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Analysis of Chicken Ribosomal RNA Genes and Construction of Lambda Hybrids Containing Gene Fragments

Abstract. *The ribosomal RNA genes (ribosomal DNA) of chicken are present in approximately 200 copies and are cleaved into two fragments of molecular weight 5 × 10^6 and 12 to 14 × 10^6 by restriction endonuclease Eco RI. Recombinant phages have been constructed in vitro by joining the smaller fragment of ribosomal DNA and the outer arms of DNA from the vector λgtWES·λC. In one of the recombinants, the coding strand of the cloned fragment is in the proper orientation for transcription with λ "early" genes; in the other two the orientation is reversed, with the coding strand in the proper position for transcription with λ "late" genes.*

Cloning of ribosomal RNA (rRNA) genes, which are present in multiple copies and specify a readily available RNA probe, can provide a convenient system for detailed analysis of eukaryotic DNA (*1*). In addition to yielding hybrid DNA which includes genes that are interesting in themselves, these experiments provide an opportunity to develop methods potentially useful for the cloning of unique sequence genes (*2*). We have employed the certified EK2 vector λgt*WES*·λC (*3*) to clone and characterize a portion of the ribosomal gene from chickens (*Gallus domesticus*).

Ribosomal RNA genes represent 0.02 to 0.12 percent of chicken DNA (*4, 5*) and are clustered. If these genes are in tandem, as are ribosomal DNA (rDNA) sequences in other eukaryotic chromosomes (*6–8*), then cleavage with restriction endonuclease should yield a number of identical fragments. Figure 1 shows results from experiments in which chicken DNA was digested with Eco RI and the resulting fragments analyzed for the presence of DNA homologous to chicken rRNA. A mixture of 18*S* and 28*S* ^{3}H-labeled chicken rRNA hybridized to fragments in two regions of the gel (Fig. 1, b and c). By comparison with parallel gels containing Eco RI digests of ^{3}H-labeled λ DNA (*9*), we estimate the molecular weights of the two rDNA fragments to be 5 × 10^6 and 12 to 14 × 10^6. The sharp band in the 5 × 10^6 dalton region and the absence of hybridization to DNA between the two bands indicated that Eco RI digestion was complete and that there were at least two sites for Eco RI in the rDNA. Other experiments (not included) showed that the smaller fragment contained sequences homologous with both 18*S* and 28*S* rRNA, whereas the larger fragment contained only 18*S* sequences. These results are similar to those found for rDNA in the amphibian *Xenopus laevis* (*10*) and in several mammals (*8*) and support the suggestion by Southern (*8*) that these two sites of Eco RI cleavage in the rDNA have been conserved since the divergence of amphibians and mammals. The sizes of the avian rDNA Eco RI fragments are closer to those of mammals (mouse, 5.2 × 10^6 and 14 × 10^6 daltons; rabbit, 6.0 × 10^6 and 12 × 10^6 daltons) than those of amphibians (*X. laevis*, 3.0 × 10^6 and 4 to 6 × 10^6 daltons) (*8, 10*). Our data suggest that the structure of the chicken rDNA is simi-

