Int. J. Dev. Biol. 50: 705-708 (2006)
doi: 10.1387/ijdb.062168as

Developmental Expression Pattern

N-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase expression during early mouse embryonic development

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ABSTRACT ***N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) is an enzyme which is known to help build up the GlcAβ1-3GalNAc(4,6-bisSO$_4$) unit of chondroitin sulfate E (CS-E). This enzymatic activity has been reported in squid cartilage and in human serum, but has never been reported as an enzyme required during early mouse development. On the other hand, CS-E has been shown to bind with strong affinity to Midkine (MK). The latter is a heparin-binding growth factor which has been found to play important regulatory roles in differentiation and morphogenesis during mouse embryonic development. We have analyzed the expression pattern of the *GalNAc4S-6ST* gene during early mouse embryonic development by whole mount *in situ* hybridization. The results show that *GalNAc4S-6ST* is differentially expressed in the anterior visceral ectoderm at stage E5.5 and later becomes restricted to the embryonic endoderm, especially in the prospective midgut region. During the turning process, expression of *GalNAc4S-6ST* gene is detected in the forebrain, branchial arches, across the gut tube (hindgut, midgut and foregut diverticulum), in the vitelline veins and artery and in the splanchnopleure layer. These results open the possibility of a role for GalNAc4S-6ST during early mouse development.**

KEY WORDS: *mouse development, AVE, endoderm development, gut tube*

N-Acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST; EC 2.8.2.33) is a Golgi-resident enzyme that transfers sulfate from 3´-phosphoadenosine 5´-phosphosulfate (PAPS) to the C-6 hydroxyl group of *N*-acetylgalactosamine 4-sulfate. *N*-acetylgalactosamine 4,6-bissulfate is a component sugar residue of the repeating disaccharide unit, GlcAβ1-3GalNAc(4,6-bisSO$_4$), in chondroitin sulfate E (CS-E) (Suziki *et al.*, 1968). CS-E was found in various mammalian cells as well as in squid cartilage (Habuchi *et al.,* 1971) and shows some activity in mast cell maturation (Eliakim *et al.*, 1986), regulation of procoagulant activity of monocytes (McGee *et al.,* 1995), promotion of neurite outgrowth (Clement *et al.,* 1999), neural adhesion through binding to Midkine (Ueoka *et al.,* 2000) and enhancement of plasminogen activation (Sakaia *et al.,* 2000). Interestingly, CS-E has been shown to bind with strong affinity to Midkine (MK) (Zou *et al.,* 2003), that together with heparin-binding growth-associated molecule (HB-GAM or pleiotrophin) forms a family of heparin-binding growth factors. These two proteins can be found during mouse embryonic development playing regulatory roles in differentiation and morphogenenetic processes (Mitsiadis *et al.,* 1995).

It has also been shown that the nucleotide sequence of human *GalNAc4S-6ST* is nearly identical to that of human B cell RAG-associated gene (*hBRAG*), which raises the interesting possibility that GalNAc4S-6ST might be involved in maturation of B cells by regulating the recombination activating gene 1 (*RAG1*) (Ohtake *et al.,* 2001). RAG1 plays an important role in V(D)J recombination and expression analysis indicates that hBRAG is coordinately expressed with RAG1 in human pro- and pre-B cell lines (Verkoczy *et al.,* 1999). The homology between human and mouse GalNAc4S-6ST might suggest a similar function.

In the present study, we show that the *GalNAc4S-6ST* gene is expressed in the anterior visceral endoderm (AVE) and is therefore likely to have an active role during early mouse development. The AVE comprises a population of primitive endoderm cells located at the distal tip of the 5.5 dpc (days post coitum) embryo, which undergo differentiation and migration to the prospective anterior region and have been reported to be involved in the establishment of the anterior-posterior (AP) axis (Rossant and

Abbreviations used in this paper: ADE, anterior definitive endoderm; AP, anterior-posterior; AVE, anterior visceral endoderm; CS-E, chondroitin sulfate; dpc, days post coitum; FGF, fibroblast growth factor; MK, midkine; RA, retinoic acid.

