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A contribution of the core-promoter and its surrounding regions to the preferential transcription of the fibroin gene in posterior silk gland extracts

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Complementation of a posterior silk gland (psg) extract to a HeLa cell extract specifically enhances the transcription of the *Bombyx mori* fibroin gene. To map the regions responsible for this enhancement, the fibroin promoter was dissected and the transcriptional function of each region was analyzed. Besides the upstream promoter element 5′ to the TATA box, two downstream elements were found to be important for the preferential transcription of the fibroin gene in the complementation system as well as in the psg extract. The minimal fibroin promoter from −37 to +10 (core-promoter) was preferentially transcribed in the psg extract, while the transcription efficiencies of other promoters like one of the *Bombyx* chorion genes and the adenovirus 2 major late promoter (Ad2MLP) were considerably lower. The transcription from the core-promoter was further enhanced when combined with either the intronic element from +156 to +454 or the upstream element. Both the upstream and intronic elements also stimulated the transcription from the Ad2MLP in an orientation independent manner. These results demonstrate that the transcription of the fibroin gene is mediated through an integration of multiple regulatory elements.

Key words: core-promoter/enhancer/fibroin gene/TATA box/transcription

Introduction

Temporal and tissue-specific regulation of gene expression is one of the major problems that must be solved in order to understand the mechanisms of development and differentiation. The regulation of the *Bombyx mori* fibroin gene is an example of such targets. In order to analyze the transcriptional regulation of this gene, a homologous cell-free transcription system has been developed from the posterior silk gland (psg) (Tsuda and Suzuki, 1981). In this transcription system the fibroin gene is preferentially transcribed when compared with the adenovirus 2 major late promoter (Ad2MLP), the mouse β-globin gene, one of the *Bombyx* chorion genes, the sericin-1 gene, and some other genes (Tsuda and Suzuki, 1981; unpublished results). The upstream element which spans a region of ~200 bp 5′ to the TATA box is known to be important for this preferential transcription in the psg extract (Tsuda and Suzuki, 1983; Suzuki *et al.*, 1986). The existence of multiple factors in the psg extract which interact with this upstream element has been demonstrated by nuclease protection and gel-retardation assays (Suzuki and Suzuki, 1988; C.-c.Hui, K.Matsuno and Y.Suzuki, in preparation). The transcription factors in the psg extract have been fractionated on a phosphocellulose column and at least three column fractions were necessary to construct faithful transcription initiation (Takiya and Suzuki, 1989). To detect the activities of specific factors which enhance the fibroin gene transcription, we have used a complementation assay consisting of a HeLa cell-free transcription system supplemented with various fractions of the psg extract. Stimulatory activities specific to the fibroin gene were detected in fraction D eluted at 1 M KCl in this complementation assay. This fraction of the psg extract enhances the transcription from the fibroin gene via the upstream element (Takiya and Suzuki, 1989). Moreover, we have found that this fraction could also preferentially enhance the transcription of a mutant lacking the entire upstream element (Takiya and Suzuki, 1989; unpublished results).

In the present study we mapped the regions in the fibroin gene promoter that are responsible for specific stimulation using both a homologous psg extract and the complementation system described above. It was shown that the core-promoter (−37 to +10) alone was specifically transcribed in the psg extract and that transcription efficiency was

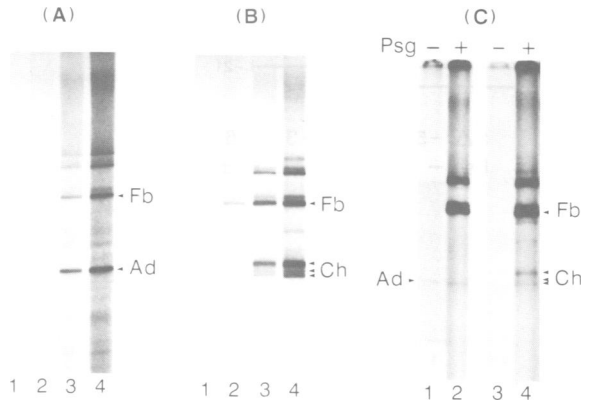

Fig. 1. Specific enhancement of fibroin gene transcription by complementation of the psg extract to the HeLa cell extract. The fibroin gene and the Ad2MLP (**A**) or the fibroin gene and the chorion gene (**B**) were transcribed together in 3 μl (**lane 1**), 4.5 μl (**lane 2**), 6 μl (**lane 3**) and 7.5 μl (**lane 4**) of the HeLa cell extract. (**C**) The fibroin gene was transcribed together with the Ad2MLP (**lanes 1** and **2**) or the chorion gene (**lane 3** and **4**) in 3 μl of the HeLa cell extract (**lanes 1** and **3**) or the HeLa cell extract supplemented with 4.5 μl of the psg extract (**lanes 2** and **4**). In (**A**) and (**C**) **lanes 1** and **2**, 0.1 μg of pFb5′Δ−238 and 0.2 μg of pM7 were used as templates, and in (**B**) and (**C**) **lanes 3** and **4**, 0.08 μg of pFb5′Δ−238 and 0.22 μg of pChΔNot were used. Transcripts were detected by a modified nuclease S1 method using single-stranded DNA probes: f1Fb38 for pFb5′Δ −238 (Fb), M13Ad4 for pM7 (Ad) and M13Ch2 for pChΔNot A gene (Ch). Relative promoter activities of the Ad2MLP, fibroin and chorion genes were 2.5:1:1.3 in the HeLa cell extract, and 1:42:3 in the complementation system. Specific transcripts are indicated by arrowheads.

increased by upstream sequences and downstream intronic elements that are enhancer-like in nature.

