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TGA1a, a tobacco DNA-binding protein, increases the rate of initiation in a plant *in vitro* transcription system

(RNA polymerase II/transcription activation/tobacco transcription factor/*Nicotiana tabacum*)

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ABSTRACT **We describe here a plant *in vitro* transcription system for class II promoters using wheat germ extract. In this system transcription is stimulated by TGA1a, a tobacco DNA-binding protein, and the stimulation is dependent on the presence of its cognate binding site upstream of the TATA box. Titration experiments showed that transcription initiation is more sensitive than transcription elongation to low concentrations of sarkosyl (*N*-lauroylsarcosine). At 0.07% sarkosyl, the formation of initiated complex is inhibited but transcription elongation is not. Under these conditions, events associated with a single round of transcription can be studied. We demonstrate that the time required for completing transcription of a 380-base-pair template is about 10 min. Addition of TGA1a increases the number of preinitiation complexes by ≈3-fold with no significant effect on the frequency of transcription initiation from a single complex or on the rate of RNA elongation. We anticipate that this *in vitro* system will be valuable for the elucidation of mechanisms that regulate transcription in plants.**

A major advance in the investigation of transcriptional control in animals has been the development of cell-free systems that mediate accurate transcription initiation on class II promoters (1, 2). These *in vitro* systems have been used to elucidate the functions of general (for review, see ref. 3) as well as gene-specific (for review, see ref. 4) transcription factors. In contrast to animal systems, transcription studies in plants have been hampered by the lack of a homologous *in vitro* system that can reconstruct some features of the transcription regulation observed *in vivo*.

Wheat germ extract (WGE) is enriched in RNA polymerase II (5) and, therefore, serves as a good source of this enzyme for *in vitro* transcription of plant class II promoters. Previously, WGE was shown to support accurate transcription initiation from the TC7 promoter of T-DNA (6). The extract also recognizes the TATA box of the cauliflower mosaic virus (CaMV) 35S promoter and initiates transcription at the correct start site (K.Y. and H.I., unpublished data). However, it was not known whether WGE would be able to mediate transcription regulation *in vitro* in response to the addition of a sequence-specific DNA-binding factor.

The activation sequence *as-1*, which contains a tandem repeat of a TGACG motif, is located between nucleotides −83 and −63 of the 35S promoter (7, 8). By using transgenic tobacco as an *in vivo* assay system, it was shown that *as-1* by itself can confer preferential expression of promoters in root (9). This result strongly suggests that *as-1* functions as a positive cis-regulatory element. ASF-1 is an *as-1*-binding factor present in tobacco nuclear extract (7). We have isolated tobacco cDNA clones that encode a DNA-binding protein for *as-1*, and this protein was named TGA1a (10). Based on the identity of their binding specificities for different TGACG-containing cis elements, we have suggested that TGA1a is a good candidate for ASF-1 (10). Because mutations in *as-1* that abolish the binding of both TGA1a and ASF-1 also cause a loss of *as-1* function *in vivo* (7, 10), TGA1a is likely to be a positive regulator of transcription through its binding to *as-1*. The combination of the *as-1* element and a TATA box constitutes one of the simplest transcription units to test for possible activation functions of TGA1a *in vitro*.

In this paper we describe the development of an *in vitro* plant transcription system that responds to the addition of a DNA-binding protein. Using this *in vitro* system, we show that TGA1a stimulates transcription by increasing the number of active preinitiation complexes.

MATERIALS AND METHODS

Construction of DNA Templates. A 69-base-pair (bp) DNA was synthesized that contained the CaMV 35S promoter (positions −44 to −4) and a polylinker consisting of *Bgl* II, *Hin*dIII, *Eco*RI, and *Xho* I sites at the 5′ end and an *Ssp* I site at the 3′ end. This DNA was inserted between the *Eco*RI and *Sac* I sites of plasmid p(C_2AT)19 (11) to obtain pP35. Synthetic DNA fragments (44 bp) containing a tandem repeat of either the wild-type or the mutant *as-1* were inserted between the *Bgl* II and *Eco*RI sites of pP35. Between the *Nde* I and *Bgl* II sites of these plasmids, a 268-bp fragment (−343 to −91 of the 35S promoter upstream region plus a linker sequence at −343 *Eco*RV–*Bgl* II; ref. 8) was inserted in the reverse orientation and the final constructs were named pUWDP and pUMDP, containing the wild-type (W) and the mutant (M) *as-1*, respectively. pP35 and p(C_2AT)19 are templates with and without the TATA box, respectively. The structures of the DNA templates are shown in Fig. 1*A*.

