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Repeated Deoxyribonucleic Acid Clusters in the Chicken Genome Contain Homologous Sequence Elements in Scrambled Order†

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ABSTRACT: Part of the repeated deoxyribonucleic acid (DNA) in the chicken genome has a clustered organization. The following description of clustered repeated sequences is derived both from analysis of DNA segments cloned in λ and from hybridization of individual cloned sequences to Southern blots of restricted total DNA. A cluster usually exceeds 20 kbp in length and consists principally, if not entirely, of repetitive DNA. Each cluster contains one copy of several different repeated sequences. The individual sequences occur several hundred times in the genome, but only once per cluster. Many of the clusters contain the same assortment of sequences but in scrambled order. In the genome, those repeated sequences that are elements of clusters occur mainly within the clustered context and are seldom, if ever, found as isolated elements flanked by nonrepeated DNA. These aspects of cluster organization suggest that the clustered sequences undergo limited rearrangement, maintaining the associations within clusters but allowing variability of sequence arrangement from cluster to cluster. The clusters that occupy the cloned DNA segments together represent at least 10% of the repetitive DNA of the chicken.

This paper concerns the structural organization of some repeated deoxyribonucleic acid (DNA) sequences in the chicken genome. Previous work on repeated sequences in eukaryotes elucidated three general patterns of organization. Some sequences have a tandemly repeating structure, with many adjacent occurrences of the same sequence element. The presence of tandem repeats has been documented for a wide variety of plant and animal genomes, where they can be a minor component or a substantial fraction of the repetitive DNA (Peacock et al., 1977; Rosenberg et al., 1978; Brutlag et al., 1978; Shmookler-Reis & Biro, 1978; Pech et al., 1979; Bedbrook et al., 1980; Donhauer et al., 1980). Other repeated DNA sequences occur as isolated elements interspersed with single-copy DNA. The alternating pattern of single-copy and repeated sequences also has wide representation in eukaryotic genomes. Although the particulars of spacing and sequence length are somewhat variable (Graham et al., 1974; Manning et al., 1975; Crain et al., 1976; Hudspeth et al., 1977; Eden & Hendrick, 1978), it is clear that this is a very basic and ubiquitous mode of repeated sequence organization (Davidson et al., 1975; Davidson & Britten, 1979). A third kind of organization consists of long arrays of different repeated sequences called "clusters". This clustered component was first identified by reassociation kinetic analysis of long DNA fragments preselected to contain at least one repeated sequence (Cech & Hearst, 1976; Marx et al., 1976; Eden et al., 1977; Rimpau et al., 1978). Such fragments sometimes contain other repeated sequences but are nearly or completely devoid of single-copy DNA. Analysis of long, reassociated DNA fragments by electron microscopy (Craig et al., 1979; Hinnebusch et al., 1980) or buoyant density (Cech & Hearst, 1976) provides additional support for the concept of genomic regions many kilobase pairs in length that are clusters of different repeated DNA sequences. Like other kinds of repeated sequence organization, this clustered mode has broad phylogenetic representation (algae, plants, insects, echinoderms, mammals, and, as reported here, birds) and can account for a substantial part of the repeated DNA in the whole genome.

A recent report documents the presence of clustered repeats in the *Drosophila* genome (Wensink et al., 1979) and, using molecular cloning, provides a clearer understanding of the substructure of clusters. They contain many of the same repeated sequences but in scrambled order from cluster to cluster. Here, we describe a similar component in the chicken genome. Using long, cloned DNA fragments, we compared the repeated sequences in different clusters. Also, individual repeated sequences isolated from clusters were used as probes to investigate the overall organization of the clustered sequences in the chicken genome.

Materials and Methods

DNAs. Total chicken DNA was isolated from the pooled blood of many individuals (*Gallus gallus* var. White Leghorn) by a procedure described previously (Eden et al., 1978). The cloned DNA fragments come from one of two sources. Some of the chicken DNA fragments used as probes were derived from a collection of clones described previously (Eden et al., 1980). They were constructed by ligation of *Eco*RI fragments of total chicken DNA into pBR322. Other larger cloned

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fragments were selected from a library of chicken DNA in charon 4a generously provided by Dr. D. Engel and constructed by the procedure of Maniatis et al. (1978). Segments of the selected phage were further subcloned in pBR322 in this laboratory. Recombinant DNA experiments were carried out under P2-EK2 containment conditions.

Enzymes. Restriction endonucleases were purchased from New England Biolabs or from Bethesda Research Laboratories and were used according to the instructions provided by the vendor. Two units of enzyme were used to digest 1 μg of DNA for 2–24 h. Bovine serum albumin (BSA)[1] was not added to the digest. DNA was deproteinized prior to gel electrophoresis or before redigestion with a second restriction enzyme.

Radiolabeling of DNA. DNA was labeled with ^{32}P in vitro by nick translation as described (Rigby et al., 1977), using [α-^{32}P]dATP and [α-^{32}P]dCTP (Amersham Searle, 300–400 Ci/mmol) as the labeled species. Specific radioactivity of DNAs ranged from 5×10^7 to 3×10^8 cpm/μg.

Southern Transfer and Hybridization. DNA fragments separated by electrophoresis through agarose slab gels (0.7–2.0%) were denatured and transferred to nitrocellulose paper according to Southern (Southern, 1975). Hybridization was carried out in sealed plastic bags at 68 °C for 24 h. The hybridization solution was 6 × SSC, 1 × Denhardt's (Denhardt, 1966) solution, and 0.1% $NaDodSO_4$. ^{32}P-Labeled DNA (2×10^6–2×10^7 cpm) was added to a 13 × 14 cm blot in 20–25 mL of hybridization solution. After hybridization, blots were repeatedly washed in 0.1 × SSC and 0.1% $NaDodSO_4$ at 52 °C. Autoradiograms were obtained by using Kodak XR-5 X-ray film and intensifying screens. Exposures were for 7 h up to 3 weeks.