Results

Specific enhancement of the fibroin gene transcription by complementing HeLa cell extract with psg extract

To assay stimulatory activity for the fibroin gene transcription, addition of crude or fractionated psg extract to HeLa cell extracts has been used (Takiya and Suzuki, 1989). In the HeLa cell extract, the *Bombyx* fibroin and chorion genes, and the Ad2MLP were all transcribed efficiently (Figure 1A and B). In these experiments, the Ad2MLP appears to be most strongly transcribed even though the upstream region of the template, pM7, has suffered a substitution mutation (Miyamoto *et al.*, 1985). A corresponding increase of transcription from these genes could be observed in an increasing amount of HeLa cell extract, suggesting that the two *Bombyx* genes were recognized similarly by the basic transcription factors in the HeLa cell extract (Figure 1B). However, upon the addition of the psg extract into the HeLa cell extract, the transcription of the fibroin gene was greatly enhanced (Figure 1C). Under the same conditions, the transcription of the Ad2MLP was scarcely affected or rather suppressed, while there was only a slight increase in transcription from the chorion gene. The fact that the *Bombyx* chorion gene and the Ad2MLP are little affected demonstrates the specificity of the enhancement of fibroin gene transcription by the psg extract.

Transcription stimulation of 5′ and 3′ deletion mutants of the fibroin gene in psg extract

The efficient transcription of the fibroin gene in the psg extract is known to be driven by its upstream element (Tsuda and Suzuki, 1983; Suzuki *et al.*, 1986). In the experiments shown in Figure 2, we mapped the regions of the fibroin gene promoter responsible for the stimulation in the complementation system by using a series of 5′ and 3′ deletion mutants. A rather unexpected result is that deletion of the upstream element from −238 to −32 only reduced the efficiency of transcription to ~50% (Figure 2A, lanes 2 and 6). pFb5′Δ−31, in which the entire upstream element of the fibroin gene is deleted, could still be enhanced considerably by the addition of the psg extract (Figure 2A, lanes 5 and 6). A further deletion of the TATA box abolished specific transcription of the fibroin gene even when the psg extract was added (Figure 2A, lanes 7−10). The transcription of 3′ deletion mutants of the fibroin gene was assayed

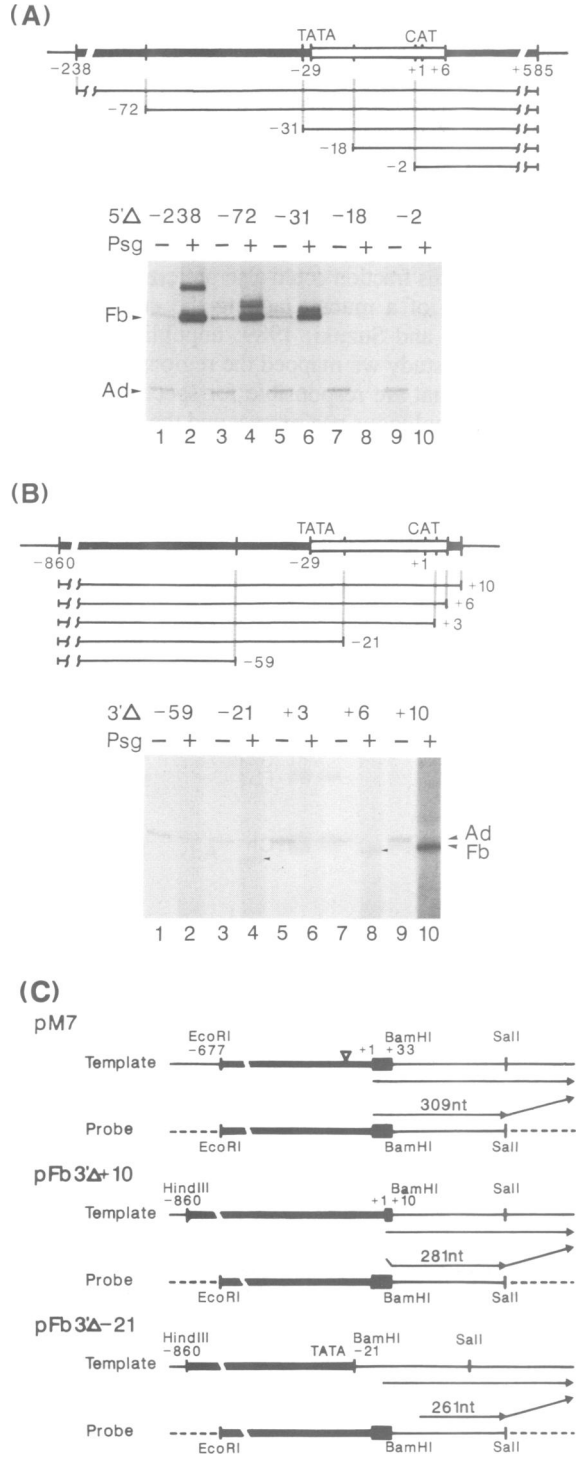

Fig. 2. Transcription of deletion mutants of the fibroin gene in the HeLa cell extract supplemented with the psg extract. The upper panels of (**A**) and (**B**) show the structures of 5′ or 3′ deletion mutants. The thick line shows the fibroin gene sequence and the open line shows the minimal promoter of the fibroin gene. (**A**) Transcription of the fibroin gene 5′ deletion mutants. Each deletion mutant (0.1 μg) was transcribed together with Ad2MLP, pM7 (0.1 μg) and specific transcripts from the fibroin gene (Fb) and the Ad2MLP (Ad) were detected with f1Fb38 and M13Ad4 respectively. With a low concentration of the template DNA, the suppression of transcription from the Ad2MLP by the psg extract was more obvious. (**B**) Transcription of the fibroin gene 3′ deletion mutants. Each deletion mutant (0.1 μg) was transcribed together with pM7 (0.1 μg) and specific transcripts from both templates were detected with M13Ad4 as shown in (C). (**C**) Scheme for the detection of transcripts from the fibroin 3′ deletion mutants. Transcripts from the 3′ deletion mutants of the fibroin gene were detected with a modified S1 nuclease assay (Hirose *et al.*, 1985) using a single-stranded DNA probe M13Ad4. The 3′ deletion mutants were linked to the *Bam*HI site of pBR322 with *Bam*HI linker at their 3′ deletion end, and the structure of the transcripts from these mutants was identical to that of the transcript from pM7 except for their 5′ end. The single-stranded DNA probe M13Ad4 is able to distinguish these transcripts. Transcription from the 3′ deletion mutants detected by M13Ad4 was sensitive to 1 μg/ml of α-amanitin (data not shown), depended on the fibroin gene promoter and was only observed under the conditions which permitted faithful transcription initiation of the fibroin gene (see panel B and the text). The black box shows the exon of the Ad2MLP or the fibroin gene, the thick line shows the upstream region, the thin line shows pBR322, the broken line shows M13DNA, the triangle on the pM7 shows the position of a point mutation at −54 and the long arrow shows the transcripts.