Gel Retardation Assays. These were carried out as described (10) except that 5% polyacrylamide gel was used. Each lane contained 0.4 ng of binding probe (4×10^4 cpm) and the indicated amount of purified TGA1a in 10 μl of binding buffer.

Preparation of WGE. Wheat germ used in this study was provided by Nisshin Flour Milling (Japan). The extract was prepared as described (6) except that the ammonium sulfate fractionation step and the final gel filtration step were modified as described below. To a supernatant (20 ml) obtained at the final step of Polymin-P fractionation (see ref. 6), 1.2 g of solid ammonium sulfate was added (15% saturation, final concentration). The solution was mixed and centrifuged. To the resulting supernatant, 2.3 g of solid ammonium sulfate was added (33% saturation), and the solution was mixed and centrifuged again. The pellet obtained by the second ammonium sulfate precipitation was dissolved in 0.8 ml of 10 mM Hepes/KOH (pH 8.0), containing 1 mM dithiothreitol. The solution was then centrifuged at 10,000 × *g* for 5 min in a

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Abbreviations: CaMV, cauliflower mosaic virus; WGE, wheat germ extract.

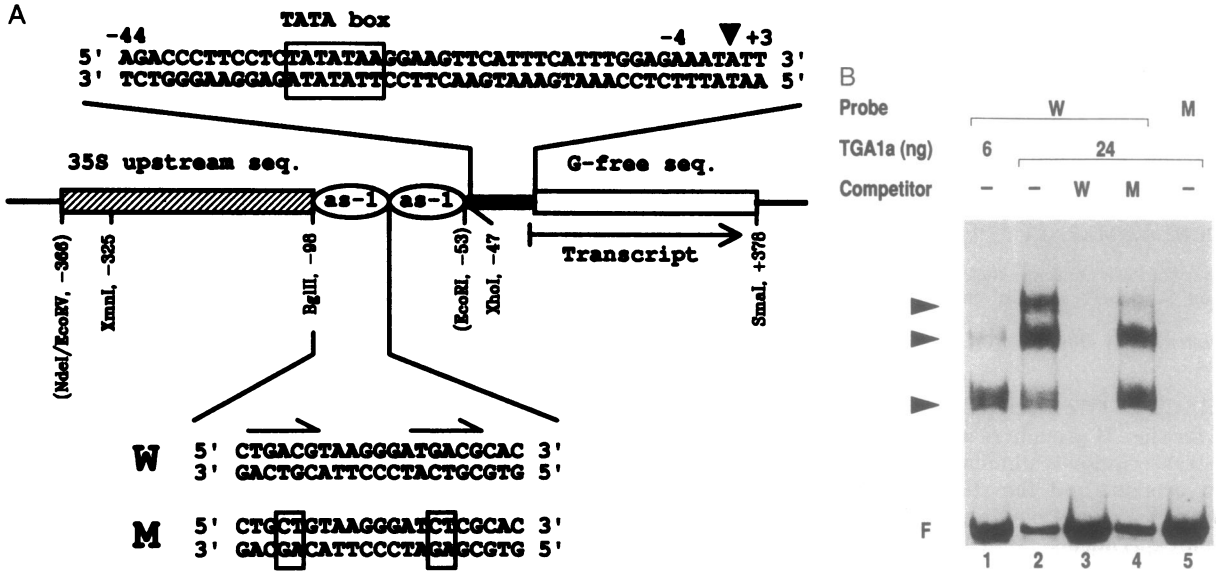

FIG. 1. DNA templates used for *in vitro* transcription and their interaction with TGA1a. (*A*) Structure of DNA templates. A tandem repeat of either the wild-type (W) or the mutant (M) *as-1* was placed upstream of the TATA-box region (−44 to −4) of the CaMV 35S promoter. Arrows indicate the repeat of TGACG motifs. Mutated nucleotides in the mutant *as-1* are boxed. The guanine (G)-free sequence and the fragment from the 35S promoter upstream region are indicated. Some restriction enzyme sites, and their positions with respect to the start site of this transcription unit, are indicated. Restriction sites that were lost during cloning processes are shown in parentheses. The transcription units were cloned into pUC13 between the *Nde* I and *Sma* I sites. A transcript of about 380 nucleotides is expected from the DNA templates. (*B*) Gel retardation assays of the upstream regions of the DNA templates. A DNA fragment from the upstream sequence of either the W promoter or the M promoter between the *Xmn* I and *Xho* I sites (−325 to −47; see *A*) was labeled and used as a binding probe. As competitors, double-stranded oligonucleotides containing a tandem repeat of either the wild-type (W) or the mutant (M) *as-1*, shown in *A*, were used at 1500-fold molar excess over the binding probes. −, No competitor; F, free DNA; arrowheads, specific DNA–protein complexes.

