protein 1; SRY, sex determining region Y; U, unknown.

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Temporal regulatory region on prosaposin

Figure 2  LUC activities in tissue extracts from transgenic mice containing 234LUC

The cerebrum (A), cerebellum (B), spinal cord (C) and eye (D) tissues were from the indicated ages. The data represent the means±S.E.M. for 2–7 animals in each age group. Nb, newborn; 3d, postnatal day 3.

and uterus/ovary. For the prenatal pups, the visceral tissues were combined into two pools: heart, lung and thymus as one pool, and liver, kidney and spleen as another pool. Organs were pulverized under liquid N$_2$ and homogenized in reporter lysis buffer (Promega). The supernatants from tissue homogenates were assayed for LUC activity using the Promega assay system and Monolight 2010 luminometer (Analytical Luminometer Laboratory, San Diego, CA, U.S.A.). The linearity of the LUC assay was verified for all samples assayed under the conditions used in these experiments. The protein concentration of each supernatant was determined using the Bradford reagent system (Bio-Rad Laboratories). The results for each tissue are the means for 2–7 animals from at least two litters at the indicated age and normalized by gene copy numbers. The non-transgenic mouse controls had no LUC activity.

Quantitative RT–PCR analysis of mRNA

Cerebrum, cerebellum, eye and spinal cord tissues were isolated from adult (6 weeks), 1 week, newborn and E18-day-old mice (n = 4–20). Total RNA was extracted using TRizol reagent. Reverse transcription of the RNA to cDNA was performed using SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen) as described previously [10]. Using cDNA as the template, PCRs were performed in the ABI Prism 7000 sequence detection system. PCR primers were designed using the ABI Prism Primer Express, version 2.0 (Applied Biosystems). Mouse prosaposin primers (forward primer, 5'-GCGACATATGCAA-ACTGTGTC; reverse primer, 5'-CACAGGTCTTCTCAGTAA) were used to generate the amplicon spanning intron 4 of the prosaposin gene in genomic DNA. Primers (18 S; forward primer, 5'-GCATGGCCGTTCTTAGTTG; reverse primer, 5'-TGAAACGCACTTGTCCCT) were used to generate internal control amplicons. In PCR, cDNA aliquots were analysed using 300 nM of each primer and 2×SYBR Green PCR master mix (Applied Biosystems). PCR amplifications were run in triplicate using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A standard curve with a titration of cDNA was performed with PCR amplification for quantification of the samples. The reaction without RT was used as negative control and showed no product in the PCR. Relative quantification of prosaposin referenced to an internal control (18 S) was used for measurement of gene expression. The data are presented as ratios of prosaposin expression in each sample (E18, newborn and 1 week) relative to that in adult.

In situ hybridization

A 0.75 kb LUC cDNA fragment, released by EcoRI and EcoRV from pGL2B, was cloned into pBluescript vector and used to generate LUC riboprobes. Radiolabelling of LUC and prosaposin riboprobes was as described previously [18]. The head of the newborn pup was cut in the midsagittal plane. The frozen
sections (7 µm) of newborn pup brains from the 2400LUC transgenic mice were post-fixed in 4% (w/v) paraformaldehyde and treated with proteinase K, followed by incubation in Tris/glycine and acetylation. Prehybridizations were at 42 °C for 15 min. The sections were hybridized (42 °C overnight) with $4 \times 10^5$ c.p.m./ml of the complementary antisense or sense probes. After hybridization, the slides were washed with SSC/dithiothreitol and then incubated in RNase A and T1. A series of high-stringency washes was performed as described previously [18]. After dehydration by graded ethanol, the slides were dipped in Kodak D19 developer and fixer. All slides were counterstained with haematoxylin and eosin. The positive signal was shown as white silver grains. The sections with sense probes were the negative controls.