Solution Reassociation Reactions and Hydroxylapatite Chromatography. Reassociation reactions using a ^{32}P-labeled chicken repeated DNA fraction, isolated as described previously (Eden et al., 1978), and sheared, unlabeled DNA from the recombinant phages were performed in 0.12 M PB and 0.1 $NaDodSO_4$ at 60 °C. Aliquots of the reaction mixtures incubated for different times were fractionated by hydroxylapatite chromatography as described (Britten et al., 1974). Radioactivity eluted from the columns was quantitated in aqueous liquid scintillation counting cocktails by using a Packard Tricarb liquid scintillation counter.

Results

Selection and Preliminary Characterization of Recombinant Phage. Initially, a cloned fragment of chicken DNA representing a single repeated sequence element was used to screen a library of chicken DNA. This fragment, 3.6 kbp in length, is repeated several hundred times in the chicken genome but is not internally repetitive. It has been fully described previously (Eden et al., 1980). Approximately 1% of the phages in the chicken library hybridize to this probe. Five of them were isolated and used as sources of DNA. For characterization of the inserted chicken DNA segments, each phage was analyzed by restriction endonuclease digestion. The pattern of restriction enzyme cleavage is different for each phage (Figure 1). Using four different restriction endonucleases, we found very few restriction fragments common to more than one phage except for vector fragments (arrows). The linear order of restriction fragments within each digest was determined by Southern transfer hybridization experiments (see Appendix). In the Appendix,[2] some representative restriction mapping data are presented together with a description of the general procedure used. All these data are summarized in Figure 1, where the restriction maps of the five phages can be compared. Since each inserted DNA segment has a different restriction map, we conclude that these phages represent five different segments of the chicken genome, each containing the repeated sequence used for screening as some part of the inserted DNA segment.

Inserted DNA in Each Phage Is a Cluster of Repeated Sequences. The recombinant phages were tested for the presence of repeated DNA sequences other than the one used for screening. By Southern transfer, phage DNA fragments separated as shown in Figure 1 were bound to nitrocellulose sheets and hybridized to a ^{32}P-labeled repeated DNA probe isolated directly from the genome. This is a complex probe representing all the sequences with more than 20 copies in total DNA, and was isolated as described previously (Eden et al., 1978). Because of the complexity of the probe, DNA fragments of a very high specific radioactivity and more total radioactivity were used in this particular experiment. This introduced considerable background noise (hybridization to traces of incompletely digested restriction fragments, and some spurious binding of radioactivity to the filters) but was necessitated by the possibility that these phages contain repeated DNA sequences that are a minor component of the probe. The autoradiograms after hybridization are shown in Figure 2A. More than 100 restriction fragments containing chicken DNA are bound to the filters. The vast majority of them hybridized to some extent with the repeated DNA probe. (Compare the complete digestion products in stained gels shown in Figure 1 with the autoradiograms in Figure 2A.) Almost all of the fragments can be clearly distinguished, and among them we found no fragment that failed to hybridize. We conclude that the inserted DNA segment in each of these phages contains several repeated sequences. In the genome, the 3.6-kbp segment of DNA we used for screening is apparently part of a much larger cluster of repeated DNA sequences.

Concurrence of Repeated DNA Sequences. We determined whether there are homologous DNA sequences in these phages other than the one used for selecting them. For the initial experiments, we used individually cloned repeated DNA sequences from a collection of DNA fragments inserted into pBR322 that has been described previously (Eden et al., 1980). This collection was the source of the original 3.6-kbp *Eco*RI fragment used for screening, which is referred to here as probe 1. We selected at random nine DNA segments other than probe 1, labeled them with ^{32}P in vitro by nick translation, and tested them for homology to the recombinant phages. For this experiment, filter-bound DNA from the set of recombinant phages was prepared and hybridized to each probe separately (data not shown). Of the nine probes, two showed homology to the phages. These are referred to here as probes 2 and 3, respectively. They are described in Table I, where their pattern of hybridization to the phages is also shown. To test for the presence of other cross-hybridizing sequences, we then isolated DNA segments directly from the recombinant phages. This was done either by subcloning of restriction fragments in pBR322 or by preparative agarose gel electrophoretic separation of restriction fragments. Probes of this second type are numbered 4–9 and are fully described in the footnote to Table I. They were tested for cross-homology by hybridization of

[1] Abbreviations used: BSA, bovine serum albumin; kbp, kilobase pairs; $NaDodSO_4$, sodium dodecyl sulfate; PB, equimolar mono- and disodium phosphate buffer, pH 6.8; SSC, standard saline citrate; bp, base pair.

[2] The Appendix contains three figures illustrating the general procedure used to develop restriction maps of the recombinant phage. The mapping data for one of them, λGg233, are presented there in detail.

