Le Doan, T., & Hélène, C. (1982) in *Structure, Dynamics, Interactions and Evolution of Biological Macromolecules* (Hélène, Ed.) pp 113–128, Reidel, Dordrecht, The Netherlands.
Montenay-Garestier, T., Takasugi, M., & Le Doan, T. (1983) in *Nucleic Acids: The Vectors of Life* (Pullman, B., & Jortner, J., Eds.) pp 305–315, Reidel, Dordrecht, The Netherlands.
Prunell, A., Goulet, I., Jacob, Y., & Goutorbe, F. (1984) *Eur. J. Biochem. 138*, 253–257.
Ramstein, J. (1977) *Biochimie 59*, 849–852.
Sarma, M. H., Gupta, G., & Sarma, R. H. (1985) *J. Biomol. Struct. Dyn. 2*, 1057–1084.
Strauss, F., Gaillard, C., & Prunell, A. (1981) *Eur. J. Biochem. 118*, 215–222.
Sturm, J. (1982) *Biopolymers 21*, 1189–1206.
Sturm, J., Schreiber, L., & Daune, M. (1981) *Biopolymers 20*, 765–785.
Toulmé, F., & Hélène, C. (1980) *J. Chim. Phys. 77*, 855–859.
Toulmé, J. J., & Hélène, C. (1977) *J. Biol. Chem. 252*, 244–249.
Trifonov, E. N. (1985) *CRC Crit. Rev. Biochem. 19*, 89–106.
Vigny, P. (1973) *C. R. Seances Acad. Sci., Ser. D 277*, 1941–1944.
Wilson, W. D., & Lopp, I. G. (1979) *Biopolymers 18*, 3025–3041.
Wilson, W. D., Wang, Y. H., Krishnamoorthy, C. R., & Smith, J. C. (1985) *Biochemistry 24*, 3991–3999.
Wu, H. M., & Crothers, D. M. (1984) *Nature (London) 308*, 509–513.
Zimmerman, S. B., & Pfeiffer, B. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 78–82.

Deoxyribonuclease I Sensitivity of the Ovomucoid–Ovoinhibitor Gene Complex in Oviduct Nuclei and Relative Location of CR1 Repetitive Sequences†

Maxwell J. Scott, Ming-Jer Tsai, and Bert W. O'Malley*
Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030
Received March 24, 1987; Revised Manuscript Received June 5, 1987

ABSTRACT: The location of CR1 middle repetitive sequences within or near the boundaries of the ovalbumin DNase I sensitive domain has suggested that CR1 sequences may play a role in defining transition regions of DNase I sensitivity in hen oviduct nuclei. We have examined this apparent relationship of CR1 sequences and transitions of chromatin structure by determining the DNase I sensitivity in oviduct nuclei of a 47-kilobase region that contains five CR1 sequences and the transcribed ovomucoid and ovoinhibitor genes. We find that three of the CR1 sequences occur within a broad transition region of decreasing DNase I sensitivity downstream of the ovomucoid gene. Another CR1 is in a region of decreased DNase I sensitivity within the ovoinhibitor gene. The fifth CR1 sequence is in a DNase I sensitive region between the two genes but which is less sensitive to DNase I digestion than the region immediately upstream from the ovomucoid gene. Thus, the CR1 sequences occur within regions of reduced relative DNase I sensitivity, suggesting that CR1s could facilitate the formation of a chromatin conformation that is less sensitive to DNase I digestion. Unexpectedly, the noncoding strand of sequences within and immediately adjacent to the 5′ end of the actively transcribed ovomucoid and ovalbumin genes was less sensitive to DNase I digestion than their respective coding strands.

It is well documented that actively transcribed genes in chromatin are in a DNase I sensitive conformation (Weisbrod, 1982). The DNase I sensitive conformation can extend beyond the transcription unit. For example, the related ovalbumin, X, and Y genes are all contained within a 100-kilobase (kb)[1] DNase I sensitive domain in oviduct nuclei (Lawson et al., 1982). At the boundaries of the domain, there is a gradual transition to a DNase I resistant conformation. The DNase I sensitive conformation appears to be unique to those tissues in which the gene is expressed (Weintraub & Groundine, 1976; Lawson et al., 1982). Furthermore, the acquisition of a DNase I sensitive state appears to precede gene expression in some developmental programs (Stalder et al., 1980; Storb et al., 1981).

In order to gain further insight into the differentiation process, we have been interested in identifying those sequences that could play a role in defining the boundaries of DNase I sensitive domains in oviduct nuclei. A possible candidate for such a regulatory sequence is the CR1 repetitive sequence. Of the three CR1 sequences in the ovalbumin domain, all occur within or near the transition regions of DNase I sensitivity (Stumph et al., 1983). Moreover, a CR1 sequence downstream of the GAPDH domain is in a region of decreased DNase I sensitivity that may correspond to the boundary of a neighboring domain (Alevy et al., 1984). There are, however, no CR1 sequences within the GAPDH domain. CR1 sequences are about 300–500 bp in length (Stumph et al., 1984) and are present at 1500–7000 copies per haploid chicken genome (Stumph et al., 1981). Two regions of about 100 and 400 nucleotides, respectively, are particularly well conserved among

† This work was supported by Grants HD08188 and HD07495 from the National Institutes of Health. M.J.S. was supported by a Hackett studentship from the University of Western Australia.

[1] Abbreviations: kb, kilobase(s); Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

0006-2960/87/0426-6831$01.50/0

CR1 sequences (Stumph et al., 1984). Oviduct DNA binding protein(s) bind(s) preferentially to the smaller of the conserved sequences (Sanzo et al., 1984), suggesting that it may be through the binding of these protein(s) that CR1s could influence structural transitions in chromatin.

The principal aim of this study is to examine further the apparent correlation of CR1 sequences and transition regions by determining the CR1 location with respect to the DNase I sensitivity of sequences around another gene that is expressed in the oviduct. For this purpose, we chose to extend our previous work on the DNase I sensitivity of the ovomucoid gene, which is specifically expressed in the oviduct (Tsai et al., 1978). The ovomucoid gene and at least 7.8 and 5.8 kb of 5′- and 3′-flanking DNA, respectively, are preferentially sensitive to DNase I digestion in oviduct nuclei (Lawson et al., 1980). In addition, we wanted to determine if the DNase I sensitivity extended upstream from the ovomucoid gene to the related ovoinhibitor gene as occurs in the ovalbumin gene family. The ovoinhibitor gene is also expressed in the oviduct (Scott et al., 1987).

We find that, unlike the ovomucoid gene, there is a region of decreased DNase I sensitivity within the ovoinhibitor gene. A CR1 repetitive sequence occurs within this region of decreased DNase I sensitivity. Similarly, three CR1 sequences occur in a broad transition region of decreasing DNase I sensitivity downstream of the ovomucoid gene. However, a fifth CR1 sequence is in a DNase I sensitive region between the two genes, but which is less sensitive than the region immediately upstream from the ovomucoid gene. Thus, the relative location of the ovomucoid gene region CR1 sequences is consistent with the proposal that CR1s may facilitate the formation of a chromatin conformation that is less sensitive to DNase I digestion in oviduct nuclei.

Experimental Procedures

Cloning of DNA in the Ovomucoid–Ovoinhibitor Gene Complex. The isolation and characterization of clones containing the 5.6 kb ovomucoid gene and about 12 kb each of 5′- and 3′-flanking DNA have been previously described (Catterall et al., 1979; Lai et al., 1979). The clone cosOM40 was isolated by screening an amplified cosmid chicken DNA library with a nick-translated 2.1 kb *Eco*RI–*Kpn*I 5′-terminal fragment of clone OM15 (Catterall et al., 1979). Construction of and procedures for screening the chicken cosmid library are as described previously (Simmen et al., 1985). Under the stringent conditions used, the probe hybridized only to unique sequences in chicken DNA. Similarly, the clone cosOM39 was isolated by screening a second chicken cosmid DNA library, of about 185 000 recombinants, using the 2.0 kb *Kpn*I–*Hin*dIII fragment from clone OI2.0 as a hybridization probe. The second cosmid DNA library was constructed in an identical manner with the first but was not amplified. Restriction mapping was performed according to established procedures (Colbert et al., 1980). Individual restriction fragments from the cosmid clones were isolated by the method of Dretzen et al. (1981). DNA fragments were subcloned into either pBR322 or the multiple cloning site of one of the riboprobe vectors pSP64, pSP65, pGEM1, pGEM2 (Promega Biotec), pSP18, or pSP19 (Bethesda Research Laboratories) using standard cloning procedures (Maniatis et al., 1982).

