Volume 9 Number 14 1981 Nucleic Acids Research

Isolation and characterization of genomic clones covering the chicken vitellogenin gene

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Received 26 May 1981

ABSTRACT

A series of overlapping recombinant clones, which cover the vitellogenin gene, has been isolated from a phage-lambda linked chicken gene library. The DNA of the overlapping clones spans 28 kb of contiguous DNA sequences in the chicken genome. Electron microscopic analysis of hybrids between vitellogenin mRNA and the genomic clones indicates that the chicken vitellogenin gene has a length of approximately 22 kb, about 3.8 times the size of the mRNA. The mRNA sequence is interrupted by at least 33 intervening sequences (introns). Comparison with the vitellogenin gene A2 from *Xenopus laevis* (Wahli *et al.*, 1980, Cell 20: 107-117) indicates conservation of the number and length of the exons during evolution. Heteroduplex analysis reveals a short stretch of sequence homology between the genes from chicken and frog.

INTRODUCTION

Vitellogenin is the 200-220 kilodalton precursor polypeptide of the major yolk proteins, lipovitellin and phosvitin. Vitellogenin synthesis occurs in the livers of all oviparous vertebrates and is induced by estrogens. Upon induction in male birds (1, 2, 3) and amphibia (4, 5) vitellogenin mRNA increases within a week from undetectable levels to 7000-35,000 molecules per cell. This strong response, which is fully reversible and not dependent on DNA synthesis, offers an attractive model for the study of estradiol-controlled gene expression in terminally differentiated cells.

The vitellogenin genes from *Xenopus laevis* have been studied extensively by Wahli and coworkers. Analysis of cDNA clones indicated that in this frog at least four different vitellogenin genes are expressed (6). Isolation of two of these genes by means of molecular cloning and analysis by electron microscopy revealed considerable differences in the non-structural parts of the

genes (7).

Recently, we and others have cloned cDNA of mRNAs coding for chicken vitellogenin (8, 9) and Very Low Density Lipoprotein II (apoVLDLII)(10), another estrogen-controlled protein synthesized in the avian liver. We used our cDNA clones to select genomic clones containing the chicken vitellogenin and VLDLII genes from a λ Charon-4A linked gene-library. The clones carrying the VLDLII gene will be described in detail elsewhere (manuscript submitted). Here, we report the isolation and characterization of overlapping clones covering a chicken vitellogenin gene. The structure of the chicken gene was compared to vitellogenin gene A2 from *X. laevis* (7).

MATERIALS AND METHODS

Screening of the chicken DNA library

The DNA library used in the present study has been described by Dodgson *et al.*(11). It consists of Lambda Charon 4A recombinants containing 15 to 20-kb inserts of chicken genomic DNA. A sample of the library was kindly provided to us by R. Axel. Screening was performed according to Benton and Davis (11), using modifications described by Maniatis *et al.*(13) and Blattner *et al.* (14). In the first screening cycle, approximately 200,000 phages were plated at a density of 80 pfu/ cm^2. *In situ* hybridization was done with a mixture of probes for vitellogenin and VLDLII sequences. The phages selected in the first cycle were subsequently screened, at much lower densities, with the separate probes to select for clones carrying vitellogenin, or VLDLII sequences. The cDNA plasmids, pVtg2-1 and pVtg2-2, complementary to different segments of the vitellogenin mRNA, isolated and described previously (8, 15) provided the probes for vitellogenin sequences. From pVtg2-1, a 1.6-kb *Msp*I-fragment, and from pVtg2-2, a 3.0-kb *Pvu*II-fragment, both containing the cDNA insert, were excised and ^{32}P-labeled by nick translation (16).

Containment

All experiments involving viable phages and bacteria containing recombinant DNA were performed under CII/EKII conditions as specified by the Netherlands *Ad Hoc* Committee on Recombinant DNA Research.

DNA preparations and characterization

Purification of phages and phage DNA was according to Williams *et al.* (17). Isolation of genomic DNA and hybridization of Southern blots (18) were essentially as described by Jeffreys and Flavell (19). The final washing step after hybridization with 5'-end labeled, fragmented RNA was in 3 x SSC, and after hybridization with nick-translated DNA in 0.5 to 1 x SSC. Isolation of specific DNA fragments from agarose gels was according to Tabak and Flavell (20). DNA digestion with restriction enzymes, DNA electrophoresis, and isolation of plasmid DNA were exactly as described in Ref. 10. DNA from λXlv128 containing the *Xenopus* vitellogenin gene A2 (Ref. 7) was a generous gift of Dr. W. Wahli.

