Transcriptional stimulation via SC site of Bombyx sericin-1 gene through an interaction with a DNA binding protein SGF-3

Kenji Matsuno, Shigeharu Takiya, Chi-chung Hui, Toshiharu Suzuki, Masakazu Fukuta, Kohji Ueno and Yoshiaki Suzuki
Department of Developmental Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan

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ABSTRACT

Three protein binding sites have been identified in the upstream region of the sericin-1 gene. Two of them, SA and SC sites, have been known as putative cis-acting elements. Using synthetic oligonucleotides of these binding sites, it was found that silk gland factor-1 (SGF-1) binds to the SA site, and silk gland factor-3 (SGF-3) binds to the SC site but not to a mutated SC site, SCM. Tissue distribution of the two factors was different. SGF-3 is present abundantly in the middle silk gland (MSG) where the sericin-1 gene is transcribed specifically but is also present in other cell types, though in a much less concentration. SGF-1 is observed very abundantly in the two parts of silk gland, MSG and posterior silk gland (PSG), but not in other cells. Templates containing multimerized SA or SC sites at -39 of the sericin-1 gene promoter were tested in MSG nuclear extracts. The SC multimer strongly activated transcription, while the mutant SCM multimer did not. The SA multimer also gave a slight stimulation of transcription. These results suggest that SGF-3 stimulates transcription through an interaction with the SC site, and SGF-1 does so weakly through the SA site.

INTRODUCTION

The sericin-1 gene encodes a silk glue protein which is expressed exclusively in the MSG of the silkworm, Bombyx mori (1, 2). The expression of the sericin-1 gene is regulated temporally as well as spatially. During larval development, the sericin-1 gene is activated and repressed and then activated repeatedly (2–4). Furthermore, it has been suggested that the sericin-1 gene expression is regulated by the titer of ecdysone and juvenile hormones (5, 6). These observations suggest that the silk gland of B. mori is a good model system to study developmental control of gene expression.

To understand the molecular basis of the transcription regulation of the sericin-1 gene and the fibroin gene, another silk gland specific gene (7), cell-free transcription systems have been developed from various tissues and cultured cells (8–10). Transcriptional analyses of 5’-deletion mutants of the sericin-1 gene indicated that two upstream regions are associated with transcription enhancement (11). In these upstream regions, three protein binding sites, SA, SB, and SC have been identified (11). In the present paper, using synthetic oligonucleotides of the binding sites, we have tested the functions of two factors binding to two of these sites, SA and SC. The results suggest that a MSG abundant factor, SGF-3 stimulates the sericin-1 gene transcription through an interaction with the binding site SC, and to a lesser extent SGF-1 stimulates through the SA site.

MATERIALS AND METHODS

Preparation of extracts

Nuclear extracts from MSGs of 2-day-old fifth instar larvae were prepared as described previously (11). Extracts from stage 25 whole embryos of B. mori were prepared according to the methods described elsewhere (10). Nuclear extracts from PSGs of 2-day-old fifth instar larvae of B. mori were prepared as described before (12). Whole cell extracts from a B. mori cell line, Bm-e21-12 were prepared according to the method of Manley et al. (13).

Gel mobility shift assay and DNase I footprint assay

Oligonucleotides covering the footprint regions identified previously (11) were synthesized and double stranded. For the gel mobility shift assay, the following binding mixtures (10 μl) were incubated for 30 min at 4°C: 12 mM HEPES (pH 7.9), 60 mM KCl, 7.5 mM MgCl₂, 1.2 mM DTT, 1 mM EDTA, 10% glycerol, 0.1 ng of radiolabeled and double stranded oligonucleotide probe, 1 μg of poly(dI-dC)poly(dI-dC), extract, and 1 μg of sonicated and denatured salmon testes DNA (for the SC oligonucleotide probe) or 1 μg of tRNA (for the SA oligonucleotide probe), in the presence or absence of 5 or 20 ng of unlabeled and double stranded oligonucleotide competitors. The mixtures were electrophoresed on 5% polyacrylamide gels in 1×TBE (50 mM Tris-borate, pH 8.3, and 1 mM EDTA) at 4°C. The gels were dried and autoradiographed. DNase I footprint assay was performed as described previously (11), in the presence or absence of unlabeled, double stranded oligonucleotide competitors. The sericin-1 gene upstream fragment, −331 to −50, was end-labeled on the antisense strand, and used as a probe.
Figure 1. Double stranded synthetic oligonucleotides of the SA, SC and a 3-bases substitution mutant of the SC site, SCM. The numbers indicate the distance in base pairs upstream of the transcription start site (+1). Overlines indicate TGTTT motifs. Substituted nucleotides are indicated by a shade.