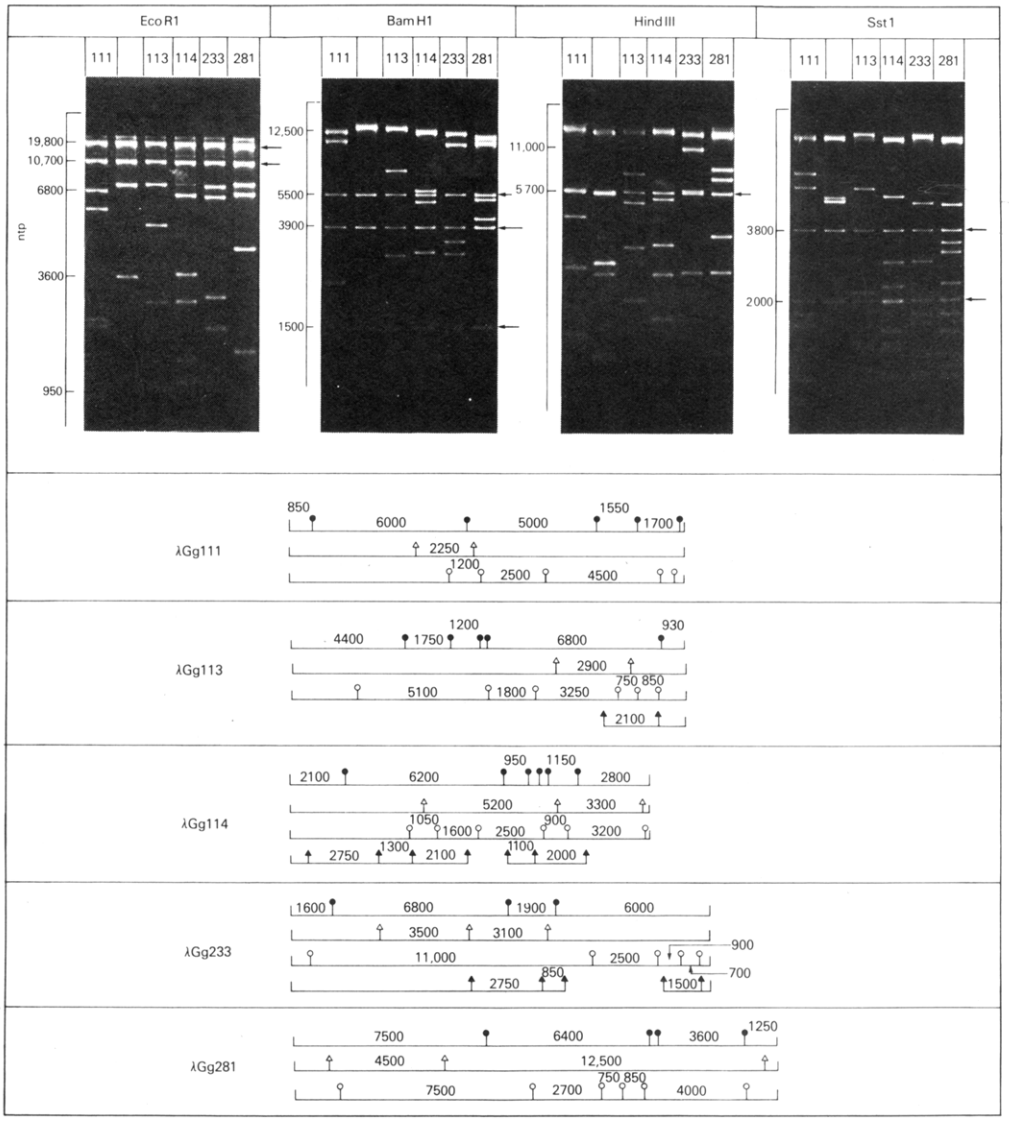

FIGURE 1: Restriction endonuclease cleavage patterns in five chicken DNA segments cloned in λ. A cloned segment of chicken DNA, 3.6 kbp in length, representing a single repeated sequence element, was used as a probe to select other genomic copies of the same sequence from a library of chicken DNA cloned in λ. The library, prepared by partial *Alu*I and *Hae*III digestion of total DNA and insertion of the DNA fragments into charon 4a by the *Eco*RI linker addition method (Maniatis et al., 1978), was the generous gift of Dr. D. Engel. Each of the five selected phages represents a different segment of the chicken genome, because a different pattern of restriction endonuclease cleavage pertains to each segment. The upper panel shows the five DNAs (designated λGg111, λGg113, λGg114, λGg233, and λGg281, respectively) digested with restriction nucleases as indicated. The fragments were separated by electrophoresis through 1% agarose gels and detected by ethidium bromide staining. The fragments derived entirely from vector DNA are indicate by arrows. In the lower panel, the positions of the restriction sites are indicated, and the lengths of the fragments are given in nucleotide pairs. The enzymes used were (●) *Eco*RI, (△) *Bam*HI, (○) *Hin*dIII, and (▲) *Sst*I. Some areas of the *Sst*I maps have not yet been established.

each of them to filter-bound DNA from the phages as described above. The results (Table I) indicate that some of them are found in recombinant phages other than the one from which they were isolated.

These results establish that the five recombinant phages selected with probe 1 have several other repeated DNA sequences in common in addition to probe 1. Apparently, these particular repeated sequences are often found in adjacent or nearly adjacent locations in the genome, since they are often found together in the 20-kbp DNA segments cloned in λ. We will refer to this adjacent occurrence of unrelated repeated DNA sequences in different locations in the genome as "concurrence". Some of the repeated DNA segments described in Table I show very strong concurrence, while others seem to occur concurrently to a lesser extent. Two of the probes did not show any concurrence (8 and 9); they were found only in one of the five phages. The frequency of association of certain sequences seems to be higher than that of others when DNA segments about 20 kbp in length are considered.

Homologous Sequence Elements Are in Scrambled Order. The location of each of the nine repeated sequence elements (probes 1–9) described in Table I was mapped in the recombinant phages. The DNA from each phage was digested with restriction enzymes. In many cases, single digestions were sufficient to map sequence locations, but double digests were also used when needed. The restriction fragments were separated by agarose gel electrophoresis and transferred to Southern blots for hybridization. In Figure 2B, we show the autoradiograms after hybridization with probe 2. The pattern of fragments that hybridized to probe 2 can be compared with the stained gels shown in Figure 1. This probe hybridized to a different pattern of restriction fragments in each phage, and to fewer restriction fragments than the total repeated DNA probe (Figure 2A). Referring to the restriction maps shown in Figure 1, we determined the location and the length of the