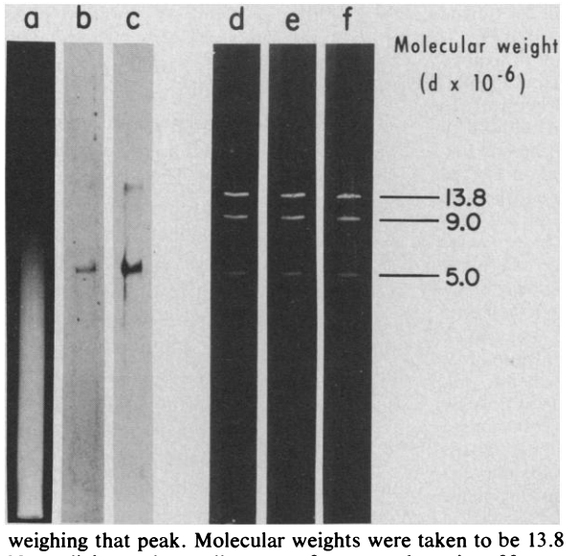

Fig. 1. Agarose gel analysis of chicken DNA and recombinant phage DNA. High-molecular-weight DNA was prepared from erythrocytes of Spafas (Norwich, Conn.) gs$^-$ chickens by a method similar to that of Chambon and co-workers (*18*). The DNA was incubated in 0.1*M* tris (*p*H 7.4), 0.05*M* NaCl, 0.005*M* $MgCl_2$, and 0.001*M* *p*-chloromercuribenzoate for 10 minutes at 37°C. Eco RI was added (2 units per microgram of DNA) and the mixture was incubated for 10 minutes. A second portion of Eco RI was then added and incubation was continued for 10 minutes. The reaction was stopped by adding one-tenth volume 10 percent sodium dodecyl sulfate and heating to 65°C for 2 to 5 minutes. Digested DNA was concentrated by ethanol precipitation before electrophoresis; such treatment causes some loss of large fragments. A 10- to 20-μg portion of the concentrated DNA was subjected to electrophoresis on 0.7 percent agarose disc gels (0.5 by 12 cm) in buffer containing 0.04*M* tris, 0.005*M* sodium acetate, and 0.001*M* EDTA (the *p*H was adjusted to 7.9 with acetic acid). Gels were stained for 1 hour or more in ethidium bromide (0.5 μg/ml). The DNA was transferred to cellulose nitrate membranes by the method of Southern (*8*). The 18*S* and 28*S* rRNA probes (specific activity ≃ 4 × 10^6 cpm/μg) were prepared from chicken fibroblasts labeled with [^{3}H]uridine. Each probe was shown to be homogeneous by polyacrylamide gel electrophoresis. The concentration of probe RNA in the hybridization mixture was approximately 0.5 μg/ml. Hybridization was detected by fluorography (*8, 19*). The stoichiometry of the recombinant DNA Eco RI fragments was determined from densitometer tracings of a photographic negative of ethidium bromide–stained gels. The area under each peak was determined by cutting out and weighing that peak. Molecular weights were taken to be 13.8 × 10^6 and 9.0 × 10^6 for the vector arms and 5.0 × 10^6 for the inserted fragment. Normalizing to the smaller vector fragment, the ratios of fragments (from largest to smallest) are: λGd1, 0.98 : 1.00 : 0.76; λGd2, 1.00 : 1.00 : 0.91; and λGd3, 1.08 : 1.00 : 0.86. Lambda Gd1 DNA, uniformly labeled with ^{32}P, was digested with Eco RI and subjected to electrophoresis on 0.7 percent agarose gels. The radioactivity in each fragment was determined by cutting out the ethidium bromide–stained bands and counting them in 5 ml of Aquasol. The counts were (from largest to smallest) 297, 154, and 80 cpm. Normalizing to the smaller vector arm, the ratio of fragments is 1.25 : 1.00 : 0.94. (a) Ethidium bromide–stained gel containing Eco RI fragments of unfractionated chicken DNA. (b) Fluorogram of Southern transfer containing Eco RI fragments of unfractionated chicken DNA hybridized with 18*S* plus 28*S* ^{3}H-labeled rRNA. In this transfer, hybridization to the 5 × 10^6 dalton fragment is apparent but hybridization to the 12 to 14 × 10^6 dalton fragment is not. (c) Fluorogram as in (b) except that before digestion with Eco RI, DNA was enriched three- to fivefold for ribosomal sequences by selecting material from the heavy side of DNA banded in a CsCl equilibrium gradient. In this case hybridization to both fragments is evident. (d to f) Ethidium bromide-stained gels of Eco RI–digested DNA from (d) λGd1, (e) λGd2, and (f) λGd3.

lar to that determined for *X. laevis* (*7, 10*). By analogy with *Xenopus*, we suppose that each unit of the tandem array consists of an 18*S* region and a 28*S* region separated by a transcribed spacer with each transcribed segment separated by a nontranscribed spacer (Fig. 2). The minimal estimated molecular weight of the complete chicken rDNA unit is 17×10^6. The hybridization data suggest that Eco RI cleaves the rDNA once within the 18*S* region and once near or at the junction of the 28*S* region and the nontranscribed spacer. Thus, the small Eco RI fragment contains part of the 18*S* region, the transcribed spacer, and most or all of the 28*S* region. The large Eco RI fragment probably contains some nontranscribed spacer, a transcribed spacer, and part of the 18*S* region. Ribosomal RNA hybridized to the small fragment in a sharp and well-defined band, but the region of hybridization to the large fragment was not always sharp. This may mean, as in the case of *X. laevis* (*11*), that the large fragment is actually a collection of fragments of varying lengths reflecting heterogeneity in the length of the nontranscribed spacers. Furthermore, this nontranscribed spacer region could contain additional Eco RI sites, generating fragments which would not be detected by hybridization to rRNA.