Preparation of DNase I Digested, Control, and Carrier DNAs. Nuclei were purified from the magnum portion of laying hen oviducts as previously described (Lawson et al., 1980). Nuclei were suspended at a DNA concentration of approximately 1 mg/mL in buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.35 M sucrose, and 40 μg/mL pancreatic DNase I (Cooper Biomedical) and incubated at 37 °C until 15–20% of the DNA was rendered soluble in cold 7% perchloric acid. Purification of what will be referred to as the DNase I digested DNA was as previously described (Lawson et al., 1980). As a source of control DNA, we used either chicken erythrocyte DNA (Calbiochem) or DNA extracted from oviduct nuclei which had not been subjected to in situ nucleolysis. Comparable results were obtained with either preparation. The high molecular weight control DNA and also herring testes carrier DNA (Sigma) were each sheared with DNase I to generate a fragment size distribution comparable to that of the DNA obtained from nuclei digested with DNase I (Lawson et al., 1980). The DNase I digested and control DNA preparations were each treated with 0.3 M NaOH according to Lawson et al. (1980) and then chromatographed on a column of Sephacryl-300 (Pharmacia). The column was eluted with 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 1 mM EDTA, and the fractions in the excluded volume were collected. Of the collected fractions, those in which a majority of the DNA was of a hybridizable size (>50 bp) were pooled, made 0.1 M in NaCl, extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v), and precipitated with 2 volumes of ethanol. On the basis of both C_0t and probe excess hybridization analysis, we estimate that the control preparations contain approximately 60% of undigested sequence.

Preparation of RNA Hybridization Probes. The conditions for in vitro transcription with SP6 or T7 RNA polymerases were similar to those described by Melton et al. (1984). Plasmid DNAs containing either the SP6 (pSP series) or the SP6 and T7 promoters (pGEM 1 and 2) were digested to completion with the appropriate restriction enzyme, phenol/chloroform/isoamyl alcohol (25:24:1 v/v) extracted, and ethanol precipitated. As recommended by Schenborn and Mierendorf (1985), restriction enzymes that generate a 3′-protruding end were not used to linearize plasmid DNA. The transcription reaction volume was 20 μL and contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each of ATP, CTP, and UTP, 0.1 mM GTP, 42 μCi of [^{32}P]GTP (410 Ci/mmol, Amersham), and 1 μg of linearized plasmid DNA. The addition of RNasin was unnecessary and indeed detrimental with some commercial preparations that were apparently contaminated with RNase. The reaction was initiated by adding 10 units of SP6 or T7 RNA polymerase (Promega Biotec), and the samples were incubated at 37 °C for 15 min. After the transcription reaction, DNase I (20 μg/mL final concentration) and carrier tRNA (80 μg) were added, and the incubation was continued at 37 °C for an additional 10 min. The RNA was extracted once with phenol/chloroform/isoamyl alcohol; the aqueous phase was removed and extracted a second time with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The RNA was precipitated once with sodium acetate (0.25 M final concentration) and 2.5 volumes of ethanol, resuspended, and precipitated twice more with ammonium acetate (2.5 M final concentration) and 2.5 volumes of ethanol. The precipitates were washed with 70% ethanol, dried, and resuspended at about 5×10^7 cpm/mL in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. To determine if the RNA was of the correct size, a 1-μL aliquot was resolved on formaldehyde–agarose gels according to the procedure of Sargan et al. (1986); the gels were dried and autoradiographed.

Solution Hybridization and RNase Treatment. Hybridizations were performed in sealed glass vials (Kontes) which, in a final volume of 20 μL, contained 10 mM Hepes (pH 7.0),

0.6 M NaCl, 2 mM EDTA, 5–10 mg/mL DNA (DNase I digested or control DNA, each plus carrier DNA in some vials), and (0.5–1.5) × 10^4 cpm of trichloroacetic acid precipitable RNA probe (specific activity ~3 × 10^7 cpm/μg). The probe is in excess of hybridizable DNA fragments under these conditions. For each RNA probe, several vials containing various amounts of either DNase I digested or control DNAs, up to 200 μg of DNA, were prepared. Carrier DNA was added to some of the vials such that the final DNA concentration was identical for all vials containing a particular RNA probe. Samples were denatured at 100 °C for 10 min and then incubated at 68 °C for 16 h. A concentration of 500 cpm/μL of a 2 kb RNA probe of specific activity 3 × 10^7 cpm/μg is sufficient to drive the hybridization to the DNA to completion over the incubation period (unpublished results) since the calculated R_0t (RNA concentration × time) is 4 times the $R_0t_{1/2}$ of the purified 1872-nucleotide [excluding the poly(A) tail] ovalbumin RNA (Roop et al., 1978). For other RNA probes, the amount added was adjusted according to the square root of the ratio of the probe length (kb) divided by 2.

Hybrid formation was determined by the addition of 300 μL of RNase digestion buffer [10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, RNase A (40 μg/mL), and RNase T1 (2 μg/mL)] followed by incubation at 30 °C for 1 h and precipitation with cold 10% trichloroacetic acid. The trichloroacetic acid precipitable material was collected on glass microfiber filters (Whatman), dried, and then resuspended in 1 mL of toluene/NCS (Amersham)/liquid fluor (New England Nuclear) (24:12:1 v/v) solution and counted in 10 mL of toluene/liquid fluor/glacial acetic acid (24:1:0.0002 v/v) solution. The contribution to the RNase-resistant counts due to acid-precipitable digestion products of the unhybridized probe was monitored by the parallel incubation of vials in which the only DNA was herring testes carrier at a final concentration identical with that of the vials containing chicken DNA. All vials were corrected for this amount which under the conditions described above rarely exceeded 3% of the input radioactivity. In a similar manner, the input radioactivity remaining after the hybridization period, which never decreased more than expected from radioactive decay, was monitored by the parallel incubation of vials containing carrier DNA. This solution hybridization procedure is both sensitive and reproducible, as shown by the small differences between the independent measurements of the sensitivity of a given fragment (see Results).

DNA Blot Hybridizations and DNA Sequencing. Plasmid DNAs were digested with various restriction enzymes, resolved on agarose gels, and transferred to nitrocellulose filters according to Southern (1975). For the detection of repeated sequences, the filters were hybridized with nick-translated total chicken genomic DNA as previously described (Lawson et al., 1982). For the detection of CR1 sequences, the filters were hybridized with a nick-translated fragment containing a CR1 sequence using the moderate stringency conditions of Levine et al. (1984). On the basis of hybridization with CR1-specific probes, subfragments were chosen and isolated for sequencing. The fragments were cloned into M13 vectors according to Messing (1983), and the DNA was sequenced by the method of Sanger et al. (1977). DNA sequences were analyzed by using the Microgenie programs from Beckman.

Results

Restriction Map of the Ovomucoid and Ovoinhibitor Genes and Their Flanking Sequences. A restriction map of the linked ovomucoid and ovoinhibitor genes and their flanking sequences is shown in Figure 1A. The relative positions of the cosmid

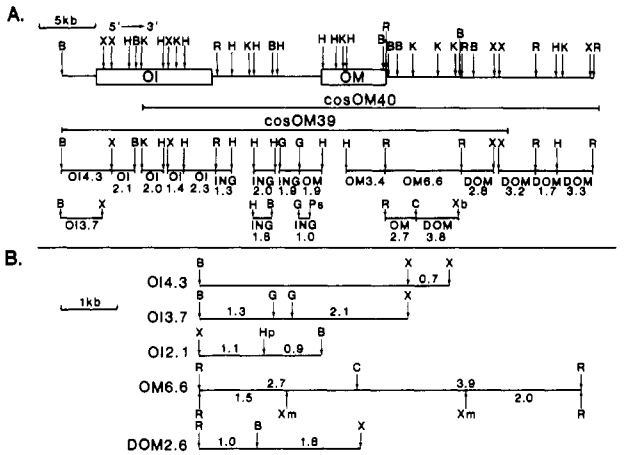

FIGURE 1: Cloning of the ovomucoid and ovoinhibitor genes and their flanking sequences. (A) The cosmid clones indicated below the restriction map were isolated from a chicken DNA library as described under Experimental Procedures. The chicken DNA inserts of the subclones that were used as templates for the synthesis of RNA hybridization probes are indicated below the cosmid clones. The subclones are designated according to the position in the gene complex (see below) and the size of the cloned DNA fragment in kilobase pairs. (B) The location of restriction sites used to linearize the indicated subclones within the chicken DNA insert is shown. RNA probes prepared from these templates are referred to in the text by the name of the clone followed in parentheses by the size of the probe in kilobases. Also shown are the restriction sites at the ends of the cloned inserts. Abbreviations: OI, ovoinhibitor; OM, ovomucoid; ING, intergenic region; DOM, downstream of ovomucoid; B, *Bam*HI; C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; Ps, *Pst*I; R, *Eco*RI; X, *Xho*I; Xb, *Xba*I; Xm, *Xmn*I.

clones isolated from chicken genomic DNA libraries are shown immediately below the restriction map. The cosmid clones span almost 50 kb of contiguous sequence. The ovoinhibitor gene was identified by hybridization analysis and sequence comparison with an almost full-length ovoinhibitor cDNA clone (Scott et al., 1987). The cloning and characterization of the related ovomucoid gene have been previously described (Lai et al., 1979).

