Participation of the Upstream Region of the Fibroin Gene in the Formation of Transcription Complex In Vitro

MASAAKI TSUDA,† SUSUMU HIROSE,‡ AND YOSHIKI SUZUKI*

Department of Developmental Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan

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The addition of exogenous histones has an inhibitory effect on fibroin gene transcription in posterior silk gland extracts. The histones probably disturb a process in complex formation, because when transcription complexes were constructed by preincubation of the templates with the extracts, the inhibitory effect of histones was greatly reduced. Transcription of a fibroin gene construct, pFl5'Δ-238, having the upstream region beyond the TATA box was relatively less inhibited than that of pFl5'Δ-44 lacking the upstream region. This tendency toward differential inhibition was observed in the silk gland extracts but not in a HeLa cell extract and persisted even after complex formation in the silk gland extracts, suggesting a specific interaction of the upstream region with some factors in the extracts. The complexes formed on pFb5'Δ-44 are probably more susceptible to the inhibitory effect of histones. On the basis of these results we propose a participation of the upstream region of the fibroin gene in the formation of stable transcription complexes at the promoter through an interaction with specific factors in the silk gland. Since the transcription-enhancing effect via the upstream region is augmented at a high histone/DNA ratio, it may mimic the in vivo situation in which the fibroin gene can be transcribed in the posterior silk gland even in the presence of excess suppressive materials.

Recent analyses of transionalional elements of type II genes by using living cells have revealed an important role of the 5'-flanking region upstream from the TATA box. The elements localized in the upstream region seem to be responsible for exerting highly efficient transcription in most of the genes tested (1, 8, 9, 15). However, the mechanism of this transcription enhancement by these elements is poorly understood. We have prepared a homologous whole-cell extract from the posterior silk glands (20), where the fibroin gene is actively transcribed. In this extract a region covering about 200 base pairs (bp) of DNA sequences upstream from the TATA box of the fibroin gene enhances transcription efficiency (20, 21). The upstream sequence can function even in an inverted orientation (21), a feature which is analogous to that of the enhancing elements of other genes characterized in vivo (16, 23). Recently, we have also shown that the proximal portion of the upstream sequence of the fibroin gene probably functions as a species-specific enhancing signal and that the distal portion appears to function in a manner specific to the silk gland tissue (Y. Suzuki, M. Tsuda, S. Takiya, S. Hirose, E. Suzuki, M. Kameda, and O. Ninaki, Proc. Natl. Acad. Sci. USA, in press).

Using in vitro transcription systems, several groups have demonstrated that the formation of transcription complex at the promoter of type I, II, and III genes is always required before the onset of transcription (2-4). The complexes are probably converted from an immature state to a mature one by a sequential assembly of several factors. This assembly step seems to be critical in determining whether the gene is actively transcribed or not. From this aspect it is important to inquire how the upstream elements could participate in complex formation at the promoter. It has been demonstrated that complex formation at the control region of the Xenopus laevis 5S RNA gene can be largely influenced by exogenously added histones (2, 7). In this study, we also tested the effect of exogenously added histones on in vivo transcription of the fibroin gene, and found an interesting phenomenon that suggests a role for the upstream element in stabilizing the process of complex formation at the promoter.

MATERIALS AND METHODS

Plasmids, enzymes, and chemicals. Construction of pFb100 plasmid DNA containing a portion, from nucleotide positions −860 (HindIII) to +585 (BglII), of the genomic fibroin gene and of a series of 5′ deletion (Δ) mutant genes, 5′Δ-238, 5′Δ-167, 5′Δ-115, 5′Δ-77, 5′Δ-52, 5′Δ-44, and 5′Δ-2, containing a portion from the respective nucleotide positions −238, −167, −115, −73, −52, −44 and −2 to +585 (BglII) of the genomic fibroin gene, has been described previously (21, 22). pFl10k has a portion of the fibroin gene (−860 to +10), whose gene body downstream from +10 was replaced by a portion (BglII to PvuII) of the herpes simplex virus thymidine kinase gene (Y. Tsujimoto, unpublished data). This pFl10k DNA was used as a transcription competitor; the transcripts synthesized on pFl10k DNA are excluded from the hybridization assay used in this report and do not disturb the analysis of transcripts on the 5′ deletion mutants of fibroin gene. Construction of rFb38, which carries the coding strand of the fibroin gene between positions −238 and +514 in single-stranded fl bacteriophage DNA, was described elsewhere (11). S1 nuclease and calf thymus histone preparation (type II) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The histone preparation was able to support nucleosome formation on DNA, at least in part, through a urea-salt step dialysis (unpublished data).