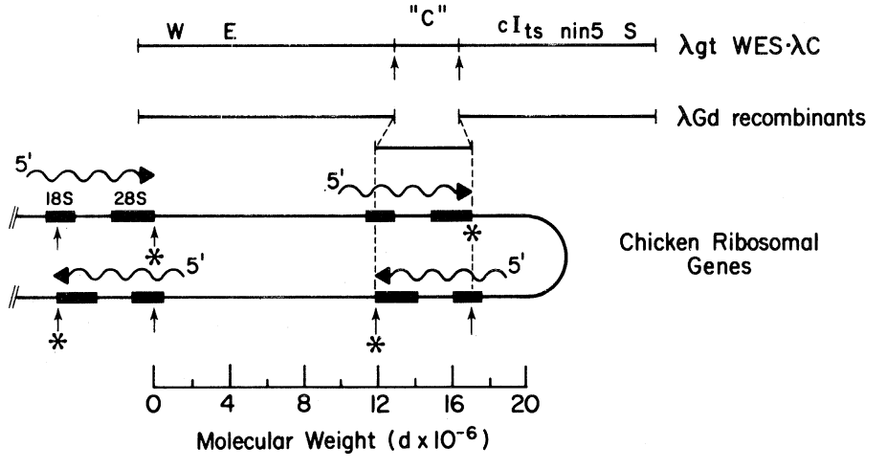

Fig. 2. Schematic showing probable structural relationships between λ vector phage DNA, λGd recombinant phage DNA's, and chicken ribosomal RNA genes. Vertical arrows indicate sites for cleaving by restriction endonuclease Eco RI. Asterisks indicate that additional Eco RI sites could occur within the nontranscribed spacer, generating fragments invisible by our assay (*20*). These fragments would be flanked by the two gene-containing fragments at the position of the asterisks. Horizontal (wavy) arrows represent RNA transcripts with the 5′ end assigned by analogy with findings for *X. laevis* (*21*).

For cloning, the 5×10^6 dalton fragment was purified by cutting out the appropriate region of a preparative gel of the Eco RI–digested chicken DNA. We extracted DNA (between 5 and 10 percent of the starting digest) from the gel segment with phenol, and subsequent hybridization analysis (*8*) confirmed that this fraction contained rRNA gene fragments and that they had been isolated free of at least 90 percent of other chicken DNA. The long arms of the Eco RI–digested EK2 vector phage DNA (see Fig. 2) were first separated from the λC fragment so that plaque formation was dependent on the addition of the chicken DNA fragments (*12*). Of approximately 200 recombinant phages tested, three were capable of hybridizing to 18*S* plus 28*S* chicken rRNA. The three recombinants λGd1, λGd2, and λGd3 were selected, plaque-purified, and grown on a special RecA host strain (see legend to Table 1), and all operations with the recombinants were carried out under P3 containment. Phage stocks were rechecked by hybridization in situ to either 18*S* or 28*S* RNA and found to be positive. Results of filter hybridization to DNA purified from two of the hybrids are presented in Table 1.

The DNA from each recombinant was digested with Eco RI and subjected to agarose gel analysis (Fig. 1, d to f). In each case three fragments were obtained; the two larger fragments (13.8×10^6 and 9.0×10^6 daltons) corresponded in size to the left and right arms of the vector (*9, 13*). The small fragments ran at the position of the 5×10^6 dalton rDNA fragments. Hybridization with DNA from such gels verified that the small fragment in each clone contained DNA homologous with the 18*S* and 28*S* rRNA sequences. The stoichiometry of the Eco RI fragments of each recombinant was determined from densitometer tracings of a photographic negative of the stained gels and verified by measurements of the isotope content of each fragment from a similar analysis of uniformly labeled (^{32}P) λGd1 DNA. The result showed (Fig. 1d and Fig. 1 legend) that the three Eco RI fragments are each present in unit amount in all the recombi-

