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An RNA polymerase II promoter containing sequences upstream and downstream from the RNA startpoint that direct initiation of transcription from the same site

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ABSTRACT **The *gfa* gene encodes glial fibrillary acidic protein (GFAP), an intermediate-filament protein expressed primarily in glial cells. We have used *in vitro* transcription studies to show that the basal level of transcription of the human gene encoding GFAP is controlled by two distinct initiators—i.e., promoter elements that direct transcription from a specific start site. One initiator is located about 25 base pairs upstream from the transcription start site, contains a TATA box, and apparently acts together with a sequence found around the transcription start site. The other initiator is located between +11 and +50 bp downstream from the transcription start site. Most of this second region overlaps with the protein-encoding sequence, which starts at bp +17. The sensitivity of transcription to α-amanitin indicates that both initiators are used by RNA polymerase II.**

Eukaryotic genes that code for a protein are transcribed by RNA polymerase II (Pol II). The control region within a few hundred bases from a transcription start site is called the promoter; it usually contains several DNA sequence elements that affect transcription. These promoter elements can be divided into two groups: initiators, which direct the initiation of transcription from a specific start site, and modulators, which influence the rate of transcription. The most common initiator is the TATA box, located about 25–30 base pairs (bp) upstream from the transcription start site (1). Recently, another initiator was found between −6 and +11 bp from the transcription start site of the murine terminal deoxynucleotidyltransferase gene and of the adenovirus major late promoter (2). Although these authors used the term "initiator" to refer specifically to this element, in this paper we use this term more generally to refer to any promoter element that determines the transcription start site. Promoter elements that have a modulating role include the GC box, the CCAAT box, and the octamer motif. The modulation element-binding proteins such as Sp1, CTF/NFI, and octamer-binding factors regulate the transcription rate presumably by interacting with Pol II basic transcription machinery (3).

To understand the molecular mechanisms of glial-specific transcription, our laboratory recently isolated the human *gfa* gene (*GFA*) (4). The product of this gene, glial fibrillary acidic protein (GFAP), is an intermediate-filament protein highly specific to astrocytes (5). The current study focuses on the cis-acting elements responsible for *GFA* basal transcription. Understanding these basal-level elements will facilitate determining how tissue specificity is controlled. We demonstrate that *GFA* contains at least two initiators, an upstream TATA box acting in concert with a sequence around the transcription start site and a downstream region having homology to the box A motif of RNA polymerase III (Pol III) gene promoters. Unlike any initiator described to date, the downstream initiator is present within the coding sequence of the gene and is recognized by Pol II.

MATERIALS AND METHODS

Plasmid DNA. Plasmids pGem-1 and pGem-3Z were purchased from Promega. Plasmid pGfaSacIB contains the 374-bp *Sac* I fragment of *GFA* in the *Sac* I site of pGem-1.

Extract Preparation and *in Vitro* Transcription. Nuclear extract was prepared by the method of Dignam *et al.* (6) from the GFAP-producing glial-like rat cell line RT4-D6 (7). Aliquots were frozen in liquid nitrogen and stored at −80°C.

In vitro transcription was performed as described by Bodner and Karin (8) except that template DNA was preincubated with 40 μg (see Figs. 2 and 3), 60 μg (see Figs. 4 and 5), or 100 μg (see Fig. 6) of nuclear extract on ice for 30 min before initiation of transcription. *In vitro* transcript was quantitated by primer extension (8), in which an end-labeled oligonucleotide complementary to a downstream region of the expected RNA transcript is used to prime DNA synthesis by reverse transcriptase. The size of the reverse transcript made thus reflects the RNA start points. Oligonucleotides used were: *GFA* oligo-B (antisense oligonucleotide positioned +178 to +201 in Fig. 1); SP6 oligo-B (5′-TATGCTTCCGGCTCGTATGTTGTG-3′, which is complementary to the pGEM-3Z sequence located 127–150 bp downstream from the multicloning *Sma* I site into which the 3′-end of the *GFA* fragments were ligated); *cat* oligo-A (5′-GCCATTGGGATATATCAACGGTGG-3′); and *cat* oligo-B (5′-TAGCTTCCTTAGCTCCTGAAAATC-3′). For quantitative analysis, pSV2cat was included in transcription reactions as an internal standard. Radioactivity in the *GFA* band, determined directly from the gel by a radioanalytic imaging system (AMBIS System, San Diego), was divided by that in the *cat* band for each lane. Each experiment was repeated two to five times with comparable results.