RESULTS
Transgenic mice and analyses of transgene temporal expression
The developmental control of prosaposin expression in vivo was investigated using four transgenic constructs (Figure 1). Each construct contained 5'-flanking sequence with up to 2400 bp of sequence 5' to the transcription-initiation site and 98 bp of 5'-untranslated region within the first exon (Figure 1). The 234LUC construct has the first 3' Sp1 and RORE sites. The 310LUC construct also includes an Sp1 cluster of three overlapping Sp1 half sites and an unknown transcription factor-binding site. This region was essential for CNS expression [25]. The 2400 bp 5' fragment was shown previously to promote visceral expression and increase CNS expression. The internal deletion construct 2400DlUC was designed to evaluate the function of RORE and Sp1 cluster by eliminating these regions. Several transgenic founders were made from each of the four constructs [25]. The transmission of the transgene to germ line occurred in 68% of founders. LUC expression was detected in approx. 45% of the lines containing transgenes. Although the levels of expression varied among the founder lines for each construct, the tissue-specific expression pattern was consistent for individual constructs. In the present study, a representative transgenic founder line for each construct (see the Materials and methods section) was used in defining the developmental regulatory elements of the prosaposin gene.

Localization of CNS developmental regulatory elements on prosaposin promoter
The construct 234LUC contains 234 bp, 5' to the transcription start site and includes one Sp1 site and a RORE. These two sites are functional ex vivo and in vivo [24,26]. Analysis of transgenic adult mice containing 234LUC showed preferential CNS (cerebrum, cerebellum and spinal cord) and eye expression [25]. In the brain tissues collected from variously aged 234LUC mice, the LUC expression was detectable at E15 (Figure 2) and increased with age. In cerebrum and cerebellum, the peak of expression occurred at 2 weeks postnatally. These peaks decreased to 20% of maximum by 3 weeks of age. The LUC activities in cerebellum were 2–4-fold higher than that in the cerebrum at all ages (Figures 2A and 2B). The peak of LUC activity in the eye was at...

Figure 3  LUC activities in tissue extracts from transgenic mice containing 310LUC
The cerebrum (A), cerebellum (B), spinal cord (C) and eye (D) tissues were from the indicated ages. The data represent the means ± S.E.M. for 2–7 animals in each age group.

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Figure 4 LUC activities in tissue extracts from transgenic mice containing 2400LUC

The cerebrum (A), cerebellum (B), spinal cord (C) and eye (D) tissues were from the indicated ages. The data represent the means ± S.E.M. for 2–7 animals in each age group.

Birth and the decrease in expression was more gradual than in the cerebellum and cerebrum (Figure 2D). In comparison, the spinal cord expression (Figure 2C) was relatively high until the postnatal period when it decreased to very low levels by weeks 3–4 and to still lower levels at older ages. The visceral tissues showed no detectable LUC activity in these 234LUC transgenic mice from prenatal to postnatal 2 weeks. These results indicate that RORE and Sp1 site within 234 bp region participate in developmental regulation of prosaposin gene in the eye, cerebrum and cerebellum. Also, different regulatory elements influence spinal cord expression.

The construct 310LUC includes an Sp1 cluster in addition to the major sites contained within 234LUC. In cerebrum and cerebellum, expression profiles for 310LUC showed the peak expression at birth, i.e. a shift of approx. 2 weeks compared with 234LUC. A rapid decrease (to 20% of the peak) then occurred in the cerebellum of embryos and adults (Figure 3B). In comparison, the cerebral expression pattern (Figure 3A) achieved a peak and a plateau for approx. 2–3 weeks and then decreased to approx. 20% of peak levels. These adult levels (as a percentage of peak) were higher when compared with the 234LUC. In adults, the patterns in the eye and spinal cord were similar to those obtained with 234LUC, and 5–10% of the peak level was found in adults (Figures 3C and 3D). The 2400LUC transgene led to very similar patterns but some differences were noted. The cerebellar pattern was very similar to that for 310LUC (Figure 4B). In the eye, the peak of expression was shifted from the newborn period to E18 (Figure 4C). In the spinal cord, the expression patterns were similar at E18 and in the newborn period for 310LUC and 2400LUC (Figure 4D). In cerebrum, a significant peak at birth was observed with the 2400LUC constructs. This was not present with the 310LUC (Figures 3A and 4A). Previously, visceral-selective expression was shown to be driven by construct 2400LUC in transgenic mice [25]. With this construct, there was little variation in LUC expression throughout development in selected tissues, including liver, kidney, spleen, lung, thymus, heart, ovaries and testes tissues (results not shown).