Table 1. Hybridization of 18*S* and 28*S* ^{3}H-labeled rRNA to recombinant phage DNA. Denatured DNA was fixed to 20-mm nitrocellulose filters (*22*). The filters were cut in half and hybridized together with either 18*S* or 28*S* ^{3}H-labeled rRNA in 1 ml at concentrations indicated in Fig. 1. Hybridization (in DNA excess) and subsequent washing and ribonuclease treatment to remove nonspecifically bound RNA were as described by Southern (*8*). For the construction of recombinants, Eco RI end fragments of vector phage DNA were purified away from the C fragment (see Fig. 2) by a two-step procedure involving preparative sucrose gradient sedimentation followed by preparative agarose gel electrophoresis of the rapidly sedimenting (longest) two fragments. In a standard transfection assay with Ca^{2+}-shocked *Escherichia coli* (*23*), this preparation yielded only one plaque-forming unit per microgram of end fragment in the absence of any addition, as opposed to 407 in the presence of added C fragment (therefore the minimal purity of end fragments was 99.8 percent). The in vitro recombination mixture (0.1 ml) contained 4.5 μg of λ vector end fragments (heated and rapidly cooled before use to disjoin molecular ends), 1.3 μg of 4 to 6×10^6 dalton chicken Eco RI fragments (isolated from a preparative gel similar to that shown in Fig. 1), 25 μg of *E. coli* transfer RNA, and 3.4 units of T4 ligase (*24*) in 0.05*M* tris (*p*H 7.4), 0.01*M* $MgCl_2$, 1.2×10^{-4}*M* adenosine triphosphate, 0.01*M* dithiothreitol, and 0.06 percent Povite bovine albumin (Biotest-Serum Institut GmbH, Frankfurt am Main). Incubation for 90 minutes at 16°C was followed by addition of 0.8 ml of 0.1*M* tris (*p*H 7.1) and 1.6 ml Ca^{2+}-shocked *E. coli* [strain 803, *rec A*, r_K^-, m_K^-, su II, su III (No. ED 8767 from the collection of N. Murray)]. After incubation on ice for 5 minutes and at 45°C for 2 minutes, the mixture was added to soft agar (15 ml) and distributed onto five Trypticase (Baltimore Biological Laboratories) agar plates. Phage from the resulting 212 plaques were screened by in situ hybridization (*2*) with purified 18*S* plus 28*S* chicken rRNA. Plaque purification and subsequent growth of hybrids was by standard phage techniques adapted to P3 containment conditions (*25*).

DNA	Duplicate tests	^{3}H hybridized (cpm)	
		18*S*	28*S*
λGd2 (0.5 μg of λGd2 DNA + 2.5 μg of λ DNA)	1	5599	6870
	2	5422	5763
λGd3 (0.5 μg of λGd3 DNA + 2.5 μg of λ DNA)	1	8069	5924
	2	6987	6947
Control (3.0 μg of λ DNA)	1	200	182
	2	360	109

nants. We verified the absence of the λC fragment (see Fig. 2) in all three recombinants by confirming the lack of *int* and *xis* gene function in the red-plaque test of Enquist and Weisberg (*14*). In addition, plating tests showed that all three recombinants failed to grow ($< 2 \times 10^{-10}$) on a *sup*0 host bacterium. These results indicated that the safety features included in the outer arms of the vector phage DNA had been conserved. These data are all consistent with simple insertion of the 5×10^6 dalton chicken rDNA fragment between the purified left and right arms of the vector phage DNA.

We determined the direction of insertion of the rDNA fragment in each recombinant by separating the left and right strands of the hybrid phage DNA by electrophoresis (*15*) and then hybridizing with 18*S* plus 28*S* rRNA (*8*). For λGd1 and λGd3 the rRNA hybridized with the right strand; for λGd2 hybridization was with the left. Thus, transcription of the rRNA sequences should be under control of the early λ phage promoter in λGd2 and of the late phage promoter in λGd1 and λGd3. Figure 2 is a model which indicates the probable structural relationships between the vector phage DNA, recombinant DNA, and chicken rRNA genes.