1.5-ml centrifuge tube. To remove metal ions and mononucleotides, the 10,000 × *g* supernatant was passed through an NAP-10 column (Pharmacia) preequilibrated with 10 mM Hepes/KOH (pH 8.0) containing 1 mM dithiothreitol. The eluate was centrifuged at 10,000 × *g* for 5 min, and 0.3 ml of glycerol was added to the supernatant and mixed. This extract was divided among 10 polypropylene centrifuge tubes (0.15 ml each) and the samples were frozen in liquid nitrogen and stored at −80°C until used.

***In Vitro* Transcription.** The standard mixture (50 μl) for the transcription assay contained 20 mM Hepes/KOH (pH 8.0), 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM $(NH_4)_2SO_4$, 0.4 mM ATP, 0.4 mM UTP, 0.04 mM CTP, 10 μCi of [α-^{32}P]CTP (specific activity, 400–800 Ci/mmol; 1 Ci = 37 GBq), 0.4 μg of DNA template, 10% glycerol, and 15 μl of WGE (final concentration, 0.27 mg of protein per ml). The assay mixture was incubated at 30°C for 20 min with or without the indicated amount of TGA1a. The reaction was terminated by the addition of 75 μl of "stop" solution (0.5% SDS/10 mM EDTA/150 mM sodium acetate with tRNA at 50 μg/ml). The mixture was extracted with a 1:1 (vol/vol) mixture of phenol and chloroform and the RNA was precipitated with ethanol. The precipitate was suspended in 5 M urea/1 mM EDTA/0.1% bromophenol blue, incubated at 95°C for 5 min, and loaded onto a 4% polyacrylamide gel containing 8 M urea. After electrophoresis, the gel was dried and subjected to autoradiography. The amounts of transcripts accumulated, corresponding to the bands marked by arrows in Figs. 2 and 5, were measured by densitometric scanning of the autoradiograms.

Purification of TGA1a. TGA1a was overproduced in *Escherichia coli* and purified by DNA-affinity column chromatography (F.K., K.Y., M. Horikoshi, R. G. Roeder, and N.-H.C., unpublished work).

RESULTS

Structure of DNA Templates. Fig. 1*A* shows the constructs we used as DNA templates in the transcription experiments. The constructs contained two copies of either the wild-type or the mutant *as-1* placed 5′ to the TATA box derived from the CaMV 35S promoter (W promoter and M promoter, respectively). Since supercoiled DNA appears to function better as a template when the 35S TATA box is used for transcription initiation (K.Y. and H.I., unpublished data), a guanine-free sequence (11) was employed in this study to facilitate the detection of the RNA products. Because we detected several TGA1a-binding sites in the vector sequence immediately upstream of the synthetic promoter, we inserted a fragment containing the −343 to −91 region of the 35S promoter, which does not contain any TGA1a-binding sites, in the reverse orientation immediately 5′ to the *as-1* site.

