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In monkey COS cells only the TATA box and the cap site region are required for faithful and efficient initiation of the fibroin gene transcription

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ABSTRACT

The DNA sequences necessary for faithful and efficient initiation of transcription of Bombyx mori fibroin gene have been studied in vivo using monkey COS cells and a SV40 origin vector. Transcriptional analysis of 5' deletion genes and of exact substitution genes including a series of single base substitution mutants indicates that the TATA box and the cap region are required for faithful and efficient initiation of the fibroin gene transcription in vivo, whereas far upstream sequences and the CAAT-like sequences are not required. In addition, single base substitution genes of the cap site region have clearly shown that there exists a strict sequence requirement of the cap region of the fibroin gene. These results define the basic promoter of the fibroin gene to the region spanning the TATA box and the cap site region.

INTRODUCTION

The Bombyx mori silk genes constitute an excellent model system for studying the control of gene expression in differentiated cells (1). Fibroin gene is actively transcribed in the posterior silk gland cells and completely suppressed in the middle silk gland cells, while sericin genes are actively transcribed in the middle silk gland cells and completely suppressed in the posterior silk gland cells. To understand the underlying regulatory mechanisms of these genes we have initiated studies on the transcriptional machineries using in vitro transcription systems and techniques of in vitro manipulation of the genes. A part of our initial effort has been concentrated on the characterization of promoter sequences of the fibroin gene (2-4). The DNA sequences required for faithful initiation of transcription of the fibroin gene in vitro have been located between nucleotide position -29 and +6 (+1 being the presumed transcription initiation site (5)), and a series of single base substitution mutants for this region has clearly shown the importance of the individual nucleotides in the TATA box and -20 region for faithful and efficient initiation of transcription of the fibroin gene (3). However, for the definition of the promoter function there could exist

some differences between in vitro systems and in living cell systems; like a structural difference of templates (for example, naked DNA vs. DNA in nucleosome structure) and/or a difference of available factors.

To date, studies on eukaryotic promoters in living cell systems have been reported on a number of different eukaryotic genes (6-15). These studies have shown that some upstream sequences from the TATA box are required for faithful and efficient initiation of transcription in vivo, and the CAAT-like sequences, one of the typical elements in the upstream sequences, could be a part of common promoter element like the TATA box.

In this paper we describe the DNA sequences necessary for faithful and efficient initiation of transcription of the fibroin gene in a living cell system. We have used a transient expression system consisting of monkey COS cells and a SV40 origin vector (13, 16). Despite a heterologous nature of the cells against the fibroin gene and replicating plasmid state, the fibroin gene has been faithfully transcribed in the simian cells. We show that in contrast to other genes, the TATA box and the cap site region are required for faithful and efficient initiation of transcription of the fibroin gene in vivo, whereas far upstream sequences and the CAAT-like sequences are not required. In addition, single base substitutions in the cap region clearly show that there exists a strict sequence requirement of the cap region of the fibroin gene.

MATERIALS AND METHODS

1. Plasmid construction

A mini-fibroin gene that lacks most part of the second exon has been constructed as shown in Fig. 1 (Y. Tsujimoto, unpublished). The SV40 origin vector, pML-SV (a gift from W. Schaffner), contains the Eco RII G fragment of SV40 at the Eco RI site of pML-1 (17) via Eco RI linkers, and lacks a part of the 72 bp repeat and the enhancer activity. The mini-fibroin gene was inserted between the Hind III sites of the pML-SV in place of the small Hind III fragment of the vector to produce pSVOFb plasmids (Fig. 1).

A series of deletion genes was constructed as follows. Fibroin DNAs suffering a series of 5' flanking sequence deletion (2) were excised with Hind III at their 5' deletion boundary and Xho I at +510, and the Hind III/Xho I fragment (-860 ∿ +510) of the wild type pSVOFb was replaced with the Hind III/Xho I fragments having a series of deletion. An internal 5' deletion gene, Δ[-58 ∿ -20], was constructed by joining a 5' deletion fibroin gene 5'Δ-19 which has Hind III linker (10-mer) at the 5' deletion end with a