Labeling of RNA

RNA was labeled following a modification of the procedure of Doel *et al.* (21). Vitellogenin mRNA (3 µg), purified as described earlier (22), was hydrolyzed for 60 min in 1 M NaOH at 0°C to an average chain length of 200-400 nucleotides. These fragments were reacted with 350 µCi[γ-^{32}P]ATP (2000 Ci/mmol; The Radiochemical Centre, Amersham, UK) and 8 units T_4 polynucleotide kinase (Boehringer, Mannheim, GFR) in a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, for 30 min at 37°C.

Hybrid formation for electron microscopy

Hybrids were formed under conditions described by Woolford *et al.* (23) with minor modifications in the incubation temperature to displace the non-coding DNA strand completely. Linear temperature gradients from 63° to 45°C with a decrease of 1°C per 20 min were used. Samples of the incubation mixture were spread from 65% (v/v) formamide, 2.1 M urea, 26 mM Tris-HCl (pH 8.5), 1 mM Na-EDTA, 40 µg/ml of cytochrome c, on a hypophase of triple-distilled water. Specimens were processed for electron microscopy, and molecules were measured as described earlier (24). ΦX174 RF DNA was used as internal length standard.

Heteroduplex formation for electron microscopy

Chimeric phage DNAs were denatured for 3 min at 80°C at a concentration of about 5 µg/ml in 70% (v/v) formamide, 0.3 M NaCl, 1 mM Na-EDTA, 10 mM Tris-HCl (pH 8.5) and were annealed in the same buffer for 90 min at 35°C, or for 60 min at 35°C followed by

30 min incubation at 30°C. Samples were spread from 38 %(v/v) formamide, 1 M urea, 90 mM Tris-HCl (pH 8.5), 60 mM NaCl, 2 mM Na-EDTA, 40 µg/ml of cytochrome c, or from 70 %(v/v) formamide, 2 mM Tris-HCl (pH 8.5), 50 mM NaCl, 9 mM Na-EDTA, 0.4 M NH_4-acetate, 100 µg/ml of cytochrome c, on a hypophase of triple-distilled water. Single- and double-stranded ΦX174 DNA were used as internal length standards.

RESULTS

Screening of the library

Earlier, our group constructed several recombinant pCR1 plasmids containing partial cDNA transcripts from chicken vitellogenin mRNA (8). Two of the plasmids, designated pVtg2-1 and pVtg2-2, have been used as probes in the present study. Plasmid Vtg2-1 covers an 1-kb segment close to the 3'-end of the 6-kb long vitellogenin mRNA whereas pVtg2-2 contains an 0.8-kb transcript complementary to a region from approximately 1.6 to 2.4 kb from the 3'-end of the mRNA (8, 15). Fragments containing the cDNA insert were excised from these plasmids (see Materials and Methods), and labeled to obtain radioactive probes for the screening of the library. The screening resulted in the isolation of four different clones, numbered 22, 23, 24 and 34, hybridizing to either one, or both cDNA sequences.

Physical map

In order to construct physical maps of the clones isolated, their DNAs were digested with restriction enzymes and subjected to agarose-gel electrophoresis combined with hybridization of Southern blots to different probes. Labeled pVtg2-1 and pVtg2-2 served as probes for the 3'-end and the mid part of the gene, respectively, thus allowing the establishment of the orientation of the gene fragments in the cloning vector. Also, labeled vitellogenin mRNA fragments were used as probe. Fig. 1 shows an example of such an experiment in which the clones 22, 23 and 24 were digested with *Eco*RI, or *Eco*RI and *Sma*I.

The physical map (Fig. 2) obtained shows that the clones contain overlapping inserts, and cover about 28 kb of the chicken genomic DNA. The 5'-end of the gene was tentatively located downstream from the upper-left *Bam*HI site since only fragments to the

