Analysis of the spatial, temporal and tissue-specific transcription of γ-*sarcoglycan* gene using a transgenic mouse

Satoru Noguchi[a,b,1,*], Eriko Wakabayashi-Takai[a,1], Toshikuni Sasaoka[a], Eijiro Ozawa[a]

[a] *Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[b] *'Inheritance and Variation' Group, PRESTO, Japan Science and Technology Corporation, Kawaguchi, Saitama, Japan*

Received 29 January 2001; revised 26 March 2001; accepted 26 March 2001

First published online 30 March 2001

Edited by Ned Mantei

Abstract To evaluate the promoter function of the 5′-flanking sequence of mouse γ-*sarcoglycan* (γ-*SG*) gene in vivo, we generated transgenic mice harboring this sequence fused with enhanced green fluorescent protein reporter gene. The reporter expression was restricted in striated muscles and particularly strong in all myofibers in skeletal muscles. Using these mice, we examine the spatial and temporal transcriptional patterns of the γ-*SG* gene during mouse skeletal muscle development. The expression of basic helix loop helix transcriptional factors preceded that of the reporter. Differences between the expression of reporter and endogenous γ-*SG* genes in non-muscle tissues suggested the existence of additional promoter elements in the endogenous gene, and the analysis of endogenous mRNAs demonstrated the existence of a novel upstream exon and promoter active in non-muscle tissues.

Key words: Muscular dystrophy; Promoter; Sarcoglycan; Transcriptional regulation; Transgenic mouse

1. Introduction

In the skeletal muscle, the sarcoglycan (SG) complex consists of four subunits, α-SG, β-SG, γ-SG and δ-SG [1]. This complex is present on the sarcolemma as a subcomplex of dystrophin and its associated protein complex [2,3]. The gene for each of these SG subunits is responsible for autosomal recessive muscular dystrophy, sarcoglycanopathy [4]. Mutation of any one gene encoding an SG subunit results in sarcoglycanopathy in which all other SG subunits on the sarcolemma are lost or greatly reduced in amount. Thus for the presence of the SG complex on the sarcolemma, it is required that all SG subunits be normally expressed.

Previously, we analyzed the expression of *SG* gene products, transcripts and proteins, during myotube differentiation in the mouse skeletal muscle C2C12 cell line [5]. In proliferating myoblasts, all SG transcripts were expressed at approximately equal levels. After myotube formation, the level of α-SG and γ-SG transcripts selectively increased, while β-SG and δ-SG remain unchanged. This transcriptional upregulation of the *α-SG* and *γ-SG* genes seems to trigger the SG complex formation because all the SG proteins accumulated almost immediately after myotube formation [6]. For the elucidation of the SG complex formation process, not only the SG subunit assembly in the post-translational step, but also the regulation of *SG* gene expression should be analyzed. Thus, we recently isolated the promoters of four mouse *SG* genes and analyzed their transcriptional regulation using the C2C12 cell line [7]. We identified a basal promoter region and two enhancer regions in the *γ-SG* promoter. Furthermore, in the upstream enhancer, we found A/T-rich and E-box elements, which are essential for transcriptional activation during myocyte differentiation.

In this study, using transgenic mice harboring a reporter gene fused with the 5′-flanking sequence of the *γ-SG* gene, we examined the promoter activity of this sequence in vivo by analyzing the spatial, temporal, and tissue-specific expression patterns of the transgene during mouse development. We found that this promoter region is sufficient to direct a strong expression in vivo in the skeletal muscle and also showed that this expression could reproduce the transcriptional pattern of the endogenous *γ-SG* gene during skeletal muscle development.