Construction of Deletion and Site-Specific Mutants. For the 5′ series of deletions, plasmid pGfaSacIB was opened at its *Eco*RI site adjacent to the upstream *Sac* I site, while for the 3′ series it was opened at its *Hin*dIII site adjacent to the downstream *Sac* I site. The opened plasmids were digested with endonuclease BAL-31 (New England Biolabs) according to the manufacturer's instructions, extracted with phenol, precipitated with ethanol, redissolved, and digested with either *Hin*dIII (5′ series) or *Eco*RI (3′ series). The products were then fractionated on a 2% agarose gel, and fragments of the desired size were recovered and subcloned into plasmid pGem-3Z, previously digested with *Sma* I and *Hin*dIII (5′ series) or *Sma* I and *Eco*RI (3′ series).

Site-specific mutations were introduced in plasmid pGfaSacIB by the method of Kunkel *et al.* (9).

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Abbreviations: Pol II and III, RNA polymerases II and III; GFAP, glial fibrillary acidic protein; *GFA*, human *gfa* gene.

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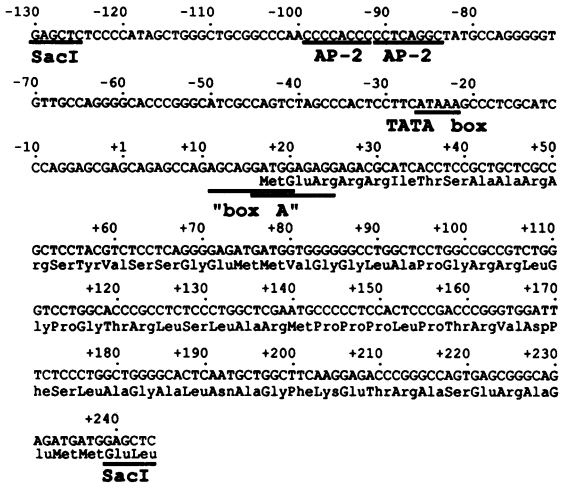


FIG. 1. Nucleotide sequence of the 374-bp *Sac* I fragment of *GFA*. The *in vitro* transcription start site determined by primer extension is at +1 (data not shown). The sequences that have homology to the AP-2 binding site, TATA box, and box A sequence (RRYNNARYGG in which R = purine and Y = pyrimidine; ref. 10) are shown.

RESULTS

The *GFA* Gene Has Two Initiators. To identify the cis-acting elements that affect *GFA* transcription within the 374-bp *Sac* I fragment (Fig. 1), a series of 5′ deletion mutants was constructed and analyzed by *in vitro* transcription (Fig. 2). A decrease by a factor of 2 was observed when the sequence between −106 and −75 was deleted, and a further reduction, by a factor of 20, occurred when the region between −38 and −16 was removed. The first region contains two repeated sequences homologous to the AP-2 binding site, 5′-CCCCAGGC-3′ (11), which occurs frequently as a modulator to enhance transcription. The second region contains a TATA box sequence, ATAAA (1) (Fig. 1). However, weak but reproducible transcription from the correct start site was still observed from an upstream deletion extending all the way to −4. A further deletion to +33 abolished any detectable transcription. These results raise the possibility that, although sequences upstream from −4 are required for efficient transcription, the region downstream from −3 suffices for correct initiation.

Transcription studies were performed with a series of 3′ deletion mutants to obtain more information about the relative importance of this downstream initiator (Fig. 3). There was a decrease by a factor of ≈6 in transcription activity when the sequence between +40 to +9 was removed, and another decrease, by a factor of 30, when the sequence between +8 and −17 was deleted. Correct initiation was abolished when the region up to −34, which includes the TATA box, was deleted. Thus, the 3′-deletion studies suggest that the region between −17 and +40 is required for efficient transcription, but that the region upstream from −18 suffices for initiation at the correct site. Together, the 5′- and 3′-deletion studies indicate the presence of two initiators for *GFA* transcription. One initiator is located upstream from

