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Promoter sequence of fibroin gene assigned by *in vitro* transcription system

(*in vitro* genetics/mutant genes)

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ABSTRACT **We have shown that the silk fibroin gene from *Bombyx mori* is faithfully transcribed in an *in vitro* transcription system of the HeLa cell extract prepared by the method of Manley *et al.* [Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gefter, M. L. (1980) *Proc. Natl. Acad. Sci. USA* 77, 3855–3859]. Using this system and a series of deletion mutants of fibroin gene, we have assigned the promoter sequence of fibroin gene. The 5′ boundary of the promoter is around nucleotide position −29, indicating that most of the T-A-T-A-A-A-A sequence (−30 to −24) is essential for the promoter function, where the transcription initiation point of fibroin gene is assigned as nucleotide position +1 [Tsuda, M., Ohshima, Y. & Suzuki, Y. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4872–4876]. The 3′ boundary is around nucleotide position +6. However, to support the efficient, faithful transcription, some additional (more than 26 but less than 41) nucleotides of nonspecific origin are required at the 5′ side of −29. Functions ascribed to the promoter region are discussed.**

The structural analysis of eukaryotic genes, including tissue-specific ones transcribed by RNA polymerase II, has revealed some interesting features of the sequence that might be involved in gene expression—for example, existence of a Goldberg–Hogness box (or T-A-T-A box) at 30 nucleotides preceding the initiation point of transcription (1–4) which has been considered as a candidate of a part of promoter (2, 4). Two recent technical developments have made *in vitro* analysis of promoter function of tissue-specific genes possible. One is establishment of *in vitro* transcription systems for the type II RNA polymerase (5, 6), and the other is *in vitro* production of mutant genes by modification of cloned wild-type genes to desired arrangements (7, 8). A preliminary report assigning the promoter of chicken conalbumin gene on the basis of the two technologies has been published (9).

Here we describe a similar effort on the assignment of the promoter of insect fibroin gene in a more comprehensive manner by an *in vitro* combination of the two technologies.

MATERIALS AND METHODS

***In Vitro* Transcription.** Preparation of HeLa cell extract and *in vitro* transcription in the extract were carried out by the procedures of Manley *et al.* (6). Quality of the extracts has been assessed by faithful transcription of the adenovirus 2 late promoter, pAd2 SmaF, cloned at the P2/EK2 level according to the recombinant DNA research guidelines of Japan. The standard 25-μl reaction mixture contained 12 mM Hepes (pH 7.9), 7 mM $MgCl_2$, 60 mM KCl, 0.2 mM EDTA, 1.3 mM dithiothreitol, 10% (vol/vol) glycerol, 10 mM creatine phosphate, 600 μM GTP and CTP, 25 μM ATP, 50 μM UTP (5 μCi of [α-^{32}P]-UTP), 10–15 μl of the extract (final, 5 mg of protein per ml), and 1 μg of cleaved plasmid DNA or 0.5 μg of purified restriction fragment. Reaction mixtures were incubated at 30°C for 60 min. The reactions were stopped by addition of 75 μl of 0.1 M NaOAc, pH 5.5/1% $NaDodSO_4$ containing 50 μg of tRNA. The samples were extracted with phenol/$CHCl_3$, 1:1 (vol/vol). RNA was precipitated with ethanol, dissolved in 50% formamide, heated at 70°C for 5 min, and chilled on ice. After addition of marker dyes, samples were loaded on a 6% polyacrylamide/7 M urea gel.

S1 Endonuclease Mapping. RNA synthesized in the HeLa cell extract in the presence of nonradioactive UTP was purified and then treated with 0.34 μg of RNase-free DNase I in 100 μl of 10 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ at 37°C for 10 min. DNA·RNA hybridization for Fig. 2 was carried out in 50 μl of 0.4 M NaCl/50% formamide/10 mM piperazinediethanesulfonic acid, pH 6.4/0.1 mM EDTA containing 1 μg of RNA per μl (sample plus carrier) and 0.7 pmol of DNA probe per ml at 37°C for 15 hr. The solution was then diluted 1:10 into S1 buffer (0.3 M NaCl/50 mM NaOAc, pH 4.6/3 mM $ZnSO_4$). Heat-denatured calf thymus DNA was added to 20 μg/ml and S1 nuclease was added to 50 units/ml. After incubation at 37°C for 30 min, DNA was recovered by ethanol precipitation and dissolved in 0.05 M NaOH/0.5 mM EDTA/5 M urea/0.025% bromophenol blue/0.025% xylene cyanol.