2. Materials and methods

2.1. Generation of transgenic mouse

The transgene vector was constructed by insertion of the rabbit β-globin intron 2 sequence and SV40 polyA site derived from plasmid pBstN [8], at the 3′-end of an enhanced green fluorescence protein (EGFP, Clontech) sequence in a 5′gsg-EGFP plasmid [7]. The 5′gsg-EGFP plasmid contains a 1.5-kbp 5′-flanking sequence extending from −1458 to +21 relative to the transcription initiation site (+1) of the mouse *γ-SG* gene, and a coding sequence for EGFP. The 5.0-kbp fragment was cut out from the vector with *Not*I and *Hin*dIII, and purified by agarose gel electrophoresis. The transgenic mice were generated by microinjection of this fragment into pronuclei of fertilized eggs of C57BL/6J mice. Transgenic animals were identified by PCR amplification or Southern blot analysis of tail DNA. The founder mice were crossed with C57BL/6J mice to produce transgenic progeny. All animal handling procedures were in accordance with a protocol approved by the National Institute of Neuroscience, NCNP, Japan.

2.2. Reverse transcription-PCR (RT-PCR)

Total RNA from various tissues of the adult transgenic mice was prepared with Trizol (Gibco BRL). Primer pairs for endogenous γ-SG

*Corresponding author. Fax: (81)-42-346 1742.
E-mail: noguchi@ncnp.go.jp

[1] Equal contribution made by both authors.

Abbreviations: BE, brain-type exon; b-HLH, basic helix loop helix; dpc, day post-coitum; EGFP, enhanced green fluorescence protein; ME, muscle-type exon; RACE, rapid amplification of cDNA end; SG, sarcoglycan

0014-5793/01/$20.00
PII: S0014-5793(01)02368-7

and G3PDH transcripts in RT-PCR were used as reported previously [5]. Reverse transcription reaction was carried out using 1 μg of total RNA with AMV Reverse transcriptase XL (Takara) at 55°C for 30 min. Amplification was carried out using LA-Taq (Takara) for 30 cycles, each cycle consisting of 94°C for 30 s, 65°C for 5 min. Primer pairs AATAACCAGCACGTTGCCCAGGAG (antisense primer), GGTGGTGCCCATCCTGGTCGAG (sense primer) for the 5′gsg-EGFP transgene, and GCGCTTCCTCCATCCGTAAATGCC (antisense primer), GTCAGGGACCGCTCCACCTGGAGC (sense primer) for brain-specific transcript of γ-SG were used. 5′-End sequences of γ-SG transcripts were examined by the rapid amplification of cDNA ends (RACE) method as described previously [7].

2.3. Observation of EGFP

Mouse embryos were dissected by removing the entire covering including the amnion, and then fixed in 2% paraformaldehyde for 120 min at 4°C. For observation of a 9.5-dpc (days post-coitum) whole embryo, or forelimb buds and hindlimb buds from 10.5-dpc to 13.5-dpc mice, fixed samples were washed with phosphate-buffered saline (PBS) and mounted on a glass slide in PBS containing 80% glycerol. For preparation of cryosections of embryos and neonatal mouse hearts, the fixed samples were immersed in PBS containing 25% sucrose for 1 day, then embedded in OCT compound (Miles) and frozen. Muscle cryosections from adult mice were prepared as described previously [9]. EGFP fluorescence in the embryos and their sections were observed under a Leica TCS SP confocal laser microscope (Leica). To visualize the 9.5-dpc embryo and isolated limbs, spontaneous fluorescence was also observed with a high sensitivity at tetramethyl rhodamine excitation (530 nm) and emission (590 nm) wavelength separated with a prism. Three-dimensional fluorescence images of limbs were reconstructed from two-dimensional scanning images using a Leica TCS-NT program. Polyclonal antibodies against Myf-5 (C-20), MyoD (M-20) and myogenin (C-12) were purchased from Santa Cruz Biotechnology.

2.4. In situ hybridization

In situ hybridization was performed according to the method of Hatanaka and Jones [10] using cRNA probes described previously [5].

2.5. Preparation and culture of primary myocytes

Primary myocytes were isolated from hindlimbs of neonatal transgenic mice and cultured as reported by Hagiwara and Ozawa [11]. On the second day after plating the cells, differentiation was induced by changing the medium to Dulbecco's modified Eagle's medium containing 1 mM hydroxylurea in 5% horse serum.

