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Specific *in vitro* transcription of conalbumin gene is drastically decreased by single-point mutation in T-A-T-A box homology sequence

(promoter/eukaryote/surrogate genetics/synthetic oligodeoxyribonucleotide/fd phage)

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ABSTRACT **A single-point mutation, consisting of a T-to-G transversion, was made in the third nucleotide of the conalbumin gene T-A-T-A-A-A-A homology sequence (the T-A-T-A or Goldberg–Hogness box). In an *in vitro* system, specific transcription of the mutant DNA was drastically decreased compared to the normal gene. This down-mutation is consistent with the idea that the T-A-T-A box is an important element for specific initiation of transcription.**

Recently, specific initiation of transcription of eukaryotic genes has been obtained *in vitro* with RNA polymerase B (or II). Initially, Weil *et al.* (1) found that the cell-free system (S100 extract) of Wu (2) conferred specificity on the transcription of the adenovirus 2 major late transcription unit DNA. With the same S100 extract, specific *in vitro* initiation of transcription on the chicken conalbumin and ovalbumin genes has been demonstrated (3). With this system, and *in vitro* genetic techniques to construct deletion mutants, it has been shown (4) that sequences, in both the conalbumin gene and adenovirus 2 major late transcription unit, located upstream from the mRNA startpoints are essential for the initiation of specific transcription. These sequences are located between −44 [i.e., 44 nucleotides upstream from the start point for the mRNA (+1), see Fig. 1] and −10 for the conalbumin gene and between −32 and −12 for the Ad2 major late transcription unit (3, 4). These two regions contain in common a sequence of 12 base pairs, 5′ C-T-A-T-A-A-A-A-G-G-G-G 3′ [the C is at position −32 (see ref. 5)], encompassing the sequence T-A-T-A-A-A-A (the so-called T-A-T-A or Goldberg–Hogness box) which is found around position −30 in most eukaryotic genes coding for mRNA and which has been postulated to be a eukaryotic promoter sequence (6–8).

Supporting this possible role is the observation that, although the adenovirus 2 deletion mutant lacking the sequences upstream from position −32 of the gene is fully active in directing specific initiation of *in vitro* transcription, a mutant with a deletion to position −29 is inactivate. This result, however, must be interpreted with some caution. In deletion mutants the DNA upstream from the deletion end point is different from the original DNA, making it impossible to rule out that the observed effects are due to some inhibitory effect of the replacing sequences rather than to an alteration of the promoter.

In order to obtain unequivocal evidence that the T-A-T-A box is implicated in the promotion of transcription, we have used *in vitro* site-directed mutagenesis to make a single-base-pair substitution in the T-A-T-A box. In this paper we report the effect on specific *in vitro* transcription of converting the second T of the conalbumin T-A-T-A box to a G.

MATERIALS AND METHODS

Synthesis of the Undecadeoxyribonucleotide d-C-T-T-T-T-C-T-A-G-A-G. The general materials and methods used for the preparation and purification of the undecadeoxyribonucleotide were as described (ref. 9 and refs. cited therein). The sequence of the purified undecanucleotide was determined by the method of Maxam and Gilbert (10). The 5′-terminal nucleotide was identified by analysis of the mixture obtained by complete digestion of the 5′-^{32}P-labeled product with snake venom phosphodiesterase (9). The sequence of the undecanucleotide was also verified by two-dimensional mobility shift analysis (11) of the 5′-labeled product.