In vitro transcription. A HeLa cell extract was prepared by the procedure of Manley et al. (13). Silk gland extracts were prepared from the posterior silk glands on day 2 of the fifth larval instar as reported previously (20). The transcription reaction and analysis of products also have been described previously (20), except that fibroin gene plasmid...
DNAs in the covalently closed circular form were used as templates. When a preincubation step was included, incubation was first carried out in the absence of nucleoside triphosphates (NTPs; ATP, GTP, CTP, UTP, and \( \alpha^-32P\)UTP) at 30°C for various periods, and then the transcription reaction was started by the addition of NTPs, followed by further incubation of 30 min at 30°C. When indicated, histones were added simultaneously with NTPs or after the preincubation. To measure total RNA synthesis, the radioactivities of acid-insoluble materials trapped on a glass fiber filter (Whatman GF/C) were counted in a toluene scintillator by sampling the reaction mixture.

**S1 nuclease mapping.** S1 nuclease mapping was carried out as described previously (11). Materials after S1 digestion were subjected to 5% polyacrylamide gel electrophoresis. After autoradiography, the bands of radioactive transcripts were cut out from the gel, and their radioactivities were measured by Cerenkov counting.

**RESULTS**

**Upstream region responsible for transcription enhancement.** In this study, we used supercoiled DNA as a template to obtain a higher transcription efficiency of the fibroin gene (11), instead of using a truncated linear DNA (21). The relationship between template activity and template form in the silk gland extract has been described elsewhere (11). Adopting a modified S1 nuclease mapping method, in which the labeled transcripts were hybridized with the coding-strand DNA probe, we first demonstrated that a broad 200-bp region upstream from the TATA box enhanced fibroin gene transcription in the silk gland extract (Fig. 1A and B). The result is essentially the same as that we obtained earlier with a linear DNA template (21).

The levels of total transcription on 5'A-238, 5'A-44, 5'A-2, or pBR322 DNA were compared with that of specific transcription from the fibroin gene promoter which could be detected by S1 nuclease mapping. The radioactivities incorporated into the acid-insoluble fraction, which are mostly sensitive to a low concentration of \( \alpha^-32P\)UTP (5 \( \mu\)g/ml), changed in parallel with the specific transcriptions (Fig. 1C).

From these data we concluded that transcription initiation other than the specific one from the fibroin gene promoter is almost repressed on the supercoiled DNA templates.

**Differential inhibition of transcription by histones.** Simultaneous addition of histones to the silk gland extract at the start of the reaction resulted in a gradual inhibition of transcription on both 5'A-238 and 5'A-44 DNA as the ratio of histones to DNA increased (Fig. 2A). However, transcription on 5'A-238 DNA was relatively less inhibited than that on 5'A-44 DNA (Fig. 2A; Table 1). Since 5'A-238 was transcribed three to four times more efficiently than 5'A-44 DNA (see, for example, Table 1), 3.3 times more \( \alpha^-32P\)UTP was used in the reaction with 5'A-44 DNA to make the intensity of the radioactive transcripts comparable with that of 5'A-238 DNA. The tendency of this differential inhibition
by histones was clearly seen when the relative template activity of 5'Δ-238 DNA to 5'Δ-44 DNA was calculated (Table 1; see also the graph in Fig. 2A). In contrast, such an apparent differential inhibition between these two DNAs was not observed in a HeLa cell extract (Fig. 2B). Since any significant enhancing effect on transcription by the upstream region of the fibroin gene was not detected in the HeLa cell extract (20, 21), we infer that this differential inhibition by histones could be caused by the activities responsible for transcription enhancement of the fibroin gene in the silk gland extract.

