A plant DNA-binding protein increases the number of active preinitiation complexes in a human in vitro transcription system

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TGAla is a tobacco DNA-binding protein that binds to the activation sequence-1 (as-1) element of the cauliflower mosaic virus 35S promoter. We have produced TGAla in Escherichia coli, purified it from bacterial extracts, and examined its effect on transcription in a human in vitro system. Addition of TGAla stimulates transcription by up to 20 times, and the stimulation is dependent on the presence of the as-1 element in the promoter. When transcription reinitiation is inhibited by 0.3 M KCl, activation is similar. Therefore, TGAla activates transcription by increasing the number of active preinitiation complexes. After formation of the preinitiation complexes in the presence of TGAla, oligonucleotides containing TGAla-binding sites do not significantly affect the stimulated level of transcription. This result indicates that a complex remains committed to the promoter site after initiation and that this complex is used repeatedly during several initiation events. Our study demonstrates for the first time that a plant factor can activate transcription in a human in vitro system and that the activation mechanism of the plant factor is similar to that of mammalian factors.

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cDNA clones obtained from screening a cDNA expression library with a probe containing the TGACG motif (Katagiri et al. 1989). On the basis of its binding specificities to different TGACG-containing cis-elements, TGA1a was suggested by us to be a good candidate for ASF-1. The high level of TGA1a mRNA in roots is consistent with the notion that this DNA-binding protein is involved in the in vivo function of as-1 that confers preferential expression in root (Katagiri et al. 1989). Because as-1 is a positive cis-regulatory element, TGA1a should be a transcription activator. Recently, we demonstrated that TGA1a functions as an activator in a wheat germ in vitro transcription system (Yamazaki et al. 1990).

Compared with in vivo assay systems, the analysis of a transcription activator using a well-characterized in vitro system allows the investigation of the activation mechanisms at the molecular level (Sawadogo and Roeder 1985a; Abmayr et al. 1988; Hai et al. 1988; Hori-koshi et al. 1988a,b; Workman et al. 1988, 1990, Klei-n-Hitpass et al. 1990). Among the various RNA polymerase II in vitro transcription systems, the one based on HeLa cell nuclear extracts is the best characterized (Dignam et al. 1983a,b). In this system, general transcription factors, TFII B, TF II D, and TFII E/F, are necessary for transcription initiation in addition to RNA polymerase II (for review, see Saltzman and Weinmann 1989).

We have investigated the function of the plant DNA-binding protein, TGA1a, in a HeLa cell in vitro transcription system reconstituted with partially purified fractions of the TFII B, TFII D, TFII E/F, and RNA polymerase II. Our results show that TGA1a can function as a transcription activator in the human in vitro transcription system and that it facilitates the formation of active preinitiation complexes.

Results

Overproduction of TGA1a in Escherichia coli

TGA1a was overproduced in Escherichia coli by the T7 expression system (Rosenberg et al. 1987). The coding region of TGA1a was cloned downstream of the T7 promoter in the expression vector pET3a (Rosenberg et al. 1987) to obtain pKT7T1A. The plasmid pKT7T1A was designed to express the full-length TGA1a protein by utilizing a Shine-Dalgarno sequence in pET3a. Sequence analysis of TGA1a genomic clones revealed that the published coding region of TGA1a (Katagiri et al. 1989) was short by 2 bases in the 5′ region (H. Fromm, unpubl.). Therefore, we reconstructed the missing two nucleotides, AT [first two nucleotides of the methionine codon ATG], as described in Materials and methods. The coding region of TGA1a is 1119 bp long, encoding 373 amino acid residues. The expression of TGA1a can be induced by the addition of IPTG in the medium. Figure 1a shows the total protein profile of the bacteria before and after IPTG induction (lanes 1 and 2). After the induction, the amount of TGA1a (band I) constitutes ~3% of the total protein. The induced cells were used as the starting material for the purification of TGA1a.