Isolation of Single-Stranded (ss) Bacteriophage fd103 DNA Containing the Noncoding Strand of the Conalbumin Pst5–Pst6 Fragment. The conalbumin Pst5–Pst6 fragment (5) was cloned into the unique *Pst* I site of the bacteriophage fd103 vector essentially as described by Herrmann *et al.* (12) except that *Escherichia coli* K514 was used for transfection. Phage plaques were screened for DNA inserts by plaque hybridization (12). The orientation of the conalbumin fragments was determined by both restriction enzyme mapping of the replicative form (RF) DNA and by hybridization of the ss-DNA to poly-(A)$^+$mRNA followed by electron microscopy (not shown). Phage was purified from the culture supernatant (prepared by centrifugation for 10 min at 12,000 × *g*). After precipitation with 3% polyethylene glycol in the presence of 0.5 M NaCl (12), the phage pellet was redissolved in 50 mM Tris·HCl, pH 7.9/1 mM EDTA (10 ml/500 ml of original culture volume) and layered onto gradients constructed with 4.8 ml of 38% (wt/wt) CsCl, 4.8 ml of 30.5% CsCl, 4.8 ml of 27% CsCl, and 9.6 ml of 12% (wt/vol) sucrose (all dissolved in 50 mM Tris·HCl, pH 7.9/1 mM Na EDTA) in a Beckman SW27 polyallomer tube (one gradient per 500 ml of original culture volume). After centrifugation for 16 hr at 130,000 × *g* and 10°C, the phage band was isolated and dialyzed against 50 mM Tris·HCl, pH 7.9/1 mM EDTA. ss-DNA was prepared by phenol extraction followed by ethanol precipitation.

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Abbreviations: ss, single-stranded; ds, double-stranded; RF, replicative form.

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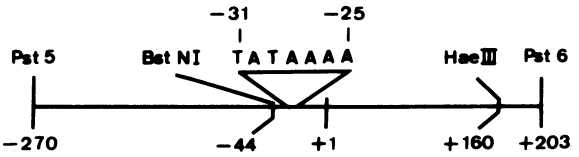

FIG. 1. Restriction enzyme map of the conalbumin Pst5–Pst6 fragment. Pst5 and Pst6 refer to *Pst* I restriction sites as defined (5). DNA sequences in the direction of transcription (downstream) are numbered with positive integers; sequences 5′ to the start point (upstream) are given negative values. +1 represents the position of the base coding for the first nucleotide of conalbumin mRNA. T-A-T-A-A-A-A is the sequence of the noncoding DNA strand between nucleotides −31 and −25 and is the conalbumin sequence equivalent to the T-A-T-A box consensus sequence found in many eukaryotic genes transcribed by RNA polymerase B (7).

Biohazards associated with the experiments described in this publication were examined beforehand by the French National Committee; the experiments were carried out under L2-B1 conditions.

RESULTS

Construction of Mutant Containing Single-Base-Pair Substitution in the Conalbumin T-A-T-A Box. It has been shown (13–17) that a specific point mutation can be obtained with a synthetic oligonucleotide differing in one base from a wild-type DNA sequence. This oligonucleotide is first hybridized to the complementary wild-type strand contained in a circular ss-DNA vector, extended with DNA polymerase I, and finally ligated with DNA ligase to complete the other strand. Transfection with these molecules gives rise to clones containing DNA with the desired sequence change. We have used a fd103 phage recombinant containing the conalbumin Pst5–Pst6 fragment (Fig. 1) and a synthetic undecanucleotide to construct a mutant containing a single base substitution in the conalbumin T-A-T-A box (Fig. 2).

The noncoding strand of the conalbumin Pst5–Pst6 fragment (Fig. 1) was cloned in fd103 ss-DNA at the position of the unique *Pst* I site of the RF DNA. The undecanucleotide, complementary to the T-A-T-A box region except for a C (boxed nucleotide in the upper left circle in Fig. 2), was syn-