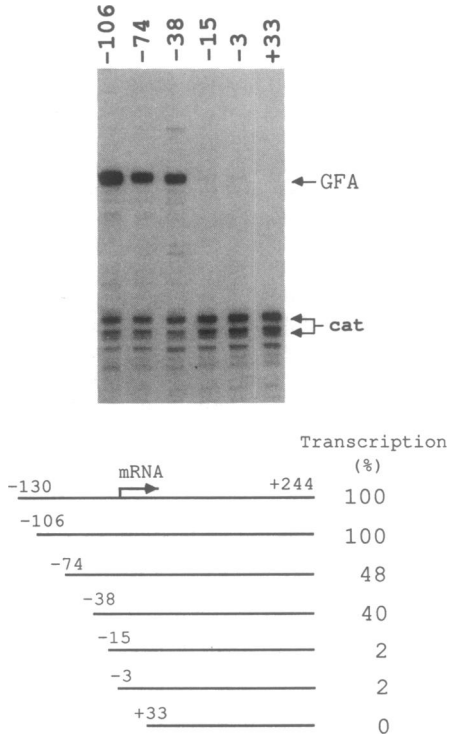


FIG. 2. Effect of 5′ deletions on *GFA in vitro* transcription. (*Upper*) *In vitro* transcription of 5′ deletion mutants. DNA of *GFA* deletion mutants (200 ng) and pSV2cat (100 ng) were cotranscribed and analyzed by primer extension using *GFA* oligo-B and *cat* oligo-A. The number of the first 5′ *GFA* nucleotide still present in the deletion is given above each lane, and the products are indicated on the right. (*Lower*) Summary of quantitative data. The results are presented as a percentage of the normalized value (see *Materials and Methods*) for the parental plasmid, pGfaSacIB, which begins at −130. (pGfaSacIB transcripts were not analyzed in the gel shown in *Upper* but were present in several other experiments.) The 5′ end of each *gfa* fragment is shown on the left.

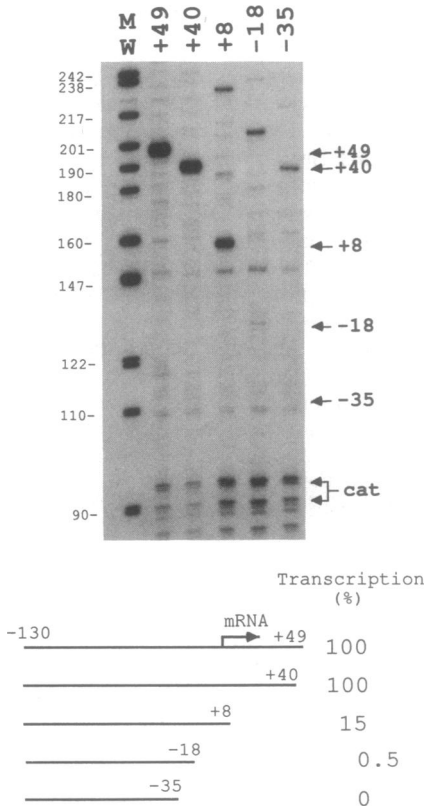
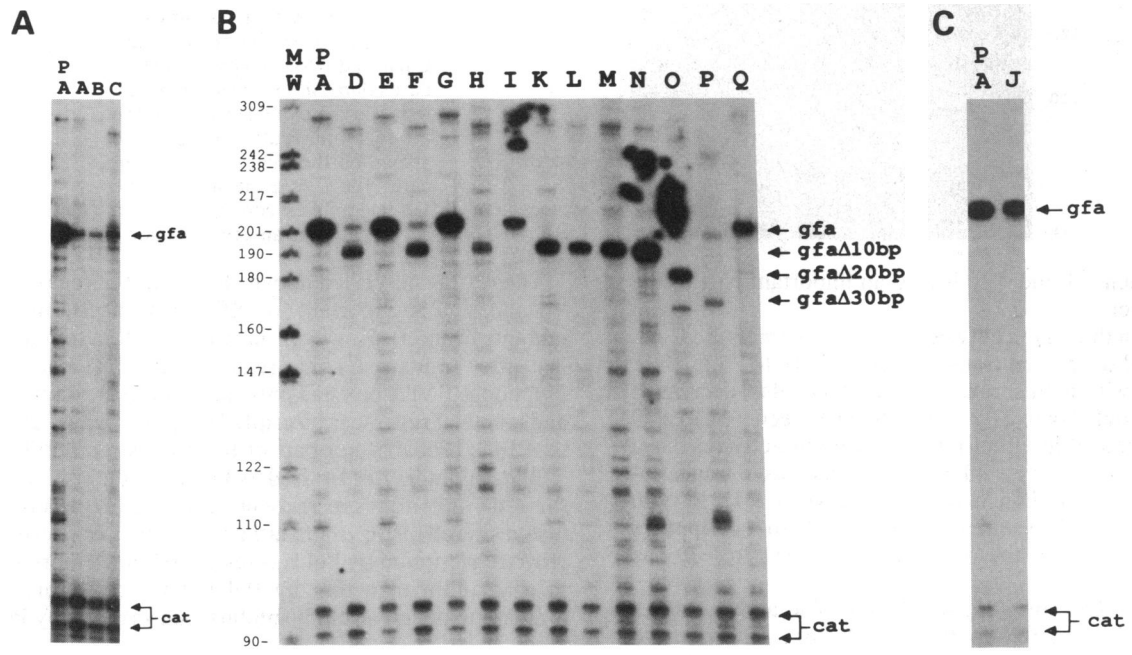