3. Results and discussion

3.1. Tissue-specific expression pattern of the transgene

We generated transgenic mice harboring the transgene 5′gsg-EGFP, which contains the 1.5-kbp mouse *γ-SG* promoter sequence and the EGFP coding sequence as a reporter gene. Two transgenic mouse lines (#59 and #66) expressing EGFP from five independent lines were used in this study. At first, we analyzed the transcription of the transgene in various tissues of adult F1 mice by RT-PCR (Fig. 1A). The endogenous γ-SG transcript was detected in abundance in the skeletal muscle of the limbs, diaphragm and heart as shown by Northern blotting which was described previously in human tissues [4] and examined in mouse tissues (data not shown). Other than these tissues, it was weakly detected by RT-PCR in the cerebellum, olfactory bulb, spinal cord, skin, lung, aorta and testis. Recently, the expression of the γ-SG transcript in the lung was reported by Durbeej and Campbell [12]. On the other hand, the 5′gsg-EGFP transcript had a more restricted distribution than the endogenous γ-SG transcript. The 5′gsg-EGFP transcript was detected in abundance in the skeletal muscle of the limbs, diaphragm, at a moderate level in the heart and at low levels in the spinal cord and testis, but not in the cerebellum, olfactory bulb, aorta and lung. From our

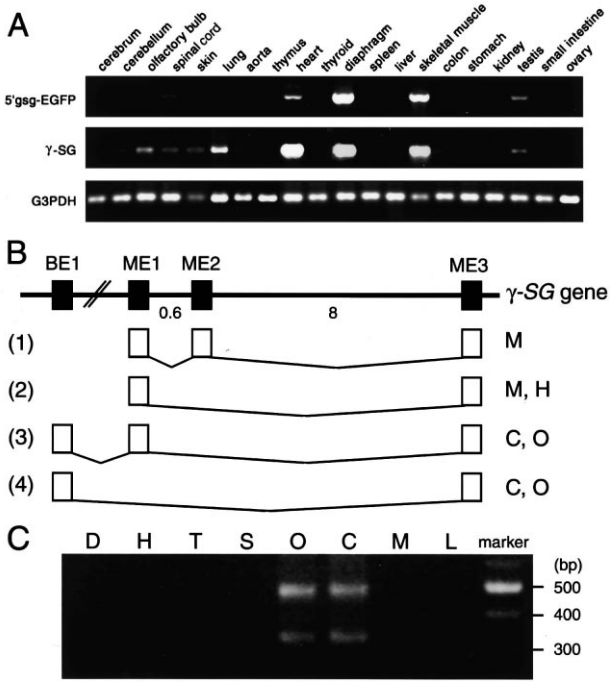

Fig. 1. Tissue-specific and spatial expression of 5′gsg-EGFP transgene. A: RT-PCR was carried out for the transcripts of 5′gsg-EGFP transgene (5′gsg-EGFP), endogenous γ-SG (γ-SG), and G3PDH as a control. The skeletal muscle sample was isolated from limbs. B and C: Identification of the brain-specific exon in γ-SG transcripts. B: 5′-Regions of muscle-type transcripts (1), (2) and brain-type transcripts (3), (4) are illustrated under γ-SG genomic configuration with intron sizes (kbp); muscle-type exon 1 (ME1), exon 2 (ME2) and exon 3 (ME3) were mapped as reported previously [7]. Transcripts (1) and (2) in the skeletal muscle (M), (2) in the heart (H) and (3) in the cerebellum (C) were isolated by RACE. BE1, brain-type exon 1. C: 5′-Region of the brain-type transcript was amplified by RT-PCR from RNA in various γ-SG-expressing tissues using antisense primer from BE1 and sense primer from ME3. The larger (475 bp) and shorter (320 bp) bands from cerebellum and olfactory bulb correspond to (4) and (3) in B, respectively. D, diaphragm; H, heart; T, testis; S, spinal cord; O, olfactory bulb; C, cerebellum; M, skeletal muscle; L, lung.