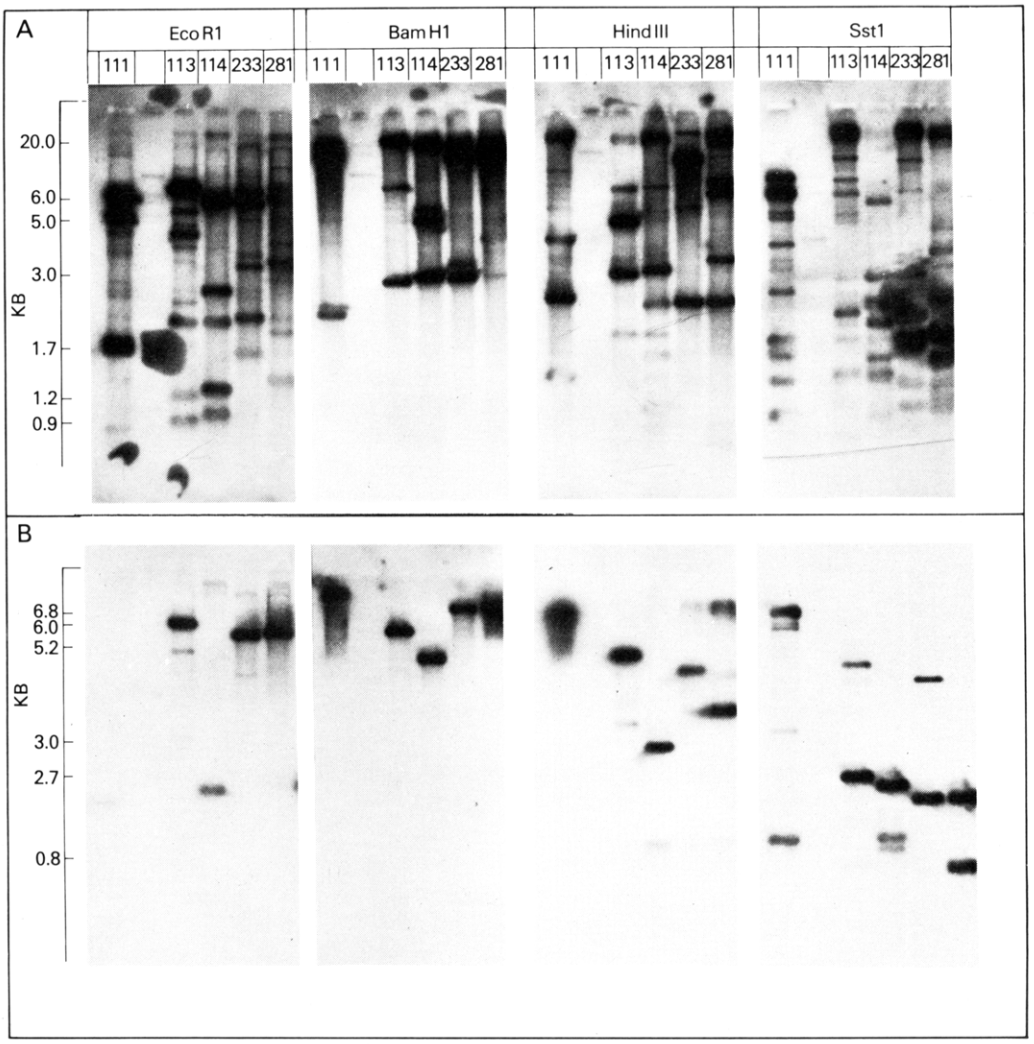

FIGURE 2: Identification and localization of repeated sequence elements within the cloned DNA segments. DNA from the recombinant phages was digested with restriction nucleases as shown in Figure 1 and transferred from gels to Southern blots. Panels A and B show the results after hybridization of duplicate blots with different probes. In panel A, we used a labeled repeated DNA fraction of the genome, isolated by partial reassociation of total DNA as described previously (Eden et al., 1978). This probe represents all the sequences with more than 20 copies in the genome. Each blot was hybridized with 1.5×10^7 cpm of probe (specific activity 3×10^8 cpm/μg). Panel B shows the results after hybridization with a single repeated DNA sequence isolated by cloning. The cloned DNA (referred to as probe 2 in the text) was labeled with ^{32}P to 1×10^8 cpm/μg, and 5×10^6 cpm were hybridized to each blot. The autoradiograms were exposed 1–2 weeks.

DNA segment homologous to probe 2 in each phage. For example, in phage λGg113, probe 2 hybridized to the 6.8-kbp *Eco*RI fragment and to the segments of the inserted DNA attached to the right arm of the vector DNA in both the *Bam*HI and the *Hin*dIII digests. This locates the homology to a short DNA segment near the right end of the inserted DNA, between the right-most *Eco*RI and *Hin*dIII sites. Probe 2 is a DNA segment 0.68 kbp in length, and it seems to hybridize to a segment of similar length in the inserted DNA of λGg113. The sequence homology to probe 2 seems to be localized to a single region of the inserted DNA in λGg113. In the other phage, probe 2 also hybridized to a short region occurring only once per insert.

This analysis was continued by using each of the other repeated sequence probes in turn. The autoradiograms are not shown, but the data are summarized diagrammatically in Figure 3. In the figure, the results for each phage are grouped together in one of the upper panels. The hybridization data are organized according to probes, as indicated by the numbers at the left. The restriction endonucleases used for DNA fragmentation are also designated in the diagram, and the restriction fragments they generate are shown schematically. The schematic diagrams correspond to the restriction maps shown in Figure 1. Fragments that hybridized to the probe are indicated by shading. The hybridization patterns were used to construct maps of the sequence order. These are shown in the lower section of each panel, where the maximum possible length and the location of DNA sequences homologous to probes 1–9 are indicated. Since probes 4–9 were isolated directly from phage λGg113 and λGg111, it was not necessary to map them in the source phage but only in the other phages. When probes failed to hybridize to a particular phage, the results were omitted from the figure, but every combination of probe and phage was tested. In general, the results with all these probes parallel those we described for probe 2. The aspects that were reproduced were hybridization to a different pattern of restriction fragments in each phage, hybridization to a single location in each insert, and hybridization to a DNA segment of approximately the same length as the probe. The most striking feature of the results is that the homologous sequence elements are in scrambled order from phage to phage. For example, probes 4 and 6 hybridize to adjacent areas in λGg233 and λGg114 but were separated by a region homologous to probe 5 in λGg113, and were even more widely separated in λGg281. Similarly, probe 7 is next to probe 3 in λGg113, but separated from it in λGg233 and λGg281. In