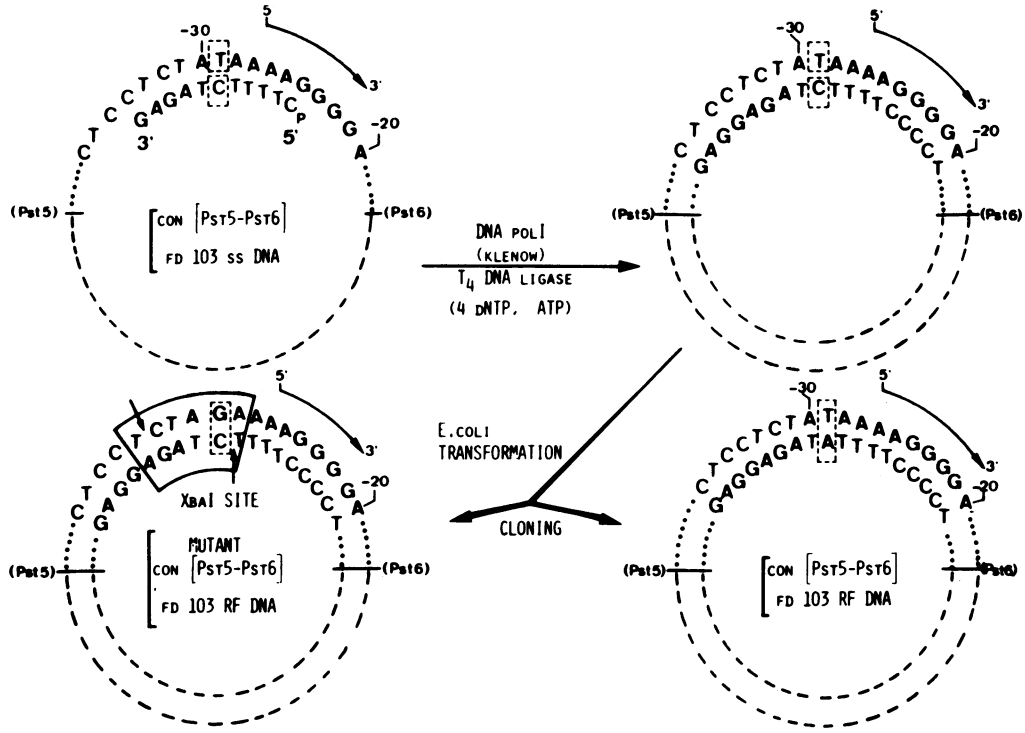

FIG. 2. Preparation of a mutant containing a single-base-pair substitution in the conalbumin T-A-T-A box. Undecanucleotide primer (100 pmol) was 5′-phosphorylated in a reaction mixture (50 μl) containing 50 mM Tris·HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 5% glycerol, 500 μCi (1 Ci = 3.7×10^{10} becquerels) of [γ-^{32}P]ATP (New England Nuclear) and 5 units of T4 polynucleotide kinase (Boehringer). After 1 hr at 37°C, additional T4 kinase (5 units) and ATP (to 100 μM) were added and incubation was continued for 30 min at 37°C. Labeled primer was isolated by chromatography on a 10-ml Sephadex G-25 column in 50 mM triethylammonium bicarbonate (pH 7.9). The primer pool was lyophilized, resuspended in water, and lyophilized a second time. A mixture (5 μl) containing 40 pmol of labeled primer, 0.6 pmol of ss-DNA fd103 recombinant (containing the noncoding strand of the Pst5–Pst6 conalbumin fragment), 40 mM Tris·HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 M NaCl, and 2 mM 2-mercaptoethanol was incubated for 3 min at 80°C and for 60 min at 0°C. A mixture (7.5 μl) containing 22 mM Tris·HCl (pH 7.5), 11 mM $MgCl_2$, 1 mM 2-mercaptoethanol, dATP, dGTP, dCTP, and TTP each at 0.83 mM, 0.4 mM ATP, 2.5 units of *E. coli* DNA polymerase I (Klenow fragment, Boehringer Mannheim), and 0.5 unit of T4 DNA ligase (BioLabs) was added and incubation was continued for 30 min at 0°C and 5 hr at 23°C. Samples (1 μl) were analyzed directly on 1% agarose gels (to check for conversion to double-stranded circles) and, after digestion with *Pst* I or *Msp* I, on 5% acrylamide urea gels (to check by autoradiography for correct priming and efficient ligation). One microliter was diluted 1:10 with 30 mM sodium acetate, pH 4.3/4.5 mM $ZnCl_2$/0.28 M NaCl and digested with 1 unit of S1 nuclease (Miles) for 15 min at 23°C. Then, 2.5 μl of 2 M Tris·HCl (pH 7.9) was added and, after extraction with phenol followed by ether and extensive dialysis against 100 mM Tris·HCl (pH 7.9), the DNA was transfected into *E. coli* K514 as described above. Approximately 10 plaques were obtained per ng of starting ss-DNA. A phage pool from approximately 200 plaques was used to infect *E. coli* KB35 and, after plating on chloramphenicol agar plates, clear lysate DNA from individual colonies was screened for the presence of an *Xba* I site in the Pst5–Pst6 conalbumin fragment. In the figure, conalbumin and fd103 ss-DNA are represented by dotted and dashed lines, respectively. The individual boxed nucleotides are mismatched (upper circles) and the boxed base pairs (lower circles) are matched. The boxed hexanucleotide sequence (lower left) is the *Xba* I recognition site. DNA polI (Klenow), DNA polymerase I (Klenow fragment); dNTP, deoxyribonucleoside triphosphate; con, conalbumin; Pst5 and Pst6, see Fig. 1 and text.