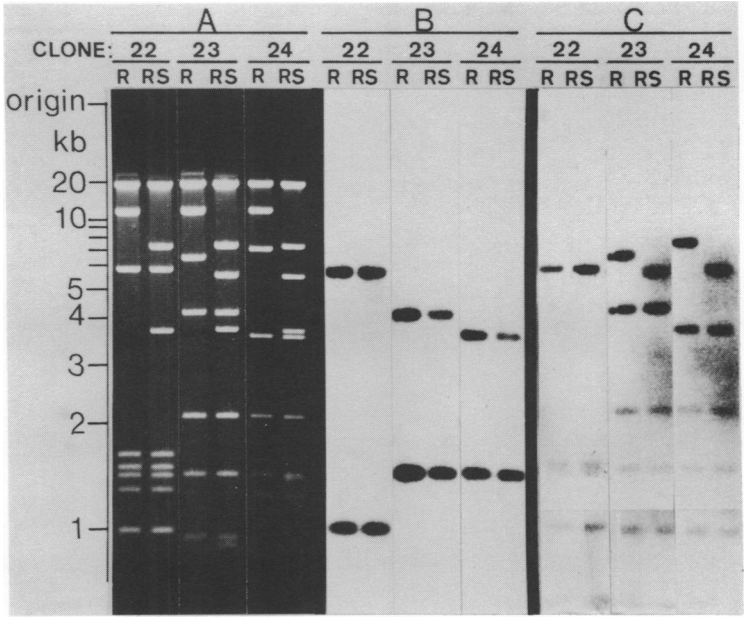


Fig. 1. Electrophoresis of restriction enzyme digests of DNA from clones 22, 23 and 24. Fragments generated by *Eco*RI (R), or *Eco*RI + *Sma*I (RS) were visualized by ethidium bromide staining (panel A), and by autoradiography of Southern blots after hybridization with cDNA plasmid pVtg2-2 (panel B) or vitellogenin mRNA (panel C). Panel C is composed of two different exposures of the same blot.

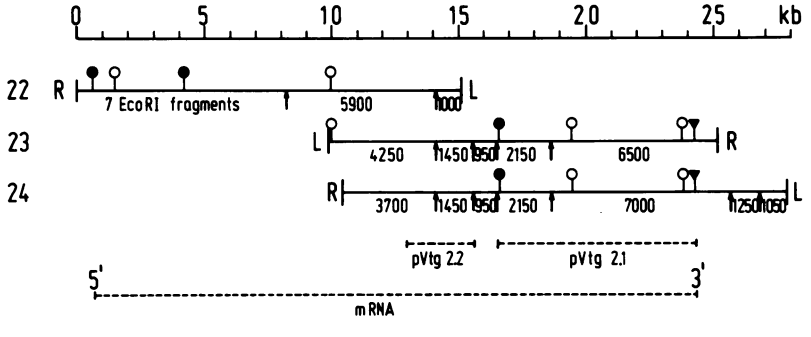
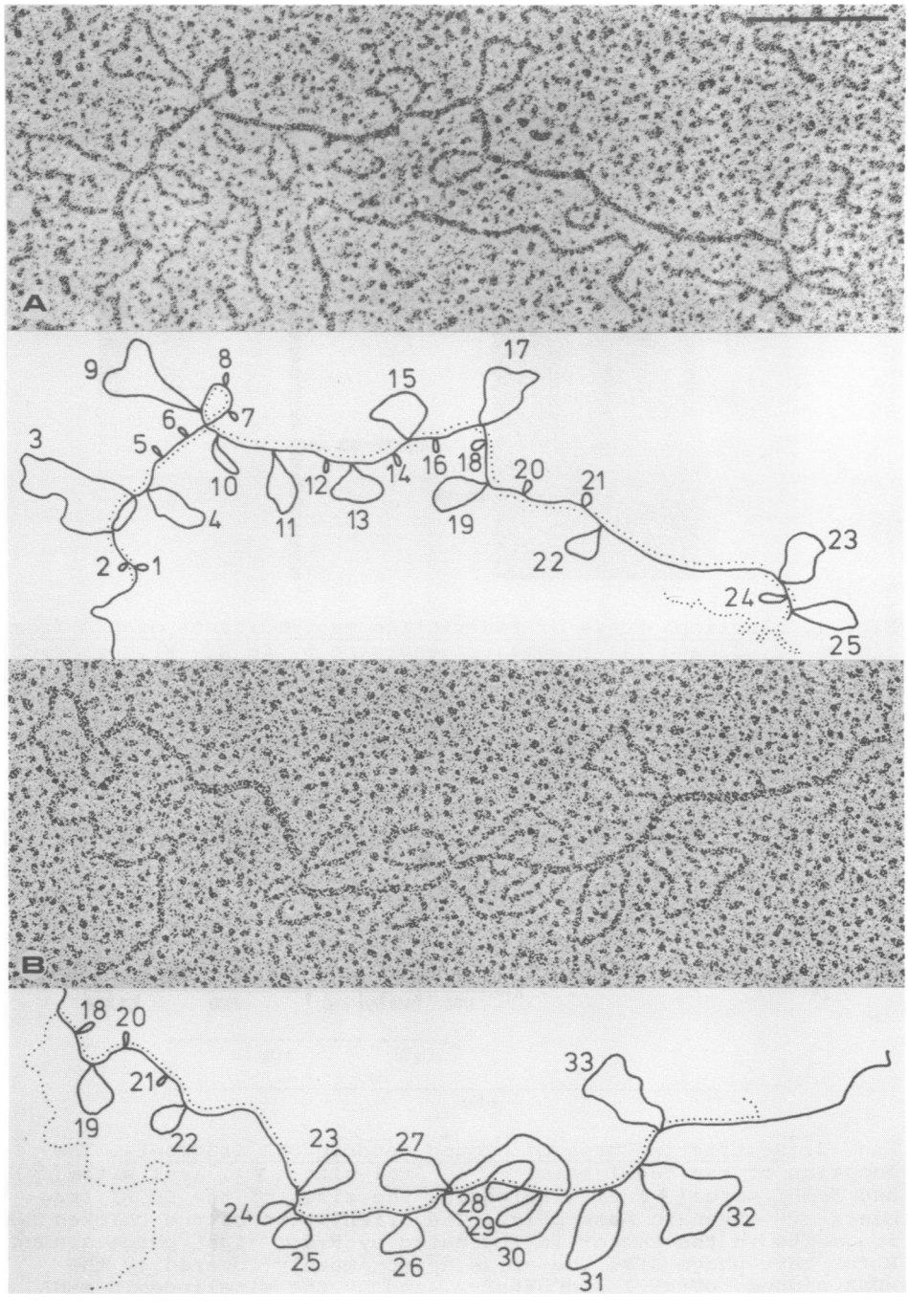