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0214-6282/2006/$25.00

Printed in Spain
www.intjdevbiol.com

Tam, 2004). The process of neural AP patterning involves the integration of various signals such as retinoic acid (RA), fibroblast growth factors (FGF) and members of the Wnt family as well as the inhibition of BMP-4, Nodal and Wnt8 signaling (Piccolo *et al.,* 1999; Glinka *et al.,* 1997; Silva *et al.,* 2003).

To elucidate the developmental aspect of GalNAc4S-6ST function, the expression pattern of *GalNAc4S-6ST* mRNA was analysed by *in situ* hybridization during mouse embryonic development.

The expression of GalNAc4S-6ST is first detected in the AVE at pre-streak stage (Fig. 1A) and continues throughout gastrulation and neurulation (Fig. 1). At mid-streak stage (Fig. 1B), in addition to the the AVE, a weak expression can be observed in the mesoderm cells arising from the anterior primitive streak. In the late-streak embryo (Fig. 1C) the expression becomes restricted to anterior definitive endoderm (ADE) corresponding to the foregut endoderm. At E7.0, expression can be found in the most rostral

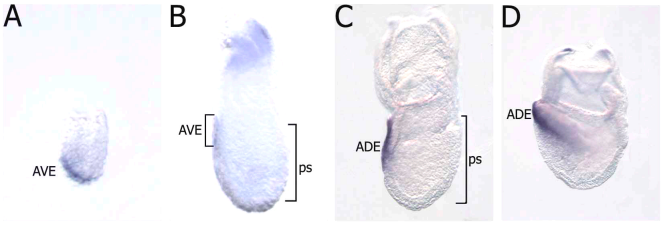

Fig. 1. Expression pattern of N-*Acetylgalactosamine 4-sulfate 6*-O-*sulfotransferase* mRNA from gastrulation to neurulation. *Whole mount* in situ *hybridization with a* GalNAc4S-6ST *DIG-labelled antisense RNA probe was performed on embryos from pre-streak to head-fold stages. Lateral view.* **(A)** *The expression of* GalNAc4S-6ST *mRNA at pre-streak stage (E5.5) is visible in the AVE.* **(B)** *Primitive streak establishment and AVE migration to the prospective anterior region. GalNAc4S-6ST is expressed in the AVE and in mesendoderm cells arising from the anterior primitive streak, as can be seen by the staining from the distal tip to the anterior region.* **(C)** *At stage E7.0, the expression of* GalNAc4S-6ST *became restricted to the anterior definitive endoderm corresponding to the presumptive foregut endoderm.* **(D)** *Expression pattern at neural plate stage.* GalNAc4S-6ST *is expressed in the definitive endoderm cells underlying the prospective head-fold. Abbreviations: ADE, anterior definitive endoderm; AVE, anterior visceral endoderm; ps, primitive streak.*

endoderm (Fig. 1D) that spread laterally underlying the prospective head-fold region during the neural plate stage and later will fuse medially originating the ventral foregut tube.

Head-fold stages are represented by E7.25, E7.5 and E8.0 embryos. At early head-fold stage (Fig. 2A) the expression of *GalNAc4S-6ST* mRNA is located to the more rostral ADE as it spreads laterally, while being completely excluded from the midline. Transverse sections of an E7.5 embryo show staining in the definitive endoderm and in the intra-embryonic mesoderm subjacent to the caudal part of the head-fold (Fig. 2 B1,B2). Some expression in the extra-embryonic mesodermal component of the visceral yolk sac, more specifically in the aggregation of tissue which will subsequently form erythroblast cells (Fig. 2 B1, red arrowheads), is also detected. There is no expression in the notochordal plate, neuroepithelium nor in the cephalic mesoderm. At the late head fold/early somite stage (Fig. 2C) the lateral edges of the ADE fold and fuse ventromedially forming the foregut pocket and the *GalNAc4S-6ST* mRNA expression follows this process. There is a strong staining in the endoderm cells lining the foregut diverticulum and in the prospective midgut as shown in Fig. 2 C1, C2 and C3.