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Figure 1. Overproduction and purification of TGA1a. (a) Total protein profiles of E. coli-expressing TGA1a. Total bacteria proteins [40 μg/lane] were separated by 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. (Lane 1) Before induction, (lane 2) after induction. In lane 2, the arrowhead indicates the polypeptide that increased in concentration after induction. (b) Purified TGA1a. A total of 10 μg of the purified TGA1a was analyzed by 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. The three bands are marked as bands I, II, and III in order of decreasing molecular weight. (c) Southwestern blot analysis of purified TGA1a. The purified TGA1a fraction [10 μg/lane] was separated by 10% SDS-PAGE, and the proteins were blotted onto a nitrocellulose membrane that was probed with a labeled hex-1 sequence. (Lane 1) No competitor, (lane 2) HW added as a competitor, (lane 3) HM added as a competitor. The positions of the polypeptide bands in a, b, and c are directly comparable. Migration positions of molecular weight markers are indicated on the right. (d) Amino-terminal sequences of bands I, II, and III. Amino-terminal sequences of bands I, II, and III [analyzed] are compared to the corresponding amino acid sequences deduced from the DNA sequence of TGA1a cDNA clone (deduced) (Katagiri et al. 1989). Comparison of the sequence of band I and the deduced sequence suggests that this band likely contains a mixture of the full-length TGA1a and the same polypeptide but without the first methionine. The sequence of band II suggests that it contains a truncated TGA1a polypeptide with its amino terminus at position 42. The sequence of band III suggests that it contains a mixture of two truncated TGA1a polypeptides with amino termini at positions 56 and 57. The schematic diagram at the bottom shows the relative positions of the translation start sites of the three bands in the TGA1a protein. The acidic region, the basic region, the leucine [L]-zipper region, and the glutamine [Q]-rich region are shown. The amino acid sequences are shown in single-letter code. (dS) Dehydroserine. Amino acids detected in the same sequencing cycles are aligned perpendicularly. However, the first amino acids of the analyzed sequences are not trustworthy, because the first sequencing cycle is often contaminated by irrelevant amino acids. The numbers indicated over the methionine codons of the deduced sequences represent the positions of the methionine codons with respect to the first methionine.
Purification of TGA1a

TGA1a was purified as described in Materials and methods. Table 1 shows each step of the purification procedure. We obtained 1.7 mg of TGA1a from a 2-liter bacterial culture at >95% purity as assessed by SDS-PAGE (Fig. 1b).

The purified TGA1a fraction contained three molecular species when analyzed by SDS-PAGE (Fig. 1b). Amino-terminal sequence analyses of these polypeptides suggested that the three bands correspond to the products of the TGA1a gene with three different amino termini in the same reading frame (Fig. 1d). Bands I, II, and III corresponded to the products that start at Met-1, Met-41, and Met-56, respectively. It is likely that Met-41 and Met-56 were used as cryptic translation start sites in E. coli.

The specific binding abilities of the three polypeptides were examined by Southwestern blot (Fig. 1c). All three polypeptides bound to the probe containing hex-1 of the wheat histone H3 promoter [Mikami et al. 1987], a region containing a specific binding site for ASF-1 and TGA1a [Katagiri et al. 1989]. It is not clear why band I gave only a weak signal, even though it was the most abundant species [see Discussion]. Binding of all three polypeptides was sequence specific; the binding was competed out by the addition of an oligonucleotide containing the wild-type hex-1 [HW] but not by an oligonucleotide containing the mutant hex-1 [HM]. HW bound TGA1a, but HM did not (see Materials and methods). This mixture of three different polypeptides was used as the purified fraction of TGA1a in subsequent experiments.

Transcription activation in a human in vitro system

The function of the purified TGA1a was assayed in a human in vitro transcription system reconstituted with partially purified fractions of TFIIIB, TFIIID, TFIIIE/F, and RNA polymerase II from HeLa cell nuclear extracts [Hai et al. 1988; Horikoshi et al. 1988b]. Hereafter, we refer to these components as the HeLa general factors. The DNA templates contained two copies of either the wild-type or the mutant as-1 sequence placed upstream of the 35S TATA box region [designated wild-type and mutant promoters, respectively; Fig. 2a]. The mutant derivative of as-1 has a very low affinity for TGA1a [Katagiri et al. 1989; Yamazaki et al. 1990]. To facilitate detection of specific transcripts, a 380-bp G-free sequence [Sawadogo and Roeder 1985b] placed 3' to the TATA box region was used as the template for RNA polymerase II.

Figure 2b shows that with the wild-type promoter, addition of 50 ng of TGA1a per assay increased transcription by ~10 times [lanes 1 and 2]. Addition of the same amount of TGA1a gave only a small stimulation with the mutant promoter [lanes 3 and 4]. This slight stimulation could result from the greatly reduced binding affinity of TGA1a for the mutant as-1. Thus, our data clearly demonstrate that TGA1a functions as a sequence-specific transcription activator in the HeLa in vitro transcription system.