(Figure 2B) using the strategy shown in Figure 2C. The transcription of pFb3′Δ+10 was stimulated by the addition of the psg extract into the HeLa cell extract (Figure 2B, lanes 9 and 10). A further 3′ deletion to +7 (pFb3′Δ+6) greatly diminished this enhancement and resulted in only weak stimulation by the psg extract (Figure 2B, lanes 7 and 8). The specific transcription of other mutants with further 3′ deletions was scarcely detected even after the addition of the psg extract, except that of pFb3′Δ−21 (Figure 2B, lanes 3 and 4). It is noteworthy that the pBR322 sequence GACGCATCGTG juxtaposed ~30 bases downstream from the TATA box of pFb3′Δ−21 is similar to the native sequence TCAGCATCAGT of the fibroin gene initiation region. These observations suggest that the structure of the transcription initiation region of the fibroin gene is important for recognition by some factors in the psg extract.

The 5′ border of the element necessary for the specific stimulation of the fibroin gene transcription in the psg extract was mapped between −31 and −18, and the 3′ border between +10 and +3 by the complementation assay. Interestingly, this region (−31 to +10) closely corresponds to the minimal promoter of the fibroin gene defined previously in psg and HeLa cell extracts (Tsuda and Suzuki, 1981; Tsujimoto *et al.*, 1981).

Both the upstream and the downstream regions of the core-promoter enhance transcription in psg extract

The observations of a weaker enhancement via the upstream element and a strong enhancement even in the absence of the upstream element described here apparently conflict with our previous results (Tsuda and Suzuki, 1983; Suzuki *et al.*, 1986). To clarify this apparent discrepancy, we further dissected the promoter region of the fibroin gene into three parts, the upstream element (UE), the core-promoter (CP) and the intron (intronic element, IE) (Figure 3A) and analyzed their functions individually. The upstream fragment from −234 to −66 of the fibroin gene (UE), which contains the entire distal element, was subcloned at position −52 of the Ad2MLP and the effect of this fragment on the transcription from the Ad2MLP was examined in the psg extract alone or in the HeLa cell extract supplemented with psg extract