Fig. 2. Restriction maps of cloned genomic DNA fragments. The location of the *Eco*RI sites (↑), *Sma*I site (▼), *Kpn*I sites (♀) and *Bam*HI sites (●) are indicated. The sizes of the *Eco*RI fragments are given in base pairs. The orientation of the chicken DNA fragments in the vector is indicated by R for right phage arm and L for left phage arm. The areas approximately covered by the cDNA clones, pVtg2-1 and pVtg2-2, and by the vitellogenin mRNA are indicated by dashed lines.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right of this site hybridize with vitellogenin mRNA. Similarly, the 3'-end of the gene must be upstream from the single *Sma*I-site in the map since only the 5.5-kb fragment, but not the 1.5-kb fragment, both generated by *Sma*I from the 7-kb *Eco*RI-fragment of clone 22, hybridizes with vitellogenin mRNA. Definite proof that all clones contain parts of the same vitellogenin gene was obtained by electron microscopy as will be discussed in the following section.

Exon-intron map

To elucidate the structure of the chicken vitellogenin gene, hybrids were formed between purified vitellogenin mRNA and DNA from clones 22 or 24, containing the 5'- and 3'-end of the gene, respectively. Two examples are shown in Fig. 3. In hybrids with clone-22 DNA, 25 introns appearing either as loops or as small knobs are seen. The single-stranded tail, extending from one end of the hybrid, represents the non-hybridizing part of the mRNA. The clone contains 4.3 kb of vitellogenin structural sequences; the intron lengths add up to 10 kb. Clone 24 contains at least 16 introns with a total length of 9.5 kb, and 17 exons with a total length of 3.4 kb. The sizes of the exons and introns are summarized in Table I.

The overlap between clones 22 and 24 is clearly demonstrated by the presence of an identical exon-intron arrangement covering the region beginning with exon 18 and extending into exon 26. The overlapping region measures 4.6 kb which is in excellent agreement with the restriction enzyme data (see Fig. 2). Combination of the data from clones 22 and 24 allows us to draw the exon-intron map of the gene (Fig. 4). We conclude that the chicken vitellogenin gene is composed of at least 34 exons, which are interspersed by introns of various lengths. The total length of the gene is 22.5 kb and the exons add up to 5.9 kb.

In clone 22, the chicken DNA segment amounts to 16 kb, as

Fig. 3. Hybrids between vitellogenin mRNA and cloned genomic chicken DNA. A hybrid of vitellogenin mRNA with clone-22 DNA, containing the 5'-proximal part of the vitellogenin gene, is shown in panel A, and a hybrid with clone-24 DNA containing the 3'-part in panel B. Our interpretation is represented in the drawings in which the DNA is a continuous and the RNA a stippled line. The introns are numbered 1 to 33 starting from the 5'-end. The bar is 0.2 μm.