We consistently detected a difference in the basal transcription level between the wild-type and mutant promoters [Fig. 2b, lanes 2 and 4]. The reproducibility of this difference suggests that a TGA1a-like activity is present in the HeLa general factors. In fact, our preparation of TFIIIB and TFIIID fractions showed specific binding activities to the hex-1 element when analyzed by gel retardation assays [data not shown]. To deplete the binding activities, the HeLa general factors were treated with the oligonucleotide HW. We found that the addition of 10 ng of HW per assay was sufficient to reduce the apparent high basal transcription level of the wild-type promoter [data not shown]. Figure 2c shows the results obtained with the depleted HeLa general factors. With this mixture, the basal transcription level of the wild-type promoter was reduced to the same level as that of the mutant promoter [Fig. 2c, lanes 2 and 4], whereas the transcription stimulation by TGA1a [50 ng/assay] remained at a similar level [cf. lanes 1 in Fig. 2b and c]. In contrast to the results obtained with the untreated HeLa general factors, no transcription activation

### Table 1. Purification of TGA1a

|       | Total volume [ml] | Total protein [mg] | Total activity $\times 10^{-6}$ [unit]$^\ast$ | Specific activity $\times 10^{-3}$ [U/mg]$^\ast$ | Yield [%] | Purification [-fold] |
|-------|------------------|--------------------|---------------------------------------------|---------------------------------------------|-----------|---------------------|
| Crude extract | 92 | 330 | 180 | 5.4 | 100 | 1 |
| (NH$_4$)$_2$SO$_4$ fraction | 4.7 | 120 | 80 | 6.5 | 44 | 1.2 |
| DE-52 | 13 | 48 | 94 | 20 | 52 | 3.7 |
| P11 | 4.2 | 8.0 | 59 | 73 | 33 | 14 |
| Mutant DNA affinity$^\ast$ | 21 | 3.3 | 27 | 82 | 15 | 15 |
| Wild-type DNA affinity$^\ast$ | 19 | 1.7 | 30 | 170 | 17 | 32 |

Starting from 6 grams [wet weight] of BL21[DE3]/plysS/pKT7T1A.

$^\ast$One activity unit is defined as the amount of activity that binds to 3% of the hex-1 binding probe [1.4 femoles] under standard assay conditions [Katagiri et al. 1989].

$^\ast$One-third of the total sample was applied to the DNA affinity columns. However, the values shown here were recalculated for the total sample.
Accurate transcription initiation from the 35S promoter in the HeLa cell system

Because the human in vitro transcription system is heterologous to the 35S promoter, we wished to ascertain whether accurate transcription initiation is retained in this system. We found that transcription initiation from the 35S promoter in the HeLa cell system (nucleotide A circled with a thick line in Fig. 4) occurred at the same site as in transgenic tobacco with respect to the position of the TATA box (Odell et al. 1985), although in the DNA template the sequence of the 35S promoter downstream of −4 is different from that of the authentic 35S promoter (Fig. 2a). Addition of TGA1a did not alter the major transcription start site; however, a minor initiation site became apparent, which was ∼30-fold weaker than the major site (Fig. 4, lane 5). Comparison of the signal strengths (Fig. 4, lanes 5 and 6) shows that TGA1a increased transcription by ∼20-fold. This amount of stimulation was similar to that obtained using the G-free sequence (Fig. 3). Because there were no other significant initiation sites between −60 and +90 (only a part of this region is shown in Fig. 4), we conclude that most transcripts shorter than 380 nucleotides, which are observed in the assays using the G-free sequence (e.g., Fig. 2c, lane 1), probably result from premature transcription termination (Sawadogo and Roeder 1985a; Carcamo et al. 1989).

by TGA1a was seen with the mutant promoter using the depleted HeLa general factors (cf. lanes 3 and 4 in Fig. 2b and c). In subsequent experiments, the depleted HeLa general factors were used.

The transcription activation level was measured at different concentrations of TGA1a (Fig. 3). With 100 ng per assay of TGA1a, we reproducibly observed 8- to 20-fold stimulation in >10 independent experiments. The amounts of transcript showed an approximately linear increase with increasing amounts of TGA1a. This result suggests that within the concentration range examined, TGA1a does not appear to activate transcription cooperatively.