Sequence-Specific Binding of TGA1a to the Upstream Sequence of the Promoter. The ability of TGA1a to bind to the upstream promoter sequence (−325 to −47; see Fig. 1*A*) was tested by gel retardation assays. TGA1a used in this study was overproduced in *E. coli* with the T7 polymerase expression system (12) and purified by DNA-affinity column chromatography (F.K., K.Y., M. Horikoshi, R. G. Roeder, and N.-H.C., unpublished work). Fig. 1*B* shows that DNA–protein complexes (arrowheads) were obtained with the upstream sequence of the W promoter but not with that of the M promoter (compare lanes 2 and 5). Because the fragment contains four copies of the TGACG motif, more than one DNA–protein complex was observed at higher TGA1a concentrations (compare lanes 1 and 2). The wild type *as-1* showed very strong competition for all the complexes (lane 3), whereas the mutant *as-1* displayed only weak competition (lane 4). These results demonstrate that the upstream sequence of the W promoter contains specific binding sites of TGA1a and that the affinity for this factor is greatly reduced in the M promoter.

Sequence-Specific Stimulation of *in Vitro* Transcription. The W and M promoters were used as templates for *in vitro* transcription with WGE. Fig. 2 lanes 8 and 9 show the transcription products obtained with the circular DNA tem-

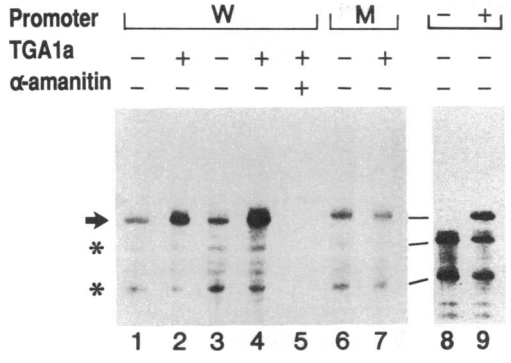

FIG. 2. *In vitro* transcription stimulation dependent on TGA1a and its cognate binding site, *as-1*. Lanes 1–7: transcription from either the W promoter or the M promoter was assayed with (+) or without (−) the addition of TGA1a (0.15 μg) or α-amanitin (1 μg/ml); 1 unit (1.65 μg) of RNA polymerase II from wheat germ (Sigma) was included for lanes 3 and 4. Lanes 8 and 9: transcription of templates with (+) or without (−) the 35S TATA-box promoter. The specific transcript (about 380 nucleotides) is marked by an arrow. Asterisks indicate transcripts arising from presumed TATA-like motifs in the guanine-free sequence.

plates with and without the 35S TATA box, respectively (see *Materials and Methods*). The production of the longest transcript (about 380 nucleotides; arrow) was dependent on the 35S TATA box. The other products may have been due to cryptic initiation sites or may have resulted from premature transcription termination in the guanine-free sequence. Among them, two major products (about 320 and 260 nucleotides; asterisks) were presumably due to cryptic initiation sites since they appeared to have been transcribed from TATA-like motifs in the guanine-free sequence. Synthesis of all of these transcripts was inhibited by a relatively low concentration of α-amanitin (1 μg/ml; lane 5), indicating that they were products of RNA polymerase II.

With the W promoter as the DNA template (Fig. 2, lanes 1–5), the addition of TGA1a increased the level of the longest transcript by 3- to 5-fold. This stimulation was observed with (Fig. 2, lanes 3 and 4) or without (lanes 1 and 2) the addition of purified wheat germ RNA polymerase II. In contrast, the addition of TGA1a suppressed the transcript level with the M promoter as the DNA template (Fig. 2, lanes 6 and 7; also see Fig. 5). These results clearly demonstrate that *in vitro* transcription from the promoter with *as-1* is stimulated by TGA1a. We have confirmed the reproducibility of these results by more than 10 independent experiments using at least five different preparations of WGE.

Separation of Transcription Initiation from Transcription Elongation. The results presented in Fig. 2 show that TGA1a increases the amounts of transcript synthesized from the W promoter. TGA1a could increase the transcript amount by increasing the number of active preinitiation complexes, the frequency of initiation from a single preinitiation complex, or the transcription elongation rate. To distinguish among these possibilities we made use of the previous observation in animal *in vitro* systems that sarkosyl (*N*-lauroylsarcosine) can block distinct steps in transcription reactions (13). We therefore examined systematically the effects of increasing concentrations of sarkosyl in our *in vitro* transcription system.