Table 1. Exon and intron lengths of the chicken vitellogenin gene

Number	exon length bp ± SD	intron length bp ± SD	Number	exon length bp ± SD	intron length bp ± SD
1	⩽50	114 ± 49	18	106 ± 20	134 ± 31
2	40 ± 17	94 ± 29	19	180 ± 34	600 ± 57
3	146 ± 17	1457 ± 143	20	149 ± 23	109 ± 29
4	251 ± 20	600 ± 86	21	220 ± 29	83 ± 34
5	160 ± 26	86 ± 17	22	174 ± 29	429 ± 57
6	151 ± 20	140 ± 46	23	714 ± 57	571 ± 29
7	157 ± 26	103 ± 23	24	69 ± 17	314 ± 29
8	140 ± 26	69 ± 29	25	97 ± 17	514 ± 57
9	169 ± 29	1029 ± 86	26	374 ± 49	457 ± 57
10	129 ± 17	486 ± 29	27	149 ± 31	686 ± 57
11	231 ± 31	657 ± 57	28	149 ± 29	523 ± 57
12	226 ± 23	94 ± 34	29	107 ± 29	429 ± 29
13	103 ± 20	571 ± 57	30	106 ± 23	1286 ± 86
14	126 ± 20	106 ± 54	31	117 ± 20	914 ± 57
15	103 ± 17	714 ± 57	32	171 ± 31	1314 ± 86
16	117 ± 17	63 ± 11	33	160 ± 34	1086 ± 57
17	189 ± 29	829 ± 57	34	371 ± 20	

This table was constructed from measurements of molecules containing parts of the gene. The number of measurements included varies from 7 to 34.

measured from heteroduplexes (see below), whereas the gene sequences measure 14.3 kb, leaving 1 to 2 kb for the 5'-flanking region. In clone 24, the length of the gene sequences is 12.9 kb and the total cloned chicken DNA fragment 18 kb, implying that about 5 kb of the 3'-flanking region are present in the clone.

From the analysis of hybrids formed between clone 23 and vitellogenin mRNA (not shown) we conclude that clone 23 extends

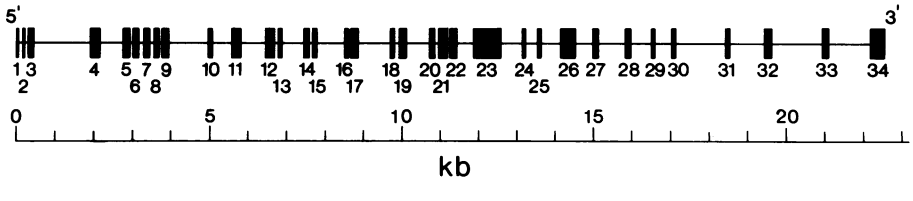


Fig. 4. Exon-intron map of the vitellogenin gene as determined by electron microscopy. This map was constructed from the exon and intron lengths summarized in Table I. Exons correspond to vertical bars and are numbered 1 to 34. Horizontal lines between the exons represent introns. All exons and introns are drawn to scale.

from exon 23 into the 3'-flanking region. The orientation of the cloned fragment is the reverse of that in clones 22 and 24 (see also Fig. 2).

The identical pattern of intron-loops in the overlapping parts already suggested that clones 22, 23 and 24 originate from the same gene. If two genes were involved, one would expect to find differences in the introns, as has been shown for the vitellogenin genes A1 and A2 from *X. laevis* (7). Moreover, heteroduplexes formed between DNA from clones 22 and 24 show a duplex region of about 4.6 kb without any evidence for the existence of non-homologous stretches (Fig. 5).