After heating at 90°C for 1 min and chilling, DNA was loaded on a 10% polyacrylamide/7 M urea sequence gel. Hybridization for Fig. 3*B* was carried out in 0.3 M NaCl/0.01 M Tris·HCl, pH 7.5/0.1 mM EDTA/80% formamide containing RNA (200 μg/ml) and DNA probe (0.4–1.0 pmol/ml) at 42°C for 40 hr. S1 nuclease digestion was carried out as described above except for the use of 18 units of S1 nuclease per ml at 42°C for 60 min.

Construction of 5′ and 3′ Deletion Mutants of Fibroin Gene and Truncated DNA Templates. From pFb29 (10) two fibroin gene subclones, pFb100 and pFb200, were derived; they contained fibroin DNA segment *Hin*dIII$_{-1}$ (at about nucleotide −850 of fibroin DNA) to *Bgl* II$_{+1}$ (+585), and *Hin*dIII$_{-1}$ to *Hpa*I$_{+1}$ (+1021), respectively, in the larger fragment of the pBR322 *Hin*dIII/*Bam*HI digest (the cleavage sites are numbered with respect to the cap site—e.g., *Hin*dIII$_{+1}$ is the first *Hin*dIII cleavage site within the gene, and *Hin*dIII$_{-1}$ is the first site preceding the gene; the nucleotide is also numbered with respect to the cap site, +1 being the first nucleotide of the fibroin gene transcription) (3).

Construction of the 5′ deletion mutants, shown in Fig. 1*A*, generally followed the procedure described by Sakonju *et al.* (7). The pFb100 DNA isolated from *Escherichia coli* GM33 dam$^-$ was cleaved at the *Bcl* I site (−171). The linear DNA (10 μg; 2.8 pmol) was digested with exonuclease III (15 units/pmol of DNA end; Bethesda Research Laboratories, Rockville, MD) in 200 μl of 6.6 mM Tris·HCl, pH 7.4/60 mM NaCl/6.6 mM $MgCl_2$/6.6 mM 2-mercaptoethanol at 23°C. Under these conditions, exonuclease III digests about 50 nucleotides per min.

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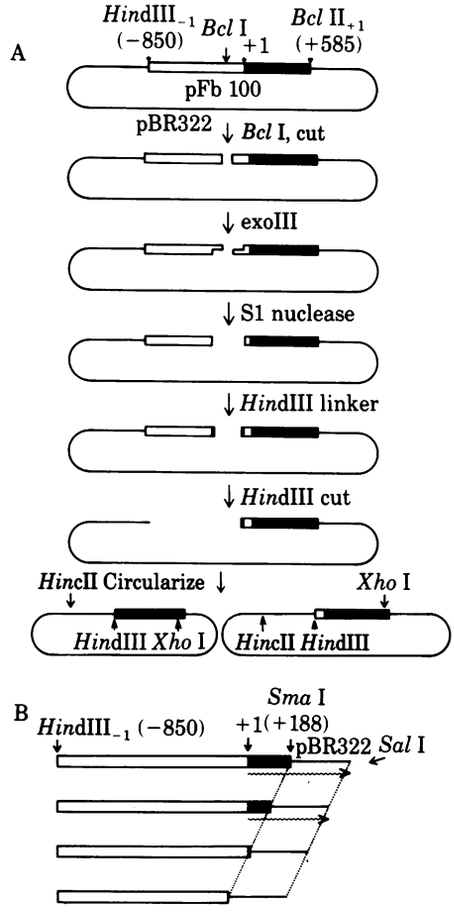

FIG. 1. Scheme for construction of 5′ deletion mutants of fibroin gene (*A*) and truncated DNA templates of 3′ deletion mutants of fibroin gene (*B*). Boxed regions, genomic fibroin DNA; open boxes, 5′ flanking sequence; solid boxes, transcribable fibroin gene region; thin lines, vector pBR322 DNA; wavy line, expected faithful transcript.