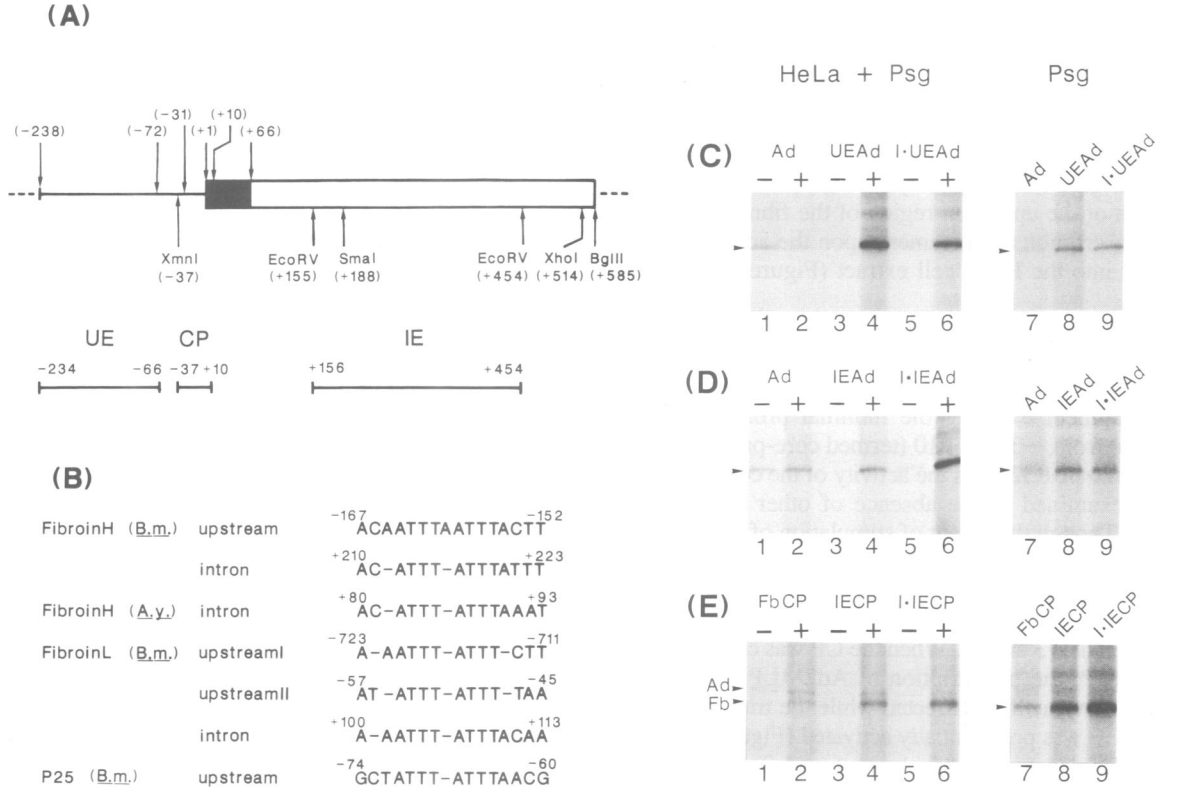

Fig. 3. Transcription stimulation of the heterologous and homologous promoters through the upstream region and the intron of the fibroin gene. (**A**) Structure of pFb5′Δ−238 and the DNA fragments used for the functional assay. The position of some relevant restriction points and deletion end points of the deletion mutants are indicated. The thin line shows the upstream region, the black box shows the first exon and the open box shows the intron. The broken line shows the region of the vector DNA. UE, the upstream element; CP, the core-promoter region; IE, the intronic element. (**B**) Homologous sequences in the fibroin gene and the fibroin light chain gene. FibroinH (B.m.), fibroin gene from *Bombyx mori* (Tsujimoto and Suzuki, 1979); FibroinH (A.y.), fibroin gene from *Antheraea yamamai* (Tamura *et al.*, 1987); FibroinL (B.m.), fibroin light chain gene from *Bombyx mori* (Y.Kikuchi and S.Mizuno, personal communication); P25 (B.m.), P25 gene from *Bombyx mori* (Couble *et al.*, 1985). (**C**) Effects of the upstream element (UE) on transcription from the heterologous promoter. The chimeric Ad2MLPs, pUEAd or pI·UEAd, and pM7 (Ad) were transcribed in 3 μl of the HeLa cell extract (−), 3 μl of the HeLa cell extract supplemented with 4.5 μl of the psg extract (+) or 4.5 μl of the psg extract alone (**lanes 7–9**). (**D**) Effects of the intronic element (IE) on transcription from the heterologous promoter. The chimeric Ad2MLPs, pIEAd or pI·IEAd, and pM7 (Ad) were transcribed in the HeLa cell extract (−), HeLa cell extract supplemented with the psg extract (+) or the psg extract alone (**lanes 7–9**). (**E**) Effects of the intronic element (IE) on transcription from the CP when inserted at an upstream position. The fibroin CP, pFbCP2 and the reconstructed fibroin gene promoters, pIECP or pI·IECP, were transcribed in the HeLa cell extract (−), HeLa cell extract supplemented with the psg extract (+) or the psg extract alone (**lanes 7–9**). In **lanes 1–6** the fibroin gene template (Fb; 0.15 μg) was transcribed together with pM7 (Ad; 0.15 μg) as an internal control. The stimulatory effects of the UE and IE were observed equally in the presence of pFb5′Δ−31 or pFbCP as an internal control (data not shown).

(Figure 3C). Since this insertion destroyed the upstream element of the Ad2MLP, its transcription in HeLa cell extract was impaired (compare Figure 3C, lane 1 with lanes 3 and 5). However, upon the addition of psg extract, a drastic increase of transcription from the Ad2MLP via the upstream element of the fibroin gene was clearly observed (Figure 3C, lanes 4 and 6). This stimulatory effect by the UE was also observed in the psg extract alone and was independent of the orientation of the UE (Figure 3C, lanes 8 and 9). Thus, the fibroin gene upstream region contains an enhancer-like element that can function in the psg extract.

DNase I footprint experiments on gel-shift complexes have shown that the sequence $^{-167}$ACAATTTAATTTA^{-155} and two other similar sequences in the upstream region of the fibroin gene were protected in the psg extract (Suzuki and Suzuki, 1988). Homologous sequences can also be found in the intron and the upstream regions of several other psg-specific genes of *B.mori* and another silkworm *Antheraea yamamai* (Figure 3B) (Couble *et al.*, 1985; Tamura *et al.*, 1987; Y.Kikuchi and S.Mizuno, personal communication). The intron fragment (IE) from +156 to +454 contains a sequence homologous to this from +210 to +223. To test the function of this fragment it was inserted at position −52 of the Ad2MLP and transcription efficiency was determined (Figure 3D). Transcription of this chimeric Ad2MLP but not the Ad2MLP itself was enhanced by the psg extract irrespective of the orientation of the insertion. The stimulatory effect of the IE was also observed with its own promoter (Figure 3E). This may be one of the reasons why the deletion of the upstream region of the fibroin gene still gives a transcription enhancement upon the addition of the psg extract into the HeLa cell extract (Figure 2A, lanes 5 and 6).

Preferential transcription of the fibroin core-promoter in psg extract

A short fragment carrying the minimal promoter of the fibroin gene from −37 to +10 (termed core-promoter) was subcloned into pBR322 and the activity of the core-promoter (CP) was examined in the absence of other fibroin gene sequences. Though the extent of stimulation of transcription from the CP was low in comparison with that of pFb5′Δ−31, a significant stimulation was observed by the addition of the psg extract into the HeLa cell extract (Figure 4A, lanes 3 and 4). When the CP was co-transcribed with Ad2MLP, the transcription of Ad2MLP was slightly suppressed or virtually unaffected while the transcription of the fibroin CP was preferentially activated (Figure 4A, lanes 5 and 6; also see Figure 3E, lanes 1 and 2).

The preferential transcription from the fibroin CP was more obvious in the transcription system composed of the psg extract alone (Figure 4B−D). When a very low concentration of the psg extract (1.5 μl) was used as shown in Figure 4C and D, lane 1, a rather high background resistant to α-amanitin was observed and no α-amanitin-sensitive transcript could be detected, while in >3 μl of the psg extract the transcript detected with M13Ad4 was completely sensitive to 1 μg/ml of α-amanitin (data not shown). In every concentration of the psg extract which gave faithful transcription (Figure 4C and D, lanes 2−5), the CP was preferentially transcribed. Thus, the fibroin CP was dominant to both the Ad2MLP and the *Bombyx* chorion gene in the psg extract and appears to be distinguished from other promoters in the psg extract.