FIG. 3. Effect of 3′ deletions on *GFA in vitro* transcription. (*Upper*) *In vitro* transcription of 3′ deletion mutants. Experiments were performed as in Fig. 2 *Upper* by using SP6 oligo-B and *cat* oligo-B as primers. The 3′ endpoints of the *GFA* sequences are given above each lane. Molecular weight standards are shown on the left, and the expected sizes of products are indicated on the right. (*Lower*) Summary of quantitative data. Values given are the percentages of the activity of plasmid +49.

−18 and is likely the commonly observed TATA box (Fig. 1). The other initiator lies downstream from −3.

To obtain further evidence for the existence of the two initiators, a series of site-specific mutations was introduced within the 374-bp *Sac* I fragment (Fig. 4). Both base replacements (mutants A and B) and a deletion (mutant C) in the TATA box decreased transcription by 90%. The remaining activity presumably can be explained by the downstream initiator. When 10-bp sequences between the TATA box and the original transcription start site were deleted (mutants D and F), two different transcripts were produced. The longer transcript initiated at +1, and the shorter initiated at +10. This is the result expected if the two initiators independently direct initiation at a fixed distance from their position. The transcript initiating from +10 would then be attributable to the TATA box, and that initiating from +1, to the downstream initiator. In addition to the displaced start site, the combined activity from the two start points was less than that from the single start site of the unaltered template. By contrast, base replacements between the TATA box and the transcription start site (mutants E and G) did not alter the initiation site and only slightly affected the amount of transcript produced. This suggests that it is the distance between the TATA box and the start site, rather than the DNA sequence of this region, that is critical for efficient transcription.

Deletion of the sequence between +1 and +10 (mutant H) diminished transcription activity to 10%, while a base substitution in this region (mutant I) reduced it to 15%. Since both of these mutations changed the transcription start site (+1) nucleotide, another mutant (mutant J) was tested in which only the sequence between +4 to +10 was altered. The effect of this mutation was less severe than that of mutant I, but it still reduced transcription to 40%. This suggests that the



D

	-20 -10 +1 +10 +20 +30 +40	Transcription (%)
PA	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	100
A	TTCTTTTTGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	10
B	TTCCCTTTGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	10
C	TT----------TCGCATCCCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	10
D	TTCATAAAG----------CCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	16
E	TTCATAAAGGGGAGCGTAGCCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	84
F	TTCATAAAGCCCTCGCATC----------GCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	20
G	TTCATAAAGCCCTCGCATCGGTCCTCGCTGCAGAGCCAGAGCAGGATGGAGAGGAGACGCATCACCTCCGC	87
H	TTCATAAAGCCCTCGCATCCCAGGAGCGA----------AGCAGGATGGAGAGGAGACGCATCACCTCCGC	10
I	TTCATAAAGCCCTCGCATCCCAGGAGCGACGTCTCGGTCAGCAGGATGGAGAGGAGACGCATCACCTCCGC	15
J	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCACTCGGTCAGCAGGATGGAGAGGAGACGCATCACCTCCGC	40
K	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAG----------AGAGGAGACGCATCACCTCCGC	28
L	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGAGCAG----------AGACGCATCACCTCCGC	32
M	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGAGCAGGATGG----------CATCACCTCCGC	34
N	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGAGCAGGATGGAGAGGAGACG----------GC	81
O	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAG----------CATCACCTCCGC	29
P	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAG----------GC	7
Q	TTCATAAAGCCCTCGCATCCCAGGAGCGAGCAGAGCCAGTCGTCCTACCTCTCCAGACGCATCACCTCCGC	27