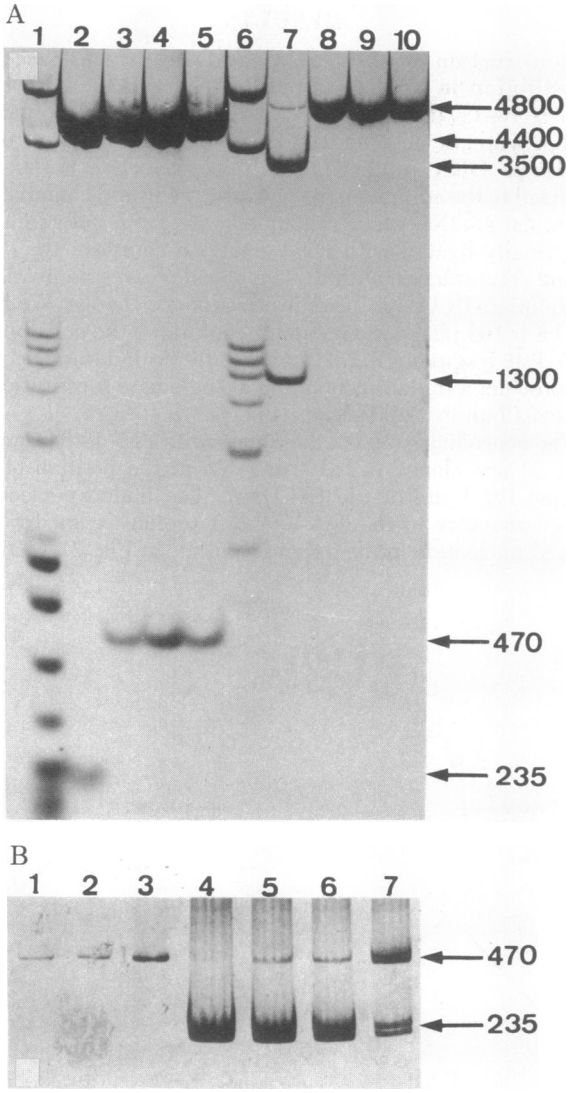

FIG. 3. Restriction enzyme mapping of mutant and wild-type recombinants. The mutant conalbumin Pst5–Pst6 fragment was cloned in the *Pst* I site of pBR322. (*A*) Purified recombinant DNA was digested with restriction enzymes and analyzed on a 1.5% agarose gel. Lanes: 1 and 6, DNA size markers; 2, 4, 7, and 9, mutant recombinant digested with *Pst* I/*Xba* I, *Pst* I, *Xba* I/*Bam*HI, and *Bam*HI, respectively; 3, 5, 8, and 10, wild-type recombinant digested with *Pst* I/*Xba* I, *Pst* I, *Xba* I/*Bam*HI, and *Bam*HI, respectively. (*B*) Mutant conalbumin Pst5–Pst6 fragment was isolated by sucrose density gradient centrifugation, digested with different quantities of *Xba* I, and analyzed on a 5% polyacrylamide gel. Lanes: 1, 2, and 3, 5, 10, and 50 pg of mutant Pst5–Pst6 fragment, respectively, before digestion; 4, 5, 6, and 7, 0.5 μg of mutant fragment digested with 20, 12.5, 7.5, and 2.5 units of *Xba* I, respectively, for 1 hr at 37°C.