For the purpose of synthesizing single-stranded RNA hybridization probes, individual fragments from within the cloned region were isolated and subcloned into the multiple cloning site of plasmids containg the SP6 or SP6 and T7 promoters. The relative positions of these subclones are shown at the bottom of Figure 1A. The subclones were designated by a letter code indicating the approximate position within the gene complex followed by the size of the cloned fragment. The letter code for the subclones is as follows: OI, contains at least some ovoinhibitor gene sequence; ING, intergenic region between the ovoinhibitor and ovomucoid genes; OM, contains at least some ovomucoid gene sequence; DOM, downstream of the ovomucoid gene. For the synthesis of discrete RNA probes, the subclones were linearized by digestion with a restriction enzyme. The location of the restriction sites that were used to linearize some plasmid DNAs within the inserted fragment is shown in Figure 1B. The probes synthesized from these templates are identified in the text by the name of the clone followed in parentheses by the probe length in kilobases. All other RNA probes were synthesized from plasmid DNAs linearized with a restriction enzyme that cuts within the multiple cloning site of the vector. As the length of these probes is the same as the inserted fragment, they are identified solely by the name of the clone.

Determination of the DNase I Sensitivity of the Ovomucoid and Globin Genes with a Probe Excess Solution Hybridization Assay. We have previously reported that the ovomucoid gene

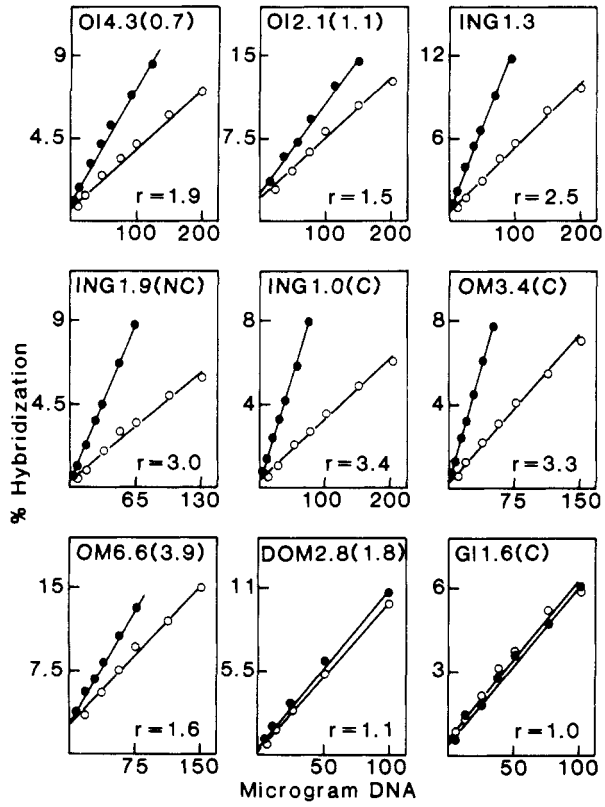

FIGURE 2: DNase I sensitivity of sequences representative of the ovomucoid–ovoinhibitor gene complex. Hybridization of DNase I digested DNA from oviduct nuclei (○) and control chicken DNA (●) with an excess of RNA probe was carried out as described under Experimental Procedures. The probe that was used is indicated at the top of each panel. Probes that were complementary to the coding strand (C) or noncoding strand (NC) are indicated for those DNA sequences where the DNase I sensitivity of both strands was determined. The chicken DNA insert of the G11.6 clone is a 1.6 kb *Eco*RI–*Bgl*II fragment which contains about 1 kb of 5′-flanking sequence and 578 bp of β-globin gene sequence as described by Dolan et al. (1983). The relative location of all other probe sequences is shown in Figure 1. The percent of the probe that hybridized is directly proportional to the amount of chicken DNA. Lines of best fit were calculated by linear regression analysis. The ratio of the slopes or relative DNase I sensitivity (r) is shown at the bottom right of each panel.

is sensitive and the β-globin gene is insensitive to DNase I digestion in oviduct nuclei (Lawson et al., 1980). This was determined by using an excess of the DNase I digested and control DNAs in the solution hybridization assay. To determine if comparable results could be obtained with a probe excess hybridization assay, single-stranded RNA probes containing portions of the ovomucoid and globin genes were prepared. The synthesized probes were shown to be the expected length for run-off transcription products by formaldehyde–agarose gel electrophoresis analysis (unpublished results). The result of the hybridization in solution of an excess of the ovomucoid and globin RNA probes to various amounts of DNase I digested or control DNA is shown in the bottom right of Figure 2. With sufficient excess of RNA probe and incubation to an appropriate R_0t (RNA concentration × time), it is possible to drive the hybridization to the complementary sequences in the chicken DNA to completion. Therefore, the amount of RNA/DNA hybrid formed is directly proportional to the amount of chicken DNA in the hybridization. A plot of the percent hybridization as a function of the amount of input chicken DNA is linear up to about 10–15% hybridization (approximately 10-fold excess probe) as over this range there is a large excess of the probe relative to the competing DNA strand of the same sense. From such plots, the relative DNase I sensitivity (r) is derived by dividing the slope of the line of the control DNA by the slope of the line of the DNase I digested DNA.

The relative DNase I sensitivities of the coding strands of the ovomucoid and globin genes were each independently measured 5 times and found to have relative sensitivity values of 3.01 ± 0.10 and 1.06 ± 0.02 (SEM), respectively. Thus, the solution hybridization assay is both quantitative and reproducible. The relative sensitivity values for ovomucoid and globin are similar to those measured previously using the chicken DNA in excess in the hybridization (Lawson et al., 1980). That is, the ovomucoid gene coding sequences are preferentially depleted in concentration in the DNA from DNase I digested oviduct nuclei. We define the coding strand of the ovomucoid and globin genes as that strand which is complementary to their respective mRNAs. Similar results with the ovomucoid and globin probes were obtained with all of the DNase I digested and control DNA preparations used in this study. The DNase I sensitivity of the noncoding strand of the globin gene is identical with the coding strand (unpublished results). That is, with this assay, it is possible to measure separately the DNase I sensitivity of both the coding and noncoding strands of a DNA sequence. Furthermore, the probe excess hybridization assay has the advantages of being a quantitative method that does not require large amounts of chicken DNA since the driver is the probe and not the DNA. Therefore, we believe the probe excess assay is superior to the assay used previously (Lawson et al., 1980).

DNase I Sensitivity of the Ovoinhibitor and Ovomucoid Genes and Their Flanking Sequences. To measure the DNase I sensitivity in oviduct nuclei of DNA sequences in the ovomucoid–ovoinhibitor gene complex, RNA probes complementary to sequences from within this region were prepared. All probes were of the expected length for run-off transcription (results not shown). The annealing data of probes that are representative of the gene complex are shown in Figure 2. The relative DNase I sensitivities derived from similar hybridization assays for sequences complementary to the various probes used in the study are listed in Table I. As for the transcribed sequences, the top and bottom strands of the nontranscribed sequences are referred to as noncoding and coding, respectively. For 12 of the probes, 2 independent measurements of the relative sensitivity were performed. The average difference between the two measurements of the sensitivity of each probe is 11.5%. This small difference is indicative of the reproducibility of the assay.

