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Specific 5′ flanking sequences are required for faithful initiation of *in vitro* transcription of the ovalbumin gene

(deletion mutants/Hogness box/eukaryotic promoter)

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ABSTRACT **An *in vitro* system [Weil, P. A., Luse, D. S., Segall, J. & Roeder, R. G. (1979) *Cell* 18, 469–484 and Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gefter, M. L. (1980) *Proc. Natl. Acad. Sci USA* 77, 3855–3859] was adapted for studying initiation of transcription of the ovalbumin gene. The DNA template was a cloned ovalbumin gene fragment that contained 5′ flanking sequences, the first structural sequence region, and a portion of the first intervening sequence. A HeLa cell crude extract was used as the source of RNA polymerase and initiation factors. Correct initiation was judged by the sizes of the transcription products generated from ovalbumin templates truncated at various positions before the 3′ end of the gene. Transcription of the specific product was carried out by RNA polymerase II, as judged from α-amanitin sensitivity. A series of deletion mutants was constructed by trimming 5′ flanking sequences of the ovalbumin DNA template by using exonuclease III. The DNAs generated were then cloned in pBR322 and used as templates to determine which sequences were necessary for initiation of transcription. Specific initiation of the ovalbumin gene was unaffected by deletion of all but 61 nucleotides of the 5′ flanking sequence but completely abolished by deletion of all but 26 nucleotides of 5′ flanking sequence. Thus, a region between 61 and 26 nucleotides upstream from the cap site, which includes the Hogness box (T-A-T-A-T-A-T) at position 32–26, is essential for the correct initiation of the ovalbumin gene. Nevertheless, natural DNA fragments containing false Hogness boxes (T-A-T-A-A-A-A and T-A-T-A-T-A-T) not normally located in the immediate 5′ flanking region of an authentic gene did not serve as promoters for initiation of transcription. These results suggest that the Hogness box is essential, but not sufficient, for specific initiation of RNA synthesis.**

The mechanisms that permit the transcription of eukaryotic genes are poorly understood. The development of DNA-dependent, cell-free, *in vitro* transcription systems would facilitate the elucidation of the steps involved in these processes. In 1978, Wu showed that accurate synthesis of a 5.5S RNA can be carried out by using a soluble extract from KB cells containing polymerase III and adenovirus 2 (Ad-2) DNA (1). Subsequently, specific transcription of *Xenopus* 5S RNA and yeast tRNA was reported in an oocyte cell-free system (2–4) and a KB cell reconstituted system (5). By the construction of 5′ and 3′ deletion mutants and an *in vitro* assay system, Bogenhagen *et al.* (6) and Sakonju *et al.* (7) showed that an internal fragment between nucleotides 41 and 87 of the 5S gene contains sufficient information to direct the initiation of specific transcription of the 5S gene by RNA polymerase III. Recently, Engelke *et al.* (8) isolated a protein factor that specifically interacts with the internal control region (nucleotides 45–96) of the 5S gene and appears to be necessary for its accurate transcription.

Contrary to the rapid progress with the polymerase III transcription system, little is known about the initiation of synthesis of mRNA by RNA polymerase II. Recently, Weil *et al.* (9) showed the selective initiation of transcription at a major late promoter of Ad-2 DNA by using crude extracts from KB cells supplemented with purified RNA polymerase II. Similarly, Manley *et al.* (10) showed the specific initiation of transcription at various late promoters of Ad-2 DNA by using a whole-cell extract from HeLa cells. We have adapted the HeLa crude enzyme system of Manley *et al.* (10) to study *in vitro* transcription of the ovalbumin gene in the hope of defining the specific features in the DNA sequences that are essential for accurate initiation. We have previously identified the transcription unit of the ovalbumin gene (11, 12), the sequences of the entire ovalbumin natural gene, and the flanking DNA bordering the 5′ end of the cap site (S. L. C. Woo, personal communication). We here report the development of an *in vitro* transcription system that provides proper initiation of the ovalbumin DNA. By using this transcription system and deletion mutants generated by exonuclease III and S1 nuclease trimming techniques (7), we found that a region upstream from the cap site that includes the Hogness box is essential for correct initiation of ovalbumin DNA.