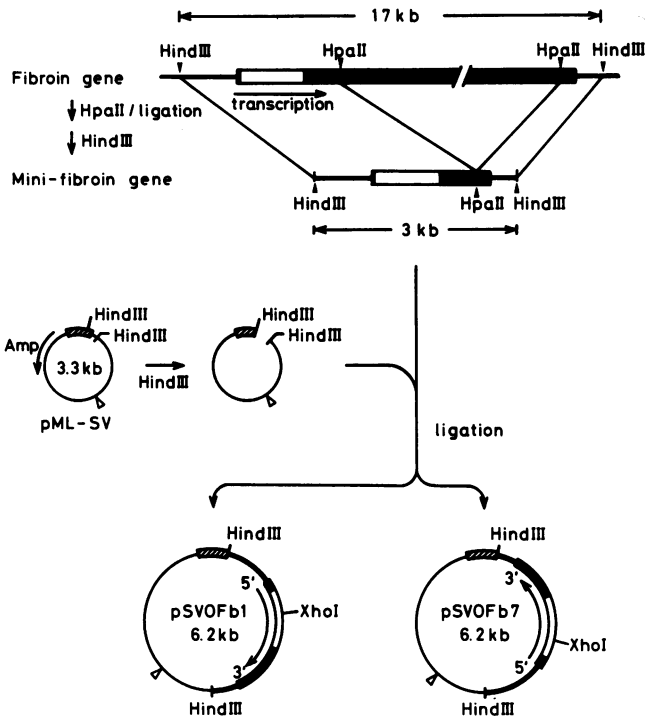

Fig. 1: Construction of pSVOFb plasmids. Solid bars: exon sequences of fibroin gene. Open bars: intervening sequences of fibroin gene. Thick lines: 5' and 3' flanking sequences of fibroin gene. Hatched bars: SV40 replication origin. Thin lines: pBR322 vector sequences. Deletion of poison sequences for replication in cells is indicated by small triangles. The mini-fibroin gene was constructed by deleting the most part of the second exon with Hpa II cut and ligation (Y. Tsujimoto, unpublished). The Hind III fragment of the mini-fibroin gene that carries 860 bp of 5' flanking sequence and 300 bp of 3' flanking sequence was inserted between the Hind III sites of pML-SV to creat the pSVOFb plasmids.

3' deletion fibroin gene 3'Δ-59 which has S1 trimmed Bam HI linker residue (3 bp) and complete Hind III linker (10-mer) at the 3' deletion end (M. Tsuda, unpublished). By a partial Hind III digestion and Xho I digestion of this deletion gene, the Hind III/Xho I fragment (-860 ∿ +510 with a deletion of -58 ∿ -20) was excised, and this fragment was used to replace the Hind III-Xho I fragment of pSVOFb.

Point mutants of fibroin gene that had been constructed on a f1 phage vector (3) were excised with Eco RI (-238) and Xho I (+510), and this EcoRI/Xho I fragment was used to replace the Hind III/Xho I fragment of

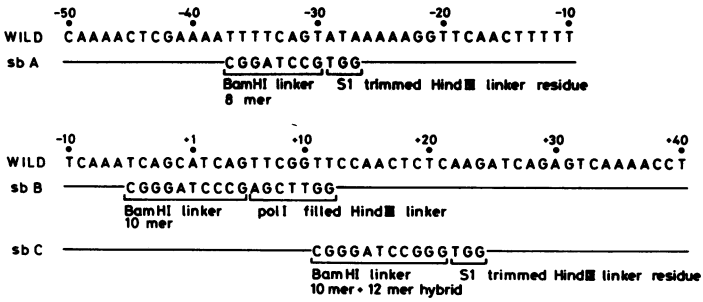

Fig. 2: The structure of multiple substitution genes. Straight lines indicate nucleotide sequence of the wild type fibroin DNA. Only the substituted nucleotides derived from linkers are shown for each gene. Sb A: The Hind III linker terminus of the 5' deletion -26 was trimmed with S1 nuclease, and joined with Xmn I digested wild-type upstream sequences that span from -38 to -860 via a Bam HI linker (8 mer). Sb B: The Hind III terminus of the 5' deletion +13 was filled in with DNA polymerase I, and joined with the 3' deletion -6 via a Bam HI linker (10 mer). Sb C: The Hind III linker terminus of the 5' deletion +25 was trimmed with S1 nuclease, and a 12 mer Bam HI linker was attached to the end. This was joined with the 3' deletion +10 that has a 10 mer Bam HI linker resulting a hybrid of the intermediate size. All multiple substitution genes carry 5' sequences up to -860 as wild type pSVOFb plasmids.

pSVOFb with a help of Hind III/Eco RI fragment adaptor taken from pBR322 DNA.

Exact substitution genes, sb A, sb B and sb C, were constructed essentially by joining a 5' deletion gene and a 3' deletion gene (2) via Bam HI linker after trimming with S1 nuclease or filling-in with DNA polymerase I at the deletion termini. The structures of these mutants at the substituted portion are shown in Fig. 2.