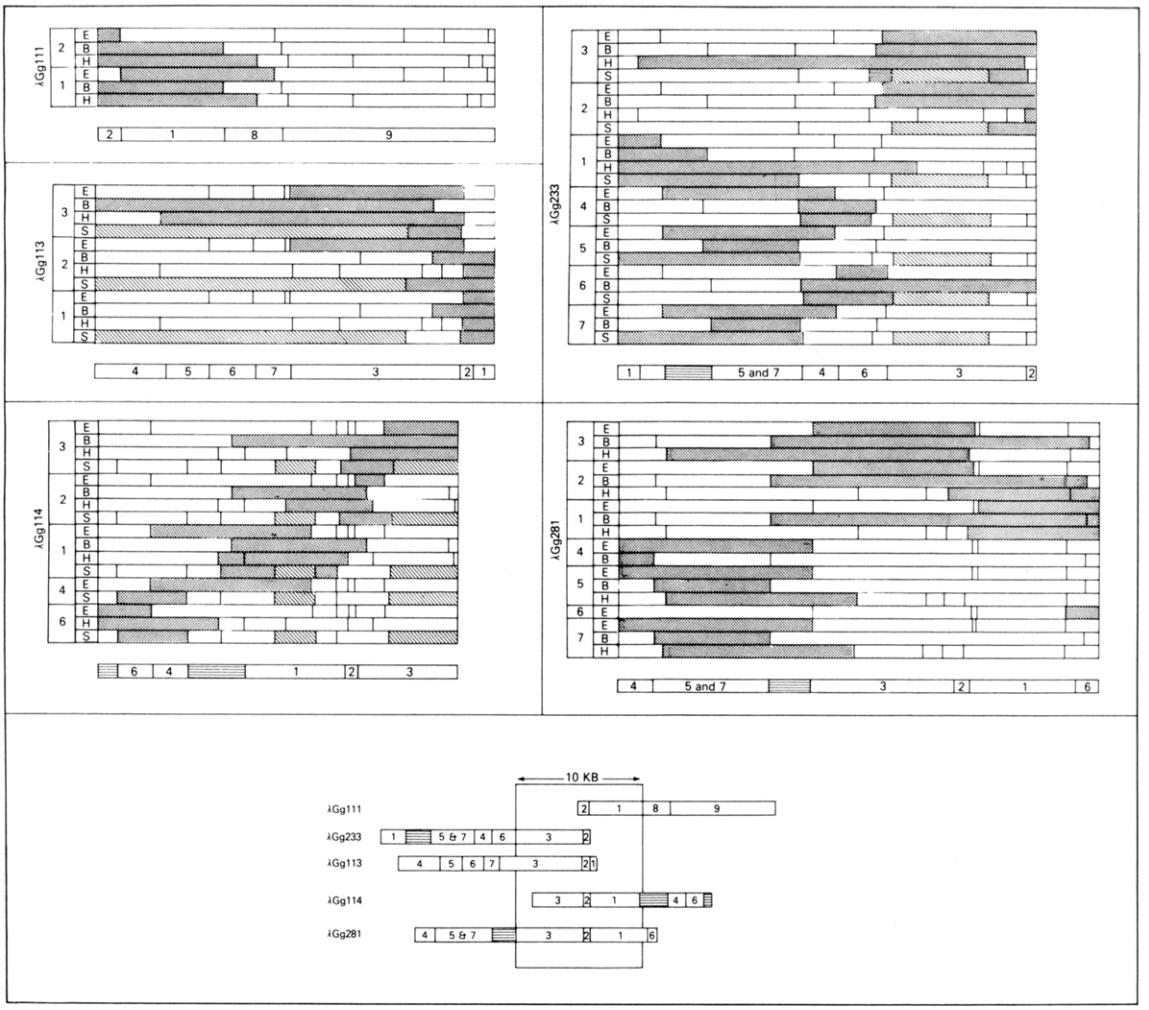

FIGURE 3: Patterns of hybridization indicate a different order of repeated sequence elements in each cloned DNA segment. This diagram summarizes the results of many hybridization experiments like the one shown in panel B of Figure 2. The basic experiment is restriction endonuclease digestion of DNA from a recombinant phage, separation of fragments by agarose gel electrophoresis, transfer of the separated fragments to blots, and hybridization with probes representing individual repeated sequence elements. We used the nine probes that are described in footnote *a* to Table I. The data are separated into five groups, each referring to one of the five recombinant phages. The restriction endonucleases used to fragment phage DNA are indicated by letters (E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sst*I), and the restriction fragments they generate are shown schematically. The hybridization results are grouped according to probes, numbered 1–9 in the figure. The restriction fragments that hybridized to each probe are indicated by shading. The diagonal slashed lines designate areas of the *Sst*I maps where the order of restriction fragments has not been determined; they were not included in the hybridization analysis. The horizontal lines show regions where no homology was detected with any of the nine probes. It is not yet known whether these regions hybridize with one another. The position and overlap of the positive restriction fragments were used to map the location of each sequence in the recombinant phages. This map of sequence order appears in the lower part of each panel. In the bottom part of the figure, the maps of sequence order are shown again in one possible alignment which contrasts the similar juxtaposition of sequences 1, 2, and 3 with variable order of the other sequences.

contrast to these sequences with different arrangements, some sequences have the same order in each phage. The region represented by probes 1, 2, and 3, about 10 kbp in length, is an example of this. The constant region was used to align the different phage inserts as shown in the lower panel of Figure 3. We draw attention to the organization of λGg233 in the context of this comparison. This phage has probe 1 widely separated from probes 2 and 3 and suggests to us that the phages constitute a circularly permuted series of repeated DNA sequences. Further work will be needed to determine this conclusively.

The comparison of these five cloned repeated DNA clusters has shown that there is concurrence of repeated sequences within the clusters and that the elements are in scrambled order from cluster to cluster. Attention was then focused on genomic DNA, to compare the structural organization of the many hundreds of copies of these sequences in total DNA with the five occurrences studied by cloning.