The majority of the ovoinhibitor gene and immediate flanking sequences (OI probes) are preferentially sensitive to DNase I digestion in oviduct nuclei. However, the sequences complementary to the OI2.1 probe, which are within the ovoinhibitor gene, are intermediate with regard to DNase I sensitivity. Furthermore, both the coding strand of the 5′ halves and the noncoding strand of the 3′ halves of the OI2.1 sequence, OI2.1 (1.1) and OI2.1 (0.9), respectively, are also of similar intermediate DNase I sensitivity (Figure 2, Table I). Since the coding strands of both OI2.1 and OI2.1 (1.1) are of relative sensitivity 1.5, we deduce that the coding strand of OI2.1 (0.9) must also have an intermediate sensitivity of about 1.5, to account for the sensitivity of the entire OI2.1 coding strand. That is, we would predict no significant difference between the relative sensitivity of the coding and noncoding strands of OI2.1 (0.9), as is the case for other fragments in the ovoinhibitor gene (OI1.4, OI2.3). The sequences between the ovoinhibitor and ovomucoid genes (ING

Table I: Relative DNase I Sensitivity of Sequences within and Flanking the Ovomucoid and Ovoinhibitor Genes

probe[a]	strand assayed	relative DNase I sensitivity[b]
OI3.7 (1.3)	coding	2.5, 2.2
OI3.7 (2.1)	noncoding	2.1, 2.1
OI4.3 (0.7)	noncoding	1.9
OI2.1 (1.1)	coding	1.5, 1.4
OI2.1	coding	1.5, 1.4
OI2.1 (0.9)	noncoding	1.5
OI2.0	noncoding	2.1, 1.8
OI1.4	coding	1.7
OI1.4	noncoding	1.9
OI2.3	coding	1.9
OI2.3	noncoding	2.1
ING1.3	noncoding	2.5, 2.5
ING2.0	noncoding	2.2, 2.2
ING1.8	coding	2.2
ING1.9	coding	2.8
ING1.9	noncoding	3.0
ING1.0	coding	3.4
ING1.0	noncoding	3.0
OM3.4	coding	2.8, 2.9, 3.2, 2.9, 3.3
OM6.6 (1.5)	coding	2.8
OM2.7	coding	2.4, 2.0
OM2.7	noncoding	2.0
OM6.6 (3.9)	noncoding	1.6
OM6.6 (2.0)	noncoding	1.6
DOM3.8	coding	1.9
DOM2.8 (1.0)	coding	1.8
DOM2.8	coding	1.4, 2.0
DOM2.8 (1.8)	noncoding	1.3, 1.1
DOM3.2	coding	1.1
DOM1.7	coding	1.2, 1.1
DOM3.3	coding	1.2, 1.0

[a] Relative location of probe sequences is shown in Figure 1. [b] Ratios of slope of control to slope of DNase I digested DNA from plots of percent hybridization vs amount of DNA as described in the text. Multiple values for a particular sequence represent results from independent experiments.

probes) are clearly sensitive to DNase I digestion.

The coding strand of the 1.5 kb *Eco*RI–*Xmn*I fragment of OM6.6, which includes all 3 polyadenylation sites of ovomucoid and about 500 nucleotides at the 3′ side of the most 3′ polyadenylation site (Gerlinger et al., 1982), is clearly sensitive to DNase I digestion (Table I). The coding and noncoding strands of OM2.7 appear to have a lower DNase I sensitivity compared to the OM6.6 (1.5) coding strand which corresponds to the 5′ half of OM2.7. This apparent decreased DNase I sensitivity is probably because OM2.7 contains an additional 1.2 kb of 3′-flanking DNA. The sequences directly 3′ to OM2.7 [DOM3.8, OM6.6 (3.9), OM6.6 (2.0), and DOM2.8, which span about 6.7 kb] are of intermediate DNase I sensitivity. Further 3′, the sequences from DOM3.2 to DOM3.3, which span about 8.2 kb, are clearly in a closed or DNase I insensitive conformation. Thus, there appears to be a gradual transition from a DNase I sensitive to insensitive conformation over about 6–8 kb of sequence directly 3′ to the polyadenylation sites of the ovomucoid gene. The DNase I sensitivities of the ovomucoid gene and immediately adjacent flanking sequences in oviduct nuclei are consistent with previous observations (Lawson et al., 1980).

The 1.1 kb *Xho*I–*Hpa*I fragment of OI2.1 contains a CR1 repetitive sequence of about 180 nucleotides. Since CR1 sequences are repeated about 1500–7000 times per haploid chicken genome (Stumph et al., 1981), as little as 1 μg of chicken DNA is sufficient, at the concentration of probe used, to hybridize all of that part of the OI2.1 (1.1) probe that is complementary to CR1 sequences (data not shown). It is for this reason the OI2.1 (1.1) DNase I digested and control DNA hybridization plots do not appear to intercept the ordinate axis at zero (Figure 2). The ordinate intercept is less than the percentage of OI2.1 (1.1) that is CR1 sequence (16%) presumably because RNase digests the looped-out RNA of mispaired RNA/DNA hybrids involving CR1 sequences, which are a heterogeneous sequence population. In support of this suggestion, when a 0.6 kb probe that was mostly CR1 sequence was hybridized to small amounts of chicken DNA (0.02–2 μg) such that the hybridization of the unique sequence in the probe was negligible, the hybridization curve plateau was 10% rather than the expected 62% (data not shown). Therefore, the OI2.1 (1.1) hybridization plots measure the relative DNase I sensitivity of only the unique chicken sequences in the 1.1 kb fragment. Moreover, the slope of the OI2.1 (1.1) control plot is identical with that of a single-copy probe of similar complexity and specific activity. Similarly, the DNase I digested and control DNA hybridization plots of OM6.6 (3.9) (Figure 2, bottom) do not intercept the ordinate axis at zero because this probe, like OI2.1 (1.1), contains a CR1 sequence.

CR1 Repetitive Sequences in the Ovomucoid–Ovoinhibitor Gene Complex. For the purpose of identifying repetitive sequences, plasmid subclones of the ovomucoid gene region were digested with various restriction enzymes, resolved on agarose gels, and transferred to nitrocellulose filters. The filters were hybridized to nick-translated total chicken genomic DNA under conditions in which only repetitive sequences can give a detectable signal (Shen & Maniatis, 1980). The autoradiograph shown in Figure 3A shows that there are five restriction fragments within the ovomucoid–ovoinhibitor region that give a signal and thus contain repetitive sequences. Filters prepared in a similar manner were hybridized at moderate stringency to CR1-specific probes. The autoradiograph shown in Figure 3B shows that a nick-translated fragment containing CR1OVb sequences (Stumph et al., 1983) hybridized to five fragments within the ovomucoid–ovoinhibitor region. A similar hybridization pattern was observed when other CR1 sequences were used as probes (unpublished observation). The five CR1-containing fragments are the same or are part of the fragments identified as containing repetitive sequences (Figure 3C). It is therefore likely that all the repeats are CR1 sequences, although it is possible that a fragment could contain more than one type of repetitive sequence. One CR1 sequence is within the ovoinhibitor gene, another is in the intergenic region, and three are downstream of the ovomucoid gene (Figure 3C). The CR1 sequence in the ovoinhibitor gene is located within the fourth intron which separates the coding sequences of the first and second Kazal domains (Scott et al., 1987).

The fragments containing CR1 sequences were isolated and sequenced. CR1 sequences were identified by homology to a CR1 consensus sequence (Stumph et al., 1984). The sequences of the five CR1s in the ovomucoid region are shown aligned to an updated consensus sequence in Figure 4. This consensus sequence is based upon the sequence data of 14 CR1s, including the CR1 sequences identified previously (Stumph et al., 1984) and the 5 ovomucoid gene region CR1 sequences. The consensus sequence has been extended an additional 45 nucleotides in the 5′ direction on the basis of homology between CR1OMb and CR1CMb (Stumph et al., 1984). Otherwise, there have been few changes made in the previous consensus sequence. The CR1 sequences closest to the 3′ end of the ovomucoid gene, CR1OMa and in particular CR1OMb, are significantly longer than the other ovomucoid region CR1s.