FIG. 4. Effects of site-directed mutagenesis on *GFA in vitro* transcription. (*A–C*) *In vitro* transcription of site-specific mutants of *GFA*. Site-specific mutations were introduced as described in *Materials and Methods* at the positions shown in *D*. Experiments were performed as in Fig. 2 *Upper* by using *GFA* oligo-B and *cat* oligo-B as reverse primers. Letters shown above the autoradiograms correspond to the names of the mutants as shown in *D*; PA is the parental plasmid pGfaSacIB. The expected sizes of the products (shown as gfa) are indicated; Δ10 bp, Δ20 bp, and Δ30 bp refer to the sizes of products expected for the *GFA* templates containing deletions in the transcribed region of 10, 20, and 30 bp, respectively. (*D*) Altered sequences and summary of quantitative data for the site-specific mutants. The name and relevant region of each plasmid are shown. Base substitutions are underlined, while the positions of deletions are marked by dashes. Activity is given as the percentage of the activity of the parental plasmid, pGfaSacIB.

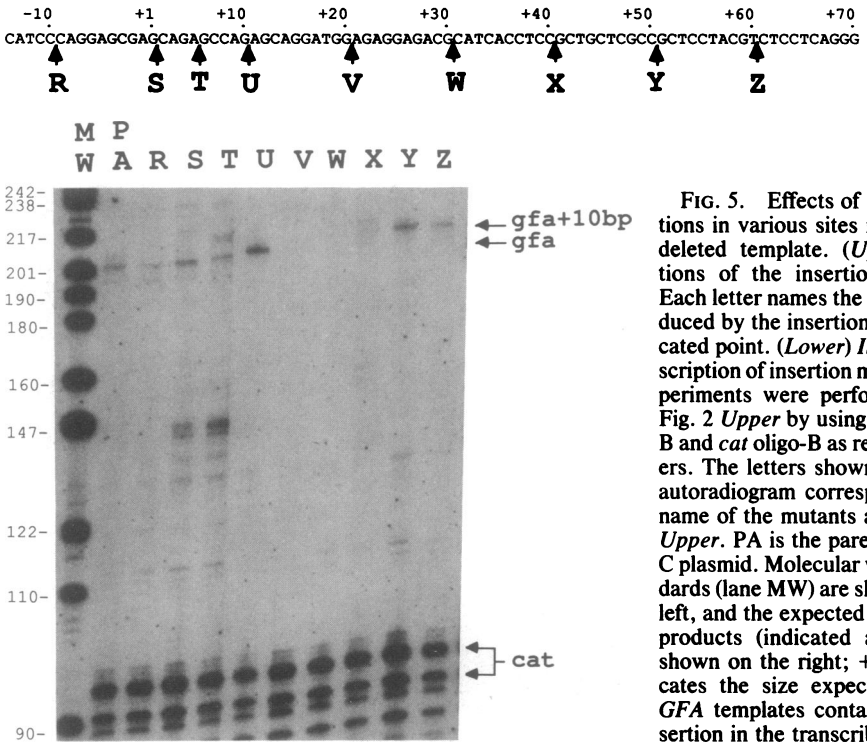


FIG. 5. Effects of 10-bp insertions in various sites in a TATA-deleted template. (*Upper*) Positions of the insertion mutants. Each letter names the mutant produced by the insertion at the indicated point. (*Lower*) *In vitro* transcription of insertion mutants. Experiments were performed as in Fig. 2 *Upper* by using *GFA* oligo-B and *cat* oligo-B as reverse primers. The letters shown above the autoradiogram correspond to the name of the mutants as shown in *Upper*. PA is the parental mutant C plasmid. Molecular weight standards (lane MW) are shown on the left, and the expected sizes of the products (indicated as gfa) are shown on the right; +10 bp indicates the size expected for the *GFA* templates containing an insertion in the transcribed region.

region between +1 and +10 is also an important part of the *GFA* promoter.