Cloned Repetitive Clusters Represent a Prevalent Component of the Genome. The inserted DNA from λGg111 and λGg113 collectively represents 1.3% of the chicken genome. This was determined by a solution hybridization experiment in which unlabeled phage DNA was hybridized to a ^{32}P-labeled probe representing sequences repeated more than 20-fold in the genome. The cloned DNA fragments were present in great excess, so that the extent of hybridization of the probe is a measure of the fraction of chicken-repeated DNA represented in the clones. Figure 4 shows these results. The closed circles are a control reaction of the probe with total DNA, and the open circles show its reaction with cloned DNA. About 10% of the probe reacted with the cloned DNA segments. The probe represents 13% of the genome (Eden & Hendrick, 1978), so the cloned sequences constitute 1.3% of the genome.

This has been confirmed by other kinds of experiments. We measured the minimum amount of total DNA required to detect these sequences by the Southern transfer format. These results are shown in Figure 5. Probes 1, 2, and 3 each detected their homologous genomic sequences within 0.3 μg or more of total DNA. The hybridization is divided among several bands in each case. The amounts required to detect a sin-

Table I: Concurrence of Repeated DNA Sequences

probe[a]	length of chicken repeated DNA represented (kbp)	hybridization to recombiant phage[b] λGg111	λGg113	λGg114	λGg233	λG281
1	3.6	+	+	+	−	+
2	0.68	+	+	+	−	+
3	7.0	−	+	+	+	+
4	2.6	−	+	+	+	+
5	1.8	−	+	−	+	+
6	1.75	−	+	+	+	+
7	1.20	−	+	−	+	+
8	2.25	+	−	−	+	−
9	8.3	+	−	−	+	−

[a] All the probes are cloned fragments of chicken DNA. By Southern transfer hybridization experiments, we have determined that there are no cross-hybridizing sequences among the nine probes (data not shown). Probes 1–3 are from the collection of chicken repeated DNA fragments inserted into the plasmid pBR322 described previously (Eden et al., 1980). Probes 4, 5, and 6 are subcloned segments of the recombinant phage λGg113 (Figure 1). For their preparation, the 4.4- and 1.75-kbp *Eco*RI fragments of λGg113 were cloned separately in pBR322. Probes 4 and 5 are the segments of the 4.4-kbp *Eco*RI fragment further separated by digestion with *Hin*dIII. Probe 6 is the 1.75-kbp *Eco*RI fragment of λGg113. Probe 8 is the 2.25-kbp *Bam*HI fragment of λGg111. Probe 9 is the large segment of the inserted DNA that remains attached to the vector right arm after digestion of λGg111 with *Bam*HI. All these DNA fragments were labeled in vitro with ^{32}P by nick translation for use as probes.
[b] Restriction fragments of the recombinant phages transferred to nitrocellulose according to Southern were hybridized to the ^{32}P-labeled probes described in footnote *a*. Probe 1 was the one used to select these phages from a library of chicken DNA cloned in λ and was referred to in our earlier work as pGg132 (Eden et al., 1980).

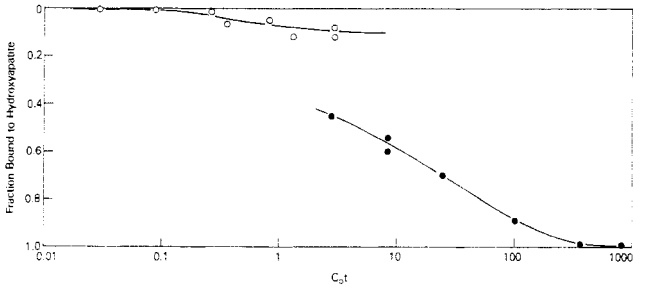

FIGURE 4: Cloned sequences account for at least 10% of chicken repeated DNA. A chicken repetitive DNA fraction was isolated directly from the genome by selection of rapidly reassociating sequences. This fraction constitutes 13% of the genome and contains sequences repeated from 20-fold to many thousandfold in total DNA. The repetitive fraction was labeled with ^{32}P in vitro by nick translation to a specific radioactivity of 3×10^8 cpm/μg. A trace of the labeled DNA was reassociated with sheared unlabeled DNA isolated from recombinant phage. We used a mixture of equal amounts of DNA from λGg111 and λGg113, which together represent all of the nine sequences described in Figure 3. The unlabeled DNA was in 50000-fold excess compared to the labeled DNA. Reassociation was measured as described under Materials and Methods. A parallel reaction mixture contained the same amount of labeled DNA and sheared, unlabeled total chicken DNA. The data have been normalized to the maximum reactivity of the probe. Self-reaction of the probe did not exceed 2%: (○) unlabeled DNA from λGg111 + λGg113; (●) unlabeled total DNA.

gle-copy DNA sequence when all the hybridization is within a single band are typically 20–50 μg of total DNA. Thus, we conclude that there are several hundred copies of these sequences in the genome.

If the chicken repetitive clusters we have studied by cloning, whose dimensions probably exceed 20 kbp, were repeated 500-fold in the genome, this would account for 1×10^7 nucleotide pairs, or about 1.0% of total DNA. This calculation agrees well with the solution hybridization result described above.

We have not yet determined whether every cluster element has the same number of copies in the genome, although the results of Southern transfer experiments indicate that most of them do have a similar representation there. However, we also have one piece of contrary evidence. We noted the frequency of positive clones detected during screening of the chicken library with each of several isolated cluster elements. Two of these hybridized to about 1% of the phages, as expected; a third probe (probe 3) does have a substantially higher representation in the library, and presumably in the genome as well (D. Sobieski, unpublished experiments).

Genomic Organization of Clustered Repeated DNA Sequences. The cloned repeated DNA sequence elements described above have been used to determine the organization of the several hundred homologous copies in the genome. With Southern transfer from agarose gels, restriction fragments of total DNA were hybridized to probes representing individual cluster elements. The result was hybridization to "bands". A band is many copies of a particular restriction fragment, all hybridizing to the probe. These multiple-copy fragments derived from the many occurrences of a repeated sequence in the genome. In Figure 6, we show the results after hybridization of a series of duplicate Southern blots to different elements of the repeated sequence clusters. All of the probes hybridize principally to bands, regardless of the restriction nuclease used to fragment total DNA.