examination, the mice of transgenic lines #59 and #66 exhibited entirely the same pattern of tissue distribution. To examine this difference between transgene and endogenous transcripts, we analyzed 5′-ends of the γ-SG transcripts in the skeletal muscle, heart, cerebellum, and lung by RACE. In the skeletal muscle and heart, the products containing only muscle-type exon (ME) 1 were obtained shown as (1) and (2) shown in Fig. 1B. No amplified product was obtained from lung RNA. In the cerebellum, a novel 5′-sequence (310 bp, GenBank/EMBL/DDBJ accession no. AB056897), linked to ME3 sequence was identified ((3) in Fig. 1B). This sequence may be included in a single exon (brain-type exon 1: BE1), because PCR amplification with the primer pairs derived from 5′- and 3′-ends of this novel sequence gave a product from genomic DNA whose sequence completely matched the sequence obtained by RACE. Using a sense primer from BE1 and an antisense primer from ME3, we surveyed the use of this BE1 in eight tissues by RT-PCR as indicated in Fig. 1C. Two bands were amplified in RNA only from the olfactory bulb and cerebellum (lanes O and C). A larger product (475 bp) contains ME1 as exon 2 while a shorter (320 bp) one does not, shown as (4) and (3), respectively, in Fig. 1B. These

results suggest that γ-*SG* gene is transcribed in these two tissues using another promoter located upstream of the muscle-type promoter.

3.2. *Spatial expression pattern of the transgene in 15.5-dpc embryo*

We used fluorescence microscopy to analyze the EGFP localization in the transgene-expressing tissues in adult and neonatal mice. Transgenic mouse lines #59 and #66 exhibited entirely the same patterns for EGFP expression in skeletal muscles. EGFP was strongly expressed in all myofibers in the skeletal muscle of the limbs and diaphragm (Fig. 2A,B). No difference in fluorescence strength was observed in the fast and slow fibers (data not shown). This suggests that the 1.5-kbp region (−1458 to +12) of the γ-*SG* promoter is sufficient to highly promote transcription in all types of skeletal muscle cells. In the heart, the number and distribution of the EGFP-expressing cells were variable in individual transgenic mice even if they were of the same line. Fig. 2C shows the heart section from the #66 transgenic mouse where EGFP was

