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Genetics

Directing transcription of an RNA polymerase III gene via GAL4 sites

(U6 RNA gene/transcription factors TFIIIC and TFIIIB)

MARIE-CLAUDE MARSOLIER*, NATHALIE CHAUSSIVERT, OLIVIER LEFEBVRE, CHRISTINE CONESA, MICHEL WERNER, AND ANDRÉ SENTENAC†

Service de Biochimie et de Génétique Moléculaire, Commissariat à l'Energie Atomique, Saclay, F91191 Gif-sur-Yvette Cedex, France

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ABSTRACT **A yeast chimeric RNA polymerase III transcription system was constructed to explore the ordered, multistep process of gene activation *in vivo*. A promoter-deficient U6 RNA gene harboring GAL4-binding sites could be reactivated by fusing the GAL4 DNA-binding domain to components of the general transcription factor TFIIIC (τ) or TFIIIB. Expression of chimeric τ138 or τ131 (but not τ95) subunits activated transcription from GAL4-binding sites located at various positions, including upstream of or within the gene. The function(s) of the B block binding domain of TFIIIC was provided by the fused GAL4-(1–147) domain. The GAL4-(1–147)–TFIIIB70 fusion protein acted at a distance like an activator of transcription. In contrast, none of the 10 different GAL4-(1–147)–polymerase subunit fusions was able to induce transcription, suggesting that RNA polymerase recruitment is not sufficient to initiate transcription.**

Initiation of transcription in eukaryotes depends on a cascade of specific interactions between promoter control regions, multiple transcription factors, and a gene-specific RNA polymerase. Transcription complex assembly on a simple yeast tRNA gene occurs in three main steps (1, 2). First, transcription factor IIIC (TFIIIC, or τ) binds to the intragenic A and B blocks. TFIIIC then promotes the binding of TFIIIB to a position upstream of the gene. The TFIIIB–DNA complex in turn recruits and directs accurate initiation by RNA polymerase III (polIII). About 25 polypeptides are part of the final assembly ($M_r = 1.5 \times 10^6$) (3). TFIIIC is organized into two interconnected globular domains, τA and τB, and is made up of 6 large subunits (τ138, τ131, τ95, τ91, τ60, and τ55) (4, 5). TFIIIB contributes 3 polypeptides [TATA-binding protein (TBP), TFIIIB70, and TFIIIB90] (6–8). polIII has 16 subunits (C160, C128, C82, C53, AC40, C37, C34, C31, ABC27, C25, ABC23, AC19, ABC14.5, C11, ABC10α, and ABC10β) (9). The genes for 18 of these 25 polypeptides have been cloned (2, 3). The role of some polypeptides has begun to emerge from *in vitro* studies: τ138 and τ95 interact with DNA and contribute to B block and A block recognition, respectively (4, 5); τ131 is thought to interact with TFIIIB70 (6) and to allow the sequential assembly of TBP and TFIIIB90 into a stable TFIIIB–DNA complex (7, 10, 11); and TFIIIB70 participates in polIII recruitment by interacting with the C34 subunit (12).

One fundamental question concerns the significance of this multistep assembly process. Is the recruitment of RNA polymerase to the promoter the main role of transcription factors? Do these successive protein–DNA and protein–protein contacts convey only positional instructions, or do they contribute functional information? Could some of these specific interactions be bypassed? We have explored the possibility of promoting transcription of a promoter-deficient gene by fusing a DNA-binding domain to individual components of the transcription system. The model system that we used is the yeast U6 RNA gene, *SNR6*, which has three promoter elements, a TATA box at −30, a weak intragenic A block, and a B block downstream of the termination signal (13–15). The TATA box and the A block contribute to start site selection. *In vitro*, the *SNR6* gene can be transcribed in the absence of the B block and TFIIIC, using purified TFIIIB and polIII (16). TFIIIC and the B block are required to relieve repression of chromatin templates (17). In this system, the distant B block displays all the properties of polII enhancer elements (15). We show that an *SNR6* gene having a mutated B block can be reactivated in yeast cells by fusing the DNA-binding domain of GAL4 [GAL4-(1–147)] to the B block binding subunit or to the TFIIIB assembling subunit of TFIIIC, or even to TFIIIB70. The extent of gene activation depended on the presence of GAL4-binding sites (upstream activating sequence bound by GAL4; UAS_G) at appropriate locations. In contrast, polymerase subunit–GAL4-(1–147) fusions could not reactivate the deficient genes.