In many cases, the hybridizing genomic restriction fragments correspond in length to the cloned fragments. For example, the 1900-bp *Eco*RI fragment of λGg233 hybridized to a prominent fragment of the same length in *Eco*RI-digested total DNA. Similarly, the 2250-bp *Bam*HI fragment of λGg111 has a clear homologue in the genome. However, most of the probes also hybridized to additional fragments whose lengths bear no obvious relationship to the length of the probe. The 1900-bp *Eco*RI fragment of λGg233 is one example, hybridizing to at least four different *Hin*dIII fragments. The probe does not contain a *Hin*dIII site, so we presume that these genomic fragments terminate within the adjacent sequences. In the inserted DNA segment of λGg233, this probe is part of an 11.0-kbp *Hin*dIII fragment (Figure 1), yet the genomic fragments to which it hybridizes are all shorter than 11.0 kbp. In the genome, this sequence has alternative neighboring repeated sequences that give rise to the different spacings of *Hin*dIII sites. The cloned representative is apparently not one of the predominant forms in this case.

To summarize the results with Southern blots of total DNA, it appears that each of these repeated sequences has a few alternative neighboring sequences in the genome that are themselves repeated sequences. We conclude this because all of the genomic restriction fragments we detect by hybridization are multiple copy, even though some of them are generated by restriction sites in sequences flanking the probe. This description accounts for the dominant kind of genomic organization of these sequences. There may be other occurrences of them with a different kind of organization, but if they are present they constitute a minor fraction of the total number of copies.

Discussion

Clustered Organization of Repeated DNA. Our present concept of the structure of chicken repeated DNA clusters is based on two kinds of experimental evidence. Some of the data

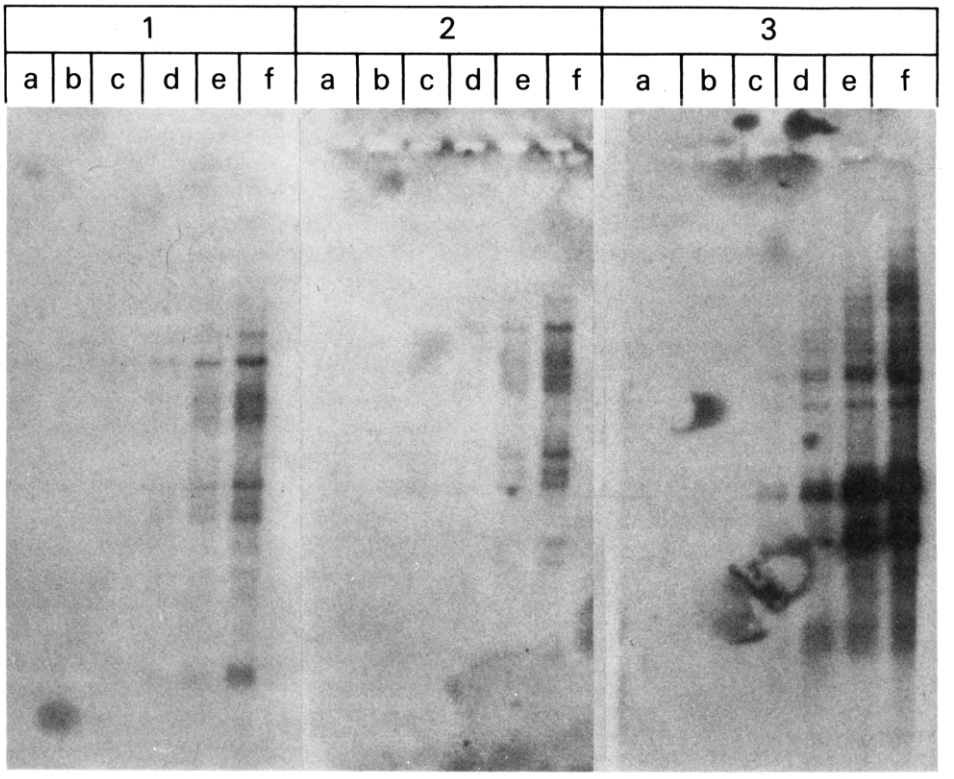

FIGURE 5: Prevalence of cloned repeated sequences in the genome. Total DNA was digested with *Hin*dIII. Increasing amounts were loaded into adjacent tracks of 0.7% agarose gel and separated by electrophoresis. The amounts of DNA were 0.01 (a), 0.03 (b), 0.1 (c), 0.3 (d), 1.0 (e), and 3.0 μg (f). After Southern transfer, the blots were hybridized with 2.5×10^6 cpm of probe. The autoradiograms after hybridization of duplicate blots with probes 1, 2, and 3 are shown. The autoradiograms were exposed for 2 weeks.