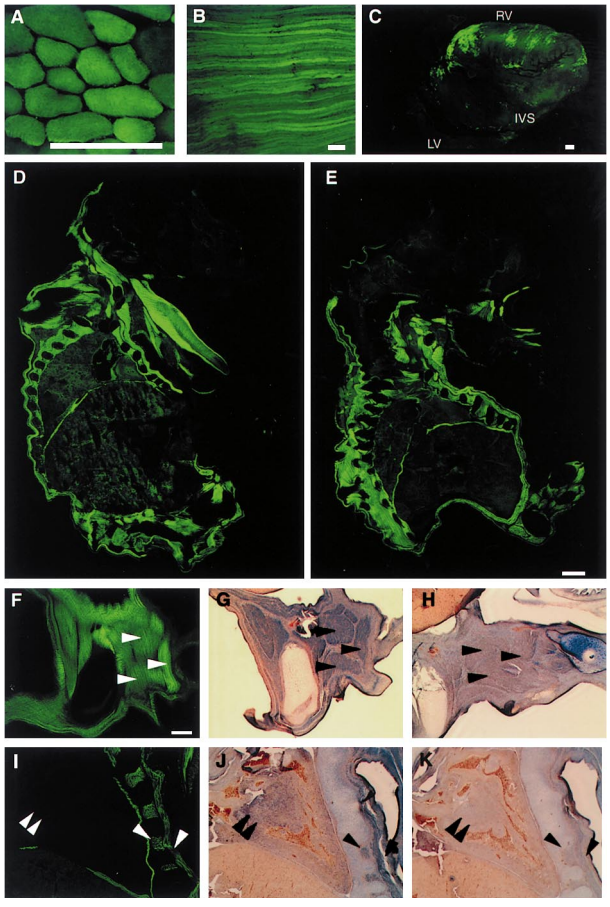

Fig. 2. Spatial expression of 5′gsg-EGFP transgene. A–C: The localization of reporter EGFP in cryosections from quadriceps muscle (A), diaphragm (B) in adult mice and heart in neonatal mouse (C). RV, right ventricle; LV, left ventricle; IVS, interventricular septum. Bars: 20 μm. D and E: The reporter EGFP expression in midsagittal (D) and parasagittal (E) sections of 15.5-dpc embryo. Bar: 500 μm. F–K: The comparison of localization of EGFP (F and I) and endogenous γ-SG transcript shown by in situ hybridization using antisense probe (G and J) and sense probe (H and K). Both EGFP and γ-SG transcript are expressed in the skeletal muscles (arrowheads), whereas EGFP was not detected in the heart in this stage (double arrowheads). Bar: 100 μm.

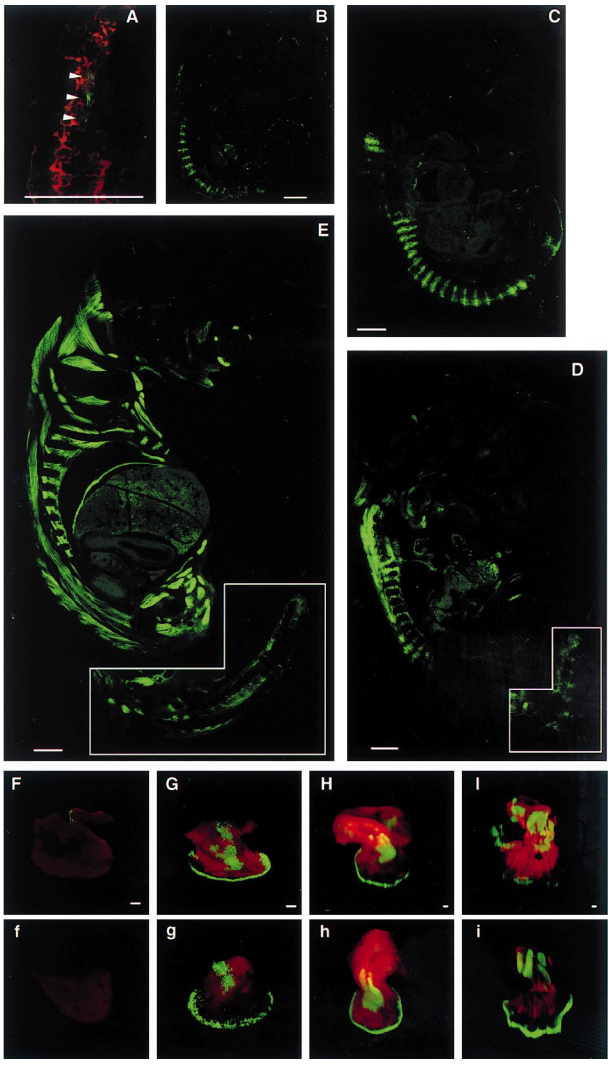

Fig. 3. Temporal expression of 5′gsg-EGFP transgene in body wall and limbs during embryogenesis. A: 9.5-dpc whole embryo was observed for EGFP expression (green) with spontaneous fluorescence (red) observed at excitation and emission wavelengths for rhodamine fluorescence to image its body. B–E: The sagittal sections through somite regions of embryos on 10.5 dpc (B), 11.5 dpc (C), 12.5 dpc (D) and 13.5 dpc (E). Insets in D and E show the EGFP expression in the tail region in the other sections. Bars: 500 μm. F–i: Three-dimensional images of EGFP fluorescence expression (green) from a dorsal view were reconstructed from several planes of two-dimensional images using a confocal laser microscope with spontaneous fluorescence images (red). Upper images, the left forelimb buds of embryos on 10.5 dpc (F), 11.5 dpc (G), 12.5 dpc (H) and 13.5 dpc (I); lower images, the left hindlimb buds on 10.5 dpc (f), 11.5 dpc (g), 12.5 dpc (h) and 13.5 dpc (i). Bars: 100 μm.