MATERIALS AND METHODS

UAS_G–U6 RNA Gene Constructs. The SNR6 constructs were derived from the pB6MΔ238/9, pB6MTATAdown, pB6MAdown, and pB6-40 plasmids previously described (15). All plasmids contain the entire region of the *Saccharomyces cerevisiae* U6 RNA gene, from position −141 to +313, cloned between the *Eco*RI and the *Bam*HI sites of the Bluescript SK vector (BSSK; Stratagene), except for the pB6-40 plasmid, which harbors a 5′ truncated fragment (from −40 to +313) cloned between the *Hin*dIII and the *Xba* I sites of BSSK. All genes had a 24-bp *Pst* I–*Xba* I DNA fragment from BSSK inserted at the *Eco*NI site, at position +73. This insertion maps at the beginning of the third loop of the predicted U6 RNA secondary structure (18). All templates also have the B block destroyed by a 2-bp deletion (13, 15). The down mutations affecting the TATA box and the A block have been described (15). One or a series of five head-to-tail UAS_G motifs (5′-CTCGGAGGACAGTACTCCG-3′) flanked by a *Hin*dIII–*Sph* I–*Pst* I linker on the 5′ side and by a *Xba* I–*Bam*HI linker on the 3′ side were extracted from G1E4T and G5E4T (kindly provided by J. Workman, Harvard Medical School, Boston) by a *Hin*dIII–*Xba* I digest, blunt-ended, and inserted at various positions: (*i*) into the blunt-ended *Acc* I site of BSSK, so that the 3′ end of the nearest UAS_G was located at position −180 relative to the transcription start site (−180–UAS_G template); (*ii*) into the blunt-ended *Acc* I site of

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Abbreviations: polIII, RNA polymerase III; TBP, TATA-binding protein; UAS_G, upstream activating sequence bound by GAL4.

*Present address: Laboratoire du Métabolisme, INRA Versailles, Route de St-Cyr, F78026 Versailles, France.
†To whom reprint requests should be addressed.

the 5′ truncated version of the U6 gene (pB6-40), so that the 3′ end of the nearest UAS_G was located at −67 (−67–UAS_G template); (*iii*) into the blunt-ended *Apa*LI site created by the TATA box down mutation (15), so that the 3′ end of the unique UAS_G was located at −40 (TATA–UAS_G template); (*iv*) into the *Sma* I site of the 24-bp insertion so that the 5′ end of the nearest UAS_G was at +88 (+88–UAS_G template); (*v*) or into the *Hpa* I site created by the 2-bp deletion in the B block (B block–UAS_G template). The constructs were verified by restriction analysis and DNA sequencing and then transferred to the multicopy vector YEp352 (19).

GAL4-(1–147) Fusion Constructions. The GAL4-(1–147)–TBP, –TFIII70, –C31, –C34, –C53, –C82, –ABC10β, –AC19, and –AC40 fusions have been described (12, 20). GAL4-(1–147)–C128 and –ABC23 were generous gifts from N. Zeckerle (University of Washington, Seattle) and D. Lalo (Saclay), respectively. The GAL4-(1–147)–τ138, –τ131, –τ95, and –C160 fusions were constructed by engineering a *Bam*HI site (τ138, τ131, and C160) or a *Bgl* II site (τ95) at −8 relative to the initiator codon. A *Bam*HI site and a *Bgl* II site were introduced after the stop codons of the τ138 and τ95 genes, respectively (21). A *Bam*HI site was already present at the 3′ end of the C160 and τ131 genes, in the plasmids pC160-7 and pCK14, respectively (22, 23), but *Bam*HI sites present in the coding parts of the C160 (position 2763) and τ131 genes (position 2310) had to be removed. These mutations were performed by oligonucleotide-directed mutagenesis (24). *Bam*HI or *Bgl* II fragments were then cloned into pMA424 (τ138, τ95, and C160) or pGBT9 (τ131) (25).