MATERIALS AND METHODS

Preparation of DNA Fragments. Restriction endonucleases were purchased from Bethesda Research Laboratories (Rockville, MD) or New England BioLabs. Assay conditions were as recommended by the supplier. DNA fragments were prepared by digestion with the appropriate restriction enzymes and separated by agarose gel electrophoresis. The band of interest was localized by staining with ethidium bromide, excised, and eluted as described (11).

***In Vitro* Synthesis and Isolation of RNA.** RNA was transcribed from ovalbumin DNA fragments by a modification of the procedure of Manley *et al.* (10) and Weil *et al.* (9).

A standard 100-μl reaction mixture contained 12 mM Hepes, pH 7.9/3–5 mM $MgCl_2$/60 mM KCl/1.5 mM dithiothreitol/10% glycerol/0.2 mM EDTA/500 μM ATP, CTP, and UTP/50 μM GTP, 50 μCi of [α-^{32}P]GTP (1 Ci = 3.7×10^{10} becquerels), 2.5 μg of DNA, and 60 μl of crude extract from HeLa cells. The HeLa crude extract was prepared according to the method of Manley *et al.* (10), except that it was dialyzed against a buffer containing 5 mM $MgCl_2$ before storage at −70°C. The reaction mixtures were incubated at 30°C for 45 min, and RNA was isolated as described (13).

Analysis of RNA by Polyacrylamide Gel Electrophoresis. RNA samples were suspended in 99% formamide, denatured by heating at 100°C for 5 min, and then loaded on a 4% gel. Gel electrophoresis was carried out at 20 mA/4 hr as described by Maniatis *et al.* (14). Autoradiograms were obtained by exposure to Kodak XR-p film with an intensifier screen at −20°C.

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Abbreviation: Ad-2, adenovirus 2.

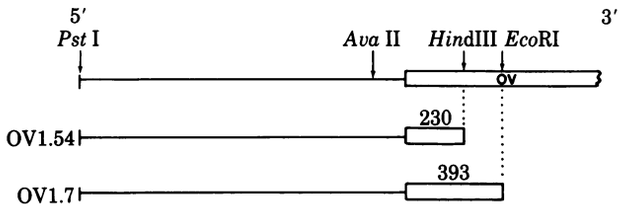

FIG. 1. Map of the 5′ end of the ovalbumin gene and its flanking sequences showing the restriction endonuclease cleavage sites.

Construction and Isolation of 5-Deleted Ovalbumin DNA. 5′-Deleted ovalbumin DNA was constructed according to the procedure of Sakonju *et al.* (7) (see Fig. 6). The blunt-ended DNAs were ligated to *Bam*HI linkers and cloned as *Bam*HI/*Eco*RI inserts in pBR322. Transformation and identification of the recombinant plasmids were carried out according to Stein *et al.* (15). Exact endpoints of the deletion were determined by DNA sequence analysis by the procedure of Maxam and Gilbert (16).

RESULTS

Transcription of Ovalbumin DNA *in Vitro*. A cloned ovalbumin gene fragment, OV1.7, that contains 5′ flanking sequences, the first structural sequence region, and a portion of the first intervening sequence (Fig. 1) was used as template. The HeLa cell crude extract, prepared according to Manley *et al.* (10), was used as the source of RNA polymerase and initiation factors. Because the 5′ end of ovalbumin mRNA is well defined (11), one should observe an RNA product 393 nucleotides long if transcription starts at the cap site, identical to the *in vivo* start site. We found a major radioactive-labeled band 393 ± 5 nucleotides long (Fig. 2), consistent with the notion that transcription of the ovalbumin DNA fragment begins at the cap site. When an internal fragment from the ovalbumin gene, OV1.8 (*Eco*RI/*Eco*RI fragment, ref. 17), was used as template, no specific product could be detected; similarly, another internal fragment of the ovalbumin gene, OV1.35 (*Hinf*I/*Hinf*I; ref. 18), did not support specific transcription of the ovalbumin gene. These results suggest that fragment OV1.7 may contain sequences that are important for initiation of transcription of the ovalbumin gene.

Synthesis of the 393-nucleotide RNA product was optimum at 22–30°C, 3–5 mM Mg^{2+}, and DNA at 25 μg/ml for 45 min.* These conditions were used for all the following experiments.