Aliquots were withdrawn at 30-sec intervals to obtain a series of deletion mutants. The exonuclease-treated DNA was purified and then digested with S1 nuclease in 150 μl of 0.3 M NaCl/50 mM NaOAc, pH 4.6/3 mM $ZnSO_4$ at 23°C for 40 min. Under these conditions, overhanging ends are quantitatively removed. Ligation of *Hin*dIII linker to the treated DNA (0.83 pmol) was carried out in 20 μl of 66 mM Tris·HCl, pH 7.6/6.6 mM dithiothreitol/0.4 mM ATP containing 150 pmol of phosphorylated *Hin*dIII linker and 3 units of ligase at 13°C for 12 hr. The DNA was purified and then digested at 37°C for 4 hr with 120 units of *Hin*dIII in 200 μl of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM NaCl/5 mM dithiothreitol/10 mM Na phosphate. The DNA was purified, and self-circularized in 20 μl of the ligation buffer.

The deletion mutants thus obtained through transformation of *E. coli* (at P1/EK1 level) contained fiborin DNAs from the *Bgl* II site at +585 to various points on the way up to the *Bcl* I site at −171.

To construct 3′ deletion mutants, pFb200 DNA was cleaved at the *Sma* I site (+188) and digested with exonuclease III and S1 nuclease as described for the 5′ deletion mutants. The DNA with flush ends was ligated to phosphorylated *Bam*HI linker. After digestion with *Bam*HI and purification, the DNA was self-circularized and used for transformation. The obtained deletion mutants contained the fibroin DNA segment from the *Hin*dIII site at −850 to various points way down to the *Sma* I site at +188 (see Fig. 1).

The exact end points of the 5′ or 3′ deletion mutants were determined by DNA sequence analysis by the method of Maxam and Gilbert (11). The deletion mutant DNA is named by its deletion end [e.g., 5′ deletion (5′Δ) −10 indicates that the 5′ flanking region is deleted except for 10 base pairs in front of the gene].

RESULTS

Accurate Transcription Initiation of Fibroin Gene *in Vitro*. We prepared HeLa cell extracts by the procedure of Manley *et al.* (6) and examined whether faithful transcription of the fibroin gene can be achieved in this system. In order to detect specific initiation of transcription on the fibroin gene, we used, as truncated templates, fibroin *Hin*dIII$_{-1}$ (−850)/*Xho* I$_{+1}$ (+514) or *Hin*dIII$_{-1}$ (−850)/*Sma* I$_{+1}$ (+188) fragment. Incubation of the *Hin*dIII/*Xho* I fragment with HeLa cell extract generated a discrete transcript of about 520 bases (see Fig. 3, lane −850). When the template was the *Hin*dIII/*Sma* I fragment, the 520-base transcript disappeared and a 190-base band was observed (data not shown). The synthesis of these specific transcripts was inhibited by α-amanitin (1 μg/ml) and the 520-base band hybridized only with the coding strand cloned in f1 phage DNA (data not shown). These results indicate that transcription of fibroin gene is initiated on the coding strand at or near the cap site.

To position the 5′ end of the specific transcript more precisely, S1 nuclease mapping (12) was carried out. Unlabeled RNA synthesized on the *Hin*dIII/*Xho* I template or purified fibroin mRNA was hybridized with the strand-separated *Taq* I$_{-1}$ (−41)/*Sau*3A$_{+1}$ (+48) fragment (coding strand) which had been labeled with ^{32}P at the 5′ end, and the protected DNA from S1 nuclease digestion was electrophoresed on a sequencing gel. Both *in vivo* mRNA and *in vitro* transcript gave the same protection bands (Fig. 2). Among these, the positions of two prominent ones corresponded to the cap site (+1) and −1. These results demonstrate that the HeLa cell extract accurately initiates transcription at the cap site (+1) of fibroin gene.