During the turning sequence, at stage E8.5 (Fig. 2D), it's possible to observe expression in the neuroepithelium of the prospective forebrain region, being particularly stronger in the dorsal aspects of the neural folds (Fig. 2 D1,D2). There are some signals of expression in the pharyngeal region of the foregut diverticulum but only in the ventral endoderm foregut diverticulum specifically in the buccopharyngeal membrane (Fig. 2 D3). At this stage GalNAc4S-6ST is also expressed in the body wall overlying the pericardial cavity as well as along the entire embryo in the vitelline veins region (Fig. 2 D,D4). A transverse section through a region where the neural tube is already closed shows staining in the endoderm continuous with the yolk sac (Fig. 2 D5). Transverse sections of the tail region reveal a strong expression in in the ventral endoderm cells of the hindgut diverticulum, in the adjacent vitelline artery and in the endoderm continuous with the yolk sac (Fig. 2 D6).

Once the turning process is completed, it became clear which cells express *GalNAc4S-6ST* (E9.5). A continuous domain of expression that extends along the ventral region of the embryo's trunk, can be seen by whole mount *in situ* hybridization (Fig. 2E), while in the forebrain expression becomes restricted to the dorsal aspect of the neural folds (Fig. 2 E1). At this stage *GalNAc4S-6ST* mRNA is detected in the epithelium of the future mandibular component of the first branchial arch (Fig. 2, E2). There is some expression in the body wall underlying the pericardial cavity adjacent to the septum transversum (Fig. 2, E4). Less pronounced but still evident is the expression in the somites at tail and heart level, being excluded from the rest of embryo body (Fig. 2 E3,E5). In the tail region, several tissues express *GalNAc4S-6ST*. There is staining in the ventral endoderm of the hindgut diverticulum adjacent to the vitelline artery, in the mesodermal cells that contact with the somatopleure layer and in the splanchnic mesoderm, also detected in the midgut region (Fig. 2 E5, E6).

The oldest embryos analyzed had reached 30-34 somite stage (E10.0) and the observed expression pattern of *GalNAc4S-6ST* is similar to that described for the previous stage (Fig. 2F). Coronal sections (Fig. 2 F1, F2 and F3) show that *GalNAc4S-6ST* continues to be ventrally expressed in the vitelline artery, in the gut endoderm, in splanchnopleure layer and in the mesendermal cells in contact to somatopleure layer. *GalNAc4S-6ST* expression is also maintained in the epithelium of the first branchial arch and in the roof plate of the forebrain (Fig. 2 F, F1).

Our results show that GalNAc4S-6ST displays a dynamic expression pattern during early mouse development. The expression is first detected in the AVE, a tissue that plays a central role in the establishment of the anterior-posterior axis. Later expression is shown to be mainly localized in the embryonic endoderm lining the gut diverticulum, which persists until E10.0. Concurrently with the turning process, *GalNAc4S-6ST* is expressed in a few, supposedly unrelated, structures such as the first branchial arch and the presumptive roof plate of the forebrain. GalNAc4S-6ST is an enzyme involved in the biosyn-