strongly expressed in nearly half of the myocardia in the right ventricle and also in small parts in the left ventricle and interventricular septum. We failed to detect EGFP in the spinal cord and testis. It was also not detected in the peripheral nerve, consistent with our previous report that ε-SG containing the SG complex found in the peripheral nerve does not have γ-SG [8].

We analyzed spatial expression patterns of the transgene in parasagittal and midsagittal sections (Fig. 2D,E, respectively) of the 15.5-dpc embryo. The EGFP was localized in the entire body specifically in the skeletal muscles, such as the extrinsic ocular muscle, back muscle, pectoral muscle, intercostal

muscle, rectus abdominis muscle, diaphragm, cutaneous muscle and tongue, including the epaxial (paraspinal and intercostal) and hepaxial (limb and abdominal) musculature. The epaxial musculature originates from the dorsal–medial dermamyotome which is primarily regulated by Myf-5, while the hepaxial musculature originates from ventral–lateral dermamyotome which is primarily regulated by MyoD [13]. The distribution matched the expression sites of the endogenous γ-SG transcript in the skeletal muscles by in situ hybridization (Fig. 2F,G,I,J, arrowheads). However, EGFP was not expressed in the heart at this embryonic stage (Fig. 2I), although the endogenous γ-SG transcript was transcribed (Fig. 2J, double arrowheads). This may be due to possible enhancers for heart expression being missing, or due to positional effects of the transgene in heart expression. Both of two transgenic mouse lines showed similar patterns in heart expression, so that a positional effect is less likely and the former possibility is more likely.

3.3. *Temporal expression pattern of the transgene during embryogenesis*

To analyze the temporal expression patterns of the transgene during mouse embryonic development, we used embryos of the F2 generation and prepared sagittal sections. In the 9.5-dpc embryo, the reporter EGFP was first expressed in elongated cells of three rostral somites (arrowhead in Fig. 3A). In the 10.5-dpc embryo, EGFP was expressed in more than 20 somites (Fig. 3B). On 12.5 dpc, EGFP fluorescence became intense in the back muscles and intercostal muscles (Fig. 3D). The rostral-to-caudal progression of the EGFP expression in the somites during embryogenesis might be related to muscle formation similar to the observation in the transgenic mice with myogenin promoter [14]. By comparing these data with the expression pattern of the endogenous γ-SG transcript during mouse embryogenesis examined by RT-PCR (Wakabayashi-Takai et al., unpublished), the transgene expression mimics the spatiotemporal expression pattern of the γ-SG transcript in the skeletal muscle.

We also analyzed the temporal expression of the transgene during embryonic limb muscle formation (Fig. 3F–I). A few EGFP-positive cells were first observed in 10.5-dpc embryo in the proximal part of the forelimb buds (Fig. 3F). On 11.5 dpc, the EGFP-positive cells spread to the central part of the forelimb buds, while EGFP began to be detected in hindlimb buds (Fig. 3G and g). Most of the EGFP-positive cells in both limb buds did not appear to be elongated. On 12.5 dpc, the EGFP-positive cells became filamentous (Fig. 3H,h). In the 13.5-dpc embryo, EGFP was expressed strongly in the proximal part (Fig. 3I,i). The onset of the transgene expression in the limb bud was 1 day later than that in the somites. Other than the expression in the musculature, an intense EGFP fluorescence was also observed in the distal ridge of the forelimb and hindlimb buds (Fig. 3G–h). This may be due to the transient ectopic expression of the transgene during development, because we could not detect a significant signal of γ-SG transcript in this region by in situ hybridization or RT-PCR amplification (data not shown) and this expression weakened and disappeared as finger formation progressed (Fig. 3I).