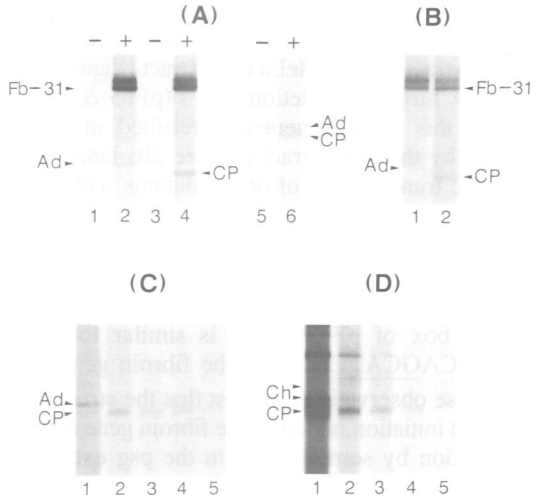

Fig. 4. Preferential transcription of the fibroin CP by the psg extract. **(A)** Transcription of the fibroin CP was compared with that of pFb5′Δ−31 or Ad2MLP (Ad) in 3 μl of the HeLa cell extract alone (−) or the HeLa cell extract supplemented with 4.5 μl of the psg extract (+). Templates: **lanes 1** and **2**, pFb5′Δ−31 (0.1 μg) and pM7 (0.2 μg); **lanes 3** and **4**, pFb5′Δ−31 (0.1 μg) and pFbCP (0.2 μg); **lanes 5** and **6**, pM7 (0.15 μg) and pFbCP (0.15 μg). Transcripts were analyzed with single-stranded probes, f1Fb38 for pFb5′Δ−31, and M13Ad4 for pM7 and pFbCP. In the complementation system, relative promoter activities of the CP and the Ad2MLP were 5:1 when pFb5′Δ−31 was used as an internal control (**lanes 2** and **4**), and 1.3:1 when these templates were co-transcribed (**lane 6**), while the Ad2MLP was a stronger promoter in the HeLa cell extract (**lanes 1** and **5**). **(B)** Transcription of the fibroin CP was compared with those of pFb5′Δ−31 and Ad2MLP (Ad) in 4.5 μl of the psg extract. Templates: **lane 1**, pFb5′Δ−31 (0.1 μg) and pM7 (0.2 μg); **lane 2**, pFb5′Δ−31 (0.1 μg) and pFbCP (0.2 μg). **(C)** Dominance of the fibroin gene to the Ad2MLP in the psg extract. The fibroin CP, pFbCP (0.15 μg), was transcribed together with Ad2MLP, pM7 (0.15 μg), in 1.5 μl (**lane 1**), 3 μl (**lane 2**), 4.5 μl (**lane 3**), 6 μl (**lane 4**) and 7.5 μl (**lane 5**) of psg extract. **(D)** Dominance of the fibroin gene to the *Bombyx* chorion gene in the psg extract. The fibroin CP, pFbCP (0.15 μg), was transcribed together with the chorion gene (Ch), pChΔNot (0.15 μg), in 1.5 μl (**lane 1**), 3 μl (**lane 2**), 4.5 μl (**lane 3**), 6 μl (**lane 4**) and 7.5 μl (**lane 5**) of the psg extract.

Preferential stimulation of transcription of the fibroin CP by phosphocellulose fraction D from psg extract

In a low concentration of psg extract, faithful transcription of the fibroin gene is undetectable and transcription initiation is mostly random (Figure 4C and D, lane 1; Takiya and Suzuki, 1989). However, when the phosphocellulose fraction D from the psg extract was added, faithful transcription of the fibroin gene was induced and random transcription was suppressed (Takiya and Suzuki, 1989). As shown in Figure 5, transcription of the fibroin CP and pFb5′Δ−31 was also induced by fraction D (Figure 5, lanes 2 and 4). Under these conditions, fraction D had virtually no effect on transcription from the Ad2MLP and the *Bombyx* chorion gene (Figure 5, lanes 2, 6 and 9). These observations indicate that the CP can act as a functional unit which is recognized efficiently by some factors in the psg extract and these factors apparently have specificity for the fibroin gene but not for the chorion gene or the Ad2MLP.

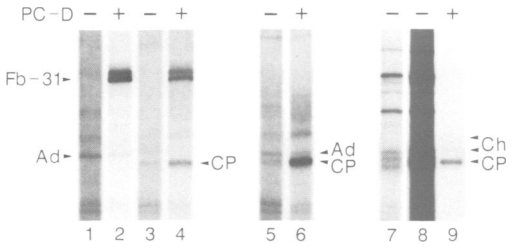

Fig. 5. Induction of the fibroin gene transcription by phosphocellulose fraction D. The fibroin CP was transcribed together with pFb5′Δ−31, Ad2MLP (Ad) or the chorion gene (Ch) in 1 μl of the crude psg extract (**lanes 1**, **3**, **5**, **7** and **8**) or the psg extract supplemented with 4.5 μl of the phosphocellulose fraction D (**lanes 2**, **4**, **6** and **9**). Template: pFb5′Δ−31 (0.1 μg) and pM7 (0.2 μg) (**lanes 1** and **2**); pFb5′Δ−31 (0.1 μg) and pFbCP (0.2 μg) (**lanes 3** and **4**); pM7 (0.15 μg) and pFbCP (0.15 μg) (**lanes 5** and **6**); pChΔNot (0.15 μg) and pFbCP (0.15 μg) (**lanes 7**, **8** and **9**). Lane 7 shows a short exposure of the autoradiogram of lane 8.

Protection of the CP region by psg extract from nuclease digestion

To identify candidates for the factors responsible for the preferential transcription of the fibroin CP, we used a DNase I footprint assay. When a DNA fragment from −238 to +65 was used as a probe, the CP, which spans ~50 bp, and the entire upstream region were protected in the psg extract (Figure 6A). The protection of the CP was also obtained with a probe DNA from −115 to +77 (Figure 6B). The details of the protected region are summarized in Figure 6D; on the coding strand, the CP region from −42 to +7 was protected with very minor sensitive sites at around −34, −22 and −5; on the non-coding strand, the TATA box region and the initiation region were protected on a fragment from −94 to +65, which were demarcated by hypersensitive sites at positions −15 and −9. Identical protection patterns were observed on the non-coding strand using a longer probe from −238 to +65 (C.-c.Hui, K.Matsuno and Y.Suzuki, in preparation).

When a DNA fragment derived from pFbCP2, which contains only the CP, was analyzed by DNase I footprinting, a remarkable similarity in the extent and efficiency of CP protection was observed: (i) protection of the CP region from −33 to +10; and (ii) protection of the downstream region with a series of hypersensitive sites. However, there are some differences in the details of the protection patterns; the hypersensitive site at −42 was shifted to −34, which is a minor sensitive site in the natural fibroin gene fragment, and the 3′ boundary of the protection was shifted from +7 to +10. Though it is not possible to compare directly the efficiency of CP protection on these two probes in a crude psg extract, our results suggest that the CP in pFbCP2 has a very high affinity for certain factors even in the absence of both the upstream and downstream sequences. These factors are the possible candidates involved in the preferential transcription of the fibroin CP.