RNA Analysis. UAS_G–U6 constructs and GAL4-(1–147) fusion constructions were introduced into *Saccharomyces cerevisiae* strain MCM564. MCM564 is a *ura3* derivative of the strain Y526 (12) obtained by selection on 5-fluoroorotic acid plates. Yeast transformation procedures, RNA extraction, and Northern blot analysis were performed as described (15), by using as a probe a DNA fragment encompassing the U6 RNA coding sequence (−120 to +122) labeled by nick-translation with [α-^{32}P]dCTP.

RESULTS

Experimental Design for a Chimeric Transcription System. Fig. 1 features the main characteristics of the modified SNR6 templates. All genes had their B blocks destroyed by a 2-bp deletion. This mutation prevents τ–DNA binding (17), inhibits *SNR6* gene transcription *in vitro* in crude extracts or on nucleosomal templates, and annihilates transcription of the U6 gene in wild-type cells (13, 15, 17). These debilitated genes had either one or a series of five head-to-tail UAS_G sequences inserted at various locations, at −180, −67, −40, +88, or +238 relative to the transcriptional start site. In the case of the UAS_G at −40, the TATA box was first destroyed to facilitate the interpretation of the results. Finally, all modified genes had a small insertion (24 bp) in the transcribed region, at position +73, to distinguish their transcripts (denoted maxi transcripts) from wild-type U6 RNAs. None of these modified U6 RNA genes, harbored on multicopy plasmids, was able to direct transcription of maxi U6 RNA *in vivo* (see below). We fused the DNA-binding domain of GAL4 (amino acids 1–147) to cloned subunits of TFIIIC, TFIIIB, or polIII. Expression of these genes, carried on multicopy plasmids, was directed by the *ADH1* promoter. We then introduced into yeast cells different combinations of UAS_G–U6 templates and GAL4-(1–147)–factor fusion proteins and monitored the level of synthesized maxi U6 RNA by Northern blotting.

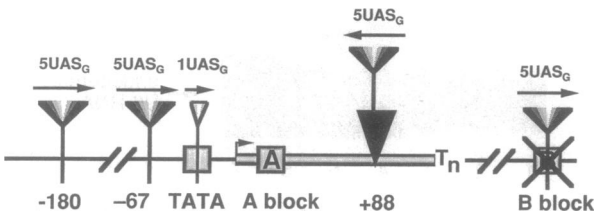

FIG. 1. UAS_G–U6 constructs. Each template had its B block destroyed (×), had a 24-bp insertion at +73 (black triangle), and harbored one or five head-to-tail UAS_G sequences at variable positions as indicated. The UAS_G shown in the TATA box indicates that the TATA box was destroyed.

GAL4-(1–147)–τ138 Directs Transcription Complex Formation on B Blockless Genes. Because τ138 interacts with DNA (4) at the level of the B block (5), we examined the ability of a GAL4-(1–147)–τ138 fusion to activate the different UAS_G–U6 templates (Fig. 2*A*). We knew that the τ138 fusion was expressed and functional since it could replace the wild-type protein to sustain cell growth (data not shown). Remarkably, GAL4-(1–147)–τ138 promoted transcription of UAS_G–U6 templates having the UAS_G sequences at different locations, at the position of the B block at +238, in both orientations (lanes 7 and 8), but also upstream, at −180 or −67 (lanes 3 and 4), as well as within the transcribed region at +88 (lane 6). The length of the transcripts was as expected for correct initiation and termination of the maxi U6 RNAs. The least effective construct was the TATA–UAS_G template (lane 5). In that case, the transcripts produced were slightly shorter, in agreement with previous observations with TATA-less U6 genes (14, 15, 26). We compared the transcription of the +88–UAS_G template (B blockless) with that of a +88–UAS_G gene retaining the normal B block, in the latter case in the absence of the GAL4-(1–147)–τ138 fusion: the transcription efficiency was similar (Fig. 2*B*), and the length of the transcripts was the same (the five intragenic UAS_G and the 24-bp insert extended the transcribed sequence by 149 bp).