3.4. *Comparison of the expression patterns of transgene and basic helix loop helix (b-HLH) proteins*

By characterization of the *γ-SG* promoter in C2C12 myocytes, we previously proposed that the b-HLH factors may work as a *trans* activator for the *γ-SG* promoter [7]. We analyzed the relationship between the expression of the transgene and b-HLH transcriptional factors. In the 12.5-dpc embryo, the localization of EGFP and MyoD was primarily the same except for EGFP expression in the distal ridge of limb buds (Fig. 4A). Under magnification, some differences between them were observed in a merged image (Fig. 4B). MyoD was detected in the weak EGFP fluorescence area in the abdominal muscles, while the transgene was expressed strongly in the filamentous myofibers with a rather weak staining of b-HLH factors in the intercostal muscles. These data suggest that there are differences in timing of expression between transgene and b-HLH factors. To confirm this, we observed their expression in earlier stage embryos. In the transverse sections of the 10.5-dpc embryos through the caudal trunk somite, the transgene was observed in a narrow region throughout the dorsal–ventral extent in myotome. On the other hand, MyoD and myogenin were observed in dorsal myotome (Fig. 4C,D). Myf-5 was expressed rather strongly in the lips of the dermatome (Fig. 4E). The localization of EGFP did not coincide with that of the b-HLH factors at the cell

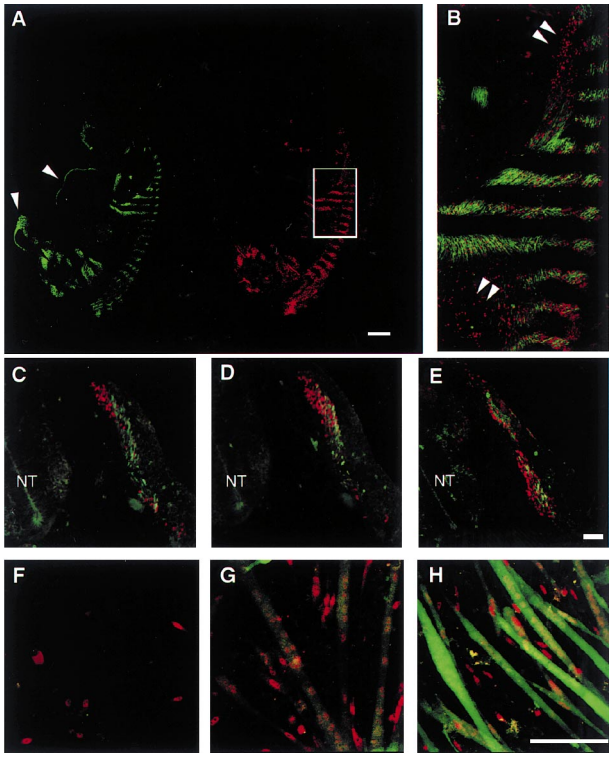

Fig. 4. Relationship between the expression of 5′gsg-EGFP transgene and that of b-HLH transcriptional factors. A: Double-fluorescence images of EGFP (green) and anti-MyoD antibody staining (red) of the sagittal section of 12.5-dpc embryo. EGFP alone was detected in ridges of limb buds (arrowheads). B: Magnified merged images of body wall regions shown in a rectangle in A. Arrowheads indicate the intercostal muscles and double arrowheads indicate the abdominal muscles. Bar: 500 μm. C–E: Merged fluorescence images of EGFP (green) and b-HLH factor antibodies (red) in transverse sections through caudal trunk somite on 10.5 dpc with anti-MyoD (C), anti-myogenin (D) and anti-Myf-5 antibodies (E), respectively. NT, neural tube. Bar: 50 μm. F–H: Double-fluorescence images of EGFP (green) and anti-myogenin antibody staining (red) in primary myocytes cultured for 1 day (F), 3 days (G) and 5 days (H) after plating the cells.