2. Cell growth and DNA transfection

COS-1 cells were maintained in Dulbecco's MEM with 10 % fetal calf serum in 10 cm plates. DNA transfection was carried out as described (13, 18). We transfected 10 μg of pSVOFb plasmid DNA per 10 cm plate containing slightly subconfluent cells. As an internal control for quantitation of fibroin transcripts, 10 μg of human β-globin gene (19) subcloned on pML-SV were cotransfected with 10 μg of pSVOFb plasmid DNA. Under this condition, cotransfection of the human β-globin DNA did not alter the transcription efficiency of fibroin gene (see Fig. 6) indicating little competition between these genes in the COS cells.

3. Primer extention and S1 nuclease mapping

Total cellular RNA was extracted after 48 hrs of DNA transfection by LiCl-urea method (20). Poly(A) RNA selection was carried out according to

the procedure described (21). Primer extention on fibroin transcripts was carried out in 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 10 mM DTT, 0.1 M KCl, 1 mM of each dNTP, 0.1 mg/ml actinomycin D and 10-20 units of AMV reverse transcriptase (Seikagaku-kogyo) in a 50 µl reaction mixture with 10 - 50 µg COS cell poly(A)$^+$ or poly(A)$^-$ RNA.

S1 nuclease mapping was carried out using 200 - 400 µg of total COS cell RNA under the conditions described (2). That S1 probes were added in excess was confirmed by the experiments using different ratios of probes to RNA. Signals on autoradiograms were scanned with a Joyce Loebl densitometer to quantitate relative activities of transcription. For quantitation of activities of mutant genes, S1 assays were carried out at 50 U/ml and 100 U/ml. We found the ratios of fibroin transcripts to β-globin transcripts were quite reproducible. Different preparations of transcript from wild type-gene, WI, WII and WIII (see Fig. 6) also showed constant ratios of fibroin transcripts to β-globin transcripts.

RESULTS

1) Faithful transcription initiation of fibroin gene in COS cells

The plasmid DNAs carrying fibroin gene used in this work are shown in Fig. 1. We used a mini-fibroin gene that was constructed from the cloned genomic fibroin gene by deleting the most part of the second exon (Y. Tsujimoto, unpublished). The vector, pML-SV, carries the replication origin of SV40 (the Eco RII G fragment), which lacks a part of the 72 bp repeat, and thus does not possess the enhancing activity (22, 23). A part of pBR322 sequences inhibitory for replication in mammalian cells was eliminated (17). The vector was cleaved with Hind III, and the mini-fibroin gene fragment was inserted between the Hind III sites in place of the small vector segment resulting in of pSVOFb1 and pSVOFb7 (Fig. 1).

These recombinant DNAs were introduced into COS cells with fine precipitates of calcium phosphate (18). After 48 hrs of DNA addition, low molecular weight DNA from the cell lysate was extracted and digested by Dpn I to sweep out non-replicated molecules (24), and analysed by electrophoresis through agarose gels (data not shown). Upon ethidium bromide staining, the two fibroin plasmids, pSVOFb1 and pSVOFb7, gave strong bands corresponding to covalently closed circular molecules, which were as strong as pML-SV plasmid, indicating that they were well replicated in COS cells (about 10,000 copies per infected cell).