Selective Initiation of Ovalbumin DNA Templates that Are Truncated at the 3′ End. To ascertain that the RNA product transcribed from the OV1.7 DNA fragment was indeed initiated at the cap site, restriction fragments of ovalbumin DNA having identical 5′ ends but varying lengths of 3′ sequences (see Fig. 2) were used as templates. If transcription initiates at the cap site and proceeds to the right toward the 3′ end of the DNA fragment, the truncated templates OV1.7 (*Pst*/*Eco*RI) and OV1.54 (*Pst*/*Hin*dIII) should give runoff RNAs that are 393 and 230 nucleotides long, respectively. We found a major RNA 395 nucleotides long in the autoradiogram when the OV1.7 (*Pst*/*Eco*RI) fragment was used as template and that reduction of the 3′ end of the template to the OV1.54 (*Pst*/*Hin*dIII) fragment led to the synthesis of a 230-nucleotide-long RNA and the disappearance of the 395-nucleotide-long product (Fig. 3). The size of the RNAs synthesized by the truncated templates closely agreed with the predicted values—395 ± 5 vs. 393 for OV1.7 and 230 ± 3 vs. 230 for OV1.54 (average values of at least six sets of experiments). These results strongly suggest that transcription initiates at a specific site *in vitro*, proceeds to the right, and terminates at the 3′ end of the truncated DNA templates. In addition, this experiment also showed that synthesis of the 393-nucleotide RNA from OV1.7 and the 230-nucleotide RNA from OV1.54 was carried out by RNA polymerase II, because the transcription of these two RNA bands was completely inhibited by α-amanitin at 2 μg/ml.

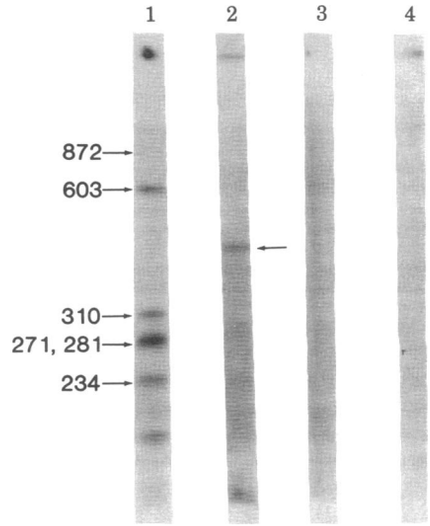

FIG. 2. Gel electrophoresis analysis of RNA synthesized *in vitro* from OV1.7 (lane 2), OV1.8 (lane 3), and OV1.35 (lane 4). RNAs synthesized by using standard reaction mixtures were denatured in formamide and loaded on 7 M urea/4% polyacrylamide gels. Autoradiograms show products synthesized by various templates. Arrows indicate positions of the specific run-off transcripts. *Hae* III-digested fragments of φX174 DNA were end labeled with [γ-^{32}P]ATP as described by Roop *et al.* (11) and used as size markers. Lane 1, markers.

Nuclease S1 Mapping of the *in Vitro* RNA Product from OV1.7. To substantiate that initiation of transcription occurs at

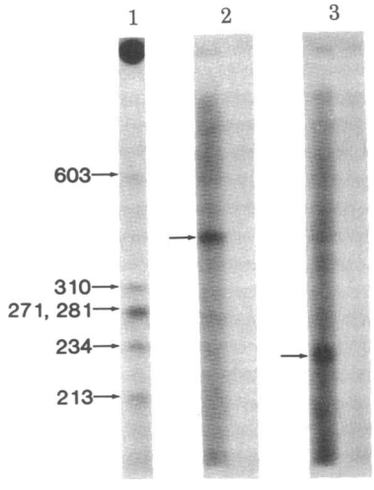

FIG. 3. Electrophoresis analysis of RNAs synthesized from truncated ovalbumin DNAs. OV1.7 (lane 2) or OV1.54 (lane 3) (2.5 μg) was used as template. Specific run-off transcripts are indicated by arrows. The α-amanitin sensitivity of transcription from ovalbumin DNA was evaluated by synthesizing RNA in the presence (right-hand side of lanes 2 and 3) of α-amanitin at 2 μg/ml. (The background observed in this figure may be due to random, nonspecific synthesis or to initiation at nicks or gaps on the DNA template.) Lane 1, markers.