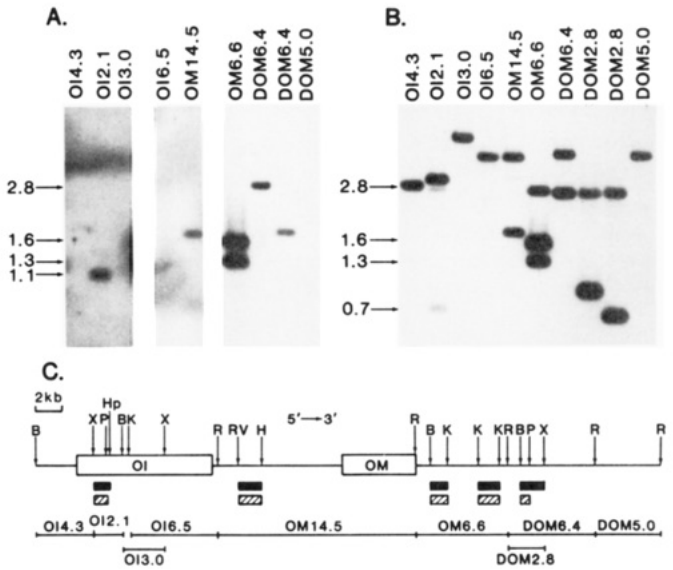

FIGURE 3: Identification of the CR1 repetitive sequences in the ovomucoid–ovoinhibitor gene complex. The plasmid subclones indicated were digested with various restriction enzymes that cut within the insert and at the vector–insert junction. In panel A, the plasmid DNAs were digested with restriction enzymes as follows: OI4.3, *Bam*HI/*Xho*I/*Hin*dIII/*Bgl*II; OI2.1, *Bam*HI/*Xho*I/*Hin*dIII/*Hpa*I; OI3.0, *Bam*HI/*Kpn*I; OI6.5, *Eco*RI/*Hin*dIII/*Kpn*I; OM14.5, *Eco*RI/*Hin*dIII/*Eco*RV; OM6.6, *Eco*RI/*Kpn*I/*Bam*HI; DOM6.4 (left), *Eco*RI/*Xho*I; DOM6.4 (right), *Eco*RI/*Xho*I/*Bam*HI; DOM5.0, *Eco*RI/*Hin*dIII. In panel B, the plasmid DNAs were digested with the same restriction enzymes with the exceptions of the following: OI2.1, *Bam*HI/*Xho*I/*Hin*dIII/*Pvu*II; DOM6.4, *Eco*RI/*Xho*I; DOM2.8 (left), *Eco*RI/*Xho*I/*Hin*dIII/*Pvu*II; DOM2.8 (right), *Eco*RI/*Xho*I/*Hin*dIII/*Pvu*II/*Bam*HI. The digested plasmid DNAs were resolved on agarose gels and transferred to nitrocellulose filters. Filters were hybridized either to nick-translated chicken DNA (panel A) or to a nick-translated fragment that contains a CR1 sequence (panel B). The arrows indicate the size in kilobases of the fragments that hybridized. The 2.8 kb *Eco*RI–*Xho*I fragment of DOM6.4 that hybridized to both probes is the chicken DNA insert of DOM2.8. The band intensities in panel A should not be directly compared as the separate strips shown represent different exposure times. The highest molecular weight band (3.0 kb or greater) in each lane of panel B is the result of hybridization of vector sequences that contaminate the CR1 probe to the vector fragment of each clone. The relative position of the restriction sites for some of the restriction enzymes that were used to digest the indicated plasmid DNAs is shown in panel C. The location of the fragments that hybridized to the chicken DNA probe is indicated by solid boxes beneath the restriction map in panel C. Similarly, fragments that hybridized to the CR1 probe are indicated by hatched boxes. The relative location of the subclones used in these DNA blot hybridization experiments is shown at the bottom of panel C: B, *Bam*HI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pvu*II; R, *Eco*RI; RV, *Eco*RV; X, *Xho*I.

The CR1OMb and CR1OMa sequences have high homology to the conserved polypurine sequence (positions 224–237) which we previously suggested may be analogous to the (+) strand primer binding site of retroviruses (Stump et al., 1984). There are two well-conserved regions in CR1 sequences (Stumph et al., 1984). The CR1OMb and CR1OMa sequences have good homology (about 70%) to the first conserved region (positions 306–405), although they are not as homologous over this region as are the ovalbumin domain CR1s (at least 80%). For the other ovomucoid region CR1 sequences (CR1OMc, CR1OMd, and CR1OMe), significant homology to the consensus begins approximately in the middle of the first conserved region. Of these, only CR1OMc has good homology (70%) to the 3′ half of the first conserved region. All of the ovomucoid region CR1 sequences, however, have high homology (75–89%, average 83%) to the second very strongly conserved region from position 467 to position 504. Oviduct DNA binding proteins bind preferentially to DNA restriction fragments that contain this strongly conserved sequence (Sanzo et al., 1984). Interestingly, at the end of this conserved region, two copies of the octanucleotide ATTCTATG are directly repeated. Over a stretch of about 60 nucleotides between the first and second conserved regions in the ovomucoid region CR1 sequences, like other CR1s (Stumph et al., 1984), there are numerous small deletions. Following the second conserved region, the ovomucoid CR1 sequences have poor homology to the consensus.

Location of CR1 Sequences Relative to the DNase I Sensitivity of the Ovomucoid–Ovoinhibitor Gene Complex. The data we have obtained concerning the relative DNase I sensitivity of the coding and noncoding strands of the ovomucoid gene region are summarized in Figure 5. Where multiple determinations of the DNase I sensitivity of a sequence were performed, we show the mean value of individual experiments. The error bars associated with each DNase I sensitivity value indicate the size of the calculated pooled standard error of the mean (Ostle & Mensing, 1975). The position and orientation of the five CR1 sequences within the ovomucoid–ovoinhibitor gene complex are shown at the bottom of Figure 5. The directional polarity of CR1 sequences is based upon limited homology of CR1U1a and CR1OVa to mammalian Alu sequences (Stumph et al., 1981). The CR1 sequences CR1OMa, CR1OMb, and CROMe all occur within the broad transition region of DNase I sensitivity downstream of the ovomucoid gene. Furthermore, CR1OMa and CR1OMe map close to the 5′ and 3′ ends, respectively, and thus appear to define the boundaries of this transition region. CR1OMd is located in a region of decreased DNase I sensitivity within the ovoinhibitor gene. There appears to be a gradual decrease in the DNase I sensitivity from both 5′ and 3′ sides toward CR1OMd. The fifth CR1 sequence is in a DNase I sensitive region between the two genes but which is less sensitive to DNase I digestion than the region immediately upstream from the ovomucoid gene. Thus, the CR1 sequences occur within regions of reduced relative DNase I sensitivity, suggesting that CR1s could facilitate the formation of a chromatin conformation that is less sensitive to DNase I digestion.

DNase I Resistance of the Noncoding Strand of Sequences within and Adjacent to the Ovomucoid and Ovalbumin Genes. For several of the DNA sequences within the ovoinhibitor gene (OI2.1, OI1.4, and OI2.3), the intergenic region (ING2.0/ING1.8, ING1.9, and ING1.0), the 3′ end (OM2.7), and downstream of the ovomucoid gene [DOM3.8/OM6.6 (3.9)], we measured the DNase I sensitivity of both the coding and noncoding strands. For each of these sequences, the DNase I sensitivity of the two strands did not differ significantly (Table I, Figure 5). However, the noncoding strands of both OM1.9 and OM3.4 were consistently more resistant to DNase I digestion than the respective coding strands [Figure 6, top; compare OM3.4 (NC) to Figure 2 OM3.4 (C)]. The 5′-terminal 1.0 kb *Bgl*II–*Pst*I fragment of OM1.9, which is all 5′-flanking sequence, corresponds to the inserted fragment of the ING1.0 clone (Figure 1A). However, both the noncoding and coding strands of ING1.0 are highly sensitive to DNase I digestion (Table I). We reasoned that since the 5′ half of the noncoding strand of OM1.9 is highly DNase I sensitive, the 3′ half must be particularly resistant to account for the observed sensitivity of the whole sequence. To test this hypothesis, we prepared a 0.75 kb RNA probe complementary to the 3′ end of the OM1.9 noncoding strand by using an OM1.9 template linearized at the *Xmn*I site (Figure 6, bottom). The annealing data obtained with this probe are shown