The amount of the transcript synthesized from fibroin promoter was calculated from either (*i*) the radioactivity of the 520-base band produced on the *Hin*dIII$_{-1}$/*Xho* I$_{+1}$ fragment and the specific radioactivity of [α-^{32}P]UTP, or (*ii*) the radioactivity of the protected bands and the specific radioactivity of the probe used for the S1 nuclease experiment. Both of these calculations gave similar values, 5–10 × 10^{-3} pmol/25-μl reaction mixture per 60 min. In our system, transcription efficiency of fibroin promoter was slightly less than that of adenovirus 2 late promoter, indicating that fibroin promoter is a rather strong promoter in the extract.

Transcription of 5′ Deletion Mutants. In order to identify the region required for the faithful initiation of the fibroin gene transcription, we constructed a series of deletion mutants of the fibroin gene. To identify the 5′ boundary of the promoter, we produced fibroin gene deleted to various extends on the 5′ side of the gene (Fig. 1*A*).

The 5′ deletion mutants whose deletion ends were located at the 5′ flanking region were tested for their ability to support the initiation of transcription in HeLa cell extract. The deletion mutant DNA was cleaved by restriction endonucleases at the sites of *Hin*cII in pBR322 and *Xho* I (+514) in fibroin DNA (Fig. 1*A*) and used as a truncated templates for *in vitro* transcription. The template DNA contained a transcribable region of the fibroin gene (+1 to +514) and a chimeric 5′ flanking sequence consisting of fibroin 5′ flanking sequence of various lengths and 484 bases of pBR322 sequence (*Hin*cII–*Hin*dIII). The faithful initiation of transcription is expected to give a transcript of 514 bases.

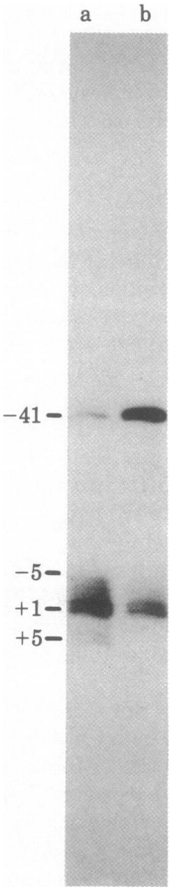

FIG. 2. S1 nuclease mapping of the 5′ end of *in vitro* synthesized RNA. Purified fibroin mRNA (lane a) or nonradioactive RNA produced on the *Hin*dIII/*Xho* I truncated template (lane b) was hybridized with the coding strand component of *Taq* I_{-1}/*Sau*3A$_{+1}$ 5′-^{32}P-labeled, treated with nuclease S1, and electrophoresed on a 10% polyacrylamide/7 M urea gel. The positions of −5, +1, and +5 were deduced from the mobilities of markers run on the same gel; the markers used were DNase I ladder produced from the *Taq* I_{-1}/*Sau*3A$_{+1}$ 5′-^{32}P-labeled probe and the *Hae* III digest of 5′-^{32}P-labeled pBR322 *Hin*fI fragment.

The transcripts produced from 5′ deletion mutants were analyzed by gel electrophoresis (Fig. 3*A*). The pFb100 DNA, which contained about 850 bases of 5′ flanking region, supported synthesis of a faithful 520-base-long transcript. Many of the transcripts seen above the faithful transcript are likely to be nonspecific. When the purified *Hin*dIII/*Xho* I (−850–+514) fibroin DNA was used as a template, the majority of the nonspecific transcripts disappeared (data not shown). Thus, most of them probably were transcripts started within the pBR322 sequence. The 5′Δ −73, −52, −44, −31, and −29 also produced the faithful transcript although the transcription promoted by 5′Δ −29 was slightly weaker than the others (about 35% reduction). On the other hand, 5′Δ −26, −22, and −2 lacked almost all, if not all, activity of faithful initiation (about 93% reduction). The 5′Δ −27 and −18 showed an intermediate promoter activity (about 80% reduction). The 5′Δ −31 and −29 produced also a minor transcript about 60 bases shorter than the faithful transcript. However, this minor product was not a transcript started at about +60 of fibroin gene but was derived from somewhere else as described below.