*Tsai, M.-J., Tsai, S. Y. & O'Malley, B. W., *Expression of Eukaryotic Viral and Cellular Genes*, Sigrid Juselius Symposium, Spring 1980, Helsinki, Finland.

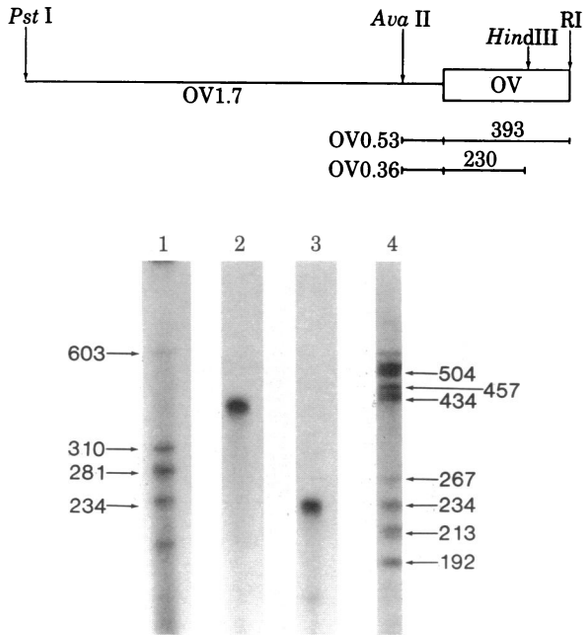

FIG. 4. Nuclease S1 mapping of the 393-nucleotide transcription product of OV1.7. [^{32}P]RNA was synthesized from OV1.7 and subjected to electrophoresis in a 4% acrylamide gel. The 393-nucleotide band was extracted according to the procedure of Maxam and Gilbert (16), and the product was then hybridized to OV0.36 (lane 3) and OV0.53 (lane 2) DNAs as described by Roop *et al.* (11), except that the NaCl content was 0.5 M and hybridization was carried out for 18 hr. After S1 nuclease digestion, the resistant [^{32}P]RNA was run in a 4% acrylamide gel and autoradiographed. *Hae* III-digested fragments of pBR322 (lane 4) and ϕX174 (lane 1) were end labeled with [γ-^{32}P]ATP as described by Roop *et al.* (11).

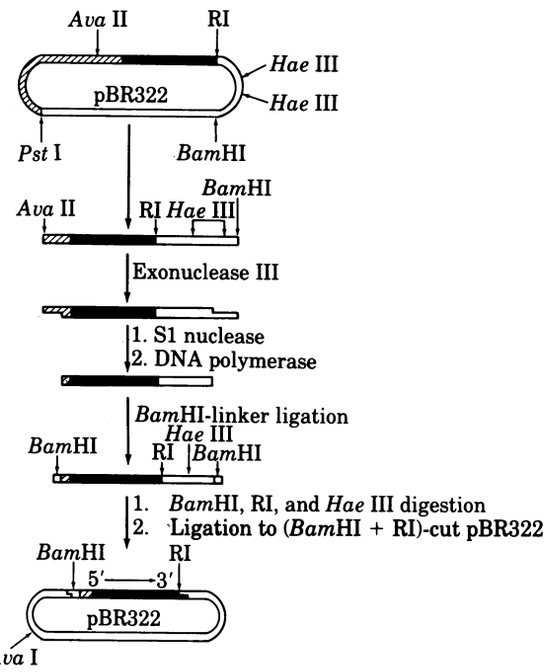

FIG. 5. Construction of 5'-deleted ovalbumin DNA. The ovalbumin gene is shown as a solid segment. The hatched segment represents the DNA sequences flanking the 5' end of the ovalbumin gene, and the open segment represents the plasmid DNA. RI, *Eco*RI.

the cap site, *in vitro* [^{32}P]RNA (393 nucleotides long) synthesized from fragment OV1.7 was isolated by preparative gel electrophoresis and hybridized to ovalbumin DNA fragments, OV0.53 (*Ava* II/*Eco*RI) and OV0.36 (*Ava* II/*Hin*dIII). After S1 nuclease digestion, the hybrid was rerun on a 4% gel. The hybrids formed should give a single band at position 393 if RNA synthesis is initiated at the cap site of the ovalbumin gene; as expected, a single band at 393 was observed (Fig. 4). However, when the 393-nucleotide RNA was hybridized to OV0.36 and digested with nuclease S1, a single band of 230 nucleotides was observed, which agrees with the predicted value if initiation occurred at cap site. Thus, initiation of synthesis of the major RNA product in this *in vitro* transcription system starts at the *in vivo* cap site.