After 48 hrs of DNA addition total cellular RNA was extracted from the

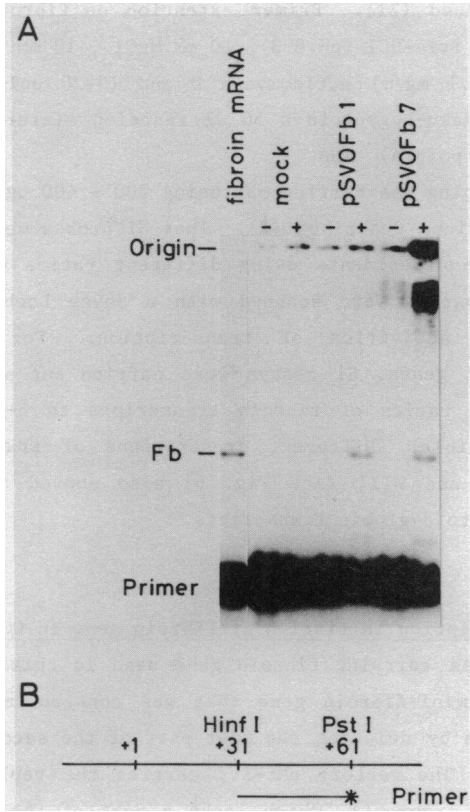

Fig. 3: Primer extention mapping of fibroin transcripts. COS cell RNAs extracted after 48 hrs of transfection with no DNA (mock transfection), pSVOFb1 DNA, or pSVOFb7 DNA were fractionated with oligo(dT)-cellulose column. -: poly(A)$^-$. +: poly(A)$^+$. The primer is Pst I - Hinf I minus strand labeled at the Pst I end(B). This primer was annealed with 50 µg of poly(A)$^-$ or poly(A)$^+$ RNA, and extended with AMV reverse transcriptase. The extension products were analysed through 10 % polyacrylamide gel containing 7 M urea. Fb: the faithful initiation signals of fibroin gene. As a standard, 5 ng of fibroin mRNA isolated from the posterior silk gland was applied.

transfected COS cells, and fibroin transcripts were analysed by primer extention mapping (Fig. 3). The two fibroin plasmids gave faithfully initiated fibroin transcripts with almost the same intensities, whereas mock transfection did not give such transcript. Fig. 3 also showed that the polyadenylation signal of the fibroin gene was functional even in the simian cells. In addition to the accurate transcripts, random transcripts initiated upstream in the vector region were also detected, which were especially strong on pSVOFb7. We tried Northern blot analyses for the fibroin tran-

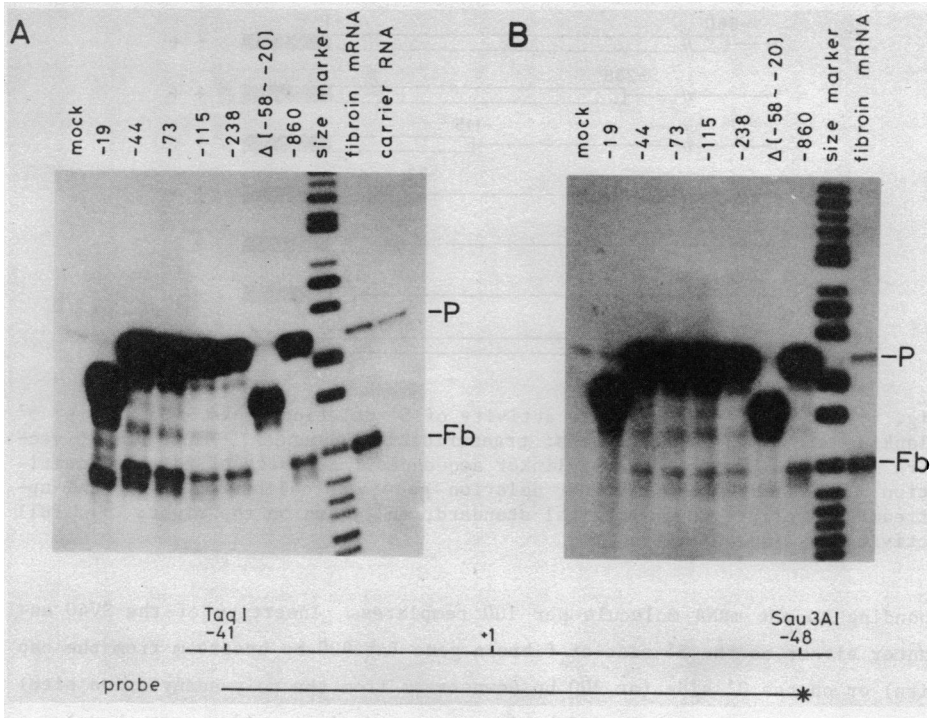

Fig. 4: S1 nuclease mapping of fibroin transcripts from 5' deletion genes. A Transcripts from 5' deletion genes of pSVOFb1. B Transcripts from 5' deletion genes of pSVOFb7. P: full length probes. Fb: the faithful initiation signals of fibroin gene. End labelled S1 probe is shown in the bottom. The probes were annealed with 200 μg of total cellular RNA, and digested with S1 nuclease (50 U/ml). As a standard 2 ng of fibroin mRNA were used. Heterogeneity in the 5' sequences between the probe and some deletion genes, Δ[-58 ∿ -20] and 5'Δ-19, gave strong protection bands shorter than the probe bands. The shorter band positions correspond to the respective divergence points. Broad bands below the bands of full length probes are S1 artifacts caused by partial melting of hybrids between strong upstream transcripts and probes. These signals were not detected by primer extention mapping (see Fig. 3). Electrophoreses were through 10 % polyacrylamide gels containing 7 M urea. As size markers 5'-terminally labeled Hinf I/Hae III digest of a pBR322 derivative was used.

scripts too, only to get a smearing pattern probably caused by these upstream random transcripts (data not shown). Faithful initiation of transcription of the fibroin gene was further confirmed by S1 nuclease mapping (data not shown, but see Fig. 4). We did not test whether splicing of the fibroin gene transcript was normally carried out in the COS cells.