Control experiments were performed to confirm the requirement for both the UAS_G and the fused GAL4 DNA-

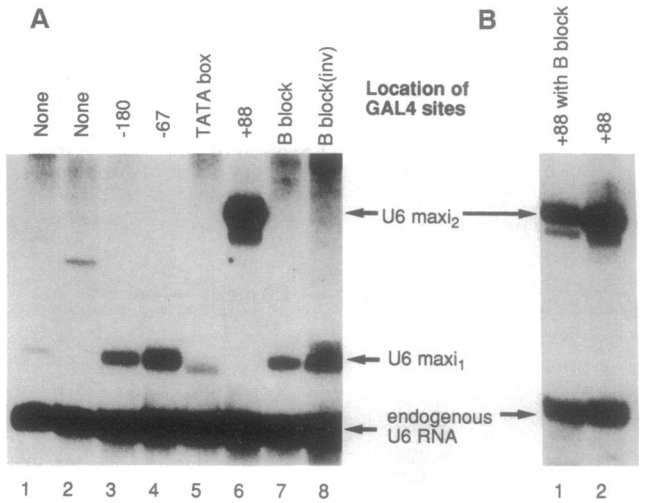

FIG. 2. Activation of the UAS_G–U6 templates by GAL4-(1–147)–τ138. (*A*) B blockless U6 templates without UAS_G (lanes 1 and 2) or harboring UAS_G at various locations as indicated (lanes 3–8) were introduced into yeast cells along with the GAL4-(1–147)–τ138 construction. The transcripts (U6 maxi$_1$ and U6 maxi$_2$) and the endogenous U6 RNA were monitored by Northern blot analysis. (*B*) Activation efficiency of τ138 when positioned via the B block or via the GAL4-(1–147)–UAS_G interaction. The U6 templates had a series of five UAS_G sequences inserted at +73 and had the B block left intact (lane 1) or destroyed (lane 2). The templates were introduced into yeast cells without (lane 1) or along with (lane 2) the pMA plasmid encoding the GAL4-(1–147)–τ138 fusion.

binding domain. While a gene lacking both UAS_G and the B block was inactive in wild-type cells (13, 15), we found that overexpression of GAL4-(1–147)–τ138 induced a faint level of transcription of the B blockless gene (Fig. 2*A*, lane 1). Because the stability of the maxi transcripts may vary with the length of the insert at +73, we mimicked the insertion of the five UAS_G sequences by a 118-bp fragment derived from the Bluescript polylinker. A background transcription induced by the τ138 fusion was again detectable but was very low (Fig. 2*A*, compare lanes 2 and 6). We also tested whether overexpression of the GAL4 DNA-binding domain, encoded by pGBT9, could by itself stimulate transcription of the various UAS_G–U6 templates. Only the −67–UAS_G construct gave a faint signal (data not shown). Therefore, we conclude that transcription was activated via the binding of GAL4-(1–147)–τ138 to the UAS_G sequences.

GAL4-(1–147)–τ131 Activates Transcription from UAS_G Sequences. τA binding to the A block via τ95 is thought to position τ131 for TFIIIB recruitment (6). We tested the GAL4-(1–147)–τ95 fusion protein for its ability to reactivate B blockless genes containing the UAS_G sequences at various locations. None of them was detectably transcribed, although the GAL4-(1–147) fusion protein was expressed, since we found that it can functionally replace the wild-type τ95 subunit (data not shown). We next explored the capacity of GAL4-(1–147)–τ131 to activate B blockless genes. No transcripts were detected in the absence of the UAS_G sequences (Fig. 3*A*, lanes 1 and 2), but maxi U6 RNAs of proper size were produced from templates harboring UAS_G sequences at −67, +88, or at the level of the TATA box (Fig. 3*A*, lanes 4–6). Surprisingly, the strongest signal was obtained with the TATA–UAS_G template, although destruction of the TATA box substantially decreases *in vivo* transcription of wild-type U6 RNA genes (14, 15). The transcripts originating from the TATA–UAS_G template were also slightly shorter, as in the case of the τ138 fusion. The integrity of the intragenic A block was required for gene transcription via the GAL4-(1–147)–τ131 fusion (Fig. 3*B*). However, one cannot exclude the possibility that the mutated RNA was made but rapidly degraded *in vivo* (for a discussion of this point, see ref. 14).