Construction of 5'-Deletion Mutants. To test whether sequences flanking the 5' end of the ovalbumin gene are important for initiation of the specific RNA *in vitro*, cloned pOV1.7 plasmid DNA was digested with *Bam*HI and *Ava* II to give a 900-nucleotide DNA fragment. This fragment was subjected to trimming by exonuclease III to generate DNAs of various lengths (7) (Fig. 5). Single-stranded DNA tails were then digested with S1 nuclease and DNA polymerase was used to fill in any residual single-strand regions. The blunt-ended DNAs were ligated to *Bam*HI linkers; digested with *Bam*HI, *Eco*RI, and *Hae* III; and then cloned to give *Bam*HI/*Eco*RI inserts in pBR322. The clones were digested with *Bam*HI, *Eco*RI, and *Hin*dIII and subjected to gel electrophoresis. The size of the *Bam*HI/*Eco*RI fragments depended on the lengths of the sequences deleted during exonuclease III treatment. The fragments were labeled at the 3' end and then subjected to sequence determination to locate the exact deletion site (16).

Transcription of 5'-Deletion Mutants. DNAs from the 5' deletion mutants were digested with *Ava* I (at position 1424 of pBR322 DNA) and *Eco*RI. The *Ava* I/*Eco*RI fragments contained the ovalbumin DNA sequences and 1.05 kilobases of plasmid sequences upstream from the ovalbumin DNA. These fragments were purified by agarose gel electrophoresis and used as templates for *in vitro* transcription. The specific run-off product of the ovalbumin DNA (395 nucleotides long) was synthesized by deletion mutants OVΔ5'(121$^-$), OVΔ5'(107$^-$), and OVΔ5'(61$^-$) (Fig. 6). Thus, deletion of any DNA sequences upstream from position 61$^-$ does not affect the initiation of transcription of the ovalbumin DNA *in vitro*. By contrast, transcription of the specific product was completely abolished when OVΔ5'(26$^-$), OVΔ5'(41$^+$), OVΔ5'(56$^+$), and OVΔ5'(86$^+$) mutants were used as templates. Some higher molecular weight products seen in the autoradiogram when deletion mutant DNA was used as template appear to result from transcription of the plasmid DNA, but no attempt was made to characterize them. The deletion of sequences between 61$^-$ and 26$^-$ completely eliminated the initiation of ovalbumin DNA, which suggests that this region of DNA is essential for initiation of ovalbumin DNA *in vitro*. Interestingly, OVΔ5' (26$^-$) contains only the last nucleotide of the "Hogness box" (T-A-T-A-T-A-T, in the case of ovalbumin) that exists in most eukaryotic genes and has been postulated to function as a possible recognition site by RNA polymerase II.

Is the Hogness Box a Sufficient Signal for Specific Initiation of Transcription of the Ovalbumin Gene? In addition to the presumptive promoter, the Hogness box (T-A-T-A-T-A-T) at the 32$^-$ position of the ovalbumin DNA, there are several similar sequences present in the OV1.7 DNA fragment. The identical sequence occurs at the 5' flanking (upstream) position 1090$^-$ (T-A-T-A-T-A-T) and similar ones occur at positions 825$^-$, 624$^-$, 607$^-$, 78$^+$, and 174$^+$. If these sequences can be recognized by RNA polymerase II as promoters, we would expect to find RNA products of OV1.7 having sizes of 1451, 1186, 985, 968, 283,