Figure 2. TGA1a activates transcription in a HeLa in vitro system. [a] Structures of DNA templates used for in vitro transcription. Two copies of either the wild-type (W) or mutant (M) form of as-1 were placed upstream of the 35S TATA region (−44 to −4). The sequences of the wild-type (W) and mutant (M) forms of as-1 are shown. A tandem repeat of the TGACG motif is indicated by arrows in W, the mutations in M are boxed. The sequence of the 35S TATA box region is also shown. The numbers indicated over the sequence represent the nucleotide positions with respect to the determined transcription initiation site (arrowhead; see Fig. 4). The 35S TATA box is overlined. G-free sequence placed downstream of the TATA region generates a 380-nucleotide-specific transcript. Details of the templates are described elsewhere (Yamazaki et al. 1990). [b] Transcription activation by TGA1a using the untreated HeLa general factors. [c] Transcription activation by TGA1a using the depleted HeLa general factors. [W] Wild-type promoter; [M] mutant promoter; [+] addition of TGA1a (50 ng); [−] no addition of TGA1a. Arrowhead indicates the specific transcript of 380 nucleotides.

Figure 3. Effects of increasing amounts of TGA1a on transcription activation. [a] An autoradiogram showing the transcripts synthesized at different concentrations of TGA1a. Arrowhead indicates the position of the specific transcript. [b] The gel bands corresponding to the specific transcripts were excised, and their radioactivities were determined by a scintillation counter. The amount of radioactivity was used to represent the amount of transcription. [W] Wild-type promoter; [M] mutant promoter.
Figure 4. Transcription initiation sites of the CaMV 35S promoter in the HeLa in vitro system. Transcription initiation sites in the in vitro system were determined by primer extension. The results of transcription reactions with [100 ng/assay] or without TGAla are shown in lanes 5 and 6, respectively. A sequence ladder of the wild-type promoter obtained with the same primer used in the primer extension is shown in lanes 1–4 as a reference. The sequence of the top strand shown in Fig. 2a is indicated on the left. The major and minor transcription initiation sites are circled with a thick line (+1) and a thin line (−2), respectively. The 35S TATA sequence is boxed.

TGA1a increases the number of preinitiation complexes

There are four possible ways that TGA1a could stimulate transcription: (1) by increasing the number of active preinitiation complexes; (2) by increasing the initiation frequency from a single preinitiation complex; (3) by increasing the elongation rate; or (4) by a combination of these possibilities. We examined whether TGA1a increases the number of preinitiation complexes under the condition of limited transcription reinitiation (Fig. 5). A high concentration of KCl (0.3 M) inhibits initiation but does not inhibit elongation in a HeLa cell RNA polymerase II transcription system (Cai and Luse 1987). Thus, when added just after the first round of initiations, 0.3 M KCl inhibits further initiation events, such that the number of transcripts corresponds to the number of active preinitiation complexes [i.e., one transcript is derived from one preinitiation complex]. We define single-round transcription as that observed under conditions in which one initiation event from each preformed preinitiation complex is allowed and multiple-round transcription as that observed when greater than a single round of transcription initiation is allowed. Although the experimental systems are not completely the same as that in this study, previous observations with the adenovirus major late promoter in HeLa cell in vitro systems showed that the first round of initiation from preformed preinitiation complexes is completed within 0.5 min after addition of nucleoside triphosphates (Hawley and Roeder 1985) and that subsequent rounds of initiations do not occur within 5 min (Hawley and Roeder 1987). In our experiments, the concentration of KCl was raised from 50 mM [standard assay conditions] to 0.3 M, 0.5 min after the initiation of the reaction. We assumed that this change in KCl concentration occurs early enough to exclude significant contributions from second rounds of initiation to the final amount of transcript.