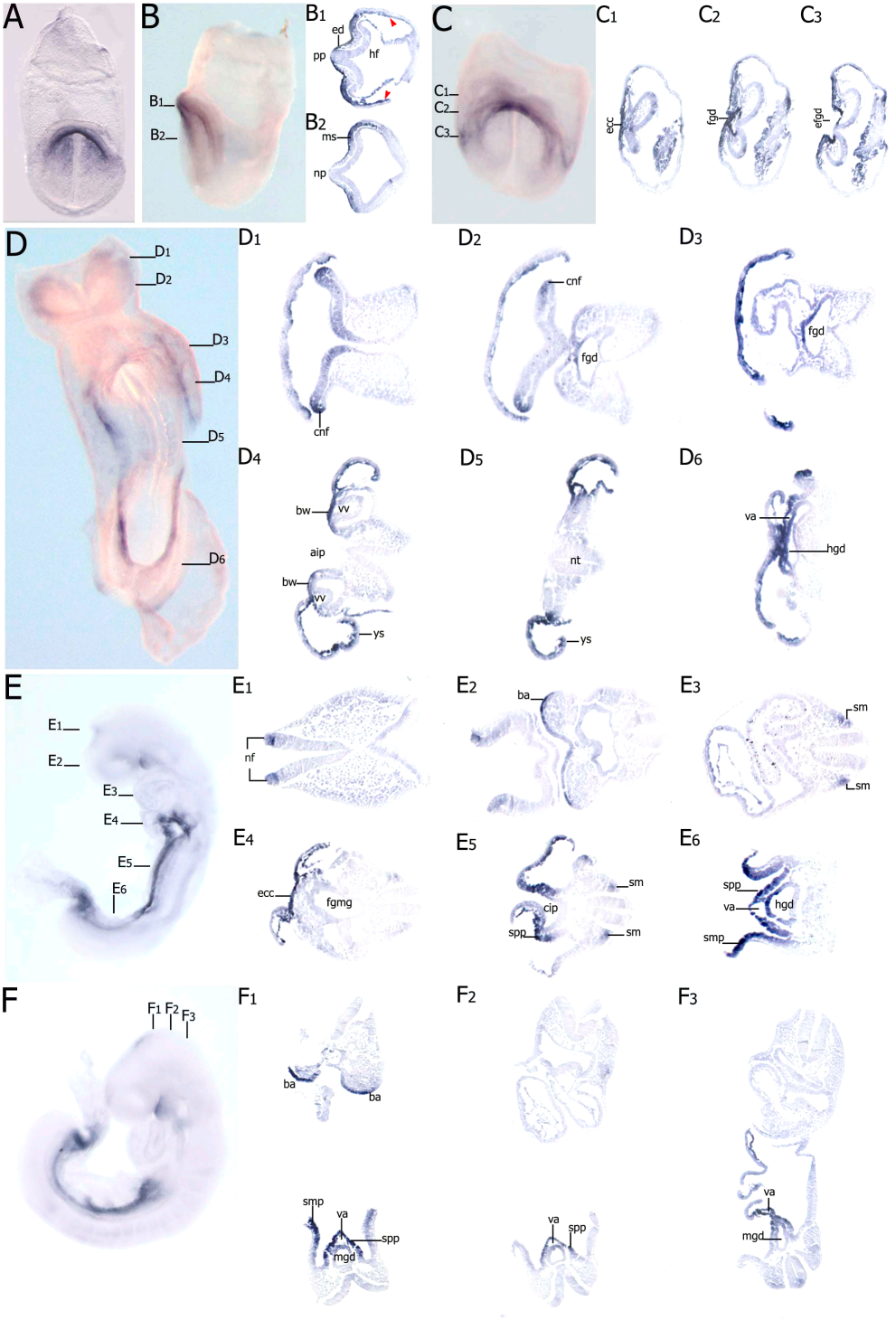

Fig. 2. Expression pattern of N-*acetylgalactosamine 4-sulfate 6*-O-*sulfotransferase* mRNA from neurulation to midgestation. *Whole mount* in situ *hybridization performed in mouse embryos at stages E7.25 – E10.0. All sections are 8-μm.* **(A)** *Anterior view. Early head-fold embryo (E7.25) reveals an expression in the more rostral ADE.* **(B)** *Lateral view. Stage E7.5. The transverse sections taken at the head-fold level show that* GalNAc4S-6ST *is expressed in the definitive endoderm cells and in the intra-embryonic mesoderm subjacent to the caudal part of the head-fold* **(B1, B2)** *as well as in the extra-embryonic mesodermal component of the visceral yolk sac (B1, red arrowheads).* **(C)** *Anterior view. Whole mount* in situ *hybridization performed at stage E8.0.* **(C1,C2, C3)** *is a sequence of transverse sections that shows a restricted expression in the definitive endoderm around the foregut diverticulum (pocket).* **(D)** *Ventral view. At stage E8.5, a dynamic pattern of* GalNAc4S-6ST *expression can be detected.* GalNAc4S-6ST *is expressed in the prospective forebrain neural folds* **(D1,D2)**, *in the foregut diverticulum* **(D3)** *and in the body wall at the pericardial cavity* **(D4)**. *In the region where the neural tube is closed,* GalNAc4S-6ST *expression is visible in the endoderm continuous with the yolk sac* **(D5)**. *Embryo tail level section* **(D6)** *shows expression in the vitelline artery and in the ventral endoderm of hindgut diverticulum.* **(E)** *Lateral view. Expression pattern of* GalNAc4S-6ST *at stage E9.0. There's expression in the prospective forebrain neural folds* **(E1)**, *in the first branchial arch* **(E2)**, *in the endocardial cells adjacent to the septum transversum* **(E4)**, *in splanchnic mesoderm, vitelline artery, hindgut diverticulum and in the endoderm in contact with the somatopleure layer* **(E6)**. *In addition, weak* GalNAc4S-6ST *expression was detected in the somites at the tail and heart region* **(E3,E5)**. **(F)** *Lateral view. Whole mount–*in situ *hybridization performed at stage E10.0 (F1-3, anterior is to the top).* **(F1,F2,F3)** *are coronal sections which show strong staining in the branchial arches in the midgut diverticulum, in the vitelline artery, in the splanchnopleure layer and in the endoderm cells in contact with the somatopleure layer. Abbreviations: ADE, anterior definitive endoderm; aip, anterior intestinal portal; ba, branchial arch; bw, body wall; cip, caudal intestinal portal; cnf, cephalic neural fold; ecc, endocardial cells; ed, endoderm; fgd, foregut diverticulum; fgmg, foregut-midgut junction; hf, head fold; hgd, hindgut diverticiulum; mgd, midgut diverticulum; ms, mesoderm; nf, neural fold; np, notochord plate; nt, neural tube; pp, prechordal plate; sm, somite; smp, somatopleure layer; spp, splanchnopleure layer; va, vitelline artery; vv, vitelline vein; ys, yolk sac.*