As outlined in the timetables of Fig. 3*A*, the DNA template and WGE were preincubated in the absence of nucleotides to arrest the reaction at the stage of preinitiation complex. After the complex was incubated with various concentrations of sarkosyl, ATP and UTP (for polymerizing the first three nucleotides, AUU, of the transcript) were added to convert the preinitiation complex to an initiated complex. The elongation reaction was resumed from the fourth nucleotide, C, by the addition of CTP. The effects of various concentrations of sarkosyl were investigated either before (timetable I) or after (timetable II) the addition of ATP and UTP. Fig. 3*B* shows that 0.06% sarkosyl completely inhibited the conversion from the preinitiation complex to the initiated complex (graph I). However, one-third of the transcriptional activity remained if sarkosyl (0.06–0.08%) was added to the reaction mixture after the polymerization of the first three nucleotides—i.e., after the formation of the initiated complex (graph II). Taken together with similar results in animal systems (13), it is likely that sarkosyl inhibits further transcription initiation when added after the formation of the initiated complex. If this interpretation is correct, the transcriptional activity that persists in the presence of 0.06–0.08% sarkosyl (Fig. 3, graph II) should represent the first round of transcription from the active preinitiation complexes formed during preincubation. In other words, the transcriptional activity obtained with this amount of sarkosyl should reflect the number of preinitiation complexes. Comparison of the time courses of the reactions with and without sarkosyl (Fig. 4, graphs 1 and 2) suggests that this concentration of the detergent has no significant effect on transcription elongation, because the kinetics of transcript accumulation during the first 5 min of these reactions are very similar. Fig. 4 graph 1 also shows that this first-round transcription is completed

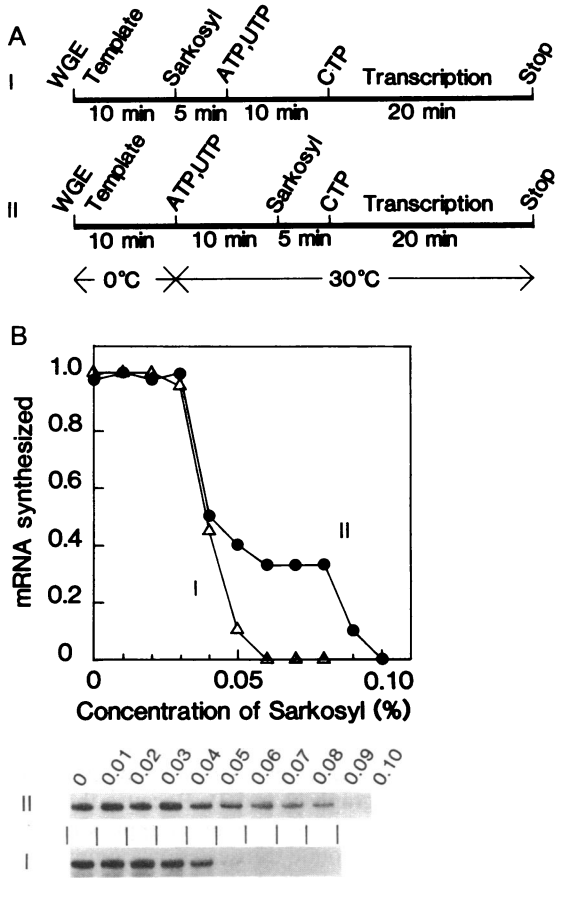

FIG. 3. Effect of sarkosyl concentration on the transcription reaction mixture. (*A*) Timetables for the transcription reaction mixtures I and II. (*B*) After preincubation of the DNA template (W promoter) with WGE, sarkosyl (0.01–0.10%) was added to the standard transcription reaction mixture before (I, △) or after (II, ●) transcription initiation. The intensity of each transcript band (about 380 nucleotides; corresponding to the band marked by an arrow in Fig. 2) on the autoradiogram (shown under the graph) was quantified by densitometric scanning. Amounts of RNA synthesized were expressed relative to that synthesized in identical reaction mixtures in the absence of sarkosyl.