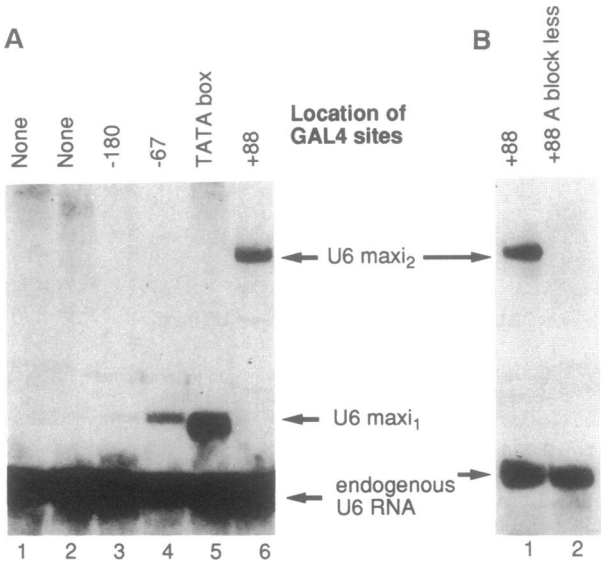

FIG. 3. Activation of the UAS_G–U6 templates by GAL4-(1–147)–τ131. (*A*) B blockless U6 templates without UAS_G (lanes 1 and 2) or harboring UAS_G at various locations as indicated (lanes 3–6) were introduced into yeast cells along with the GAL4-(1–147)–τ131 construction. The transcripts (U6 $maxi_1$ and U6 $maxi_2$) and endogenous U6 RNA were monitored by Northern blot analysis. (*B*) Activation by GAL4-(1–147)–τ131 of the +88–UAS_G template with an intact A block (lane 1) or a mutated A block (lane 2).

It is remarkable that in all these experiments, no RNA species other than those of the expected size were produced, which again indicates a normal pathway of TFIIIB assembly.

TFIIIB70 but Not TBP Fusion Proteins Can Activate Transcription from UAS_G Sequences. We investigated the possibility of bypassing the τ-dependent TFIIIB assembly process by directly recruiting a TFIIIB component, TFIIIB70 or TBP, via a UAS_G sequence. Overexpression of GAL4-(1–147)–TFIIIB70 or GAL4-(1–147)–TBP did not activate B blockless templates devoid of UAS_G elements (Fig. 4, lanes 1 and 2, and data not shown). In the presence of the GAL4-(1–147)–TFIIIB70 fusion, maxi U6 RNA transcripts were produced from templates harboring UAS_G elements upstream of the start site, in the TATA box, at −67, or even better at −180 (lanes 3–5). Transcription was also detectably activated from position +88 (lane 6). UAS_G sequences located further downstream (+238), in the B block, were ineffective (lane 7). As in previous cases, the transcripts generated from the TATA–UAS_G template were shorter. When the A block of the −180–UAS_G and the +88–UAS_G templates was destroyed, these genes were no longer detectably transcribed in the presence of the GAL4-(1–147)–TFIIIB70 fusion (data not shown). We examined whether the transcription of the B blockless U6 templates could be triggered by the GAL4-(1–147)–TBP chimeric protein. The two templates tested had the UAS_G inserted at −67 or at −40 (TATA–UAS_G). No maxi transcripts were observed.

GAL4-(1–147)–Polymerase Subunit Fusions Fail to Activate Transcription. Since the function of TFIIIB is to recruit RNA polymerase to the transcription start site, we wondered whether this recruitment step could be bypassed by grafting a specific DNA-binding site onto the polymerase. Almost all the yeast polIII subunit genes have been cloned (9), and we tested 10 different GAL4-(1–147)–subunit fusion constructs (subunits C160, C128, C82, C53, AC40, C34, C31, ABC23, AC19, ABC10β). None of these subunit fusions was able to activate transcription of three B blockless templates with the UAS_G at −180, −67 or −40 (data not shown).