**Effect of template preincubation on transcription inhibition by histones.** Several groups have already demonstrated that in the absence of NTPs, preincubation of DNA templates in crude cellular extracts or reconstituted transcription systems composed of partially purified transcription factors results in the formation of transcription complexes at the promoter (4, 5, 12, 18). We also obtained results indicating that transcription complexes can be formed during such a preincubation step that they can be stably maintained even when competitor DNAs are added after the preincubation step (data not shown). Even in the presence of histones, the specific transcripts became clearly detectable when both DNAs were preincubated with the extract (Fig. 3, lanes 3 to 5 and 8 to 10), while only a trace amount of transcripts was detected if the preincubation step was omitted (lanes 2 and 7). These results indicate that once transcription complexes are formed in the preincubation step, the inhibitory effect of histones drastically decreases. This suggests that histones probably inhibit the process of complex formation at the promoter.

A 15-min preincubation of 5'Δ-238 DNA with histones (Fig. 4, lane 2) further decreased the transcription efficiency when compared with a control in which histones were simultaneously added with the extract (Fig. 4, lane 1). This decrease of transcription efficiency was more severe with 5'Δ-44 DNA (lanes 3 and 4). These reductions were not due to activities affecting template conformation, such as a DNA-nicking activity, because the incubation of DNA with histones did not alter the conformation of supercoiled DNA (data not shown). In addition, preincubation of the extract with histones before the addition of DNA did not decrease

**FIG. 2.** Effect of simultaneous addition of histones with DNA at the start of incubation. Transcription on 5'Δ-238 DNA or 5'Δ-44 DNA was carried out in posterior silk gland extract (A) or HeLa cell extract (B). Histones were added at a ratio (wt/wt) of 0.5, 1, 1.5, or 2 to a fixed amount of DNA (40 μg/ml) from the beginning of the reaction. The ratio zero means that no histone was added. After determining the radioactivities of specific transcripts, we calculated and plotted the relative efficiency of transcription. Symbols: ○, 5'Δ-238 DNA; ●, 5'Δ-44 DNA; ▲, the specific transcript; △, the faithful transcripts which are continued along the entire plasmid region or unfaithful transcripts which are started somewhere other than the cap site; these transcripts give a protection band whose length covers all the hybridizable portion on the φ1Fo38 DNA probe, 752 bp for 5'Δ-238 DNA, or 558 bp for 5'Δ-44 DNA. In the transcription of silk gland extract (A), 3.3 times more [α-32P]UTP was used in the reaction of 5'Δ-44 DNA.

**TABLE 1.** Relative template activity of 5'Δ-238 DNA to 5'Δ-44 DNA in the presence of exogenous histones

<table>
<thead>
<tr>
<th>Ratio of histones/DNA*</th>
<th>5'Δ-238</th>
<th>5'Δ-44</th>
<th>Relative enhancement (5'Δ-238/5'Δ-44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>625</td>
<td>170</td>
<td>3.7</td>
</tr>
<tr>
<td>0.5</td>
<td>420</td>
<td>86</td>
<td>4.9</td>
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<tr>
<td>1.0</td>
<td>266</td>
<td>39</td>
<td>6.8</td>
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<tr>
<td>1.5</td>
<td>149</td>
<td>15</td>
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</tr>
<tr>
<td>2.0</td>
<td>70</td>
<td>3</td>
<td>23</td>
</tr>
</tbody>
</table>

* Weight/weight ratios. Zero means no addition of histones.