Figure 2. A gel mobility shift experiment with the SC and SA oligonucleotide probes. The SC oligonucleotide (lanes 1–8) and the SA oligonucleotide (lanes 9–14) probes were assayed for binding factor with MSG extract (5 μg). Lanes 1 and 9, no extract; lanes 2 and 10, no competitor; lanes 3 and 13, 50-fold molar excess of unlabeled SC oligonucleotide; lanes 4 and 14, 200-fold molar excess of unlabeled SC oligonucleotide; lanes 5 and 6, 50and 200-fold molar excess of unlabeled SCM oligonucleotide, respectively; lanes 7 and 11, 50-fold molar excess of unlabeled SA oligonucleotide; lanes 8 and 12, 200-fold molar excess of unlabeled SA oligonucleotide, is contained in the reaction mixtures. Lane M is a size maker, 32P labeled pBR322 HindI digests.

Construction of plasmids

Oligonucleotides were phosphorylated, annealed and ligated as described by Vinson et al. (14). After ligation, the oligonucleotides were phosphorylated again, and the catenated products in the ranges of 200 to 500 bp were purified by electrophoresis on a low-melting-temperature agarose gel. The purified DNAs were ligated to the position −39 of the sericin-1 gene 5’-deletion mutant, pSr300 5’Δ-38 (11). The plasmids pSrSa, pSrSC, pSrSCM and pSrNc (see Fig. 6) were inserted with the multimerized SA, SC and SCM (see Fig. 1), and a non-specific 200bp EcoRI fragment, respectively. The constructions of these plasmids were confirmed by dyeoxy sequencing with pBR322 EcoRI primer (Promega).

In vitro transcription

In vitro transcription was performed as described previously (15). As an internal control, pFbCP2 (12), containing the fibroin gene core promoter region −37 to +10, was used. An S1 nuclease assay was performed as described before (15), except that 280 units of S1 nuclease were used. As a probe, single stranded phage DNA of M13Srl (15) and M13CP (12) were used. For quantitation of transcriptional activity, bands of a resulting autoradiogram were scanned with an LKB 2202 UltroScan laser densitometer (9).

RESULTS

Factors binding to the oligonucleotides of footprint regions of the sericin-1 promoter

Previously, we have identified three protein binding sites in the upstream region of the sericin-1 gene. Three footprint regions, −202 to −183, −145 to −137 and −103 to −85 were referred to as SC, SB and SA sites, respectively (11). Transcriptional analysis of 5’-deletion mutants of the sericin-1 promoter in vitro...
Figure 4. Relative activity of SGF-1 and SGF-3 in the extracts from expressing- and nonexpressing-cells. Gel mobility shift assays were performed with various extracts, using the SA oligonucleotide probe (A) and the SC oligonucleotide probe (B). The retarded bands were excised and their radioactivities were measured. Radioactivity of free probe was defined as 100%. Experiments were done in duplicate. The filled circles are MSG, the open circles are PSG, the filled triangles are e-21 and the open triangles are embryos.

Analysis on the fibroin gene upstream binding factors has demonstrated the presence of five sequence specific DNA binding proteins: SGF-1, -2, -3, -4, and FBF-A1 (C.-c. Hui, K. Matsuno and Y. Suzuki, submitted). Based on the results of competition experiments, the factors binding to the SA and SC sites were found to be SGF-1 and SGF-3 respectively, which have also been identified as the fibroin gene binding factors (C.-c. Hui, K.Matsuno and Y.Suzuki, submitted). Effect of these double stranded oligonucleotides in the competition of the DNase I footprints on the SC and SA sites was tested. Comparing lanes 1 and 2 in Fig. 3, two footprint regions, SC and SA, were observed by the addition of the MSG extract. The SC footprint was competed effectively with the SC oligonucleotide (lanes 3 and 4) without affecting the SA footprint, but not with the SCM (lanes 5 and 6) or SA (lanes 7 and 8) oligonucleotide. The SA footprint was competed effectively with SA oligonucleotide (lanes 7 and 8) without affecting the SC footprint, but not with the SC (lanes 3 and 4) or SCM (lanes 5 and 6) oligonucleotide. Cross-competition between the SC and SA sites was not observed under these conditions. These results indicate that the factors binding to the SC and SA sites are different; consistent with the results of the gel mobility shift assay (Fig. 2).

The relative activity of SGF-1 and SGF-3 in sericin-1-expressing and -nonexpressing cells

Assuming that SGF-1 and SGF-3 are involved in regulating the tissue-specific expression of the sericin-1 gene, the relative binding activities of SGF-1 and SGF-3 in four different extracts were determined. Radioactivities of the retarded bands were measured at the various protein concentrations of the extracts from MSGs, PSGs, whole embryos at stage 25, and a cell line,
e-21, and percentages of the band over the probe were shown in Fig. 4. The binding activity of SGF-1 to the SA oligonucleotide is 5—10 times higher in the silk gland extracts, MSG and PSG, than in the embryonic and e-21 extracts (Fig. 4A). The binding activity of SGF-3 to the SC oligonucleotide is 4—10 times higher in MSG extract than in other extracts tested (Fig. 4B). An example of the gel shift experiments is shown in Fig. 5. The e-21 extract gave two additional bands indicated by the open triangles (Fig. 5 lanes 4 and 8). The lower one was near to the complexes of SGF-1 and SGF-3 but the position was not exactly the same. These results suggest that SGF-1 is more concentrated in the silk gland, and SGF-3 is more concentrated in the middle part of the silk gland (MSG) than in the non-expressing-tissues or -cells.