From these results we have calculated template utility of pSVOFb DNAs. Faithful fibroin transcripts were about 100 copies per infected cell, corre-

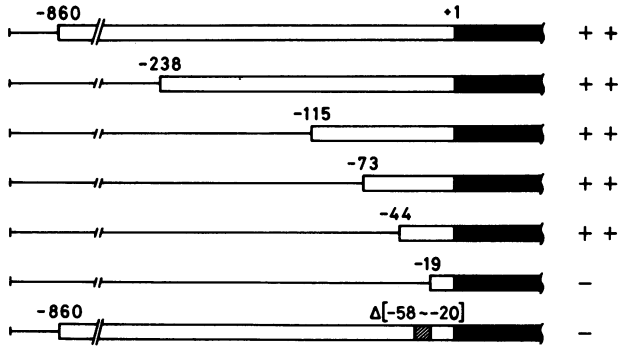

Fig. 5: Structure and template activity of 5' deletion genes. Open bars: 5' flanking sequences. Solid bars: transcribable sequences. Thin lines: vectors sequences. Hatched bars: linker sequences. Activity of faithful initiation of transcription of each deletion gene was calibrated using the upstream transcripts as an internal standard, and shown on the right. ++: full activity. -: no activity.

sponding to one mRNA molecule per 100 templates. Insertion of the SV40 enhancer either on the 5' side of fibroin gene (at 900 bp upstream from the cap site) or on the 3' side (at 300 bp downstream from the poly-adenylation site) did not increase the level of fibroin gene transcription (data not shown).

2) Activity of 5' deletion genes

To delimit the upstream 5' sequences necessary for the faithful initiation of transcription of the fibroin gene in COS cells, genes with sequential deletion which had been constructed on pBR322 (2) were recloned on pML-SV to make 5' deletion pSVOFb plasmids. To control the effects of vector sequences ligated just upstream of the deleted fibroin genes, we made deletion genes both for pSVOFb1 and for pSVOFb7. Transcripts of these mutants were analysed by S1 nuclease mapping (see Fig. 4).

On pSVOFb7, deletion genes 5'Δ-860, -238, -115, -73 and -44 gave faithful transcripts of almost the same intensities, whereas a deletion 5'Δ-19 did not give faithful transcripts (Fig. 4B). We further tested a deletion gene Δ[-58 ∿ -20] that carries 13 bp linker sequences in place of the natural sequences between -58 and -20 (see Fig. 5). This internal deletion gene gave no faithful transcripts at all. Results of deletion on pSVOFb1 DNA were essentially the same as those on pSVOFb7 DNA (Fig. 4A). However, general enhancement was caused on the whole transcripts when deletions from 5' direction on pSVOFb1 DNA were extended beyond -115. The effects might be caused by the SV40 replication origin located 5' side of deletion genes on this ori-

entation. The deletion 5'Δ-19 of pSVOFb1 lost an activity to produce faithful transcripts, while incorrectly initiated transcripts were produced (Fig. 4A).

Taking the upstream transcripts represented by strong protection bands of probe size as an internal control, activities of the 5' deletion genes were calibrated and summarized in Fig. 5. These results revealed that all the machineries responsible for faithful initiation of transcription of the fibroin gene were included in the very proximal sequences that were no farther than -44. Sequences between the nucleotide -44 and -19, which include the TATA box, were indispensable for expression of fibroin gene in COS cells.

3) Effect of exact substitution in the immediate 5' sequences

To characterize a nature of the immediate 5' sequences more precisely, we have prepared exact substitution genes for this region (see Fig. 2 and Fig. 7). The mutants have multiple flocked substitutions or single base substitutions in the immediate sequences. However, total spacing, the upstream sequences (to -860 for sb A, sb B and sb C, to -238 for single base substitution mutants) and the downstream sequences are exactly conserved. We transfected these mutant genes onto COS cells together with human β-globin gene, which served as an internal control for quantitation of fibroin transcripts. Promoter activities of each mutants were assayed by S1 mapping (Fig. 6), and intensities of faithful transcripts of fibroin and β-globin genes were measured with a densitometer tracing of autoradiograms. Fig. 7 summarizes the relative activities of the substitution genes.