* Radioactivities of the 514-bp bands shown in Fig. 2A. For 5'Δ-44 DNA the measured radioactivities were divided by a factor of 3.3, because 3.3 times more [α-32P]UTP was added to the reaction.
**FIG. 3. Effect of template-preincubation before histone addition.**

The experimental schedule is illustrated in the top panel. After 5'Δ-238 DNA or 5'Δ-44 DNA (40 μg/ml) was preincubated in the extract for 4 (lanes 3 and 8), 9 (lanes 4 and 9), or 18 (lanes 5 and 10) min, transcription was carried out in the presence of histones (ratio, 2) for 30 min. Transcription was also started in the presence of histones without the preincubation step (lanes 2 and 7). After autoradiography (middle panel), the 514-bp bands were cut out, and the relative efficiency (%) of each transcription to that carried out in the absence of histones without the preincubation step (lanes 1 and 6) is plotted in the bottom panel. Symbols: O, 5'Δ-238 DNA; ●, 5'Δ-44 DNA.

transcription efficiency when compared with a control in which the extract was preincubated in the absence of histones (data not shown). This fact excludes the possibility that histones could directly inactivate transcriptional factors. These data support the notion that the binding of histones to DNA, probably at the promoter region, inhibits transcription.

**Differential inhibition observed after preincubation.** Complex formation appeared to be achieved efficiently once the preincubation was started (see the graph in Fig. 3). However, the recovery of transcription by an 18-min preincubation was significantly lower with 5'Δ-44 DNA than with 5'Δ-238 DNA (see the bottom panel of Fig. 3). To confirm this observation further, we carried out transcription in the absence or presence of histones after a preincubation of 20 min. A stronger inhibition by histones with 5'Δ-44 DNA than with 5'Δ-238 DNA could be observed even after the process of complex formation (Fig. 5), which suggests the decrease of intensity of specific bands from lane 1 to lane 2 with that from lane 5 to lane 6. Therefore, there appears to be some form of complexes which are stably maintained to support transcription even in the presence of histones, and another form of complexes whose transcriptional ability is easily inactivated by the addition of histones. On the basis of these results, we infer that the proportion of the unstable complex is higher with 5'Δ-44 DNA than with 5'Δ-238 DNA and that the higher template activity of 5'Δ-238 is probably because of a higher proportion of stable complexes.

An additional preincubation with histones for 8, 18, or 30 min before the addition of NTPs did not alter the level of transcription when compared with that obtained when histones and NTPs were added simultaneously after the preincubation step (Fig. 5B, compare lanes 10 to 12 with lane 9 for 5'Δ-238 DNA and lanes 14 to 16 with lane 13 for 5'Δ-44 DNA). This further supports the existence of such stable complexes on both templates and the much higher proportion of these complexes on 5'Δ-238 DNA.

**Timing of complex-inactivation.** Histones do not appear to exert their inhibitory effect on the elongation step because specific transcription on both DNAs after the preincubation step increases linearly at least up to 30 min (data not shown). When histones were used after the addition of NTPs to the preincubated templates, their inhibitory effect was greatly reduced on both templates (Fig. 5A, compare lanes 3 and 4 with lane 2 and lanes 7 and 8 with lane 6). An incubation of only 10 min in the presence of NTPs almost rescued the templates from inhibition by histones (Fig. 5A, compare lane 4 with lane 1 and lane 8 with lane 5). Since unstable complexes have been found to form stable complexes rapidly after the addition of NTPs (10), our results suggest that the effect of histones is probably at the step before stable complex formation.

**FIG. 4. Effect of the preincubation of histones with DNA.** After 5'Δ-238 DNA was preincubated under transcription reaction conditions in the presence (lane 2) or absence (lane 1) of histones (histones-to-DNA ratio, 1:1) for 15 min, posterior silk gland extract was added and incubated for another 30 min. To the control (lane 1), histones were simultaneously added with the extract. A similar experiment was done with 5'Δ-44 DNA (lanes 3 and 4), using 3.3 times more [α-32P]UTP. 514, 514-bp band.

**DISCUSSION**

**Mode of transcription inhibition by histones.** Exogenously added histones bind to DNA so that the DNA can be protected from micrococcal nuclease digestion, whether the silk gland extract is included or not (data not shown). Simultaneous addition of histones to the fibroin gene tem-
plate and the extract inhibited transcription (Fig. 2A), and prebinding histones to the DNA before the addition of the extract further decreased transcription efficiency (Fig. 4). Therefore, it seems plausible that histones inhibit transcription by binding to the promoter region as well as other regions of the DNA. A random occupation of promoters by histones could account for the transcription inhibition observed when histones were added at the start of the reaction (Fig. 2), because preincubation of the templates with the extract before the addition of histones recovered most of the transcription (Fig. 3). Under such an inhibitory condition where histones bind to DNA, the upstream region might rescue the complex formation at the promoter, giving rise to the differential inhibition between 5′Δ-238 and 5′Δ-44 DNAs.