**SGF-3 is a transcription factor**

To test the possibility whether SGF-1 and SGF-3 are transcription factors, the multimers of the SA, SC and SCM oligonucleotides were inserted at -39 of the sericin-1 gene promoter (Fig. 6). These plasmids were transcribed in a MSG extract and the transcription activities of these promoters were standardized with that of the internal control. The insertion of five repeats of the SC oligonucleotide efficiently stimulated transcription 8.8-fold (compare Fig. 7 lane 3 with lane 1). However, the insertion of five repeats of the mutated SC site, the SCM oligonucleotide, did not stimulate transcription (only 1.2-fold; compare lane 4 with lane 1). By competition experiments in gel mobility shift and DNase I footprinting assays, the SCM oligonucleotide is known to have a reduced ability to bind SGF-3 (Figs. 2 and 3). The observations that the SC multimer, but not the SCM multimer, can stimulate transcription suggest that SGF-3 is responsible for this transcriptional stimulation activity. The insertion of five repeats of the SA oligonucleotide activated transcription 2.8-fold (compare lane 2 with lane 1), while the insertion of a non-specific, 200 bp EcoRI fragment hardly affected transcription (0.9-fold) (compare lane 5 with lane 1). Thus, SC oligonucleotide and to a lesser extent SA oligonucleotide activated transcription specifically.

**DISCUSSION**

In the present study, we have demonstrated that two factors, SGF-1 and SGF-3, bind to the SA and SC sites, respectively, of the sericin-1 gene upstream, and the SC site stimulates transcription through an interaction with SGF-3 in vitro. The oligonucleotide of the SA site contains two repeats of TGTTT motif, which had been proposed as a recognition sequence of a ubiquitous binding factor, SGF-1, in several silk gland genes (C.-c.Hui, K.Matsuno and Y.Suzuki, submitted). The TGTTT

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**Figure 5. Relative activity of SGF-1 and SGF-3 in the extracts from expressing- and nonexpressing-cells.** Using the SC oligonucleotide probe (lanes 1—4) and the SA oligonucleotide probe (lanes 5—8), a gel mobility shift assay was performed with the extracts (10 µg) from MSG (lanes 2 and 6), PSG (lanes 3 and 7) and e-21 (lanes 4 and 8). Lanes 1 and 5 do not contain extract. Lane M is a size marker, 32P labeled pBR322 HinfI digests. An arrowhead and an arrow shows the band formed with SGF-3 and SGF-1, respectively. Open triangles indicate the unidentified band formed in the extract from e-21.

**Figure 6. Schematic representation of templates for in vitro transcription.** The constructions of the templates are shown schematically. The pSrSA, pSrSC and pSrSCM plasmids contain five repeats of the SA, SC and SCM site oligonucleotide, respectively. The pSrNC contains a non-specific EcoRI fragment, about 200 bp. All DNA fragments are inserted into the EcoRI site at -39 of the sericin-1 gene 5’-deletion mutant, pSr300 5’Δ-38 (11). Open arrow, SA site; hatched arrow, SC site; filled arrow, SCM site.
Oct-2 are respectively tissue-specific transcription factors that activate expression of the genes specifying pituitary and lymphocyte phenotypes (17, 18). It is known that Pit-1 and Oct-2 can bind to the octamer sequence (19), and SGF-3 also can bind to the octamer sequence, with a comparable affinity to the SC site (C.-c.Hui, K.Matsuno and Y.Suzuki, submitted). However, the meaning of this observation is not clear at this time. The multimer of the SA oligonucleotides activated transcription only 2.8-fold (Fig. 7). It was demonstrated that SGF-1 specifically binds to the SA oligonucleotide (Figs. 2 and 3). Therefore, SGF-1 probably stimulates transcription through an interaction with the SA site. The transcriptional activity of the internal control promoter was suppressed when it was co-transcribed with pSrSa and pSrSC which were transcribed efficiently (Fig. 7 lanes 2 and 3). The transcriptional suppression of the internal control promoter can be explained by a competition for the transcriptional machinery with the active promoters, pSrSa and pSrSC. We have demonstrated that two distinct proteins bind to the sericin-1 gene upstream elements and stimulate transcription in vitro. Interestingly, these two factors have been known to bind also to the upstream elements of the fibroin gene. In addition to SGF-1 and SGF-3 reported in this study, FBF-A1, SGF-2, SGF-4 (C.-c.Hui, K.Matsuno and Y.Suzuki, submitted) and PSG specific factors (20) have been identified as the enhancer binding proteins of the fibroin gene. However, molecular image of differential regulation of the sericin-1 and fibroin genes remains still obscure. Presently, we consider that the differential expression of the two genes is a result of cooperative function of these factors. Recent observation that these two genes are differentially transcribed in MSG and PSG extracts (10) encourages us to analyze the cooperative function.

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