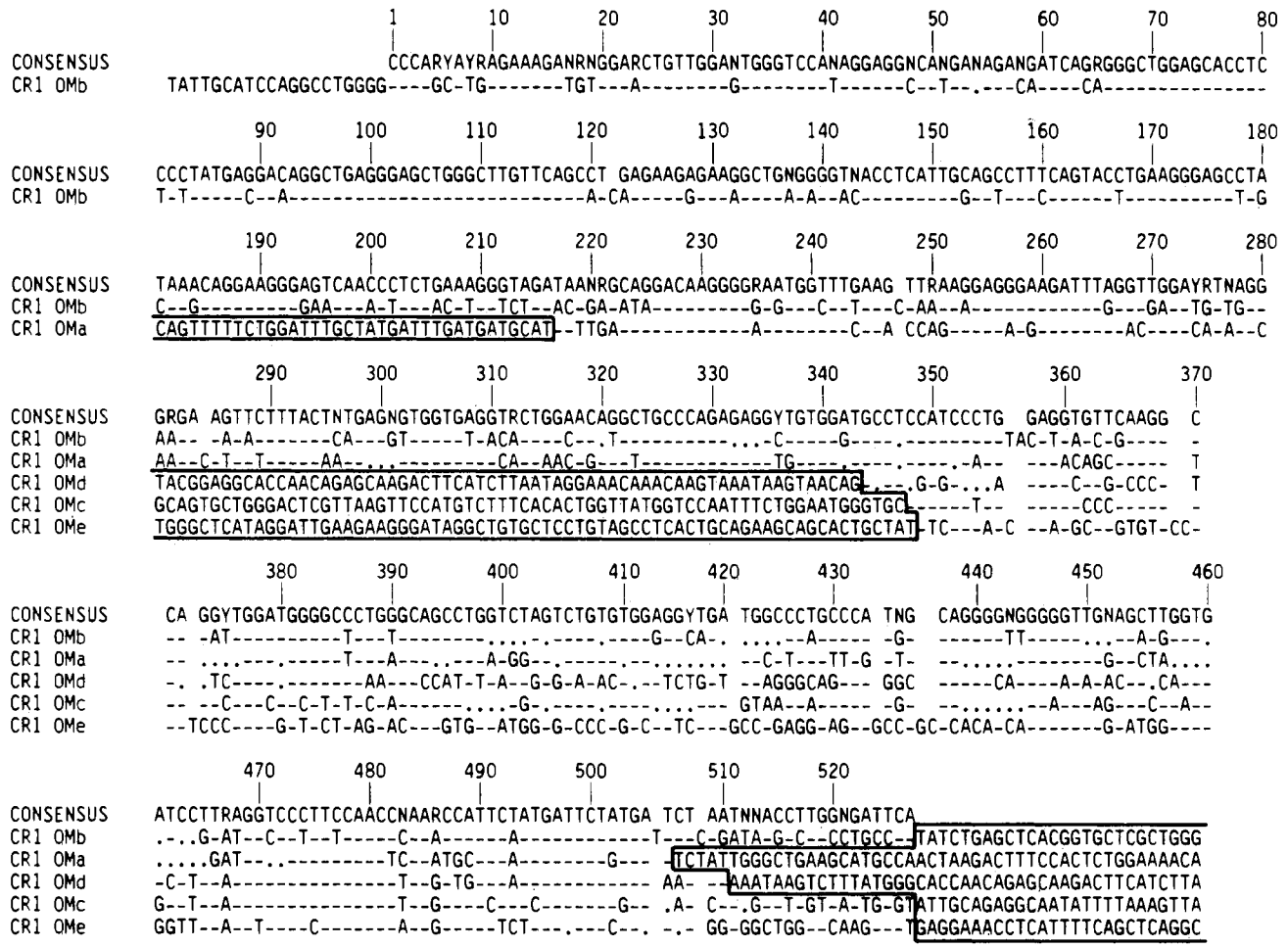

FIGURE 4: Comparison of the five ovomucoid region CR1 sequences to the consensus. A consensus CR1 sequence deduced by alignment of 14 CR1 sequences is shown at the top line of each row of sequence data. At any one position in the consensus, the nucleotide shown was present in at least 50% of CR1 sequences. The purines (R) and pyrimidines (Y) indicated were present in all the CR1 sequences. The numbering refers to the nucleotide position in the consensus. Dashes indicate identity of the particular ovomucoid CR1 sequence to the consensus at any given position. Dots indicate the absence of a nucleotide at a particular position. Gaps in the consensus were introduced when the nucleotides were present in a minority of CR1 sequences. The boxed nucleotides were not homologous to the consensus. N is any nucleotide.

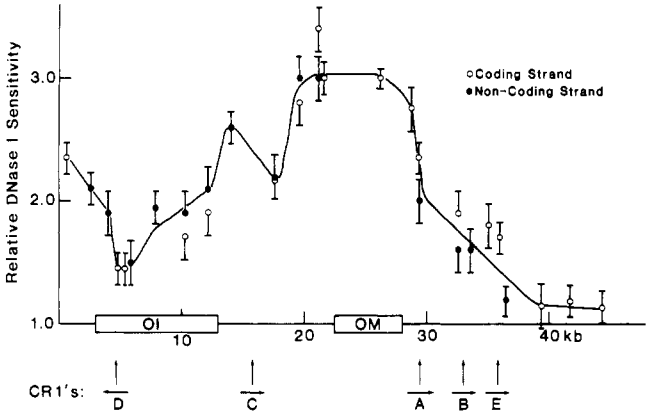

FIGURE 5: Summary of DNase I sensitivity and CR1 location in the ovomucoid–ovoinhibitor gene complex. Each point represents an independent experiment or the mean of separate experiments using a specific probe and is positioned relative to the map at the midpoint of the probe sequence. The error bars indicate the size of the pooled standard error of the mean. The vertical arrows (labeled A–E) indicate the midpoint and the horizontal arrows the orientation of the CR1 sequences.

in Figure 6 (middle). As predicted, the 3′ end of the noncoding strand of OM1.9 is resistant to DNase I digestion.

We were interested in determining if the resistance of the noncoding strand to DNase I digestion was a general feature of the promoter regions of actively transcribed genes in the oviduct. For this purpose, several restriction fragments from the ovalbumin promoter region were cloned into the riboprobe vectors. A restriction map showing the position of these clones relative to the transcription initiation site is shown at the bottom of Figure 6. Like OM1.9, the noncoding strand of OV1.5, which contains the transcription initiation site but is mostly 5′ flanking, is more resistant to DNase I digestion than the coding strand (Figure 6, middle). Furthermore, the noncoding stand of the 523 bp *Ava*II–*Eco*RI fragment of OV1.7 (Woo et al., 1981; Heilig et al., 1982), which overlaps with the 3′ end of OV1.5, is insensitive to DNase I digestion (Figure 6, bottom). However, the noncoding and coding strands of OV0.45, which is directly 3′ to OV1.7, are both equally sensitive to DNase I digestion.

The decreased DNase I sensitivity of the noncoding strand of the ovalbumin and ovomucoid promoter regions was not detected previously (Lawson et al., 1980). This was probably because the double-stranded DNA probes that were used hybridize both to the DNase I sensitive coding strand and to the noncoding strand, of which most of the latter is DNase I sensitive in the large DNA fragments that were assayed (Lawson et al., 1980). In regard to this, DNA excess or C_0t hybridization analysis has confirmed the decreased DNase I sensitivity of the noncoding strand of a fragment within the ovalbumin gene (unpublished results). Since the DNase I resistance of the noncoding strand appears to reflect a localized unusual chromatin structure within DNase I sensitive regions,

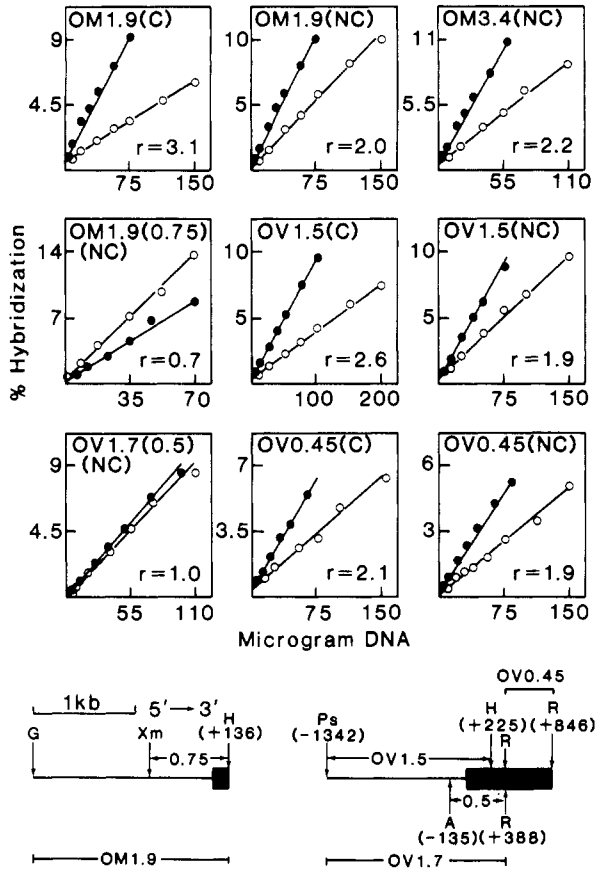

FIGURE 6: DNase I sensitivity of the coding and noncoding strands of sequences within and adjacent to the ovomucoid and ovalbumin genes. The relative DNase I sensitivity (r) was assayed as described under Experimental Procedures using the probes indicated at the top of each panel and either DNase I digested DNA from oviduct nuclei (O) or control DNA (●). Probes were complementary to either the coding (C) or the noncoding (NC) strand of a DNA sequence. The position of the cloned chicken DNA inserts used as templates for RNA probe synthesis relative to the ovomucoid and ovalbumin genes is shown at the bottom left and bottom right, respectively. The transcribed regions are represented by solid bars. The exact positions of restriction sites known from sequence data are shown relative to the transcription initiation sites of the genes: A, *Ava*II; G, *Bgl*II; H, *Hin*dIII; Ps, *Pst*I; R, *Eco*RI; Xm, *Xmn*I.

these hybridization experiments have been presented separately from the annealing data for the majority of the probes in the ovomucoid gene region. Thus, the noncoding strand of DNA sequences near the transcription initiation site of the highly transcribed ovomucoid and ovalbumin genes appears to be as insensitive to DNase I digestion in oviduct nuclei as is the transcriptionally silent β-globin gene.