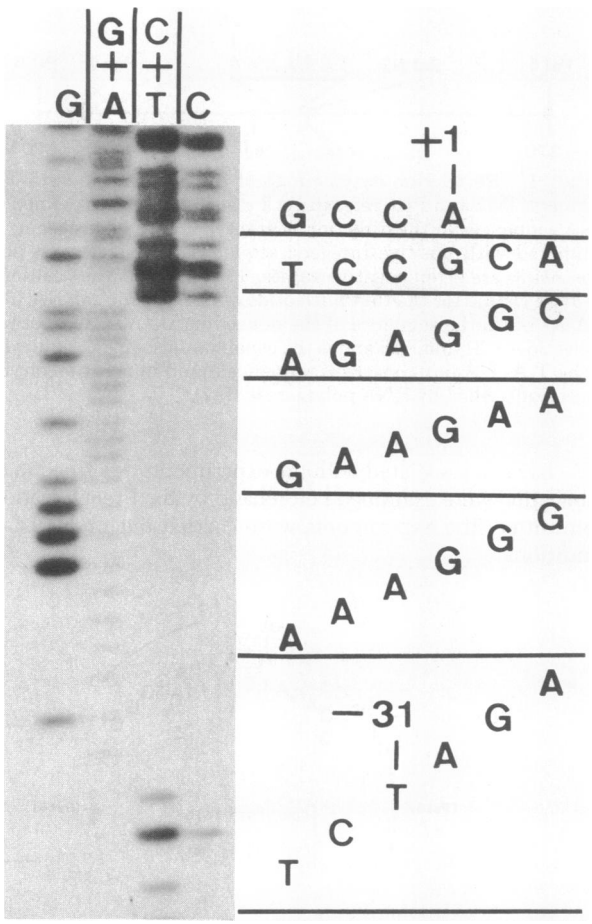

FIG. 4. Sequence of mutant DNA. The *Bst*NI (−44) to *Hae* III (160) fragment (Fig. 1), 5′-end-labeled at the *Bst*NI site, was subjected to sequenced determination by the method of Maxam and Gilbert (10) and electrophoresed on a 20% acrylamide/urea gel. −31 and +1, number of nucleotides from the mRNA start point (see Fig. 1).

thesized by the triester method, hybridized to the ss-DNA, and used to synthesize the complementary strand (13–17) (see legend to Fig. 2). After cloning, RF DNAs from 50 individual colonies were screened for the presence of an *Xba* I site, which is conveniently formed by the mutation (see Fig. 2). The DNA of two colonies contained an *Xba* I site. The mutant Pst5–Pst6 fragment was isolated from the fd103 RF DNA and inserted into the *Pst* I site of pBR322. Recombinants corresponding to the two original clones were selected and on subsequent characterization were found to give essentially identical results.

Characterization of the Conalbumin T-A-T-A Box Mutant. Figs. 3 and 4 demonstrate that the conalbumin mutant differs from the wild-type DNA by the change of the second T of the T-A-T-A box to a G. Fig. 3 compares the restriction enzyme digestion patterns of mutant and wild-type DNAs. Only the mutant, and not wild-type pBR322 recombinant, contained an *Xba* I site (Fig. 3A, lanes 7–10). Only the Pst5–Pst6 fragment (470-base-pair band) from the mutant DNA contained an *Xba* I site (giving the bands at 235 base pairs). To show that the mutant DNA was not contaminated with wild-type fragment we digested 0.5 μg of mutant Pst5–Pst6 fragment with increasing amounts of *Xba* I (Fig. 3*B*, lanes 4–7). Comparison of lane 4 (corresponding to the largest amount of *Xba* I tested) with lanes 1–3 shows that >99% of the mutant Pst5–Pst6 fragment contained the expected *Xba* I site. The T→G transversion was the only modification observed in this region, as shown by the sequence analysis in Fig. 4.