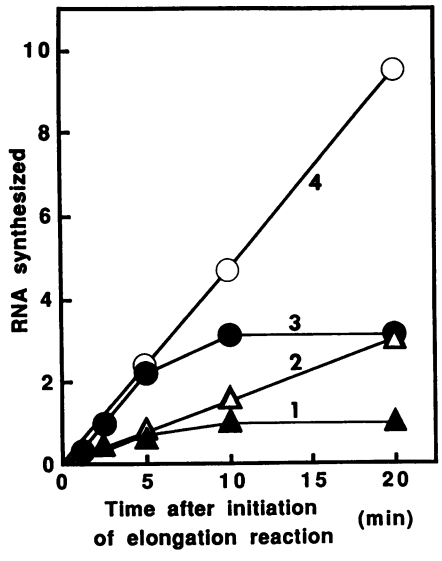

FIG. 4. Effect of TGA1a on single- and multiple-round transcriptions. DNA templates (W promoter) were preincubated with WGE in the presence (graphs 3 and 4) or absence (graphs 1 and 2) of TGA1a (0.15 μg per assay). Sarkosyl (final concentration, 0.07%) was added after transcription initiation as shown in timetable II of Fig. 3*A* (graphs 1 and 3) or was not added at all (graphs 2 and 4). After addition of CTP, transcription elongation was terminated by adding the stop solution at the times indicated. Amounts of specific transcript (about 380 nucleotides) synthesized were expressed relative to that synthesized in the reaction with sarkosyl and without TGA1a in 20 min.

within 10 min, since transcript accumulation reaches a plateau after 10 min. These two characteristics in the time course of transcript accumulation are also conserved when the reaction mixtures contain TGA1a (Fig. 4, graphs 3 and 4). Taken together, these results indicate that regardless of TGA1a, 0.07% sarkosyl inhibits transcription reinitiation but not transcription elongation. In either the presence or the absence of TGA1a, multiple rounds of transcriptions result in an ≈3-fold higher amount of transcripts accumulated than a single round of transcription after 20 min (compare graphs 1 and 2 or 3 and 4 in Fig. 4). This 3-fold difference implies that, on the average, three rounds of transcription are completed in 20 min in the absence of sarkosyl.

Increased Formation of Transcription Preinitiation Complex in the Presence of TGA1a. We exploited the differential sensitivity of the two transcription steps to 0.07% sarkosyl to characterize single-round transcription in our system. Using the program given in timetable II of Fig. 3*A*, we determined the amounts of transcripts synthesized 1, 2.5, 5, 10, and 20 min after the resumption of transcription elongation by the addition of CTP. Fig. 4 shows the effects of TGA1a on single- and multiple-round transcriptions. Addition of TGA1a increased the amount of transcript in single-round transcription by ≈3-fold (compare graphs 1 and 3). Because this stimulation rate in single-round transcription is approximately the same as that in multiple-round transcription (compare graphs 2 and 4), we conclude that TGA1a stimulates transcription principally by increasing the number of active preinitiation complexes (for details see *Discussion*).

Effect of TGA1a Concentration on Transcription Initiation. We next examined the effect of TGA1a concentrations on transcription. Fig. 5 shows that with the W promoter, transcription increased with increasing concentration of TGA1a up to 0.15 μg per assay and no further increase was seen at higher concentrations. In the case of the M promoter, the transcript level gradually decreased with increasing TGA1a concentration.

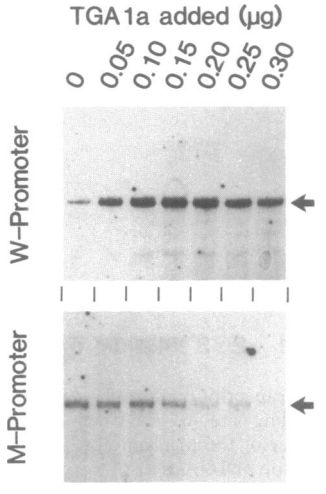

FIG. 5. Effect of TGA1a concentration on a single round of transcription. DNA templates (W promoter and M promoter) were incubated with WGE and various concentrations of TGA1a. Sarkosyl (0.07%) was added after transcription initiation as shown in timetable II of Fig. 3*A*. Twenty minutes after addition of CTP, transcription elongation was terminated by adding the stop solution. Bands corresponding to the specific transcript (about 380 nucleotides) are marked by arrows.