The recombinants reported here are being used to study the processing of precursor RNA to mature 18*S* and 28*S* rRNA. Cloning of the fragment of molecular weight 12 to 14×10^6 will provide a DNA segment with alternating transcribed and nontranscribed regions, which should serve as a useful model for understanding the organization and regulation of other eukaryotic genes.

Cloning the rRNA gene has provided insight into the requirements for cloning other genes, including those present in a single copy. The fraction of chicken DNA used to construct these recombinants was approximately 5 to 10 percent of the total genome. Three out of about 200 (1.5 percent) of the recombinants contained the 5×10^6 dalton rDNA fragment, which suggests that this fragment makes up about 0.075 to 0.15 percent of the total chicken genome. Knowing the amount of DNA per cell [2.4×10^{-6} μg (*16*)] and the minimal molecular weight of the rDNA unit (17×10^6), we estimate from our data that there are between 215 and 430 copies of the rRNA genes per chicken genome. These values are in line with the data of Sinclair and Brown (*4*), which suggest that there are approximately 175 to 350 copies of the rRNA genes, and with the estimate of Baluda and co-workers (*17*) that there are 210 to 218 copies of the 28*S* gene. Therefore, in our experiments, the production of a specific recombinant was proportional to the fraction of DNA of interest present in the whole genome. This indicates that the cloning of a single-copy gene will require approximately a 200-fold amplification of the methods of detection or gene purification employed in this study.

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3 January 1977

Clones of Individual Repetitive Sequences from Sea Urchin DNA Constructed with Synthetic Eco RI Sites

Abstract. *Interspersed repetitive sequences were isolated from sea urchin DNA by renaturing to low* C_0t *followed by treatment with nuclease S1. Synthetic Eco RI sites were ligated onto the repetitive sequence elements, which were then inserted at the Eco RI site of plasmid RSF2124 and cloned. The repetitive sequences can be excised from the plasmid with Eco RI for further study.*

To facilitate studies of individual repetitive DNA sequence families, we have constructed recombinant DNA plasmids containing repetitive DNA from *Strongylocentrotus purpuratus* (*1*). Our object was to obtain cloned sequences terminated at the ends of interspersed repetitive sequence elements (*2*), rather than where restriction enzyme sites happen to fall. This was accomplished through the use of synthetically prepared Eco RI restriction sites that were ligated to the repetitive DNA fragments. The product of the ligation reactions was cloned to provide a source of plasmids from which individual repetitive DNA sequences could be reisolated in relatively large amounts.

Repetitive DNA duplex was prepared as follows (details may be found in the legend to Fig. 1). Sea urchin DNA sheared to a single-strand weight mean length of about 2000 nucleotides was renatured to C_0t 40 (C_0 is the initial concentration of nucleotides in moles per liter, and t is the time in seconds) and then treated with single-strand specific S1 nuclease. Previous studies (*2, 3*) have shown that by this point most repetitive sequences in the sea urchin genome are fully renatured. The nuclease digestion removes the nonrepetitive regions flanking the interspersed repetitive sequences, since these remain single-stranded at C_0t 40 (*2*). The resistant DNA sequence consists almost entirely of repetitive duplexes. We have shown earlier that a majority of the S1 nuclease resistant duplexes are 300 to 400 nucleotides in length, and that this is the characteristic length of interspersed repeats in the sea urchin genome (*2, 3*). The resistant repetitive duplexes are expected to have 3′-hydroxyl and 5′-phosphoryl termini (*4*).

A symmetrical decamer containing the Eco RI restriction site was synthesized by a triester chemical synthesis method (*5*). The structure of this decamer is shown in Fig. 1. The 5′-hydroxyl termini of the Eco RI decamers were labeled with ^{32}P, using T4 polynucleotide kinase. The decamer was covalently linked to the repetitive sea urchin DNA duplexes by blunt-end ligation with T4 DNA ligase (*6*). The expected products of the blunt-end ligation are the repetitive sea urchin DNA fragments bearing covalently linked Eco RI decamers, plus polymerized Eco RI decamers. The ligated mixture was next digested with Eco RI endonuclease. Eco RI treatment should yield the repeti-