To confirm that the 520-base-long transcript produced from individual 5′ Δ mutants is the faithful transcript, we carried out the S1 nuclease mapping. Each transcript produced from 5′Δ −44, −31, −27, and −26 was hybridized with the *Hin*dIII (−850)/*Hpa* II (+189) fragment that had been labeled with [γ-^{32}P]ATP and polynucleotide kinase at the *Hpa* II site. After S1 nuclease digestion, the protected DNA was run on a 6% polyacrylamide/7 M urea gel (Fig. 3*B*). 5′Δ −44 and −31 showed a protected fragment which was identical in size to that protected by fibroin precursor RNA (lanes b, c, h, and i). The 5′Δ −27 also showed a protected fragment equal in size to that with the fibroin precursor RNA although the extent of protection was less than that by 5′Δ −44 (lanes c and d). This decreased pro-

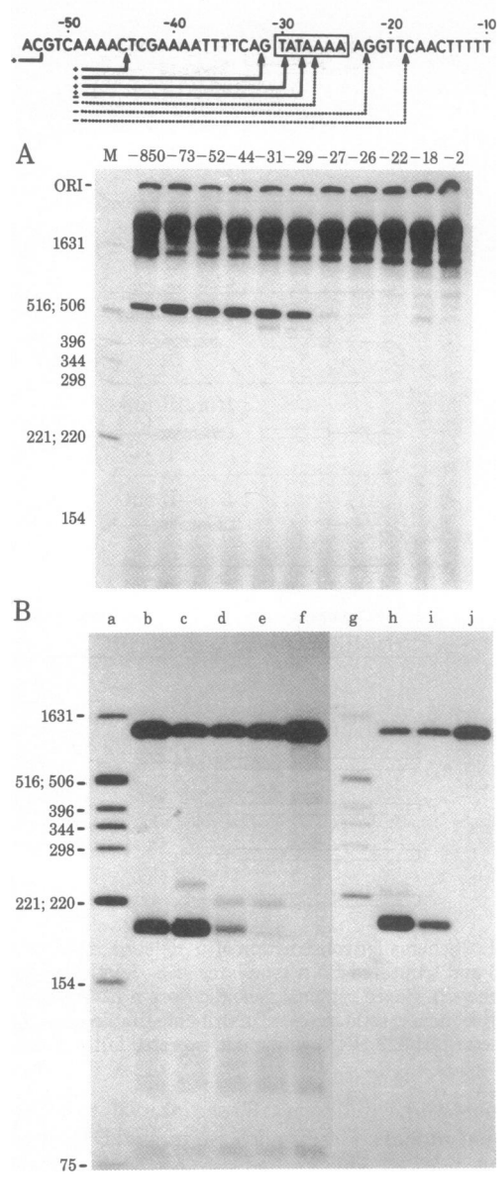

FIG. 3. Transcription of 5′ deletion mutant DNAs (*A*) and S1 nuclease mapping of the transcripts from 5′ deletion mutants (*B*). (*A*) The diagram is a summary of the experiment. On the nucleotide sequence, the boxed region represents T-A-T-A box. The arrows indicate the deletion ends of respective deletion mutant DNA. Those deletion mutants that support the efficient, faithful transcription are indicated as + and by a solid line. Those that do not are indicated as − and by a dashed line. The radioautogram is of the transcripts produced *in vitro* from the 5′Δ mutant DNA and run on a 6% polyacrylamide/7 M urea gel. Lane M is the size marker, the end-labeled pBR322 *Hin*fI digest. The sizes of the fragments are given in bases (13). The numbers shown above the autoradiograph represent the respective deletion end of mutant DNA used as template. (*B*) The 5′ ^{32}P-end-labeled restriction fragment *Hin*dIII$_{-1}$/*Hpa* II$_{+1}$ (+189) was hybridized with the transcripts produced from 5′Δ mutant DNA: lane c, −44; d, −27; e, −26; h, −31; f and j, tRNA; b and i, total RNA extracted from the posterior silk gland (3). After S1 nuclease digestion, resistant material was run on a 6% polyacrylamide/7 M urea gel. Lanes a and g are size markers, the end-labeled pBR322 *Hin*fI digest. The experiments for lanes a–f and for g–j were carried out separately. The band near the top of the gel arose by DNA·DNA reassociation. The faint bands ≈220 bases long (lanes c, d, and e) are the ones protected by transcripts that started somewhere upstream from +1. However, the size of the protected DNA does not indicate the size of the transcript but only reflects that the nucleotide sequences of the probe and the respective transcript produced from 5′Δ mutant DNA diverge at the respective deletion end. The size of the protected DNA exactly corresponds to the distance between the respective deletion ends and the labeled end of the probe.