thesis of glycosaminoglycans and the activity of several growth factors, such as the FGF class, depends on the interaction with proteoglycans via their glycosaminoglycans side chains. It can be argued that GalNAc4S-6ST may play a role in differentiation and morphogenenetic processes during embryonic development by serving as a regulatory point in the production of CS-E, which has been shown to interact and modulate the activity of the heparin-binding growth factor MK.

Experimental Procedures

Gene expression profiling using GeneChips ® (Affymetrix ®) led to the identification of several transcripts differentially expressed in the mouse AVE (Filipe *et al.*, unpublished results). Among them, mouse *GalNAc4S-6ST* mRNA was identified as being upregulated in the AVE gene pool. The full-length clone of mouse GalNAc4S-6ST (accession number: AV322573), containing the entire coding sequence as well as the 5' and 3'-UTR, was acquired from RZPD® (4631426J05Rik). Mouse embryos were obtained by crossing B6SJL/F1 hybrids maintained on a 7pm to 5am dark cycle and mated overnight. Noon of the day on which a vaginal plug was observed was considered 0.5 dpc. Embryos were dissected from the uterus in PBS and staged by morphological landmarks (Downs and Davies, 1993). Embryos were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated through a graded methanol series and stored at -20°C until use. Whole mount *in situ* hybridization and antisense-probe preparation were performed as previously described (Belo, 1997). Digoxigenin labeled *GalNAc4S-6ST* mRNA probe was synthesized by linearizing the AV322573 clone with *Eco*RI and transcribing with T7 RNA polymerase. The probe was run on a 2% agarose gel before use to verify yield and length. After staining, embryos were refixed in 4% paraformaldehyde and photographed using a Leica DFCM20 digital camera. Some embryos were embedded in paraffin and sectioned at 8 μm using a Leica RM2135 microtome. The sections were examined and photographed using a Leica DM MB2 microscope and a Leica DFCM20 digital camera.

Acknowledgements

We thank Vera Lucas-Teixeira and Lisa Gonçalves for critically reading of this manuscript. Ana M. Salgueiro and M. Filipe are recipients of F.C.T. BI and PhD fellowships, respectively. This work was supported by research grants from F.C.T. and IGC/Fundação Calouste Gulbenkian to J. A. Belo, where he is a Principal Investigator.

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Received: 7th April 2006
Reviewed by Referees: 18th May 2006
Modified by Authors and Accepted for Publication: 1st June 2006
Published Online: 4th October 2006