The construct 2400LUC has an internal deletion of the Sp1 cluster and RORE sites. Deletion of this region decreased the LUC expression in CNS tissues and the eye by >10-fold relative to 2400LUC [25]. When driven by 2400LUC, the expression peak in the newborn period was obliterated in cerebellum, eye and spinal cord (Figure 5). In addition, in cerebellum and spinal cord, the increase in expression towards the newborn period was missing, whereas very high levels were obtained in the eye. In cerebrum, the pattern of expression was similar to that obtained with the 234LUC constructs, except for E15 where the expression was nearly 50% of the peak at 2 weeks of postpartum age (Figure 5A).

Taken together, these results demonstrate the importance of the RORE and the Sp1 cluster in mediating the temporal regulation in CNS. Deletion of this region removed a developmental enhancement activity. Additional elements within the extended sequence 5' to 310 bp do not appear to be critical to the developmental regulation for visceral tissues.
**Figure 5** LUC activities in tissue extracts from transgenic mice containing 2400ALUC

The cerebrum (A), cerebellum (B), spinal cord (C) and eye (D) tissues were from the indicated ages. The data represent the means ± S.E.M. for 2–7 animals in each age group. LUC activities in these tissues were approx. 10% of those from the corresponding tissues from 310LUC transgenic mice.

**Temporal expression of endogenous prosaposin RNA**

The natural promoter function was evaluated by studying the temporal expression pattern of endogenous prosaposin mRNA in eye, spinal cord, cerebellum and cerebrum collected from the mice at various ages (Figure 6). The mRNA levels were quantified by RT–PCR. For correction of minor variations in the input amounts of mRNA, the mRNA levels were normalized to 18 S RNA, which was quantified in parallel in each experiment. In Figure 6, set 1 refers to the mRNA level in the adult mice. The amount of mRNA from younger mice is presented as a ratio relative to that in the adult.

The prosaposin mRNA expression profile was different in these organs. The level in cerebellum was constant, whereas those in spinal cord and eye increased from embryonic to adult stages. The cerebrum showed an increase from E18 to 1 week and then a decrease by approx. 2-fold in adults. When compared with the reporter transgene expression, these results indicate that the major early developmental expression elements for cerebrum are present in 310LUC. However, additional regulatory elements outside the 2400 bp promoter fragment are required for the developmental control of the prosaposin locus in cerebellum, eye and spinal cord.

**The cellular localization of 2400LUC expression**

In adult mice, the cellular localization of LUC from the promoter constructs (Figure 1) corresponded closely to that for prosaposin [25]. The cellular expression pattern in the head of newborn mice was compared with 2400LUC and the endogenous prosaposin by in situ hybridization analysis (Figure 7). The sections were cut from midsagittal plane. Using the prosaposin riboprobe, the endogenous prosaposin mRNA had generalized expression over whole brain section. The signals were detected in the neurons of the cerebral cortex, mature Purkinje cells and the granular layer of cerebellum (Figure 7A), the trigeminal ganglion (Figure 7B), the epithelial cells of choroid plexus, and neurons of brain stem (Figure 7C) and hippocampus (Figure 7D). With LUC riboprobes, the transgene expression driven by the 2400 bp promoter fragment was generalized and was above background signals in cerebellum, cerebral cortex (Figure 7E) and hippocampal (Figure 7H) regions. Strong LUC signals were in several restricted regions, including the chondrocytes of the developing skull (Figure 7E), the trigeminal ganglion (Figure 7F) and the neuronal clusters in the brain stem (Figure 7G). LUC expression was also found in the epithelial cells of inner ear and the immature fibroblast cells of connective tissues around developing teeth (results not shown). Endogenous prosaposin mRNA was expressed in the inner ear and developing teeth, but was undetectable in the skull. This result indicates that the promoter fragment (2400 bp) is able to drive region-specific expression at birth.