However, the differential inhibition was clearly observed even when the preincubation step was included (Fig. 3 and 5A). It is difficult to explain this type of transcription inhibition only by a random occupation of the promoter by histones, because most of the complexes had already been constructed during the preincubation step (Fig. 3). Since no significant decrease of the transcription efficiency was detected with 5′Δ-238 and 5′Δ-44 DNA after a prolonged preincubation with histones (Fig. 5B), there appear to be some transcriptionally active complexes which are resistant to the inhibition of histones after the preincubation step. These results (Fig. 3 and 5A) imply that besides these transcriptionally active complexes, there are immature complexes which are readily inactivated by the addition of histones before transcription elongation begins (Fig. 5A). The extent of the differential inhibition observed under the preincubation condition (Fig. 3 and 5) seems to be large enough to account for that obtained by the simultaneous addition of histones at the start of the reaction (Fig. 2). Thus, the differential inhibition between 5′Δ-238 and 5′Δ-44 DNAs might primarily be caused by this mode of transcription inhibition by histones. Transcriptional factors assemble stepwise on the promoter in constructing a transcriptionally active complex through an immature one (4–6, 10). In our case, the transcription complexes that are readily inactivated by histones might represent earlier steps of the assembly and the stable complexes that are resistant to histones might represent later steps. The upstream region of the fibroin gene seems to promote or ensure the process of complex formation under inhibitory circumstances including the presence of histones.

The extent of transcription enhancement via the upstream region of the fibroin gene in the silk gland extracts varies from batch to batch. It tends to be higher when activities suppressing transcription are stronger (M. Tsuda and Y. Suzuki, unpublished data). Moreover, an increase of transcription enhancement via the upstream region is also observed when the concentration of DNA in the cell-free system is lowered (Suzuki et al., in press). This suggests that suppressing materials in the posterior silk glands can confer some degree of differential activity on various templates. Since endogenous histones were undetectable in the silk gland extracts (M. Tsuda, unpublished data), the presence of inhibitory substances other than histones is expected. Factors that suppress transcription of the chorion gene, but not of the fibroin gene, have been detected in silk gland extracts (Y. Suzuki, unpublished data). However, the nature of these inhibitory substances awaits further investigations.

**Possible role of the upstream region in vivo.** Mattaj et al. (14) have reported that an enhancerlike sequence of the...
Xenopus U2 gene facilitates the formation of stable transcription complexes when microinjected into Xenopus oocytes. Several protein factors which specifically bind to the upstream sequence of type II genes are also responsible for transcription enhancement in vitro (5, 17). We have also detected such activities in a partially purified fraction of the silk gland extract which enhance the transcription efficiency depending on the upstream region of the fibroin gene (Suzuki et al., in press). On the basis of these findings together with those described in the present paper, we can speculate that some protein factors that interact specifically with the upstream sequences of the fibroin gene could lead to or stabilize the formation of transcription complexes at the promoter. In the cell-free system with the silk gland extracts, a function of such specific factors could produce active complexes on the promoters of 5'-Δ-238 DNA more frequently than on those of 5'-Δ-44 DNA, giving rise to a higher template activity on 5'-Δ-238 DNA. This function associated with the upstream region of the fibroin gene appeared to become more prominent when histones were exogenously added to the extract (Table 1). Since we can imagine that inhibitory substances such as histones and other nonhistone proteins are abundantly associated with chromatin, factors interacting with the upstream sequences might, at least in part, play an important role in vivo in establishing active transcription complexes at the promoter. To clarify these points it is necessary to purify the components responsible for both the enhancement and the inhibition and to reconstruct a transcription system reflecting the in vivo regulation.

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LITERATURE CITED