tection by RNA produced from 5′Δ −27 compared to that from 5′Δ −44 is consistent with the result shown in Fig. 3*A*.

Although the 5′Δ −26 seemed to be able to support a small amount of faithful transcription (Fig. 3*A*), the S1 nuclease mapping shown in Fig. 3*B* (lane e) indicates that the major starting point of the transcript produced from 5′Δ −26 is not +1 but around +5. Thus, the nucleotide sequence around −26 seems to be involved in the selection of an accurate starting point of transcription as well as the maintenance of the transcription efficiency. The S1 nuclease mapping experiment also showed that the minor transcript (about 60 bases shorter than the accurate transcript) produced by 5′Δ −31 did not result from initiation at a site about 60 bases downstream from +1 (Fig. 3*B*, lane h).

From these results we conclude that the 5′ boundary of the promoter is around −29.

Transcription of 3′ Deletion Mutants. The 3′ deletion mutant DNAs (Fig. 1*B*), whose deletion ends were located within the gene or in the 5′ flanking region, were assayed for their ability to support faithful initiation of transcription. The 3′ deletion DNA was cut with restriction endonucleases at the *Hin*dIII$_{-1}$ site (−850 in the 5′ flanking region of the fibroin gene) and also at the *Sal* I site in the pBR322 sequence downstream from the gene. This truncated template DNA contained the 5′ flanking sequence up to −850 at the 5′ side and the pBR322 sequence (*Bam*HI to *Sal* I, 275 base pairs) at the 3′ side with or without a little bit of transcribable fibroin gene sequence between them. Therefore, the size of the faithful transcript produced from each deletion mutant varied in parallel with the extent of deletion of each mutant. The transcripts produced from 3′ deletion mutants were analyzed in 6% polyacrylamide/7 M urea gel.

The 3′Δ +67, +10, +8, and +6 supported transcription (Fig. 4). The RNAs transcribed from these four 3′Δ mutants were the same as those expected from faithful transcription of the individual mutants (Fig. 1*B*). The heterogeneous transcripts seen above the faithful one were nonspecific transcripts as described for the 5′Δ mutants. 3′Δ +67, +10, and +8 showed a similar efficiency of faithful initiation; 3′Δ +6 showed slightly less efficiency. 3′Δ +3, −1, −6, −15, and −21 did not support efficient transcription although, in every case, a small amount of transcript was made (about 10%). The sizes of these faint transcripts indicate that, on these 3′Δ mutants, the transcription always started at the site about 30 bases downstream from nucleotide −30 of the flanking sequence of the fibroin gene.

Thus, the 3′ boundary of the promoter is located around +6 within the gene and the DNA sequence close to the 5′ end of the gene is required for the efficient transcription.