Deletions in the region between +11 to +30 (mutants K, L, M, and O) all decreased transcription activity to 30%. Base substitution within this region (mutant Q) also decreased transcription activity to 27%. A deletion between +31 to +40 (mutant N) had little effect on transcription activity, but a deletion between +11 to +40 (mutant P) decreased transcription more severely than a deletion between +11 to +30 (mutant O). Thus, the sequence between +31 to +40 appears to be important when the region between +11 to +30 has been removed.

Mapping of the Downstream Initiator. The above studies suggest that in addition to the TATA box, an extensive downstream region contributes to efficient initiation of *GFA* transcription. To determine the location of the downstream initiator, the 10-bp DNA sequence 5′-TTCCAAGGTT-3′ was inserted at various points (Fig. 5) into a template from which the TATA initiator had been deleted (mutant C, Fig. 4). The following results would be expected for insertions placed upstream, within, or downstream of the downstream initiator. An insertion occurring either upstream or downstream of the initiator should not prevent initiation of transcription. However, since a downstream insertion would reside between the transcription initiation site and the beginning of the reverse transcript used to detect GFA RNA, the length of the reverse transcript formed should be increased by 10 nucleotides. An upstream insertion should not change the size of the reverse transcript if the initiator prompts initiation at a fixed distance from its location. While we cannot predict whether an insertion within the initiator would lead to a transcript of altered length, we expect it would severely reduce the amount of transcript produced. The results obtained (Fig. 5) are consistent with these expectations. Insertions just after +20, +30, and +40 (mutants V, W, and X) severely depressed initiation, indicating that the downstream initiator spans this region. Insertions further downstream (mutants Y and Z) did not affect the quantity of transcript, but the reverse transcript produced was 10 nucleotides longer. Upstream insertions (mutants R, S, T, and U) had little effect on either the amount or the size of the reverse transcripts.

The results described above show that the region between +1 and +10 is important for efficient transcription (Fig. 4, mutants H and I). However, insertions within (mutant T) and just after (mutant U) this region in the plasmid lacking a functional TATA box had only modest effects on the amount and length of reverse transcript. Thus, the region between +1 to +10 is probably not part of the downstream initiator.

Both Initiators Are Utilized by Pol II. To determine whether the *GFA* initiators are recognized by Pol II or Pol III, we tested the effect of α-amanitin on *in vitro* transcription. *In vitro* transcription by Pol II is about 100 times more sensitive to α-amanitin than that by Pol III; in particular, 1 μg of α-amanitin per ml strongly inhibits transcription by Pol II but

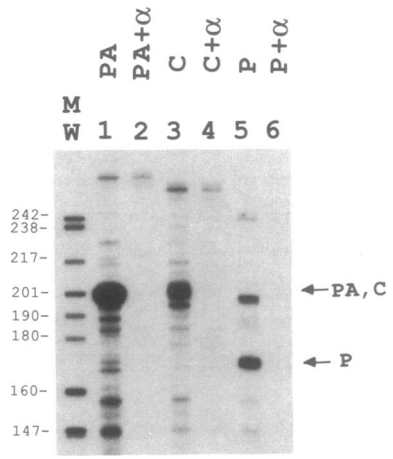


FIG. 6. Effect of α-amanitin on *in vitro* transcription of *GFA*. *In vitro* transcription was performed in the absence (lanes 1, 3, and 5) and in the presence (lanes 2, 4, and 6) of 1 μg of α-amanitin per ml by using as templates 500 ng of plasmids pGfaSacIB (lanes 1 and 2), mutant C in Fig. 4 (lanes 3 and 4), or mutant P in Fig. 4 (lanes 5 and 6). RNAs were analyzed by primer extension with *GFA* oligo-B as primer. Molecular weight standards (lane MW) are shown on the left, and the expected positions for *GFA* transcripts from each template are shown on the right. (The strong upper band in lane 6 is due to initiation from a start site not utilized *in vivo*.)

does not significantly affect transcription by Pol III (12). The sensitivity to α-amanitin was examined by using a template that contains both initiators (the wild-type pGfaSacIB plasmid), one that contains the downstream initiator but no TATA box (mutant C in Fig. 4), and one that contains the TATA box but lacks most of the downstream initiator (mutant P in Fig. 4). Transcription from all three templates was almost completely inhibited by 1 μg of α-amanitin per ml (Fig. 6). Thus, we conclude that both the TATA box and the downstream initiator are utilized by Pol II.