Discussion

The complementation assay using a small amount of HeLa cell extract supplemented with psg extract or various fractions of a psg extract has been shown to be useful for the detection of some stimulatory activities for the fibroin gene transcription (Takiya and Suzuki, 1989). Since this complementation assay offers higher sensitivity for these stimulatory activities (Figure 1), it was used in this study together with the psg extract alone for the dissection of the promoter region of the fibroin gene.

In the present study, we showed that a 47 bp region carrying the minimal promoter of the fibroin gene was sufficient for preferential transcription in the psg extract. This preferential activation of transcription from the fibroin CP was also observed in the complementation assay. Under these conditions, the transcription of other type II genes, such as the Ad2MLP and one of the *Bombyx* chorion genes, were suppressed or virtually unaffected. These results show that the fibroin CP is recognized by some transcription factors in the psg extract distinctly from other type II promoters. The fibroin CP and the transcription initiation region have been shown by *in vivo* and *in vitro* transcription systems to be important for general promoter function as with other type II promoters (Tsuda and Suzuki, 1981; Tsujimoto *et al.*, 1981; Tokunaga *et al.*, 1984; Takiya *et al.*, 1985). However, here we have demonstrated that the CP itself could be transcribed preferentially in the psg extract.

Recently, a small element carrying the TATA box and the transcription initiation region has been discovered to possess an intrinsic function for specific regulation in several genes. Promoter segments close to the TATA box of the chick conalbumin gene (−44 to +62) and the ovalbumin gene (−56 to −1) were sufficient to confer their liver cell- and oviduct tubular gland cell-specificity (Dierich *et al.*, 1987). The human gastrin gene fragment from −17 to +57 shows a considerable cell type specificity in transfection experiments (Theill *et al.*, 1987). The 15 bp sequence containing the TATA box of the glycoprotein C gene of herpes simplex virus was demonstrated to be sufficient to specify a late gene type behavior of expression (Homa *et al.*, 1986, 1988; Shapira *et al.*, 1987). The TATA box of the human heat shock protein 70 gene itself could confer a specific responsiveness to the E1A product (Simon *et al.*, 1988). There are other examples of regions close to the transcription initiation region of some genes, including the *Drosophila* homeotic genes, which have essential functions for their transcription (Talkington and Leder, 1982; Coen *et al.*, 1986; Hultmark *et al.*, 1986; Seiler-Tuyns and Paterson, 1987; Biggin and Tjian, 1988; Perkins *et al.*, 1988; Soeller *et al.*, 1988; Smale and Baltimore, 1989). These lines of evidences suggest that the basal promoter elements of the type II genes, including the TATA box and transcription initiation region, can possess specific as well as general functions.

In the psg extract, interaction between the transcription machinery and the TATA box and −20 region of the fibroin gene was suggested by the experiments using single-base substitution mutants in *in vitro* transcription assays (Hirose *et al.*, 1982, 1984). The DNase I footprint analyses shown in Figure 6 revealed an ~40 bp protection covering the entire CP. These factors interacting with the CP are the likely candidates responsible for its preferential transcription. At least one of the factors which interacts with the TATA box region was detected in the phosphocellulose fraction D of the psg extract (unpublished results). The protection patterns of the CP and the natural fibroin promoter in the psg extract were almost identical, but the protected region of the CP