Nonspecific Extra DNA Sequence Is Required for Transcription Initiation. As described above, the 5′ boundary of the promoter is located around −29. Thus, the nucleotide sequence beyond −29 does not play an important role in the initiation of faithful transcription. However, the template DNA always contained an additional DNA segment, about 480 base pairs, composed of *Hin*dIII linker and pBR322 sequence at its 5′ side. Therefore we attempted to determine how much 5′ flanking DNA is required for the initiation of transcription. We utilized the set of 5′Δ mutants. These were cleaved at the sites of *Hin*dIII (deletion end) and *Xho* I (+514) and used as truncated templates for *in vitro* transcription (Fig. 1*A*). The RNAs produced from the individual mutants were analyzed by gel electrophoresis. The template DNAs from pFb100, 5′Δ −140, −73, −68, and −67 were active but those from 5′Δ −52, −44, and −31 were almost, although not completely, inactive (Fig. 5). The 5′ flanking sequence of 5′Δ −67 was enough for transcription but that of 5′Δ −52 was not. 5′Δ −67 contained a 5′ flanking sequence of 70 bases of double-stranded DNA with 4 bases of single-stranded DNA at the proximal end; 5′Δ −52 had 55 bases of double-stranded DNA and 4 bases of single-stranded DNA.

DISCUSSION

Because the T-A-T-A-A-A-A sequence at −30 to −24 of the fibroin gene is also found in many other eukaryotic genes transcribed by RNA polymerase II, at corresponding sites with or

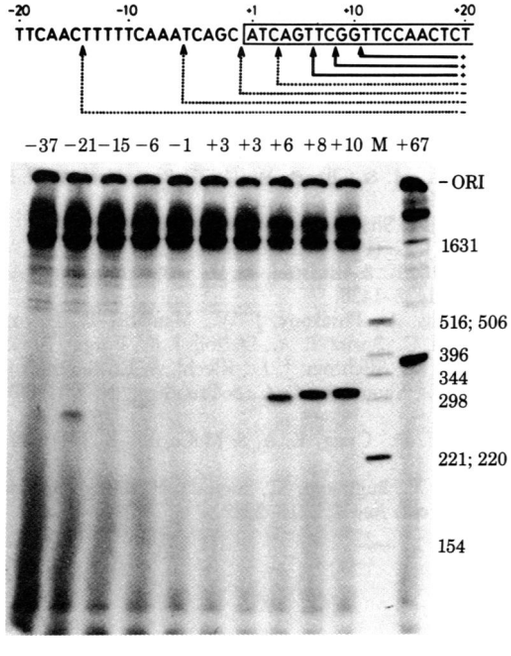

FIG. 4. Transcription of 3′ deletion mutant DNAs. The boxed sequence in the upper part indicates the transcribable region of the fibroin gene. Other features are essentially as in Fig. 3*A*.

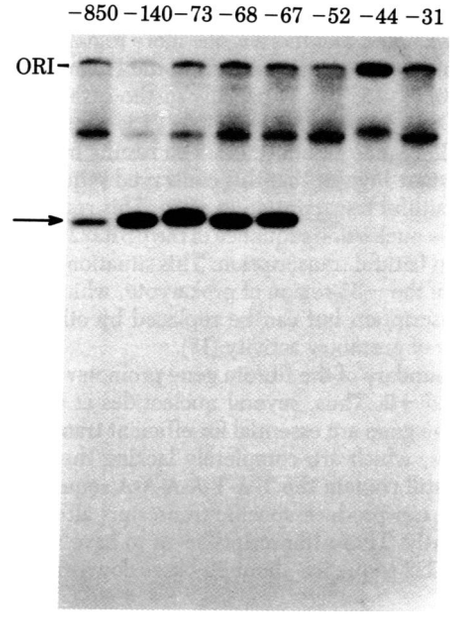

FIG. 5. Transcription on template DNAs having various lengths of 5′ flanking sequence. The 5′Δ mutant DNA shown on each lane was digested with *Hin*dIII and *Xho* I and used as truncated template in the transcription system *in vitro*. The transcripts produced were run on a 6% polyacrylamide/7 M urea gel and autoradiographed. The arrow indicates the position of the faithful transcript.

without minor variation, and is similar to the prokaryotic promoter sequence (Pribnow box sequence), it most likely is the eukaryotic promoter sequence (2). In this study we applied *in vitro* genetics to identify the region required for the faithful initiation of the fibroin gene transcription. We constructed *in vitro* a series of deletion mutants and examined whether they could support faithful transcription in the *in vitro* transcription system. This approach has been used to locate the region required for the transcription of the 5S RNA gene transcribed by RNA polymerase III (7, 8).