In the analysis shown in Figure 5, preinitiation complexes were formed by preincubation of the DNA template with the HeLa general factors in the presence and absence of TGAla. The transcription reaction was then initiated by the addition of NTPs (Fig. 5a). Addition of KCl (final 0.3 M) at 0.5 min before the addition of NTPs (Fig. 5a, time point I) almost completely inhibited subsequent transcription initiation (Fig. 5b, lanes 1 and 2), whereas addition of the same concentration of KCl at 0.5 min after the addition of NTPs (Fig. 5a, time point II) did not. In this latter case, accumulation of the transcript terminated within 10 min (Fig. 5b, lanes 3–8). The only transcript that accumulated was 380 nucleotides (Fig. 5, lanes 3, 4, and 6–8). The appearance of only this full-length transcript suggests that our conditions the second round of transcription is indeed inhibited (Sawado-godo and Roeder 1985a; Carcamo et al. 1989). We found that 0.3 M KCl appeared to affect the elongation rate slightly (Fig. 5b, cf. lanes 5 and 11). After a 5-min incubation at 0.3 M KCl, the average length of the transcripts was ~310 nucleotides (Fig. 5b, lane 5). On the other hand, after a 5-min incubation at 50 mM KCl [standard assay conditions] most of the transcripts were full length (380 nucleotides; Fig. 5b, lane 11). Considering this difference in the transcript size [shorter transcripts contain less radioactivity], the number of transcripts produced after a 5-min incubation at 0.3 M KCl was approximately the same as that produced at 50 mM KCl. This similarity in the number of initiated transcripts at early times of the transcription reaction also supports the notion that a single round of transcription was observed in these experiments. At both KCl concentrations, the addition of TGAla caused about an eightfold stimulation of transcription [cf. solid and open squares for 50 mM KCl or solid and open circles for 0.3 M KCl in Fig. 5c]. Thus, the degree of stimulation by TGAla was almost the same under single- and multiple-round transcription conditions. This indicates that TGAla stimulates transcription principally by increasing the number of preinitiation complexes rather than by increasing either the initiation frequency from a single preinitiation complex or the elongation rate.

TGA1a-dependent preinitiation complexes remain committed during multiple-round transcription

Relevant to the mechanism of action of TGA1a, we then investigated whether the TGA1a-dependent preinitiation complexes remain committed to the initial templates during multiple rounds of transcription. If so, this
Figure 5. TGA1α stimulates a single round of transcription in vitro. (a) The timetable for the experiments. (H Factors) HeLa general factors; (NTPs) ribonucleoside triphosphates. (b) Transcription stimulation by TGA1α is observed in both single- and multiple-round transcriptions. The transcription reaction was carried out with (+, 100 ng/assay) or without (−) TGA1α. The final concentrations of KCl and the times when the concentration was changed from 50 mM KCl (standard conditions) to 0.3 M KCl are indicated. (t) Incubation time as indicated in a. Arrowheads indicate the 380-nucleotide-specific transcript. (c) The transcripts shown in b were quantified by measuring the amounts of radioactivity of the corresponding bands by a scintillation counter. The amount of radioactivity was used to represent the amount of transcription. (○) 0.3 M KCl at t, −TGA1α; (●) 0.3 M KCl at t, +TGA1α; (□) 50 mM KCl, −TGA1α; (■) 50 mM KCl, +TGA1α. Results obtained in the same experiment are shown.

would raise the possibility that TGA1α is required only transiently to stimulate transcription. For the study of template commitment of preinitiation complexes, it is desirable that the transcription system is capable of efficient reinitiation. As shown in Figure 5c, the absolute level of transcripts at 40 min postinitiation was eightfold higher under multiple-round transcription conditions than under single-round transcription conditions in both the presence and the absence of TGA1α (cf. solid square and circle, or open square and circle). This indicates that efficient reinitiation occurs under multiple-round transcription conditions (standard assay conditions). The occurrence of large amounts of discrete shorter transcripts under multiple-round transcription conditions (e.g., Fig. 5b, lane 14) also suggests efficient reinitiation, because these shorter transcripts are thought to represent the premature terminated products of latter rounds of transcription on the same G-free sequence template (Sawadogo and Roeder 1985a; Carcamo et al. 1989).

We first investigated the requirement of TGA1α before and after initial preinitiation complex formation by addition either of an oligonucleotide (HW) that contains a TGA1α-binding site or an oligonucleotide (HM) that does not bind TGA1α [Fig. 6]. Preinitiation complexes were formed, and the reaction was arrested at this stage by preincubation in the absence of NTPs. Transcription was initiated by the addition of NTPs, and the transcripts generated in 20 min were analyzed (see the timetable in Fig. 6). Lanes 1 and 2 show the level of transcript without and with transcription stimulation by TGA1α, respectively. The addition of 200 ng of HW per assay before the preincubation abolished the transcription stimulation by TGA1α [lane 3], but the same amount of HM decreased the transcript only slightly [lane 4]. In contrast, neither HW nor HM significantly affected the activation by TGA1α when added after the preincubation [lanes 5 and 6; NTPs were added 5 min after the addition of the oligonucleotides]. These results show that TGA1α is required during the formation of preinitiation complex to stimulate transcription, as expected from the above-described action of TGA1α in increasing the number of preinitiation complexes. They also raise the possibility that TGA1α may not be needed in the subsequent steps, because the addition of HW after preinitiation complex formation did not significantly affect the overall level of transcription activation. However, we cannot exclude the possibility that TGA1α may persist and/or be required after preinitiation complex formation because it could form a very tight complex [with the HeLa general factors] that becomes resistant to competition by HW. To assemble the HW-resistant complex,
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The purified TGA1α fraction contains three related polypeptides