Discussion

The genes for the related Kazal serine proteinase inhibitors ovomucoid and ovoinhibitor are closely linked and expressed in the oviduct. By evolutionary analogy with the ovalbumin multigene family, we expected the ovomucoid and ovoinhibitor genes to be contained within the same DNase I sensitive domain in oviduct nuclei. Indeed, the coding strand of the ovomucoid gene, part of the ovoinhibitor gene and the region between the genes, is sensitive to DNase I digestion. However, part of the ovoinhibitor gene is intermediate with regard to its DNase I sensitivity in oviduct nuclei. This may accurately reflect the structure of the ovoinhibitor gene in this region in each oviduct cell nucleus. Alternatively, it is possible that this region is DNase I sensitive in one subpopulation of cells and is in a DNase I insensitive conformation in the other fraction of oviduct cells, the combination of which would produce the observed overall intermediate DNase I sensitivity. Implicit in the latter model is that the ovoinhibitor protein is only expressed in those cells in which the gene is DNase I sensitive. We are currently testing this prediction by in situ hybridization of an ovoinhibitor-specific probe to oviduct tissue sections. In any event, the decreased DNase I sensitivity within the ovoinhibitor gene probably does not occur simply because of an apparent moderate level of expression (unpublished results) since the moderately transcribed X and Y genes as well as genes apparently transcribed at low rates are fully sensitive to DNase I digestion in oviduct nuclei (Lawson et al., 1980; Garel et al., 1977).

A major purpose of this undertaking was to determine if CR1 sequences in the ovomucoid gene region were within or near regions of transition from a DNase I sensitive to insensitive conformation. Of the five CR1 sequences in a 47 kb region including the ovomucoid gene, three occur in a broad transition region of decreasing DNase I sensitivity that appears to begin immediately downstream of the ovomucoid gene polyadenylation sites. In addition, CR1OMd occurs in the region of decreased DNase I sensitivity within the ovoinhibitor gene. Moreover, there appears to be gradual transitions in the chromatin structure from the DNase I sensitive configurations near the 5′ and 3′ ends of the transcription unit to the less sensitive region within the gene.

The presence of four of the five ovomucoid region CR1s, the three CR1s in the ovalbumin domain and a CR1 downstream of the GAPDH DNase I sensitive domain (Alevy et al., 1984), within or near regions of decreased or intermediate DNase I sensitivity could be purely coincidental. Alternatively, this correlation may indicate that CR1 sequences play a role in determining the higher order chromatin structure of certain gene regions in oviduct nuclei. If CR1 sequences do have such a role, they are clearly not essential for defining transition regions as no CR1s occur in the GAPDH domain (Alevy et al., 1984) and furthermore no repetitive sequences occur near the 5′ transition region of the lysozyme domain (Jantzen et al., 1986). However, just as there are multiple promoter elements, so there may be several different elements capable of defining a transition region of DNase I sensitivity.

If CR1 sequences do have a functional role in determining chromatin structure, what mechanisms could be involved? As CR1 sequences are located within or near transition regions of DNase I sensitivity, CR1s could conceivably facilitate the formation of either an open or a closed chromatin conformation. Either process could involve the previously reported oviduct proteins that bind preferentially to DNA fragments containing the strongly conserved sequence which is near the 3′ end of the CR1 consensus (Sanzo et al., 1984). The intermediate DNase I sensitivity around CR1OMd in the transcribed ovoinhibitor gene is consistent with the hypothesis that CR1s facilitate closed chromatin formation. A similar mechanism has been suggested to explain the transcription silencing properties of a rat repetitive sequence. In transfection experiments, the presence of this repetitive sequence in any orientation, either upstream or downstream of a globin gene that is linked to the SV40 enhancer, resulted in decreased globin expression (Laimins et al., 1986). We are currently examining whether CR1 sequences can act as transcription silencers.

The location of CR1OMc in the DNase I sensitive intergenic region suggests that CR1 sequences alone are not sufficient to define a transition region of DNase I sensitivity. CR1OMc is, however, associated with a reduction of DNase I sensitivity,

consistent with the hypothesis that CR1s may facilitate the formation of a conformation that is less sensitive to DNase I digestion. It is possible that CR1OMc may be adjacent to a sequence which facilitates open chromatin formation and overrides any effect the CR1 may have on the DNase I sensitivity of the region. The ovomucoid and ovoinhibitor genes are estimated to have diverged from a common ancestor at least 250 million years ago (Scott et al., 1987). The tight linkage of these genes over this time suggests that they may share regulatory sequences, such as a sequence which facilitates open chromatin formation. The ovoinhibitor gene is also expressed in liver whereas ovomucoid is not (Scott et al., 1987). Furthermore, the sequences immediately downstream of the ovomucoid gene are DNase I insensitive in liver (Lawson et al., 1980). It would be of interest to determine whether the putative transition region of DNase I sensitivity downstream of the ovoinhibitor gene in liver nuclei occurs near CR1OMc.

An unexpected result of this work was that the noncoding strands of the sequences around the initiation sites of the actively transcribed ovomucoid and ovalbumin genes are insensitive to DNase I digestion in oviduct nuclei. Similarly, for both a fragment within the ovalbumin gene (unpublished results) and also a fragment within the ovomucoid gene, the noncoding stand is less sensitive to DNase I digestion than the coding strand. Therefore, the apparent preferential DNase I resistance of the noncoding strand does not appear to be peculiar to the promoter regions of these genes. However, for one DNA fragment within the ovalbumin gene and for several fragments in the ovomucoid gene region, no difference in the DNase I sensitivity of the coding and noncoding strands was observed. These results suggest that the noncoding strand is preferentially protected from DNase I digestion and that the DNA may have a single-stranded conformation in certain regions within actively transcribed genes and their immediately adjacent 5'-flanking sequences. In regard to this suggestion, digestion studies with single-strand DNA-specific nucleases (Han et al., 1984) as well as in situ hybridization to chromosomes that were not denatured (Henikoff, 1981) suggest that the actively transcribed *Drosophila* hsp 70 gene and adjacent flanking sequences are in a single-stranded conformation in heat-shocked cells. It should be noted that DNase I digests both single-stranded and duplex DNA, although the enzyme apparently digests the latter at about a 4-fold greater rate (Kurnick, 1954).

The noncoding strand is presumably protected from nuclease attack by the binding of nuclear proteins, for example, matrix or transcription factors. In regard to the latter, the *Xenopus* transcription factor TFIIIA binds predominantly to the noncoding strand of the 5S ribosomal RNA genes and is suggested to remain bound to the noncoding strand during the passage of RNA polymerase (Sakonju & Brown, 1982). Clearly, more studies need to be done to determine if there are any common sequences in the regions where the noncoding strand is preferentially protected, whether oviduct proteins bind to these regions, and what is the relationship of the transcription rate to strand differences in DNase I sensitivity.

Added in Proof

Baniahmad et al. (1987) have shown that a CR1 sequence from the chicken lysozyme gene region is a transcription silencer.

Acknowledgments

We thank Serlina Robinson and Craig Wilkinson for technical assistance, Cheryl McCarthy for preparation of the manuscript, and Dr. Martin Alevy for many helpful discussions.

Registry No. DNase I, 9003-98-9; ovomucoid–ovoinhibitor, 62449-23-4.

References

Alevy, M. C., Tsai, M.-J., & O'Malley, B. W. (1984) *Biochemistry 23*, 2309–2314.

Baniahmad et al. (1987) *EMBO J. 6*, 2297–2303.

Catterall, J. F., Stein, J. P., Lai, E. C., Woo, S. L. C., Dugaiczk, A., Mace, M. L., Means, A. R., & O'Malley, B. W. (1979) *Nature (London) 278*, 323–327.

Colbert, D. A., Knoll, B. J., Woo, S. L. C., Mace, M. L., Tsai, M.-J., & O'Malley, B. W. (1980) *Biochemistry 19*, 5586–5592.