The 5′ boundary of the promoter of the fibroin gene is located at around −29 and the 3′ boundary is around +6. The 5′ boundary of the promoter of chicken conalbumin gene and adenovirus late gene has also been determined to be at around −30 (9) by use of the *in vitro* transcription system developed by Weil *et al.* (5). Thus, the sequence around −30 plays an important role in the initiation of the transcription by RNA polymerase II.

In the case of the fibroin gene, the first residue, T, of the T-A-T-A-A-A-A sequence can be deleted (actually replaced by G residue) without a severe reduction in transcription. The second and third residues are important because deletion of the A-T sequence results in a severe reduction of the fibroin gene transcription. In our system, deletion of T-A-T-A of the sequence almost abolished fibroin gene transcription; a small amount of fibroin gene transcript with a mislocated starting point was produced. A similar observation has been reported by Grosschedl and Birnstiel (14) who used microinjection of sea urchin histone gene into *Xenopus* oocyte nuclei. Thus, the sequence around the T-A-T-A-A-A-A sequence not only controls the efficiency of transcription but also seems to play a role in the selection of the accurate starting point. Although 5′Δ −22 and −26 did not support faithful transcription, 5′Δ −18 supported an intermediate level of faithful transcription. This would be explained by the fact that the pBR322-derived nucleotide sequence G-A-T-A-A-G which is similar to the Hogness box sequence T-A-T-A-A-A (−30 to −25) appears at −29 to −24 on 5′Δ −18 DNA [note that the T residue at −30 of wild-type gene can be replaced with G without severe reduction of faithful transcription (Fig. 3*A*).

It has been noted that there is one more region of homology, although not so strong, upstream from the Hogness box (about −70 to −80) (4, 15, 16). Especially in the case of the fibroin gene, the DNA sequence at −85 to −71 is similar to the prokaryotic −35 region sequence (2). The results from analysis of the 5′ Δ mutant suggest that this conserved sequence is not so crucial for faithful transcription *in vitro*. This region can be replaced by the nucleotide sequence of the pBR322 origin without any effect on faithful transcription. This situation might be similar to that of the −35 region of prokaryote, which is important for the transcription but can be replaced by other sequences without loss of promoter activity (17).

The 3′ boundary of the fibroin gene promoter is determined to be around +6. Thus, several nucleotides at the 5′ end sequence of the gene are essential for efficient transcription. The 3′Δ mutants, which are completely lacking the transcribable region but still contain the T-A-T-A-A-A-A sequence of the fibroin gene, can produce specific transcripts although they do so inefficiently. These transcripts seem to have starting points on the pBR322 sequence about 30 bases downstream from the nucleotide position −30 of the fibroin DNA. This result is consistent with the inference deduced from the analysis of the 5′Δ mutants that the sequence around T-A-T-A-A-A-A plays a role in the selection of the starting point of the transcription. A similar observation has been obtained by Corden *et al.* (9). The recognition of the T-A-T-A box sequence by transcription machinery would put the transcription initiation about 30 bases downstream.

Thus, the distance between the T-A-T-A box region and the region around the starting point, both of which are essential for efficient initiation, seems to be important. This is consistent with the fact that, among many eukaryotic genes, the T-A-T-A box sequence is found at the highly conserved position around −30.

Although the nucleotide sequence around −29 is shown to be important, the template DNA always contained a common pBR322 sequence of about 484 base pairs at the 5′ side of the fibroin gene flanking sequence. Our result indicates that the 5′ flanking sequence of 74 bases on the noncoding strand (70 bases on the coding strand) is enough for the transcription but 55 bases on the noncoding strand is not enough. This suggests that some DNA length but not specific sequence beyond −29 is required for efficient transcription.

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