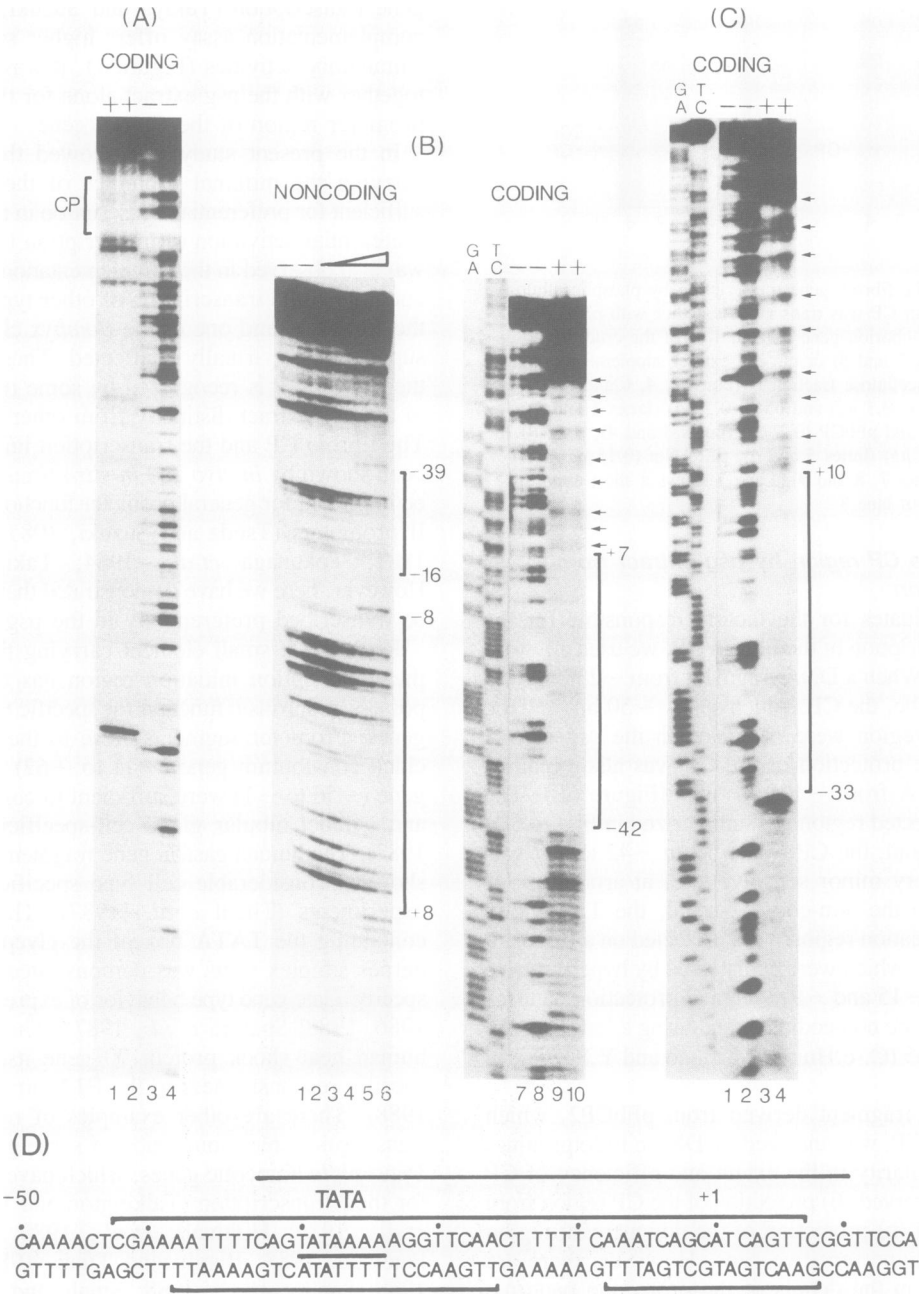

Fig. 6. DNase I footprinting of the CP region. **(A)** The coding strand of the fibroin gene from −238 to +65 was incubated with 210 μg of the psg extract (+) and digested with DNase I for 60 s (**lane 1**) or 90 s (**lane 2**). As a control, the probe was incubated with 40 μg of BSA (−) and treated with 20 ng (**lane 3**) or 50 ng (**lane 4**) of DNase I for 60 s. **(B)** The non-coding strand from −94 to +65 (**lanes 1−6**) and the coding strand from −115 to +77 (**lanes 7−10**) were assayed. **Lanes 1**, **2**, **7** and **8** are controls including 40 μg of BSA. **Lanes 3−6** included 30, 90, 150 and 210 μg of the psg extract respectively and the samples were treated with DNase I for 60 s. **Lanes 9** and **10** are the same as lanes 1 and 2 in (A). **(C)** The coding strand of a fragment containing the CP derived from pFbCP2, which is flanked by pBR322 sequence, were assayed. **Lanes 1−4** are the same as lanes 7−10 in (B). **(D)** Summary of the protection of the CP region. Nucleotide sequence of the natural fibroin promoter is shown. The protected regions obtained for the natural fibroin gene probe are marked with brackets and for the pFbCP probe with the line above the sequence.

was shifted several nucleotides downstream, and the transcription efficiency from the CP was very low when compared with that of the natural fibroin promoter. This may be interpreted as the difference in the protein−DNA and protein−protein interactions in the natural fibroin promoter and the CP that might be affecting the function of the transcription machinery and the efficiency of transcription from these promoters. An interaction of the HeLa cell TFIID with the adenovirus E4 promoter is known to be altered by transcription activation factors bound to upstream elements (Horikoshi *et al.*, 1988a,b).

The addition of the phosphocellulose fraction D to a small amount of crude psg extract specifically induced transcription of the fibroin gene (Figure 5; Takiya and Suzuki, 1989). Fraction D contains a stimulatory activity which functions through the upstream element (Takiya and Suzuki, 1989), but the specific induction of fibroin gene transcription by fraction D could still be observed with a deletion mutant

which lacks the entire upstream region (Takiya and Suzuki, 1989). The specific induction of transcription from the CP by fraction D is consistent with the above observations. These results suggest that the CP itself is an important element for the specificity of fibroin gene transcription. Because the dominance of the fibroin CP is observed not only for the Ad2MLP but also for the *Bombyx* chorion gene, the preferential transcription of the CP is unlikely to be due to a species-specific factor.

Though the fibroin CP was specifically transcribed in the psg extract, the transcription efficiency was very low when both the upstream and the downstream regions were deleted. The upstream region ~200 bp 5′ to the TATA box of the fibroin gene is known to be important for transcription in the psg extract (Tsuda and Suzuki, 1983; Suzuki *et al.*, 1986). When the upstream fragment (UE) was inserted upstream of the Ad2MLP, transcription from Ad2MLP was enhanced in the psg extract. However, the transcription efficiency of this chimeric Ad2MLP was still considerably lower than that of the natural fibroin gene (unpublished results). Here, we have shown that a fragment of ~300 bp in the intron of the fibroin gene (IE) also possesses an enhancer-like function. Within this region, a sequence homologous to that in the upstream element of the fibroin gene as well as in the intron and upstream regions of other psg-specific genes can be seen. At present we cannot exclude the possibility that there are other important elements in the intron outside of the +156 to +454 region.

The combination of the CP and IE was transcribed more efficiently than the combination of the Ad2MLP and IE (Figure 3D and E). Similarly, a combinational effect of the CP and the upstream region could also be observed (compare Figure 2B, lanes 9 and 10 with Figure 4A, lanes 5 and 6). The co-operation of multiple regulatory elements is probably important for the specificity and efficiency of fibroin gene transcription. There are several examples of how such complex interactions between regulatory sequences are required for correct and regulated gene expression: only the complex combinations of regulatory elements in the adenovirus 2 E2aE promoter can confer the Ela responsiveness (Zajchowski *et al.*, 1987); tissue-specific expression of the *Drosophila* alcohol dehydrogenase gene is regulated by synergistic interactions between multiple promoter elements (Fischer and Maniatis, 1988); synergism between the immunoglobulin enhancers and promoters has also been reported (Garcia *et al.*, 1986). The complex organization of small promoter elements in the upstream regions and enhancers of many type II genes in eukaryotes suggests that interactions between many different transcription factors bound to these units might be necessary for appropriate gene expression (Hatzopoulos *et al.*, 1988; La Tangue and Rigby, 1988). In combinations with other regulatory elements, the TATA box and the transcription initiation region of the fibroin gene, here termed CP, probably serves a specific role for the regulation of fibroin gene transcription.