Comparative analysis of vitellogenin genes from chicken and *X. laevis*

A comparison between the vitellogenin genes from chicken and

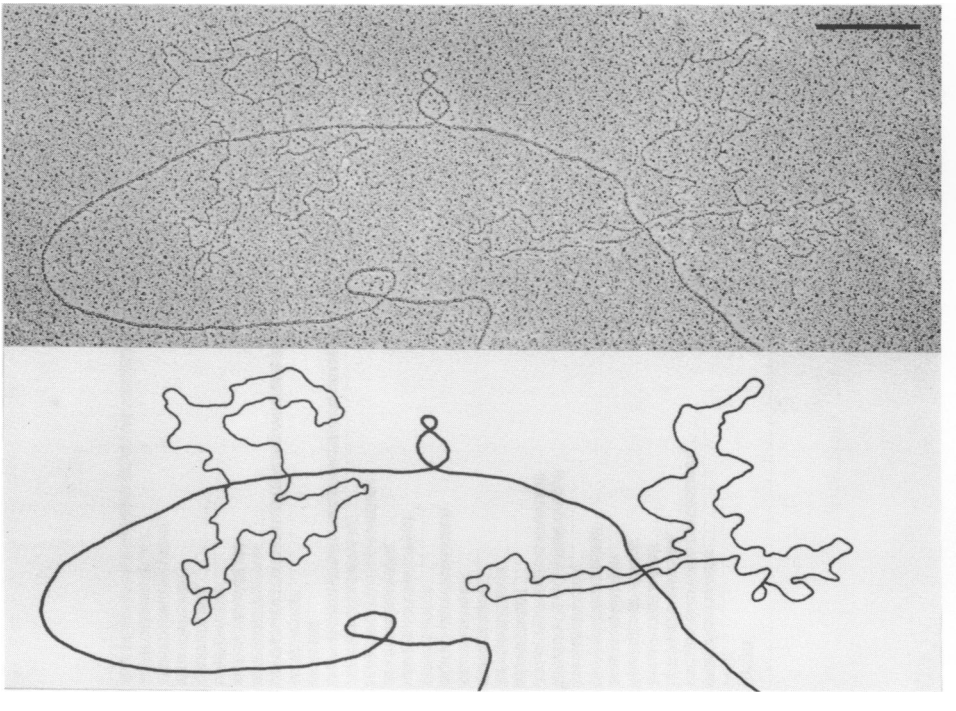


Fig. 5. Heteroduplex of two overlapping genomic chicken clones. Electron micrograph of a heteroduplex molecule between clone 22 and clone 24. The overlapping part is seen as a double-stranded region bounded by two large single-stranded loops representing chicken DNA sequences present in only one of the clones. The bar is 0.3 µm.

X. laevis is interesting from an evolutionary point of view. As a first approach, we compared the intron-exon maps of both genes, using the data on the vitellogenin gene A2 of *X. laevis* determined by Wahli *et al.*(7;and personal communication from the authors). In both species the vitellogenin genes are split into 34 exons. A comparison in pairs, of exons with the same numbers, shows no similarity between both genes (Fig. 6; correlation coefficient $\rho = -0.13$). However, when each exon in the chicken gene is compared to the one-lower numbered exon of the *X. laevis* gene a significant resemblance is apparent ($\rho = 0.89$), especially in the first 24 exons ($\rho = 0.97$). No similarity in the lengths of the introns was observed.

Heteroduplexes were formed between λXlv128 (Ref. 7), which contains the *X. laevis* vitellogenin gene A2, and either chicken clone 22 or clone 24. The vitellogenin gene fragments are in these three clones in the same orientation with respect to the Charon arms. Fig. 7 shows examples of heteroduplex molecules; in both combinations, a homology region of 290 ± 50 base pairs

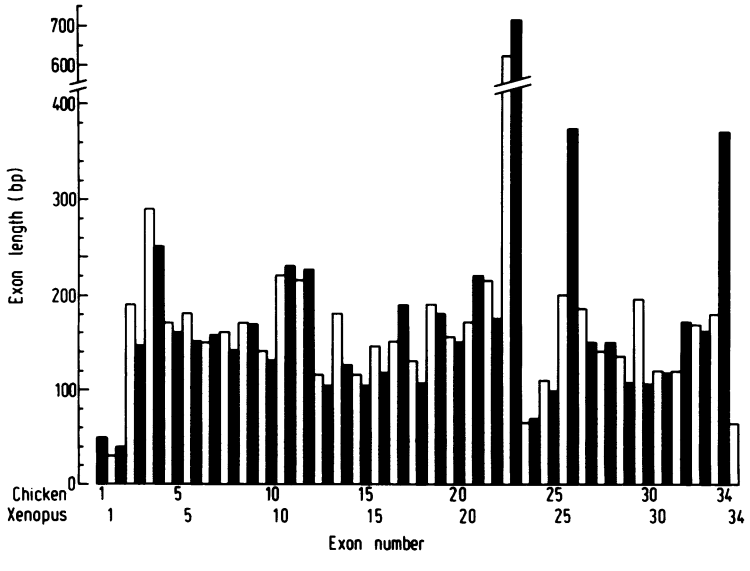


Fig. 6. Comparison of the exon lengths of chicken and *Xenopus* vitellogenin genes. Values for chicken exons (black bars) are from Table I, and for *Xenopus* exons (open bars) from Wahli *et al.* (7; and personal communication from the authors).