We found that the purified fraction of TGA1α contained three polypeptides with different translation start sites (Fig. 1b). The two shorter polypeptides probably result from use of two other methionine codons (Met-41 and Met-56) as translation start sites (Fig. 1d) in E. coli. All of the three polypeptides are likely to be active in DNA binding, because they were purified by two different DNA affinity chromatography steps. However, the full-length TGA1α (Fig. 1, band I) showed only weak binding activity in Southwestern blot analysis (Fig. 1c). This result could be explained if there is differential renaturation of the three polypeptides after denaturation by SDS. We found that after denaturation in guanidine hydrochloride, the renatured full-length TGA1α barely recovered its binding activity. In contrast, a shorter derivative of TGA1α (containing only the DNA-binding domain) readily recovered most of its binding activity (K. Seipel, unpubl.).

We do not know whether all three TGA1α derivatives are equally active in transcription activation. Although the two shorter derivatives lack a portion of the acidic region that is presumably an activation domain, they retain the entire region of another putative activation domain, the glutamine-rich region (see below).

Transcription activation mechanism of TGA1α

Taking advantage of an in vitro transcription system, we demonstrated that TGA1α increased the number of preinitiation complexes and that the TGA1α-dependent preinitiation complexes remained committed to the templates under multiple-round transcription conditions. Figure 7 summarizes the activation mechanism of TGA1α. When preinitiation complexes are formed with HeLa general factors [H factors] on the template with the wild-type TGA1α-binding site [Template [W]], the number of complexes is increased by TGA1α. This step of activation is inhibited by oligonucleotide HW, which contains the TGA1α-binding site. It is not known whether TGA1α remains associated with the preinitiation complexes or not. Transcription is initiated by the addition of ribonucleoside triphosphates [NTPs], and the preinitiation complex is changed into an elongation complex. This initiation step is inhibited by 0.3 M KCl. For reinitiation of transcription, some part of the preinitiation complex that remains committed to the template after the first round of initiation is reused for the next round of initiation. This step circumvents the HW-sensitive step.

It is of interest to know which factors are involved in the part of the preinitiation complex that remains committed to the template and is reused for reinitiation of transcription. The absence of TGA1α in this complex would indicate that TGA1α is required only transiently to increase the number of initial preinitiation complexes and that the resulting preinitiation complexes can initiate transcription several times. In studies of the adenovirus major late promoter, preferential utilization of...
templates used in the first round of transcription was reported [Hawley and Roeder 1987], and when analyzed by footprinting assays in the presence of its sequence-specific activator upstream stimulating factor [USF], some complex minimally containing USF and TFIID was found to remain at the promoter site after initiation [Van Dyke et al. 1988]. In our case, a similar complex including TGAla might remain at the promoter site, although this complex is resistant to HW. To address this question, it may be possible to isolate the template-associated complex, because of the apparent stability of the complex during several rounds of initiations, for further biochemical characterization. The idea that sequence-specific DNA-binding proteins might be needed for the establishment but not for the maintenance and continued utilization of active preinitiation complexes was also suggested from studies of adenovirus E4 promoter activation by ATF [Hai et al. 1988, Horikoshi et al. 1988b]; this idea was best demonstrated for yeast 5S promoter activation by ATP (Hai et al. 1988; Horikoshi et al. 1988b), USF (Workman et al. 1990), and the progesterone receptor [Klein-Hitpass et al. 1990]. Preinitiation complexes formed in the presence of TGAla were resistant to the oligonucleotide containing TGAla-binding site (Fig. 6). Similar stabilities of preinitiation complexes were observed with ATF [Hai et al. 1988], USF [also known as major late upstream transcription factor, Carcano et al. 1989], and the progesterone receptor [Klein-Hitpass et al. 1990]. Therefore, our demonstration that TGAla also acts in the same manner to stimulate transcription strengthens the notion that transcription activation mechanisms are conserved between plants and animals.