DISCUSSION

Previous work has shown that WGE is able to support accurate transcription of the TC7 promoter (6) and the 35S promoter of CaMV (K.Y. and H.I., unpublished data). In this paper, we demonstrate that under appropriate conditions transcription in the *in vitro* system is responsive to the addition of the tobacco DNA-binding protein TGA1a (10). The stimulatory effect of TGA1a is sequence-specific, since only the promoter containing its cognate binding site (*as-1*) can respond. Transcription (marked by asterisks in Fig. 2) from presumed TATA-like motifs in the guanine-free sequence is not stimulated by the addition of TGA1a. This observation may imply that the effect of TGA1a is not transduced to a presumed TATA-like motif at about 150-bp downstream from its binding sites beyond the 35S TATA box. Therefore, transcription activation in this case may require a close interaction between TGA1a and the RNA polymerase complex.

Mikami *et al.* (14) have reported that wheat germ contains a DNA-binding activity (HBP-1b) specific for *as-1*. We did not detect any significant difference in transcript accumulation between the W promoter (Fig. 2, lane 3) and the M promoter (lane 6) in the absence of added TGA1a. Our results therefore suggest that WGE prepared by the method described here does not contain a significant amount of endogenous transcription activator specific for the *as-1* element.

Our *in vitro* transcription mixture contains DNA template and mononucleotides in saturating concentrations. In preliminary optimization experiments we found that the concentration of RNA polymerase II may be the rate-limiting factor in the reaction mixture. To increase the basal transcription level, we supplemented the WGE with purified wheat RNA polymerase II (Sigma) in one series of experiments (Fig. 2). The RNA polymerase II-supplemented WGE gave a higher transcription efficiency than the WGE alone. Moreover, the system retained accurate transcription initiation and was responsive to TGA1a.

To further characterize the function of TGA1a, we attempted to separate transcription initiation from the subsequent reactions of RNA chain elongation. We found that these two steps can be resolved by the anionic detergent sarkosyl (Fig. 3*B*). At sarkosyl concentrations >0.06%, the

formation of the initiated complex was completely inhibited. However, the reactions subsequent to transcription initiation were unaffected even at 0.08% sarkosyl. We therefore used 0.07% sarkosyl to prevent the formation of new initiated complex without affecting the subsequent steps of the first round of transcription. At this sarkosyl concentration, it is then possible to study events for a single round of transcription.

We investigated the molecular mechanism of TGA1a activation of transcription by comparing its effect on single- and multiple-round transcriptions. TGA1a does not appear to affect elongation rate since the time required to complete a single round of transcription is not changed by the addition of TGA1a (Fig. 4, graphs 1 and 3). Thus TGA1a should stimulate transcription principally by increasing the number of preinitiation complexes and/or by increasing the frequency of initiation from a single complex. We found that the addition of TGA1a stimulates transcription by approximately the same rate in both single- and multiple-round transcriptions (compare graphs 1 and 3 or 2 and 4 in Fig. 4). Similar results were obtained with varying concentrations of TGA1a (data not shown). Because the amount of transcripts in a single round of transcription is correlated with the number of preinitiation complexes, these observations suggest that TGA1a stimulates transcription mainly, if not exclusively, by facilitating the formation of preinitiation complexes. Indirectly the results also indicate that it is unlikely that TGA1a changes the initiation frequency.

We observed a decrease in the transcript level at higher concentrations of TGA1a (0.2 μg per assay) with both the W and the M promoter (Fig. 5). This decrease may be explained by "squelching" (15), in which the activation domain of unbound TGA1a titrates out basic transcription factors or RNA polymerase II in the reaction mixture.

In the current work we have demonstrated that WGE can support transcription activation *in vitro* that is dependent on TGA1a and its cognate binding sites. Extension of the *in vitro* transcription system we have developed here to the investigations of other combinations of DNA-binding proteins and their cognate binding sites would be useful for the elucidation of the mechanism of transcription regulation in plants. This system will enable the direct functional assay of not only sequence-specific DNA-binding proteins but also general transcription factors.

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