DISCUSSION

In vitro transcription analysis of the 374-bp DNA fragment of *GFA* (Fig. 1) has revealed that there are several DNA regions affecting the transcription rate. The farthest region upstream is a sequence between −100 and −85 that has homology to the AP-2 binding site (11, 13). The next upstream region, between −26 and −22, is a TATA box. TATA boxes act as initiators in many eukaryotic systems (1). In our study its role as an initiator is indicated by the effects of 5′- and 3′ deletions on transcriptional activity and by the proportional displacement of the transcription start site by an internal deletion placed between the TATA box and the normal transcription start site.

A third region, containing the sequence between +1 and +10, appears to be part of a TATA-associated initiator complex. In the absence of this region, the TATA box by itself exhibits very low activity (compare lanes +8 and −18 in Fig. 3 *Upper*). A few other TATA-associated initiator complexes have been described in which a region proximal to the transcription start site ("start region") is required together with the TATA box for efficient transcription from the correct site (14–18). For one of these, the adenovirus major late promoter, the start region by itself acts as an initiator (2). Although the start region of *GFA* is not homologous to that of the adenovirus major late promoter, we cannot exclude the possibility that the *GFA* start region itself also acts as an initiator.

A fourth cis-acting region is the downstream initiator located between +11 and +50. This region contains two overlapping sequences homologous to the box A consensus sequence (10). However, sequences required for full functioning of the *GFA* downstream initiator extend beyond the region of box A homology, and the element is used by Pol II rather than by Pol III. Both this downstream initiator and the TATA-associated initiator complex can direct transcription independently from the same start site. When both are present they act synergistically, initiating much more strongly than either alone (Fig. 4).

Compound promoters containing an upstream TATA-like element and a downstream sequence with homology to box A also have been described for the genes for 7SK RNA (19, 20), MYC (21), Epstein–Barr virus-encoded small RNA (EBER) (22), and U6 small nuclear RNA (23–25). The TATA-like sequence of the 7SK RNA gene acts as a Pol III gene initiator. Although the function of the box A is unclear, the observation that mutations in the TATA-like sequence do not completely eliminate transcription (21) suggests the presence of another initiator. The *MYC* gene has a promoter structure similar to that of the 7SK RNA gene, but its TATA-like sequence is recognized by both Pol II and Pol III (26); the role of its box A sequence has also not been determined. The EBER gene contains a typical Pol III gene promoter composed of a box A and box B in addition to an upstream TATA-like sequence; both of these initiators are recognized by Pol III. The U6 small nuclear RNA gene is similar to the 7SK RNA gene in having a TATA-like sequence and a downstream box A-like sequence and in being transcribed primarily by Pol III. However, in this case the TATA-like sequence is not an initiator but determines the RNA polymerase specificity of an initiator located further upstream (27, 28). Mutations in the U6 TATA-like sequence switch the preference of the upstream initiator from Pol III to Pol II (28). The function of the downstream box A-like region of the U6 RNA gene is unclear.

While the *GFA* promoter is similar in structure to each of the bipartite promoter elements just discussed, it differs in having a downstream initiator that is recognized by Pol II rather than by Pol III. The downstream initiators recognized by Pol II that have been described previously reside in the *Drosophila* genes hsp22 (29), engrailed (30), Ultrabithorax (31), and Antennapedia P2 (32).

The *GFA* downstream initiator is also unique because it is located almost entirely within the protein-coding region of the gene. To our knowledge, an initiator being present in a protein-coding sequence has not been reported previously.

This manuscript is dedicated by Y.N. and M.B. to the memory of a great geneticist, coauthor Dr. Ernst Freese. We thank M. Imagawa for advice on *in vitro* transcription, C. Wu and A. P. Wolffe for critically reading the manuscript, and D. Schoenberg for editing the manuscript.

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