Although we have shown here that TGAla functions as a transcription activator, the protein domain responsible for this activation remains to be identified. We have proposed previously that an acidic region (amino acid residues 20–67) and a relatively glutamine-rich region (amino acid residues 259–347) of TGAla may serve as activation domains [Katagiri et al. 1989] because such motifs are known to activate transcription in yeast and animals [Ptashne 1988, Mitchell and Tjian 1989]. In the case of GAL4, an acidic region also functions as an activation domain in plants [Ma et al. 1988]. Further analyses of various deletion mutants of TGAla in the human in vitro transcription system will greatly facilitate functional dissection of this plant transcription factor.

Materials and methods

Plasmids and bacteria

The expression vector pET3a [Rosenberg et al. 1987] was used for the expression of TGAla. A double-stranded oligonucleotide shown below was first synthesized and cloned into the SacI–KpnI site of pBluescript II KS+ (Stratagene) to obtain pKE1. Unique restriction sites and important codons within the oligonucleotide region of pKE1 are indicated.

The 1.2-kb EcoRI–XhoI fragment of XhoI [Katagiri et al. 1989], which contains the entire TGAla-coding region except 2 bp at the 5’ end, was cloned into the EcoRI–XhoI site of pKE1 to obtain pKT1A. In this context, the methionine codon in the oligonucleotide region of pKE1 (shown above) became the first methionine codon of the TGAla reading frame. Sequence analysis of TGAla genomic clones revealed that the open reading frame starting from this methionine codon is identical to the authentic TGAla open reading frame [H. Fromm, unpubl.]. The NdeI–BamHI fragment of pKT1A was cloned into the NdeI–BamHI site of pET3a to obtain pKT7T1A. The pKT7T1A was transformed into the host bacteria BL21(DE3)/plysS, and the transformant BL21(DE3)/plysS/pKT7T1A was used for the overproduction of TGAla.

The plasmids pUWDP and pUMDP containing the wild-type and mutant promoters, respectively [Yamazaki et al. 1990], were used as the DNA templates in the in vitro transcription assays.

Figure 7. Transcription activation by TGAla. A proposed model showing the sequence of events in transcription initiation [Hawley and Roeder 1987] and the activation mechanism of TGAla. The oligonucleotide HW inhibits the increase in the number of the preinitiation complexes. The conversion of the preinitiation complex into the elongation complex is inhibited by 0.3 M KCl. The reinitiation of transcription involves a complex that bypasses the HW-sensitive step. It has not been ascertained whether TGAla is released after formation of the preinitiation complex. Abbreviations are the same as in Fig. 5.
Overproduction and purification of TGA1a

BL21(DE3) ployS/pKT7T1A was cultured in M9ZB medium at 37°C until the A₆₀₀ reached 0.4. IPTG was added to the culture to a final concentration of 0.4 mM and the bacteria were incubated at 37°C for 2 hr before harvesting. The following procedure was performed at 4°C. The bacteria (6 grams wet weight) were suspended in 80 ml of buffer E [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride], lysed by two cycles of freeze-thaw, and then extracted with 1 M NaCl. The crude extract was adjusted to 40% saturation of ammonium sulfate, and the precipitate was collected. The precipitate was suspended in buffer A-0.04 [buffer E plus 20% glycerol, 0.1% Nonidet P-40, 40 mM KCl] and dialyzed against the same buffer. The fraction was applied onto a DE-52 [Whatman] column [bed volume 33 ml] equilibrated with buffer A-0.04 and the flowthrough fractions were pooled. The DE-52 fraction was then applied onto a P11 [Whatman] column [bed volume 5 ml] equilibrated with buffer A-0.04. After consecutive washings with buffer A-0.04 and buffer A-0.2 [same as A-0.04 except 0.2 M KCl], the column was eluted with buffer A-0.5 [same as A-0.04 except 0.5 M KCl], and the peak protein fractions were pooled. The P11 fraction was passed through two DNA affinity chromatography steps to remove a nonspecific activity of transcription stimulation in the fraction, which was observed when analyzed in a wheat germ system. K. Yamazuki et al., (unpubl.). The buffer of the fraction was exchanged to the same buffer. The column was eluted with buffer B-0.2 [20 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.2 M KCl] by gel filtration on Sephadex G-25 [Pharmacia], and one-third of the total fraction was applied onto the mutant DNA affinity column [bed volume 5 ml] equilibrated with the same buffer. The column was eluted with buffer B-0.4 [same as B-0.2, except 0.4 M KCl] and the eluant was applied onto the wild-type DNA affinity column [bed volume 5 ml] equilibrated with buffer B-0.4. The column was eluted with buffer B-0.8 [same as B-0.2, except 0.8 M KCl], and the eluted fractions were pooled as the purified fraction of TGA1a.