DISCUSSION

We have fused the DNA-binding domain of GAL4 to 15 different components of the yeast polIII transcription system

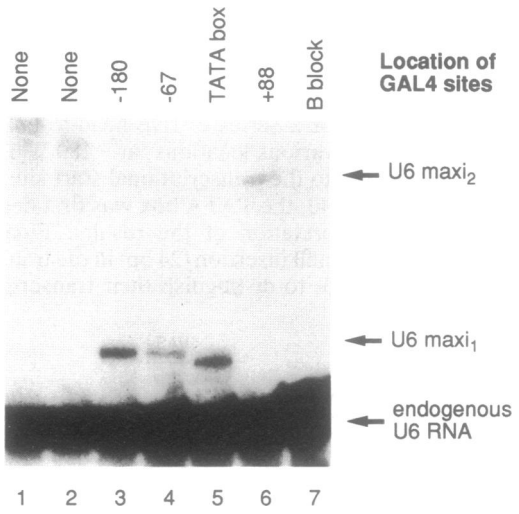

FIG. 4. Activation of the UAS_G–U6 templates by GAL4-(1–147)–TFIIIB70. B blockless U6 templates without UAS_G (lanes 1 and 2) or harboring UAS_G at various locations as indicated (lanes 3–7) were introduced into yeast cells along with the GAL4-(1–147)–TFIIIB70 construction. The transcripts produced from the U6 templates (U6 $maxi_1$ and U6 $maxi_2$) were monitored by Northern blot analysis.

to gain insights into the mechanism of gene activation. We found that several fusion proteins could activate transcription of a promoter-deficient U6 RNA gene in a manner dependent upon the presence of UAS_G sequences at appropriate locations. Here we consider two possibilities: (*i*) that the fusion proteins acted alone, or, as it seems more likely, (*ii*) that the fusion proteins assembled into their normal multimeric complexes.

The τ138 subunit of TFIIIC intervenes in the primary step of gene activation by binding to the B block (4, 5). This specific DNA recognition step could be replaced by an unrelated (GAL4–UAS_G) interaction system, much like in the case of polII activators (27). This result makes unlikely the idea (28) that B block binding by τB causes a structural change in the factor favoring the subsequent binding of τA domain to the A block. UAS_G sequences were effective at various positions, including upstream of the initiation site (see Fig. 5). This result illustrates the flexibility of the transcription machinery. In striking contrast, B-block-deficient genes could not be reactivated by GAL4-(1–147)–τ95 fusion, whatever the position of the UAS_G sequences. This observation suggests either that A block binding by τA cannot be replaced by an unrelated DNA–protein interaction or that a correct positioning of τ95 is critical and that this positioning could not be achieved with the GAL4-(1–147)–UAS_G interactions. In our current view, τ95 associates with the A block and thereby helps to position τ131 properly for TFIIIB assembly. Surprisingly, however, we found that GAL4-(1–147)–τ131 could activate transcription from various places, including from an intragenic position, at +88 (see Fig. 5); the transcripts were always of the correct size, and gene activation required the integrity of the A block, at least in the case of the +88–UAS_G template. Therefore, transcription probably occurred through the normal TFIIIB assembly pathway, after relocation of TFIIIC onto the A block. In the above discussion, we assumed that the τ138, τ131, and τ95 fusion polypeptides were integrated into and acted via the whole TFIIIC factor. This is a reasonable assumption since each of these three chimeric subunits was able to sustain normal cell growth in the absence of the corresponding wild-type polypeptide. Nevertheless, the possibility remains that the τ131 fusion protein could activate TFIIIB assembly by itself, depending on the position of the UAS_G.

We demonstrated that GAL4-(1–147)–TFIIIB70, but not –TBP, can promote transcription of B blockless genes. A direct explanation would be that τ function can be bypassed by positioning TFIIIB70 on the DNA, either alone or preassembled into TFIIIB. Several observations argue against this interpretation. First, the TFIIIB70 fusion protein activated transcription from different places (see Fig. 5). In addition, transcripts of the correct size were generated in each case. Since the integrity of the A block was needed for gene activation (again assuming no indirect effect on RNA stability), we believe that the TFIIIB70 fusion helped recruit TFIIIC to the A block, which, in turn, properly assembled TFIIIB, either by relocating the bound TFIIIB70 fusion or by recruiting new TFIIIB components. In fact, the TFIIIB70 fusion acted more as an activator than as an initiation factor. The fact that an essential transcription factor can activate transcription at a distance, like an activator, provides support for the idea that enhancers and promoters are functionally and evolutionary related (29).