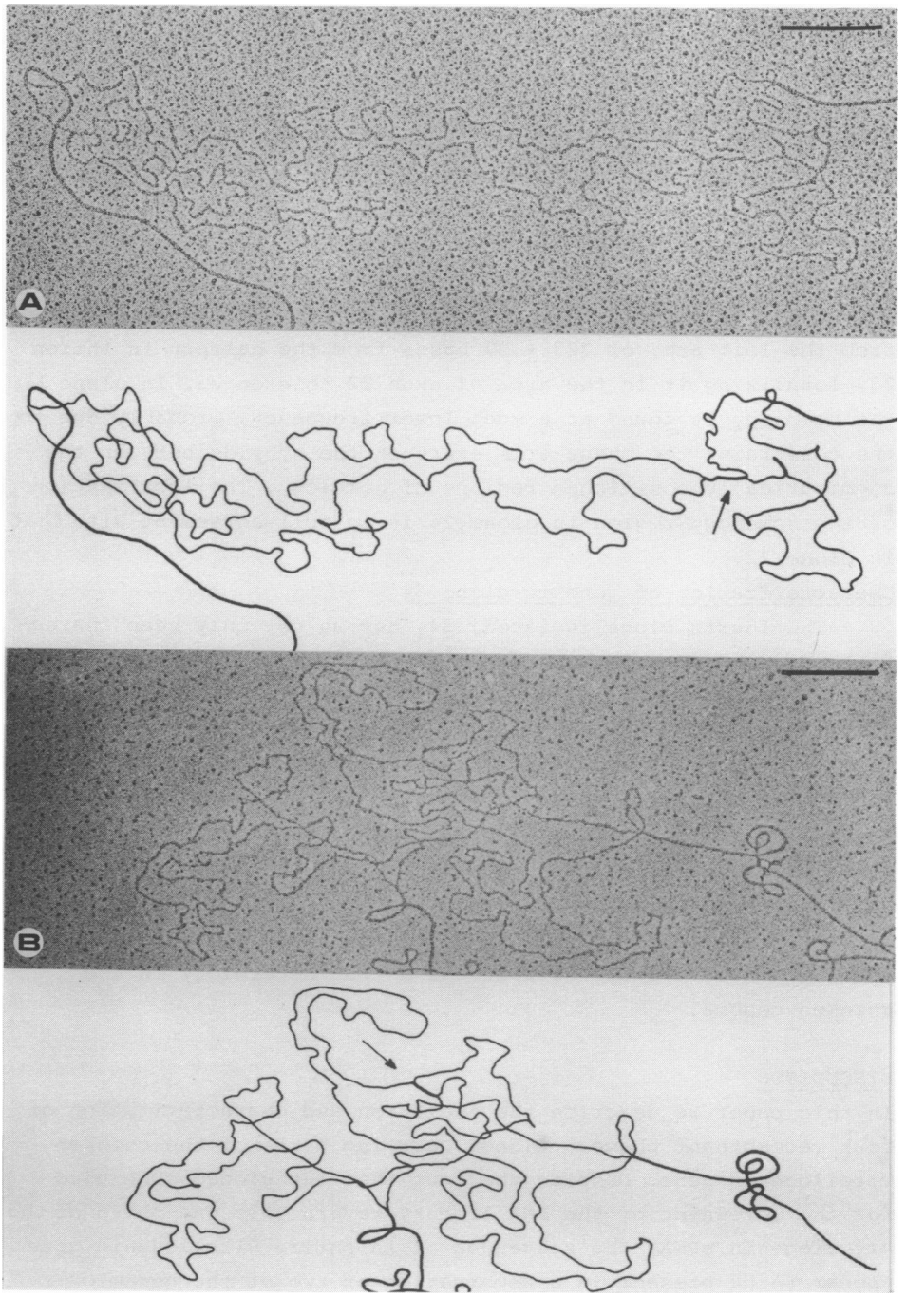
(S.D.) is observed. One of the strands between the homology region and the left phage arm is identical in both heteroduplex combinations and therefore, most likely, is *Xenopus* DNA. Close to the homology region, a large hairpin, presumably the hairpin present in intron 23 originally observed by Wahli *et al.* (7), is clearly visible (see Fig. 7, panel A). Eighteen heteroduplexes between clones 22 and λXlv128 were measured to localize the homology region. In chicken clone 22, this region is situated 1.5 kb from the left vector arm, which places it in exon 23 and/or intron 23. In λXlv128, we find the homology region at 6.1 kb from the left arm, or 325 ± 50 bases from the hairpin in intron 23, localizing it in the area of exon 22 to exon 23. In clone 24, the homology is found at a much lower frequency, probably due to the constraint the phage arms exert on the hybrids between the asymmetrically positioned regions of homology. The localisation of the homology region in clone 24 is in full agreement with that in clone 22.

Characterization of genomic clone 34

The fourth clone isolated, 34, has as yet only been characterized preliminarily. From hybridizations with ^{32}P-labeled vitellogenin mRNA and cDNA clone pVtg2-1, we infer that clone 34 contains 3'-proximal gene sequences. Electron microscopy of hybrids between mRNA and clone-34 DNA revealed an exon-intron arrangement which is very similar to the gene region, between intron 31 and the 3'-end, in clone 24. However, Southern-blot hybridizations showed that the 3'-flanking sequences in clone 34 and 24 are completely non-homologous (data not shown). Further experiments are necessary to decide whether this anomalous finding is caused by a cloning artifact, or reflects a true heterology in the chicken genome.