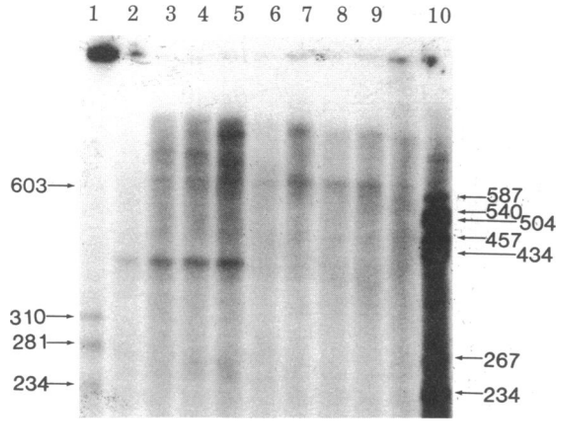

FIG. 6. Electrophoresis analysis of transcripts from cloned 5′-deleted ovalbumin DNA. The 5′-deleted ovalbumin DNA (Δ5′) is a series of clones in which the direction of deletion is from the 5′ end toward the 3′ end. The precise end point of deletion is determined by DNA sequencing. For instance, 121^- indicates that the ovalbumin flanking DNA is deleted to the left of nucleotide 121^-, which itself is 121 nucleotides upstream from the cap site of the ovalbumin gene. Autoradiograms show *in vitro* transcribed [^{32}P]RNA. Lanes: 2, φX174; 3, OVΔ5′(121^-); 4, OVΔ5′(107^-); 5, OVΔ5′(61^-); 6, OVΔ5′(26^-); 7, OVΔ5′(41^+); 8, OVΔ5′(56^+); 9, OVΔ5′(86^+). Size markers are *Hae* III fragments of φX174 (lane 1) and pBR322 (lane 10) DNAs ^{32}P-end labeled as described by Roop *et al.* (11).

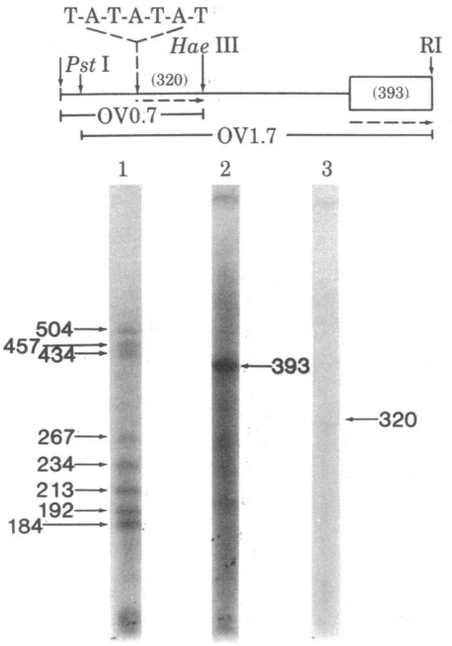

FIG. 7. Electrophoresis analysis of RNA synthesized *in vitro* from OV0.7 (lane 3) and OV1.7 (lane 2). Fragment OV0.7 was isolated from pOV1.7 and digested by *Hae* III and *Eco*RI restriction enzymes as illustrated in the map. [^{32}P]RNAs were synthesized and analyzed as described in Fig. 2. Lane 1, markers.

and 187 nucleotides, respectively.

However, the only major RNA product found was 393 nucleotides long (see Figs. 2, 3, and 6). We conclude that specific transcription of the ovalbumin gene is directed solely by the Hogness box at the −32 position. One might argue that the RNA polymerase or other factors in our *in vitro* system may concentrate on the strong promoter (at position −32) and leave weak ones unattended. Thus, we obtained a DNA fragment restricted by *Hae* III (OV0.7) that lacked most of the Hogness boxes, including the one at position −32.† We might expect that, without competition from a stronger promoter, the RNA polymerase could concentrate at these promoter regions if it is possible for them to be used. We found that no major RNA band with the predicted sizes (310 and 155 nucleotides) was synthesized from this DNA template (OV0.7) (Fig. 7). Therefore, the Hogness box at position −32 is the only one that can be used as a promoter for *in vitro* RNA synthesis; none of the others are effective. Taken together, our results suggest that the Hogness box is essential, but not sufficient, for specific initiation of RNA synthesis by the ovalbumin gene.