**DISCUSSION**

Prosaposin is expressed in all cell types at various levels [18,20,21,28,29]. Developmental characterization of prosaposin...
mRNA expression has been studied by in situ hybridization and Northern-blot analysis [18,20,21,30]. In CNS, ubiquitous low-level expression was observed at E12.5. By E14 the brain showed areas of expression above the background [18,20,23]. However, dramatic differential expression of prosaposin mRNA in the CNS was found only in postnatal mice [18,21]. In the present study, transgenic mice were used to evaluate the role of control elements in the promoter region, including RORE and a 5' Sp1 cluster on the developmental expression of prosaposin. This area of the promoter had been shown to drive CNS-specific expression in vivo [25,26]. By analyses of LUC expression in CNS and eye tissues throughout development, the interaction of transcription factors binding to RORE and 5' Sp1 sites in the constructs 310LUC and 2400LUC appeared to be maximized at birth. Deletion of RORE and the Sp1 cluster in 2400DLUC led to near baseline expression without a peak in the newborn period. 234LUC contained RORE and 3' Sp1 sites, but the latter site alone produced no detectable reporter gene activity [25]. Thus most of the promoter function could be attributed to the RORE, particularly the peak at 1–2 weeks in the cerebrum and cerebellum. Compared with 310LUC, the delay of peak expression by 2 weeks in 234LUC could be attributed to the absence of factors at the 5' Sp1 cluster. Consequently, the RORE and 5' Sp1 cluster appear to be necessary for producing the peak activity at birth. The expression profile of endogenous prosaposin mRNA in eye, spinal cord and cerebellum showed an increasing trend throughout development with highest level in adult. Interestingly the expression profile in cerebrum correlates with that of the transgene constructs, whereas that in the cerebellum, eye and spinal cord did not. This indicates the existence of additional regulatory elements that account for these differences, i.e. more 5' or possibly within 15 kb first intron [22,23].

Three phases of LUC transgene expression were noted for all constructs: ascending, peak and descending phases. The ascending phase may correspond to cell proliferation and an increasing number of differentiated cells in the brain. The rapid descending phase implicates elements required for maintaining the level of prosaposin expression. These temporal changes also could reflect a change in cell type composition and/or the amount of transcript in each cell. In situ hybridization analysis revealed the region-specific expression in the brain at birth, driven by the 2400 bp promoter fragment. This isolated promoter fragment was able to drive high-level expression in the trigeminal ganglion. Thus the ganglion is a cell type that could contribute to the peak activity. In most brain sections, prosaposin mRNA showed generalized expression and 2400LUC also drove low LUC expression. Such expression patterns are consistent with 2400LUC expression in the adult brain. The concordant expression of LUC and prosaposin RNAs were localized in Purkinje cells of the cerebellum and the neurons of the brain from adult mice with the 2400LUC transgene [25].

The factors that bind to RORE include the RORz and Rev-erbA (reverse erythroblast A) family. These factors are required for cerebellar development [31–33]. Also, their expression pattern in brain tissues closely resembles that of prosaposin: (1) RORz is expressed in Purkinje cells and thalamus, (2) high-level expression

Figure 6 Expression of endogenous prosaposin mRNA

Mouse endogenous prosaposin mRNA expression profiles in cerebrum (A), cerebellum (B), spinal cord (C) and eye (D). Prosaposin mRNA amounts were normalized to 18 S RNA for each sample at the indicated age and are presented as a ratio relative to the adult level. The data represent the means±S.E.M. for multiple animals assayed in triplicate using RT–PCR.
of Rev-erbA is found in hippocampus, cerebral cortex, Purkinje cells and granular cells in cerebellum and (3) the peak of RORα RNA expression in brain is between postnatal weeks 1 and 2 [34]. A burst of Rev-erbα expression has been found in the second week postnatally [31]. The correlation of peak activity for prosaposin and these orphan receptor-related proteins at the same period during development suggested their importance in the regulation of prosaposin expression. Furthermore, when the promoter fragment was not in the context of the gene, the RORE plus 5'Sp1 cluster led to peak expression in the neonatal period.
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