***In Vitro* Transcription of the Conalbumin T-A-T-A Box Mutant.** By S1 nuclease mapping and by the "run-off" transcription method it has been shown (3) that specific initiation occurs *in vitro* on conalbumin gene DNA. In the "run-off" assay, RNAs of discrete sizes are produced on restriction enzyme DNA fragments by run-off termination whenever specific initiation occurs. From the length of the run-off transcripts, the position of the 5′ end of the RNA can be deduced. As expected (see Fig. 1), a major run-off transcript of ≈200 nucleotides (see band labeled with an arrowhead in Fig. 5*A*, lane 3, and also refs. 3 and 4) is obtained with the Pst5–Pst6 fragment as a template. Similarly, for the *Hae*III-digested Pst5–Pst6 fragment, a specific run-off fragment of about 160 nucleotides was obtained (Fig.

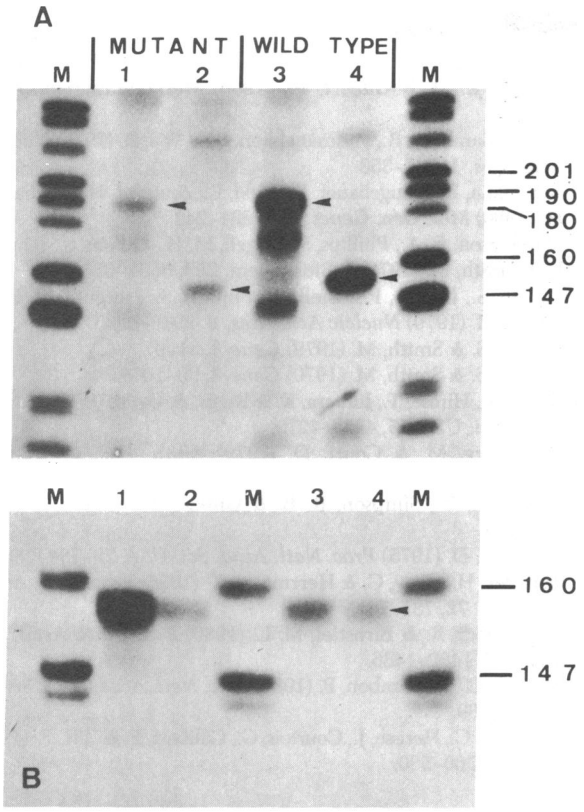

FIG. 5. *In vitro* transcription of wild-type and mutant conalbumin gene DNA. The wild-type and mutant conalbumin DNA fragments were transcribed *in vitro* as described (3) by using purified calf thymus RNA polymerase B and HeLa cell S100 extract, except that the nucleotide concentrations were 100 μM ATP, 100 μM GTP, 100 μM UTP, and 10 μM CTP, and creatine kinase (0.13 mg/ml) and creatine phosphatase (0.1 mM) were included in the reaction. (*A*) ^{32}P-Labeled RNA was on a 5% polyacrylamide/urea gel. Lanes: 1 and 3, 0.5 μg of mutant and wild-type Pst5–Pst6 fragments, respectively; 2 and 4, 0.5 μg of mutant and wild-type Pst5–Pst6 fragments digested with *Hae*III, respectively (see Fig. 1). (*B*) The RNA synthesized on Pst5–Pst6 fragment digested with *Hae*III was electrophoresed on a 5% polyacrylamide/urea 40-cm thin gel (0.3 mm). Lanes: 1, RNA synthesized on the wild-type fragment; 2 and 4, RNA synthesized on the mutant fragment; 3, one-fifth of the amount of RNA run in lane 1. M, ^{32}P-labeled DNA size markers. The arrowheads point to the bands discussed in the text.

5*A*, lane 4; Fig. 5*B*, lanes 1 and 3). The RNA run-off transcripts appeared to be slightly shorter than expected perhaps because of termination of transcription slightly before the end of the fragment or because of slight differences in the electrophoretic properties of DNA and RNA. Specific run-off transcripts of identical mobility, but weaker intensity, were obtained (Fig. 5*A*) for the mutant Pst5–Pst6 fragment that was intact (lane 1) or was digested (lane 2) with *Hae*III.