DISCUSSION

In this paper we describe the isolation and characterization of four recombinant phage λ clones carrying parts of the chicken vitellogenin gene. Despite the fact that the cloned cDNA used for the screening of the DNA library covers only one-third of the vitellogenin mRNA, the sequences of an entire vitellogenin gene appear to be present in a combination of two of the genomic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clones selected. Clone 22 contains the 5'-half of the gene, whereas the remaining part is present in the overlapping clones 23 and 24. Clone 24 extends 4 to 5 kb into the 3'-flanking region.

Our conclusion that clone 22 contains the region encoding the cap site of the mRNA is based on the following observations: 1. In the electron micrographs of hybrids between clone-22 DNA and vitellogenin mRNA we never detected a RNA tail beyond exon 1. 2. Certain restriction-enzyme fragments containing the region adjacent to the right phage arm did not hybridize with labeled mRNA. However, we can not entirely exclude the possibility that, due to the limited resolution of the electron microscopy, we have missed a small 5'-proximal exon. Moreover, the cap of the mRNA may have reduced the labeling of fragments from the 5'-part of the mRNA-probe.

The sum of the exon lengths, 5.9 kb, is somewhat less than the mRNA length determined earlier by gel electrophoresis and electron microscopy (about 7 kb; see Ref. 22) and by analysis of overlapping cDNA clones (6.6 kb; see Ref. 25). The discrepancy may to some extent be due to the use of different methods, but is in our opinion more probably a consequence of the lack of adequate RNA markers (22). Irrespective of these differences, the length of the vitellogenin gene of chicken is equal to that of *Xenopus* where the mRNA length has also been found to be somewhat larger (7).

The number of exons in the vitellogenin genes of chicken and *Xenopus* are equal. A correlation in the length distribution of the exons of both species is only observed when the comparison starts with *Xenopus* exon 1 and chicken exon 2. This apparent shift may simply be due to a misinterpretation of the hybrid structure at the ends of the genes. Therefore, we paid special attention to the possible presence of an additional intron-loop in exon 34, which is in chicken much longer than in *Xenopus*. Although we did not find any evidence, we can at this stage not completely exclude that we have missed one or more introns or

Fig. 7. Heteroduplex of genomic vitellogenin clones from *Xenopus* and chicken. Heteroduplex molecules between *X. laevis* clone λXlv128 containing the entire A2 gene, and chicken clone 22 (panel A) or clone 24 (panel B). The 0.3-kb homology region is indicated by arrows. The bar is 0.3 μm.

exons. If no more introns and exons are found, *Xenopus* and chicken have probably both lost one intron close to the opposite ends of the gene.

The finding of a region of homology between the chicken and *Xenopus* vitellogenin gene suggest that this sequence has been under strong selective pressure in the course of evolution, and might code for an important funtional domain. As possible candidates we like to suggest phosvitin, the serine-rich part, which is released from vitellogenin upon deposition into the egg yolk, or a site recognized by the receptor on the oocyte membranes. However, many other possibilities could be envisaged. A definite solution should come from sequence analysis.

In contrast to *Xenopus* where a family of at least four vitellogenin genes has been demonstrated (6,7) the situation in chicken is less clear in this respect. Evidence has been presented for vitellogenin heterogeneity in the chicken at the level of protein (26) and mRNA (25). However, we have no convincing evidence for the existence of more than one chromosomal gene. Clone 34, which differs in the 3'-flanking gene region from the other clones, might contain a part of another vitellogenin gene. This would however imply conservation of intron lengths, in this part of the genes, unlike the situation in *Xenopus*. Therefore we reckon with the possibility that clone 34 has resulted from an artifact during the cloning.

ACKNOWLEDGEMENTS

We are very grateful to Drs. R. Axel, J.B. Dodgson. J. Strommer and J.D. Engel for providing us with the DNA library. We thank Dr. F.R. Blattner for Charon phages and bacterial strains. We would like to express our gratitude to drs. W. Wahli and G.U. Ryffel for their gift of λXlv128 DNA and for making additional data on vitellogenin exon lengths available to us. We would like to thank Drs. P. van den Boogaart and B. Wieringa for vitellogenin cDNA clones and vitellogenin messenger RNA. We thank Mr. K. Gilissen for printing the photographs, Ms. A. Rijnsent for skilful technical assistance, and Mr. J. van den Heuvel and Mr. N.H.J. Panman for constructing equipment and for making the drawings.

This investigation was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.), with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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