Activities to produce faithful fibroin transcripts were significantly impaired with drastic substitutions over the TATA box (sb A). This result was in good agreement with the results of 5' deletion genes described above. Single base substitutions of the TATA box sequence showed deleterious effects too, especially significant for substitutions of the nucleotide -29 and -28 which accompanied apparent alterations in the initiation site. These results revealed the importance of the nucleotides in the TATA box for faithful and efficient initiation of transcription of fibroin gene *in vivo*.

Besides the TATA box, multiple substitutions at around the cap site region (sb B) completely abolished faithful transcription indicating a cap site requirement of fibroin gene. This was further comfirmed with single base substitutions at -10, -7, -4 and +1. Among these mutants a substitution of the nucleotide -4 (from C to T) greatly repressed template activity. The effect of this mutation was so strong as to be comparable to the effects of single base substitutions in the TATA box. This indicates a strict sequence

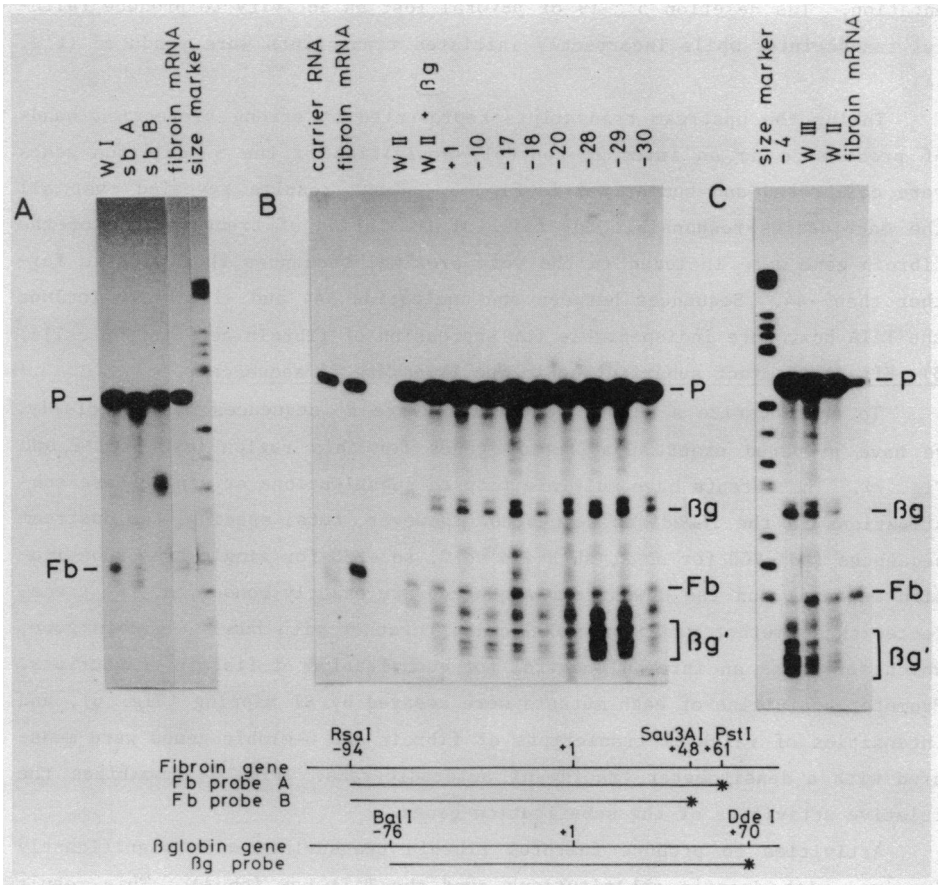

Fig. 6: S1 nuclease mapping of transcripts from exact substitution genes. P: full length probes. Fb: the faithful initiation signals of fibroin gene transcription. βg: the faithful initiation signals of β-globin gene transcription. βg': irregular transcription signals of β-globin gene. WI, WII and WIII are different preparations of wild type transcripts. Size markers are 5'-terminally labeled Hae III digest of a pBR322 derivative. Panel A S1 mapping of transcripts from multiple substitution genes. End labeled probes (Fb probe A, see bottom) were prepared for each gene. The broad bands below band P in the lane sb B may be S1 artifacts. Panels B and C S1 mapping of transcripts from single base substitution genes. Fb probe B and βg probe (see bottom) were mixed and annealed with total cellular RNA, and digested with S1 nuclease. Bands above βg signals are S1 artifacts caused by the strong protection bands of probe size. These bands are also detected with silk gland mRNA when exposed for a long time. Activities of faithful signals of fibroin transcripts and β-globin transcripts were quantitated by densitometer tracing of autoradiograms.