Electrophoresis of the run-off transcript obtained with the mutant Pst5–Pst6 fragment digested with *Hae*III next to the wild-type transcript on a 5% polyacrylamide/urea sequencing gel (Fig. 5*B*, lanes 1/2 and 3/4) demonstrated that both RNAs had the same mobility. Therefore, it appears that the transcription start site is the same for wild-type and mutant conalbumin genes. By scanning various exposures of the autoradiograms we found that specific transcription on the mutant fragment was about 5% of that of wild-type. Similar results were obtained by using various DNA concentrations (10–50 μg/ml in the assay) and DNA preparations (from both the fd103 and pBR322 recombinants).

DISCUSSION

We show here that a single-base-pair transversion (T-to-G) at position −29 in the T-A-T-A box drastically decreases the efficiency of specific transcription of conalbumin DNA. This down-mutation, together with previous results (3, 4), demonstrates that the T-A-T-A box is an important element for initiation of specific *in vitro* transcription. Therefore, it appears that the T-A-T-A box fulfills at least one of the criteria used to define prokaryotic promoters. However, we cannot definitely conclude that the T-A-T-A box is part of a eukaryotic promoter region because prokaryotic promoters were defined as regions of the DNA that are indispensable for initiation of transcription and to which RNA polymerase binds (see refs. 18 and 19 and refs. therein).

The T-A-T-A box shares both sequence and functional homologies with the Pribnow box (18, 19) of prokaryotic promoters. This prokaryotic homology sequence, first observed by Pribnow (20) and by Schaller (21), is related to 5′ T-A-T-A-A-T-G 3′ and is located about 10 base pairs upstream from the mRNA start point. The effects of various mutations in the Pribnow box have been reviewed (18, 19). Strikingly, all A·T-to-G·C base substitutions are promoter-down mutations, and a G·C-to-A·T base substitution is a promoter-up mutation, suggesting that the Pribnow box mutations are exerting some effect on local DNA melting. However, because A·T-to-T·A base changes also influence transcription (see refs. 18 and 19), this is probably not the major effect.

These observations raise the question of whether the mutation we have constructed in the T-A-T-A box prevents a similar DNA-opening event in eukaryotic transcription or decreases the affinity of RNA polymerase B or factor(s) in the S100 extract for this DNA sequence. In preliminary mixing experiments using crude S100 extracts, we have been unable to demonstrate any competition between wild-type and mutant conalbumin DNAs for factor(s) present in the *in vitro* transcription system. However, because we cannot rigorously prove that the DNA is in excess over essential factor(s) in the system, we cannot draw any firm conclusion about the mechanism by which the T-to-G transversion affects the efficiency of specific *in vitro* transcription of the conalbumin gene. Studies of the interaction of purified RNA polymerase B and factor(s) present in the S100 extract with the T-A-T-A box region will be necessary to answer this question.

Point mutations have unequivocally demonstrated the *in vivo* promoter role of the Pribnow box. Although it is tempting to speculate that the T-A-T-A box will also exhibit a promoter role *in vivo*, no direct evidence is available at the present time to support this assumption. Two reports dealing with this problem have been recently published. First, Grosschedl and Birnstiel (22) found that for the sea urchin histone H2A gene injected into *Xenopus* oocytes, deletion of the T-A-T-A sequence did not abolish transcription. Instead, a number of new start sites of lower efficiency were generated. Second, Benoist and Chambon (23) showed that a recombinant plasmid, constructed by inserting simian virus 40 early genes into pBR322, expressed the early genes when introduced into eukaryotic cells. A recombinant with a 60-base-pair deletion, which removed the T-A-T-A box, still expressed the simian virus 40 early genes.

Along the same lines it should be recalled that there are two notable exceptions to the universal presence of the T-A-T-A box; the papovavirus late genes and the adenovirus early region 2 genes (24). These exceptions are accompanied by the occurrence of multiple start sites, suggesting that there may be more than one class of promoter for RNA polymerase B. However, the above *in vivo* experiments with deletion mutants do not exclude that the T-A-T-A box plays an essential role *in vivo*. For

the experiments of Benoist and Chambon it is possible that, in the mutant lacking the T-A-T-A box, minor promoters, initiating upstream from the major cap sites, were responsible for the transcription of the early genes. In the experiments of Grosschedl and Birnstiel, one cannot exclude that some of the sequences replacing the deleted DNA are mimicking the original T-A-T-A box function.

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