Dolan, M., Dodgson, J. B., & Engel, J. D. (1983) *J. Biol. Chem. 258*, 3983–3990.

Dretzen, G., Bellard, M., Sassone-Corsi, P., & Chambon, P. (1981) *Anal. Biochem. 112*, 295–298.

Garel, A., Zolan, M., & Axel, R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4867–4871.

Gerlinger, P., Krust, A., LeMeur, M., Perrin, F., Cochet, M., Gannon, F., Dupret, D., & Chambon, P. (1982) *J. Mol. Biol. 162*, 345–364.

Han, S., Udvardy, A., & Schedl, P. (1984) *J. Mol. Biol. 179*, 469–496.

Heilig, R., Muraskowsky, R., & Mandel, J.-L. (1982) *J. Mol. Biol. 156*, 1–19.

Henikoff, S. (1981) *Chromosoma 83*, 381–393.

Jantzen, K., Fritton, H. P., & Igo-Kemenes, T. (1986) *Nucleic Acids Res. 14*, 6085–6099.

Kurnick, N. B. (1954) *J. Am. Chem. Soc. 76*, 417–424.

Lai, E. C., Stein, J. P., Catterall, J. F., Woo, S. L. C., Mace, M. L., Means, A. R., & O'Malley, B. W. (1979) *Cell (Cambridge, Mass.) 18*, 829–842.

Laimins, L., Holmgreen-Konig, M., & Khoury, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3151–3155.

Lawson, G. M., Tsai, M.-J., & O'Malley, B. W. (1980) *Biochemistry 19*, 4403–4411.

Lawson, G. M., Knoll, B. J., March, C. J., Woo, S. L. C., Tsai, M.-J., & O'Malley, B. W. (1982) *J. Biol. Chem. 257*, 1501–1507.

Levine, M., Rubin, G. M., & Tjian, R. (1984) *Cell (Cambridge, Mass.) 38*, 667–673.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Ostle, B., & Mensing, R. W. (1975) in *Statistics in Research*, 3rd ed., pp 289–300, Iowa State University Press, Ames, IA.

Roop, D. R., Nordstrom, J. L., Tsai, S. Y., Tsai, M.-J., & O'Malley, B. W. (1978) *Cell (Cambridge, Mass.) 15*, 671–685.

Sakonju, S., & Brown, D. D. (1982) *Cell (Cambridge, Mass.) 31*, 395–405.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Sanzo, M., Stevens, B., Tsai, M.-J., & O'Malley, B. W. (1984) *Biochemistry 23*, 6491–6498.

Sargan, D. R., Tsai, M.-J., & O'Malley, B. W. (1986) *Biochemistry 25*, 6252–6258.

Schenborn, E. T., & Mierendorf, R. C. (1985) *Nucleic Acids Res. 13*, 6223–6236.

Scott, M. J., Huckaby, C. S., Kato, I., Kohr, W. J., Laskowski, M., Jr., Tasi, M.-J., & O'Malley, B. W. (1987) *J. Biol. Chem. 262*, 5899–5907.
Shen, C.-K. J., & Maniatis, T. (1980) *Cell (Cambridge, Mass.) 19*, 379–391.
Simmen, R. C. M., Tanaka, T., Ts'ui, K. F., Putkey, J. A., Scott, M. J., Lai, E. C., & Means, A. R. (1985) *J. Biol. Chem. 260*, 907–912.
Southern, E. (1975) *J. Mol. Biol. 98*, 503–517.
Stalder, J., Groudine, M., Dodgson, J. B., Engel, J. D., & Weintraub, H. (1980) *Cell (Cambridge, Mass.) 19*, 973–980.
Storb, U., Wilson, R., Selsing, E., & Walfied, A. (1981) *Biochemistry 20*, 990–996.
Stumph, W. E., Kristo, P., Tsai, M.-J., & O'Malley, B. W. (1981) *Nucleic Acids Res. 9*, 5383–5397.
Stumph, W. E., Baez, M., Beattie, W. G., Tsai, M.-J., & O'Malley, B. W. (1983) *Biochemistry 22*, 306–315.
Stumph, W. E., Hodgson, C. P., Tsai, M.-J., & O'Malley, B. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6667–6671.
Tsai, S. Y., Roop, D. R., Tsai, M.-J., Stein, J. P., Means, A. R., & O'Malley, B. W. (1978) *Biochemistry 17*, 5773–5780.
Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 193*, 848–856.
Weisbrod, S. (1982) *Nature (London) 297*, 289–295.
Woo, S. L. C., Beattie, W. G., Catterall, J. F., Dugaiczyk, A., Staden, R., Brownlee, G. G., & O'Malley, B. W. (1981) *Biochemistry 20*, 6437–6446.

Dynamic Interaction between Components of Hexaprenyl Diphosphate Synthase from *Micrococcus luteus* BP-26†

Ichirou Yoshida, Tanetoshi Koyama, and Kyozo Ogura*
Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai, 980 Japan
Received February 26, 1987; Revised Manuscript Received June 3, 1987

ABSTRACT: Hexaprenyl diphosphate synthase from *Micrococcus luteus* BP-26, which has been known to be dissociated into two essential components, designated as components A and B, during hydroxyapatite chromatography [Fujii, H., Koyama, T., & Ogura, K. (1982) *J. Biol. Chem. 257*, 14610–14612], was also resolved similarly by Sephadex G-100 or DEAE ion-exchange chromatography. Each component takes various self-aggregated forms. The apparent molecular mass of component B estimated by gel filtration on Superose 12 varied depending on its concentration, ranging from ~18 to 49 kilodaltons (kDa). On the other hand, the apparent molecular mass of component A varied depending on not only its concentration but also the ionic strength of the medium, ranging from ~13 to 24 kDa. When a mixture of components A and B preincubated in the presence of Mg^{2+} but in the absence of substrate was subjected to Superose 12 gel filtration, they were eluted at positions identical with those observed when they were chromatographed individually. In contrast, when a mixture of components A and B incubated in the presence of Mg^{2+} and substrates was filtrated on Superose 12, the elution positions were markedly changed, showing that an ~24-kDa aggregate of component A (designated as *A*) and an ~27-kDa aggregate of component B (designated as *B*) were the major species. Evidence was also obtained to show that farnesyl diphosphate (FPP) binds to the components to form an aggregate, *A*–*B*–FPP–Mg^{2+}, which probably represents an intermediary state of enzyme catalysis.

Prenyltransferases catalyze the head-to-tail condensation between isopentenyl diphosphate (IPP)[1] and an allylic diphosphate to generate various prenyl diphosphates. The early investigations on the biosynthesis of bacterial isoprenoid compounds have led to the conclusion that the prenyl diphosphates are intermediates of bacterial isoprenoid compounds such as prenyl side chains of the respiratory quinones and sugar-carrier lipids (Poulter & Rilling, 1981).

Seven prenyltransferases that catalyze the condensation of isoprene units to give long-chain prenyl diphosphates whose chain lengths are longer than C_{20} have so far been separated from various bacteria. They can be classified into two groups according to the reactions they catalyze. One is the group of enzymes that catalyze the formation of (*Z*)-prenyl chains [(*Z*)-polyprenyltransferase], and the other group catalyzes the formation of (*E*)-prenyl chains [(*E*)-polyprenyltransferase]. Undecaprenyl diphosphate synthase (Kurokawa et al., 1971; Keen & Allen, 1974; Baba & Allen, 1978, 1980; Takahashi & Ogura, 1982; Muth & Allen, 1984) is representative of the former, producing undecaprenyl diphosphate which is the precursor of well-known bacterial sugar-carrier lipid for cell wall biosynthesis. (*Z*)-Nonaprenyl diphosphate synthase (Ishii et al., 1986) is also one of the (*Z*)-polyprenyltransferases. (*E*)-Polyprenyltransferases include hexaprenyl diphosphate (C_{30}) (Fujii et al., 1982), heptaprenyl diphosphate (C_{35}) (Takahashi et al., 1980), octaprenyl diphosphate (C_{40}) (Fujisaki et al., 1986), solanesyl diphosphate (C_{45}) (Sagami et al.,

† This work was supported by Grants-in-Aid for Scientific Research 58470133 and 60303006 from the Ministry of Education, Science and Culture of Japan and by the Asahi Glass Foundation for contribution to industrial technology.
* Author to whom correspondence should be addressed.

[1] Abbreviations: FPP, (2*E*,6*E*)-farnesyl diphosphate; IPP, isopentenyl diphosphate; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

0006-2960/87/0426-6840$01.50/0