The DNA affinity columns were prepared essentially as described (Kadonaga and Tjian 1986), using HW and HM double-stranded oligonucleotides that contain the wild-type and mutant hex1 sequences, respectively. Concatemerized HW and HM were coupled to CNBr-activated Sepharose 4B [Pharmacia] to prepare the wild-type and mutant DNA affinity columns, respectively. hex1 is a cis-element [-180 to -160] of the wheat histone H3 promoter (Mikami et al. 1987). It contains one copy of the TGACG motif that binds to TGA1a (Katagiri et al. 1989). The nucleotide sequences of HW and HM are shown below.

| HW | 5' CGAAGCTTCGCCCAGTCACCAACTC 3' |
| HM | 5' CGAAGCTTCGCCCACGCTCAATC 3' |

The TGACG motif of HW is underlined, and the mutations in HM are shown in bold letters. HW binds TGA1a, whereas HM does not when analyzed as competitors in gel retardation assays (data not shown).

**In vitro transcription assays**

Partially purified fractions of RNA polymerase II, TFII B, TFII D, and TFII E/F were prepared as described (Horikoshi et al. 1988b). The activities of factors were RNA polymerase II, 0.7 units/μl; TFII B, 0.85 units/μl; TFII D, 1.5 units/μl; and TFII E/F, 0.7 units/μl. The reaction mixture [25 μl/assay] contained 10 mM Tris-HCl [pH 8.0], 40 mM HEPES-KOH [pH 7.9], 50 mM KCl, 2 mM MgCl₂, 10% glycerol, 1 mg/ml of BSA, 8 mM DTT, 100 μM ATP, 100 μM UTP, 20 μM CTP, 5 μCi [α-32P]CTP (800 Ci/mmole), 50 μM 3'-O-methyl GTP, 0.15 unit of RNase T1 [Pharmacia], 0.8 unit of RNasin [Promega], 0.2 μg of DNA template, 0.5 μl of TFII B, 0.5 μl of TFII D, 0.5 μl of TFII E/F, 1 μl of TFII H, 0.25 μl of RNA polymerase II, and the indicated amount of TGA1a. The standard incubation was at 30°C for 20 min. After the incubation, the reaction was terminated by the addition of 75 μl of stop solution [0.5% SDS, 10 mM EDTA, 150 mM sodium acetate, 50 μg/ml of tRNA]. After extraction with phenol and chloroform, the transcripts were analyzed on a 6% sequencing gel. For the depleted HeLa general factors, the mixture of TFII B, TFII D, TFII E/F, and RNA polymerase II was preincubated with 10 ng/assay of HW at 30°C for 30 min before the reaction. In experiments involving a preincubation step, nucleoside triphosphates and the indicated components were omitted from the preincubation mixture. The components indicated in the figures were added according to the timetables. The reactions were stopped, and the transcripts were analyzed as described above. Amounts of transcripts were measured by determining the radioactivity in excised gel bands using a scintillation counter.

**Primer extension assay**

In vitro transcription with [100 ng/assay] or without TGA1a was carried out under standard assay conditions, except that [α-32P]CTP was omitted from the reaction mixture. The transcripts were analyzed by primer extension as described (Hai et al. 1988), using an oligonucleotide primer complementary to +94 to +115 of the template, pUWDP. The amount of the primer used was in excess to that of the transcripts. For reference, pUWDP was sequenced with the same primer using a Sequenase sequencing kit [U.S. Biochemicals].

**Amino-terminal sequencing of protein**

The three polypeptides in the TGA1a preparation were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membrane (Matsudaira 1987), and sequenced by a gas-phase sequencer (Model 477A). Both sequencers were equipped with on-line PTH analyzers (Model 120A) and data analysis modules (Model 900A).

**Southwestern blot**

Southwestern blot analysis was performed essentially as described (Miskimins et al. 1985). A 112-bp fragment containing hex1 was labeled by Klenow fill-in and used as the binding probe. The binding mixture contained 5 ng/ml of the probe [7 × 10⁶ cpm/ml] and 10 μg/ml of poly[dI-C]. As a competitor, 18 ng/ml of either HW or HM was added to the binding mixture.

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