The advantage of the present *in vivo* system is to incorporate all parameters of gene regulation, including chromatin repression. U6 transcription on chromatin templates requires TFIIIC and the presence of the B block (17). How was chromatin repression relieved in the present chimeric systems on B blockless genes? This function was probably provided in part by the GAL4 DNA-binding moiety, which can cause chromatin disruption (30) and thereby provide DNA access to the fused factor subunits. One may have anticipated a problem in two cases, when the UAS_G sequence lay in the vicinity of the TATA box (TFIIIB binding site) and when five UAS_G sequences were inserted within the transcribed sequence. Léveillard *et al.* (31) found that the DNA binding protein GCN4 strongly repressed transcription of a tRNA gene *in vitro* when prebound within the TFIIIB binding region (−23 to −36) but that GCN4 binding further upstream (−43) had no effect. The TATA–UAS_G template harbors a UAS_G sequence extending from −40 to −59 and was the most efficient of all the constructs tested with the τ131 or TFIIIB70 fusions (see Fig. 5). Hence we believe that after the initial GAL4 fusion protein–TATA–UAS_G template binding step, the factors could be repositioned properly. The case of the template with internal UAS_G sequences is also interesting, as they mimic the normal situation in tRNA genes where the B block is intragenic (32). While at least one UAS_G had to be occupied to initiate transcription complex formation, the GAL4-(1–147)–UAS_G interaction apparently did not oppose the progression of the RNA polymerase.

Our failure to trigger transcription by GAL4-(1–147)–polymerase subunit fusions suggests that the process of RNA

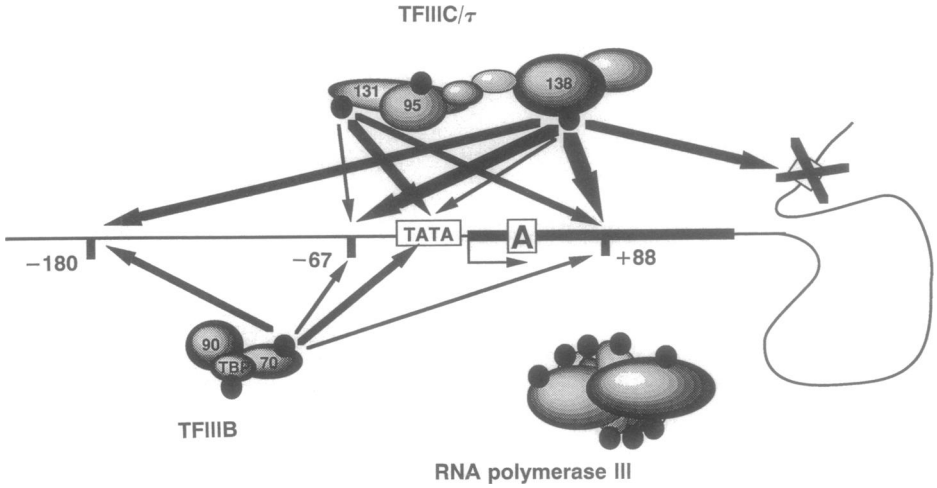

FIG. 5. The diagram summarizes the level of gene activation obtained with various tagged subunits of TFIIIC and TFIIIB according to the location of the UAS_G. The grafted GAL4-(1–147) moiety is represented by a small closed circle. The thickness of the arrows is roughly proportional to the activation efficiency. The absence of an arrow denotes no activation. In this scheme, we hypothesize that fusion proteins are integrated into multiprotein complexes, which may not always be the case.

polymerase assembly is crucial for initiation. Ten different subunit fusions were tested and the *in vivo* expression of six of them has been demonstrated (12, 20). At least in one case (C34), the chimeric subunit was able to functionally replace the wild-type subunit, so we believe that at least some of the fusion proteins were incorporated into polIII enzymes, which thus could bind the UAS_G sites nonproductively. In contrast, GAL4-(1–148)–T7 RNA polymerase is able to initiate at a promoter and to transcribe efficiently *in vitro* while remaining anchored to a UAS_G (33).

We conclude that all the known functions of the B block binding site of TFIIIC (DNA recognition, antirepression, polymerase read-through) can be replaced by the DNA-binding domain of a polII activator. In contrast, A block binding appears to be critical. Another conclusion is that transcription complex assembly does not necessarily require a strictly ordered cascade of interactions. Finally, recruiting RNA polymerase on the DNA is probably not the only role of basal transcription factors in gene activation.

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