level in this stage. Based on these observations, we assumed that the b-HLH factors appear and pass away rather prior to the expression of EGFP. To clarify this relation, we made primary cultures of myocytes from limbs of the neonatal mice and found that myogenin was strongly expressed, whereas EGFP was relatively weakly expressed in early stage elongating mononucleated myocytes on the third day of culture (Fig. 4G). Myogenin expression became weaker in large multinucleated myotubes on the fifth day in contrast to the strong expression of EGFP (Fig. 4H). The expression of b-HLH factors preceded that of EGFP and this temporally reciprocal expression strongly supports our previous in vitro observation that the reporter expression is regulated by b-HLH transcriptional factors during skeletal muscle development.

The above data show that our promoter may be useful for gene therapy. Recently, gene transfer into SG-deficient animals was attempted [15,16]. In these cases, the cytomegalovirus early promoter and truncated muscle creatine kinase promoter were used for the expression of the transferred gene. However, the expression pattern of the transferred gene should reproduce the spatial, temporal and tissue-specific expression patterns of the endogenous one. In particular, the formation of the SG complex in the skeletal muscles may be regulated by the enhancement of *α-SG* and *γ-SG* transcription. Therefore, the use of the *γ-SG* promoter seems to be advantageous for the exogenous SG expression in therapeutic attempts to restore the SG complex in the striated muscles.

Acknowledgements: The authors would like to thank Dr. Michihiro Imamura of our laboratory for discussion and encouragement, as well as Drs. Masayoshi Mishina (The University of Tokyo) and Minoru Kimura (Tokai University) for the plasmid pBstN. This work was supported by a grant from the COE program of the Ministry of Health and Welfare, Japan.

References

[1] Yoshida, M., Noguchi, S., Wakabayashi, E., Piluso, G., Belsito, A., Nigro, V. and Ozawa, E. (1997) FEBS Lett. 403, 143–148.
[2] Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) Nature 345, 315–319.
[3] Yoshida, M. and Ozawa, E. (1990) J. Biochem. (Tokyo) 108, 748–752.
[4] Ozawa, E., Noguchi, S., Mizuno, Y., Hagiwara, Y. and Yoshida, M. (1998) Muscle Nerve 21, 421–438.
[5] Noguchi, S., Wakabayashi, E., Imamura, M., Yoshida, M. and Ozawa, E. (1999) Biochem. Biophys. Res. Commun. 262, 88–93.
[6] Noguchi, S., Wakabayashi, E., Imamura, M., Yoshida, M. and Ozawa, E. (2000) Eur. J. Biochem. 267, 640–648.
[7] Wakabayashi-Takai, E., Noguchi, S. and Ozawa, E. (2001) Eur. J. Biochem. 268, 948–957.
[8] Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindostrom, J., Takahashi, T., Kuno, M. and Numa, S. (1984) Nature 307, 604–608.
[9] Imamura, M., Araishi, K., Noguchi, S. and Ozawa, E. (2000) Hum. Mol. Genet. 9, 3091–3100.
[10] Hatanaka, Y. and Jones, E. (1998) J. Comp. Neurol. 395, 296–309.
[11] Hagiwara, Y. (1994) Dev. Growth Differ. 36, 141–148.
[12] Durbeej, M. and Campbell, K.P. (1999) J. Biol. Chem. 274, 26609–26616.
[13] Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S.J. and Rudnicki, M.A. (1997) Development 124, 4729–4738.
[14] Cheng, T.-C., Hanley, T.A., Mudd, J., Merlie, J.P. and Olson, E.N. (1992) J. Cell. Biol. 119, 1649–1656.
[15] Cordier, L., Hack, A.A., Scott, M.O., Barton-Davis, E.R., Gao, G., Wilson, J.M., McNally, E.M. and Sweeney, H.L. (2000) Mol. Ther. 1, 119–129.
[16] Xiao, X., Li, J., Tsao, Y.P., Dressman, D., Hoffman, E.P. and Watchko, J.F. (2000) J. Virol. 74, 1436–1442.