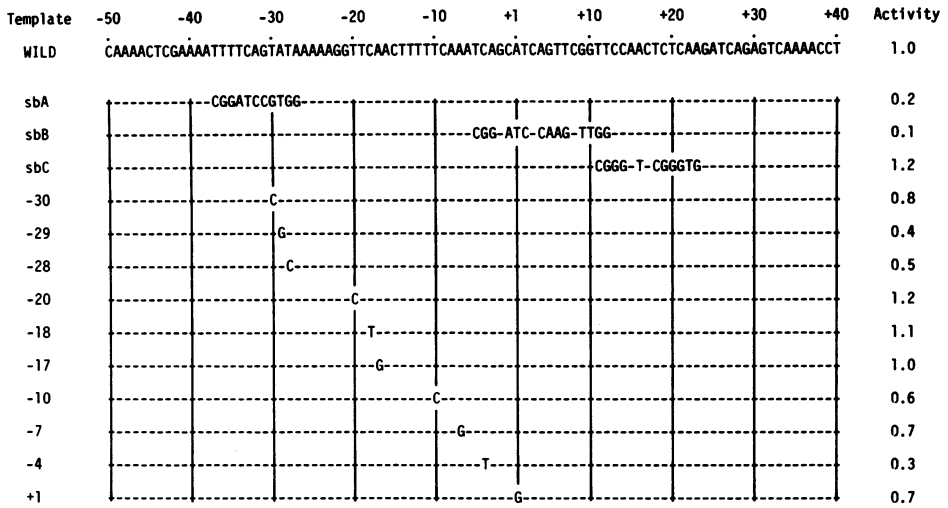

Fig. 7: Structure and template activity of exact substitution genes. Only the nucleotides different from the wild type sequences are indicated. Dashed lines indicate sequences identical to wild type. Relative transcription activities compared to wild-type gene (1.0) are shown.

requirement of the cap region of the fibroin gene like the TATA box.

Hirose et al. (3) have shown the importance of the -20 region of the fibroin gene for faithful and efficient transcription using in vitro transcription systems. However in COS cells, we could not detect any deleterious effects of single base substitutions of this region. Multiple base substitution mutant at around position +15 (sb C) also showed a normal level of transcription activity.

DISCUSSION

We have studied a transient expression of a cloned B. mori fibroin gene transfected on monkey COS cells. Despite the heterologous circumstance and the replicating plasmid state, the fibroin gene was faithfully transcribed and poly-adenylated. We have tested fibroin plasmids, pSVOFb1 and pSVOFb7, that have fibroin DNA inserts of alternative orientations with respect to the SV40 replication origin. The efficiency and the faithfulness of the fibroin transcripts did not depend on the orientation. In addition, the SV40 insert of pSVOFb plasmids does not have the functional enhancer. Thus, in this system, the faithful transcription initiation of the fibroin gene is solely depended on the fibroin gene promoter.

The CAAT-like sequences were not required for fibroin gene expression in COS cells

S1 nuclease mapping of the 5' deletion genes showed that sequences proximal to -44, which harbor only the TATA box and the cap region, suffice for faithful and efficient initiation of transcription of fibroin gene in COS cells. Upstream sequences of fibroin gene include duplicated CAAT-like sequences at position from -93 to -85 (26). The CAAT-like sequences have been shown to be required for maximal expression of α-globin gene in COS cells (13) and β-globin gene in HeLa cells (10, 11) or 3T6 cells (7). However, removal of the CAAT-like sequences of the fibroin gene did not impair the promoter activity. Grosschedl and Birnstiel (8) have shown that, in the case of sea urchin histone H2A gene, deletion of the CAAT-like sequences leads to a slight stimulation of transcription. Likely, in the case of HSV tk gene (12), while the 5' side of the CAAT-like sequences are required for efficient and faithful transcription, most of the CAAT-like sequences can be substituted without significant reduction of promoter activity. It is noteworthy that in the same COS cells, the CAAT-like sequences of α-globin gene were required for expression but those of fibroin gene were not. This seems inconsistent with an idea that general factor(s) for the CAAT-like sequences might exist in COS cells. Or the apparent CAAT-like homology might have little meaning for promoter function at least for fibroin gene.

The TATA box is required for fibroin gene expression in COS cells

Deletion or substitution of the TATA box profoundly impaired promoter function indicating the requirement of the TATA box for faithful and efficient expression. This has been confirmed with single base substitution genes of the TATA box. The results are in good agreement with previous *in vitro* studies of the fibroin gene promoter using HeLa cell extract or silk gland extract (2, 3, 4). The results are also consistent with *in vivo* studies on other eukaryotic promoters (7, 10, 12, 26, 27). However, the early gene promoter of SV40 is an exception (6): it does not require the TATA box for efficient expression *in vivo*. In this case, the viral enhancer could provide different molecular situation around the promoter, or feed back regulation by T antigen, product of this gene, might act to control the transcriptional efficiency. We argue that the TATA box is a general element for faithful and efficient initiation of transcription of eukaryotic genes both *in vitro* and *in vivo*.