DISCUSSION

We have shown that specific transcription of ovalbumin DNA can be initiated *in vitro* by using cloned ovalbumin DNA and a crude total cell extract from HeLa cells. Initiation of *in vitro* transcription appears to be accurate, as judged by S_1 mapping and by the sizes of the RNA products synthesized from various ovalbumin DNA templates truncated at the 3′ end. Thus, *in vitro* initiation of transcription occurs at a unique site, the *in vivo* cap site, and proceeds to the right of the 3′ end of the ovalbumin DNA fragment.

The efficiency of the *in vitro* initiation of transcription for the ovalbumin gene is comparable to that of the Ad-2 late promoter as described by Manley *et al.* (10) and Weil *et al.* (9). It was calculated that ≈2 fmol of specific ovalbumin RNA transcripts were synthesized in a 50-μl reaction mixture in a 45-min period; i.e., approximately 0.01 molecule of RNA transcript is made per molecule of DNA in an hour. This is extremely slow as compared to the *in vivo* rate of transcription, but that is not surprising because pertinent regulatory factors are likely to be missing in such heterologous *in vitro* transcription systems. The fact that accurate initiation of transcription can be obtained in these systems using various eukaryotic genes suggests that a universal signal important for initiation *in vivo* may be encoded in the 5′ flanking sequence to the gene.

The studies on transcription of 5′ deletion mutants indicate that the deletion of DNA sequences between positions 61^- and 26^- completely abolishes the initiation of synthesis of the specific product. The ovalbumin gene, like many other eukaryotic genes (Ad-2, globin, simian virus 40, histones, conalbumin), contains a conserved DNA sequence, T-A-T-A-T-A-T (Hogness box), 26–32 nucleotides upstream from the cap site. Our results imply that a limited region of 5′ flanking DNA that includes the Hogness box and the 29 nucleotides upstream from it is required for *in vitro* initiation for the ovalbumin gene. Recently, Wasylyk *et al.* (19) observed that flanking DNA between positions 8^- and 44^- of the conalbumin gene are essential for initiation of transcription *in vitro*. Taken together, these results suggest that the conserved DNA sequences, the Hogness box, is the presumptive promoter necessary for *in vitro* initiation of polymerase II gene products. However, in view of the low efficiency of the *in vitro* transcription system, these results do not exclude the possibility that other DNA regions are also important for specific interaction with regulatory proteins.

It should be noted (see Fig. 5) that the deletion of different lengths of the 5′ flanking sequence upstream from the Hogness box has a *polar* (gradient) effect on the rate of initiation of the specific 395-nucleotide product. Deletion of all sequences upstream from position 61^- gives rise to the highest level of transcription of this product, although the parental OV1.7, which

† The identical box (T-A-T-A-T-A-T) at 1090^- and a potential box (T-A-T-A-T-T-A) at 825^- remain present in the OV0.7 fragment.

has the longest flanking ovalbumin DNA sequences, has the lowest level of specific transcription. Similarly, Grosschedl and Birnstiel (20) have recently reported that deletion of the conserved sequences upstream from the Hogness box of the histone H2A gene also enhances the rate of synthesis of the H2A gene product. Whether these sequences can function as binding sites for specific inhibitors of transcription is, at present, speculative.

The observation that 5′ flanking sequences are potential promoters for initiation of transcription of the ovalbumin and other eukaryotic genes suggests that the recognition sites for eukaryotic polymerase II and polymerase III may be very different at the level of the DNA. Polymerase III appears to require internal DNA sequences and a protein factor that interacts with 5S genes in the internal gene region between positions 41^+ and 85^+ (6–8).

Recently, we have also been able to obtain accurate initiation of transcription of ovomucoid gene fragments *in vitro*. However, when we transcribed an ovomucoid DNA fragment containing the cap site, 154 nucleotides of the gene sequence, and only 10 nucleotides of the 5′ flanking sequences—i.e., the Hogness box was deleted—no specific run-off RNA product was detected. Again, this observation is consistent with the hypothesis that the Hogness box is necessary for specific initiation of transcription *in vitro* by RNA polymerase II.

Finally, we found that other Hogness box-type sequences in OV1.7 DNA do not serve as a meaningful signal for initiation of a specific RNA product *in vitro*. Thus, it is likely that other genomic sequences in addition to the Hogness box are essential for specific initiation of gene transcription.

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