Materials and methods

Nuclear extract

Crude nuclear extracts from the posterior silk glands were prepared by the method of Tsuda and Suzuki (1981) with several modifications. Posterior silk glands (usually 70–100 pairs) stored at −80°C were thawed in ~10 ml extraction buffer (40 mM Tris–HCl pH 7.9, 5 mM $MgCl_2$, 12.5% sucrose, 25% glycerol and 3 mM DTT), and the glands were minced with scissors for ~10 min until the tissue fragments were ~1 mm^3. The glands were minced for a further 5 min after addition of another 10 ml of the same buffer, homogenized twice in a 15 ml Dounce type homogenizer (Kontes) with a loose-fitting pestle (A type) and centrifuged at 5000 r.p.m. for 5 min in a Sorvall HB-4 rotor. The supernatant and the white material covering the nuclear pellet was discarded by pipetting. The crude nuclear pellet was suspended in ~20 ml of the buffer, and homogenized for 13 strokes. After adjusting the volume of the suspension of 40 ml, 4 ml (one-tenth volume) of saturated $(NH_4)_2SO_4$ was added with gentle stirring. The mixture was stirred for 30 min at 4°C, and then centrifuged at 50 000 r.p.m. for 4 h in a Beckman 60 Ti rotor. About two-thirds of the upper part of the supernatant was recovered and the proteins were precipitated by the addition of solid $(NH_4)_2SO_4$ (0.33 g/ml). The precipitate was redissolved in a small volume (1–1.2 ml) of dialysis buffer (20 mM HEPES pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol and 2 mM DTT). After dialysis against the same buffer the lysate was clarified by centrifugation at 45 000 r.p.m. for 60 min in a Beckman type 50 rotor. The supernatant was pooled and stored in small aliquots or recovered as several tiers and stored at −80°C.

HeLa whole cell extract was prepared by the method of Manley *et al.* (1980) with minor modifications. Phosphocellulose fraction D of the psg extract was prepared as described (Takiya and Suzuki, 1989). Protein concentrations of the HeLa cell extract, psg extract and phosphocellulose fraction D used here were 17, 31 and 0.9 mg/ml respectively.

Transcription

In vitro transcription was performed as described (Tsujimoto *et al.*, 1981) except that the concentration of ATP was increased to 600 μM. Covalently closed circular DNA templates were used at the concentration of 24 μg/ml except for the experiments in Figure 2 (16 μg/ml). RNA was analyzed with a modified S1 nuclease assay (Hirose *et al.*, 1985).

Template and single-stranded probe DNAs

The fibroin gene 5′ deletion mutant pFb5′Δ−238 (Tsuda and Suzuki, 1983) and other 5′ and 3′ deletion mutants (Tsujimoto *et al.*, 1981) were as described. pAd500 contains sequences from −677 to +33 of the Ad2MLP (Tsuda and Suzuki, 1983). pM7 is a derivative of pM677 (Miyamoto *et al.*, 1984) with its upstream region partially damaged by a point mutation (Miyamoto *et al.*, 1985). A subclone of *Bombyx* chorion genes, pChΔNot was prepared by inserting the 3.15 kb *Eco*RI fragment of pB23 (Mitsialis and Kafatos, 1985) into pBR325, which contains a pair of chorion genes (A and B), followed by deletion of the internal 0.42 kb *Not*I fragment within the coding region of the A gene. A subclone of the fibroin CP, pFbCP, was constructed by inserting the *Xmn*I (−37)–*Bam*HI (+10) fragment of pFb3′Δ+10 between the *Eco*RV and *Bam*HI sites of pBR322. Another fibroin core-promoter plasmid, pFbCP2, was constructed from pFb3′Δ+10 of which sequences between −860 and −38 were deleted; the plasmid was digested partially with *Xmn*I in −37, cleaved with *Hin*dIII, filled with T4 DNA polymerase and self-circularized. Chimeric plasmids of the fibroin sequences and Ad2MLP were constructed by insertion of the fibroin DNA fragments into the *Sma*I site at −52 of pM7. pUEAd and pI·UEAd were made by insertion of the upstream DNA fragment from −234 to −66 of the fibroin gene with right and inverted orientations respectively. pIEAd and pI·IEAd were made by insertion of the *Eco*RV fragment from +156 to +454 of the fibroin gene intron with right and inverted orientations respectively. pIECP and pI·IECP were made by insertion of the *Eco*RV fragment from the fibroin gene intron into the filled *Cla*I site of pFbCP2 which was 47 nt upstream from the initiation site of the fibroin gene. The single-stranded DNA probe f1Fb38 carries the fibroin gene from −238 to +510 (Hirose *et al.*, 1985), and M13Ad4 carries the *Eco*RI–*Sal*I fragment of pAd500 (Hirose and Suzuki, 1988). M13Ch2 contains the 1 kb *Hpa*II fragment from pChΔNot including the promoter regions for both the chorion A and B genes, and the coding strand of the A gene (downstream to +325) was cloned on the viral strand.

DNase I footprinting

Footprinting reactions contained the same components as the transcription mixture, except that nucleoside triphosphates were omitted. The reactions (25 μl) contained up to 15 μl of extract, 1–5 fmol of end-labeled DNA probe and 1 μg of poly(dA-dT), 10 μg of tRNA, 1 μg of pdN_5 and 2 mM potassium phosphate (pH 7.0) as carriers. The extract was incubated for 10 min on ice with carriers before the addition of the DNA probe. Binding was allowed to proceed for 15 min on ice and then for 5 min at room temperature. DNase I (500 ng in 2 μl) was added and incubated for 60 or 90 s at room temperature. Reactions were stopped by the addition of 30

μl of the stop solution (200 mM NaCl, 50 mM EDTA, 1% SDS, 20 μg/ml sonicated salmon sperm DNA) and 10 μg of proteinase K. After incubation for 30 min at 45°C, DNA was extracted with phenol–chloroform, precipitated with ethanol and analyzed on a 6% polyacrylamide sequencing gel. The *Eco*RI–*Pst*I fragment of pFb5′Δ–238, the *Hin*dIII–*Hph*I fragment of pFb5′Δ–115 and the *Rsa*I–*Bam*HI fragment of pFb3′Δ+65 were used as probes.

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