The cap site region is required for fibroin gene expression in COS cells

The faithful transcription initiation of the fibroin gene was completely

abolished by multiple base substitutions at around the cap site. Single base substitutions in the cap site region also significantly reduced the transcription activities confirming the requirement of the cap region in COS cells. A substitution of the nucleotide -4 from C to T severely reduced the transcriptional efficiency; this substitution reduces melting energy at the cap region. This indicates a strict sequence requirement of the cap region of fibroin gene in COS cells.

Tsuda and Suzuki (29) have detected an efficient and specific initiation of transcription on fibroin gene at the nucleotide +25 when a twisted DNA template was incubated with purified RNA polymerase II. They have noted a homology between the sequences around +25 and the sequences around the cap site,

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-10          +1          +10
 .           .           .
 TCAAATCAGCATCAGTTCGG
     ***  ** **
 AACTCTCAAGATGAGAGTCA
 .           .           .
+15         +25         +34
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The strong down mutation at -4 (C → T) disrupts the left core of this sequence homology, and seems to reduce an affinity of the cap region to RNA polymerase II. Our results are also consistent with *in vitro* studies of deletion fibroin gene where the 3' boundary of fibroin gene promoter was located at around +6 (2, 4). Requirement of the cap region for faithful and efficient initiation of transcription has also been reported in the case of conalbumin gene (30), α-globin gene (31) and sea urchin histone H2A gene (8). It has been shown that replacement of the cap region of a *Dictyosterium* actin gene with the cap region of fibroin gene raised the level of actin gene transcription by six fold (S. Takiya, personal communication). It also suggests the importance of the cap region for faithful and efficient transcription initiation. Although we can not find apparent sequence homology between the cap regions of eukaryotic genes, these results suggest that specific structure of the cap region may be favored by RNA polymerase II to initiate transcription efficiently.

Using *in vitro* transcription systems Hirose et al. (3) have studied the effects of single base substitutions of the immediate 5' flanking sequences described here. The effects by substitutions in the TATA box region, agree well with those observed in the present study. In the *in vitro* transcription systems, no deleterious effect was detected for single base substitutions at around the cap region, while significant negative effects were detected for substitutions at around the -20 region. In COS cells, no essential nucle-

otide was found at around -20 region. One of possible factors that might cause these discrepancies between the in vitro and in vivo systems is template topology; truncated linear templates were used in the cell-free systems, while in COS cells templates are replicating plasmids packaged into nucleosome structure like SV40 minichromosome (32). Tsuda and Suzuki (29) have shown that no specific initiation was detected on linear templates with RNA polymerase II alone suggesting that RNA polymerase II could have a stronger affinity to cap regions of twisted form. Although we have little information on the fine structure of DNA packaged into nucleosome configuration, it might resemble twisted DNA structure. It is interesting to test in vitro activities of templates with single base substitutions using twisted DNA molecules and to compare the results with those in COS cells.

Heterologous systems vs. homologous systems

As COS cells are heterologous circumstances for fibroin gene, it is moderate at present to consider the promoter elements described here as the basic machineries that assure faithful and basal level expression in vivo. As noted above, upstream sequences farther than the nucleotide -44 are not required for maximal expression of fibroin gene in this system. Concerning this point, the level of expression should be taken into consideration. Template utility is about 3×10^6 fold higher in the silk gland cells (30,000 messages / gene / 48 hrs in the silk gland cells (33) vs. one message / 100 genes / 48 hrs in COS cells). However, it seems unlikely that species distance made the expression of fibroin gene inefficient, since the expression level of the fibroin gene is no lower than human β-globin gene in COS cells (see Fig. 6). Rather it is probable that upstream sequences of fibroin gene could not play a role in the heterologous circumstances owing to absence of tissue specific factors. In this connection, it is interesting that upstream sequences (-238 to -53) farther than -44 are required for maximal transcription initiation of the fibroin gene in a silk gland extract but not in a HeLa cell extract (4, 34). To reveal such specific factors and DNA sequences responsible for the highly efficient expression in the silk gland cells, trials to introduce manipulated fibroin genes into the silk gland cells and to reconstruct a cell-free transcription system reflecting the in vitro fibroin gene regulation are underway.

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