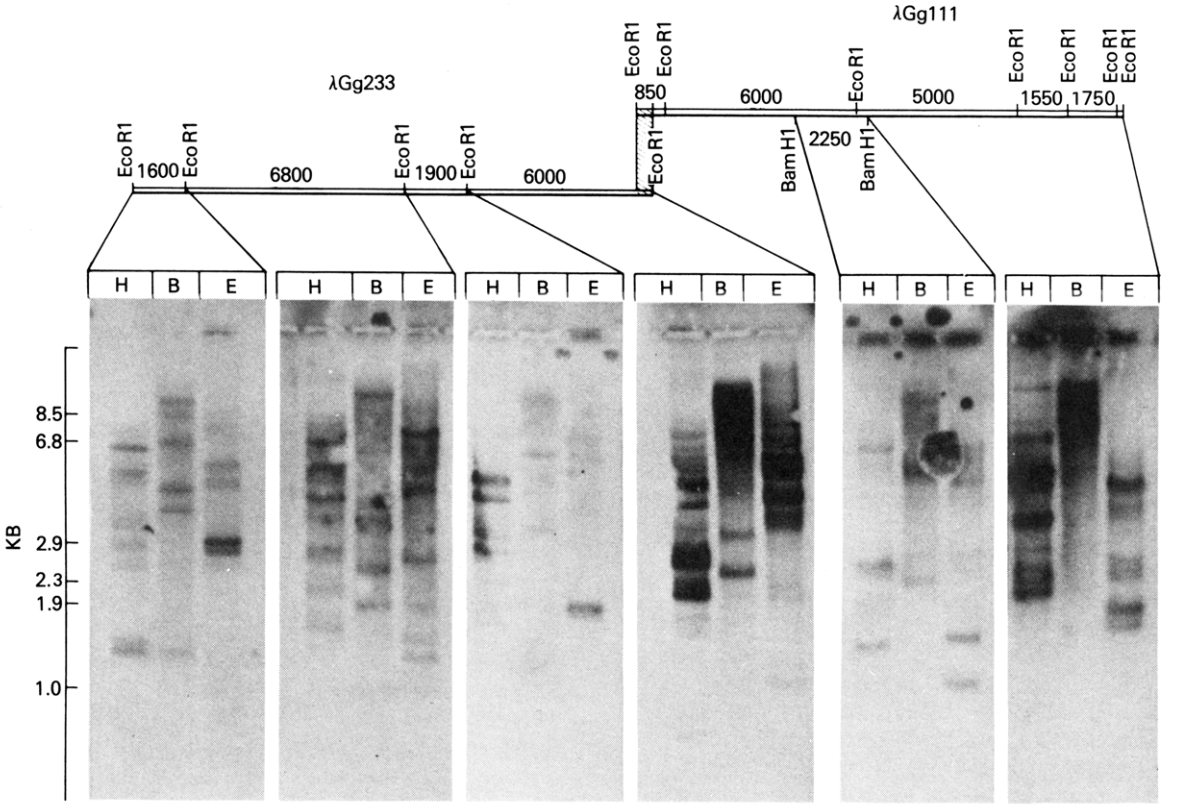

FIGURE 6: Genomic organization of cloned repeated DNA sequences. Total DNA digested with *Hin*dIII, *Bam*HI, and *Eco*RI, respectively, was separated by electrophoresis through 0.7% agarose gels. A 10-μg sample of DNA was loaded per track. After Southern transfer, each blot was hybridized with a probe representing a different repeated DNA segment, isolated from the DNA cloned λGg233 or λGg111 as shown. A sample of 2.5×10^6 cpm of each probe was used, and the autoradiographic exposure was adjusted to yield similar intensity. The lengths of the probes are indicated in nucleotide pairs, and the lengths of genomic DNA fragments detected by hybridization are indicated at the left.

was collected by comparing different segments of chicken DNA cloned in λ. Other information was obtained by hybridization of cloned DNA fragments to Southern blots of total DNA. Here, we briefly summarize these results.

Each of the DNA segments cloned in λ contains many repeated DNA sequences. We conclude this because every part of the inserted DNA hybridizes with the labeled repeated DNA fraction isolated directly from the genome. The reciprocal experiment, where every labeled segment of the cloned DNA hybridizes to multiple-copy genomic sequences, confirms this. It is possible that these 20-kbp cloned segments consist entirely of repeated DNA. We have not directly excluded the possibility that they also contain single-copy sequences, but it is unlikely that they contain any single-copy segments long enough to constitute full protein-coding genes.

We describe the DNA segments inserted in λ as clusters of many different repeated DNA sequence elements rather than as a single, large repeated sequence for several reasons. First, each part of the inserted DNA hybridizes to a different pattern of restriction fragments of total DNA. Second, the comparison of different cloned segments shows that they have homologous sequences but in scrambled order. Third, the cluster components have somewhat different copy numbers in the genome. These facts are incompatible with interpretation of the cloned segments as single repeated sequence elements.

The data clearly indicate that these repeated sequences are structurally distinct both from tandemly repeating and from interspersed repeated sequences. First, the possibility of tandem repetition was tested in part by cross-hybridization of different areas of a single inserted DNA segment. The results indicate that each sequence is represented only once per insert. In addition, hybridization of parts of these cloned to Southern blots of total DNA digested with a variety of restriction endonucleases failed to show a series of fragments whose lengths are integral multiples, which would be an indication of tandem repetition. The cloned DNA segments could include some short regions where there is tandem repetition, but for the most part they consist of an array of different sequences rather than many tandem copies of the same sequence. Second, it is clear that these sequences do not resemble interspersed repeated sequence elements. In the interspersed mode of organization, each member of a family of repeated sequences is flanked by different single-copy sequences in the genome. The heterogeneity of flanking sequences usually generates great variety in the length of the restriction fragments of total DNA that hybridize to an interspersed repeated sequence probe. In Southern transfer experiments with restricted total DNA, the hybridization result is a broad "smear" rather than hybridization to fragments of discrete lengths. The repeated sequences we describe here show a very different result, with hybridization mostly limited to a few very prominent restriction fragment lengths.

The clusters described here may be parts of even larger repeated sequence arrays in the genome. We are unable to measure their actual dimensions because they usually exceed the lengths of fragments that can be cloned in λ. Further efforts to locate clones containing a cluster boundary were unsuccessful (R. Cuevo, unpublished experiments), which implies that most clusters are significantly longer than 20 kbp. The formal possibility remains that there is a very long-range tandemly repeating structure whose basic unit exceeds 20 kbp. However, we have now considered 15 different cloned clusters and find no two of them to be alike in terms of sequence order. Thus, if a tandemly repeating structure exists, it must encompass many sequence variants, in which case the term "tandem" becomes less appropriate. Taken together, the present evidence points more strongly to variability of sequence order within clusters than to homogeneity and justifies a clear distinction between the clustered and tandem modes of organization.

Concurrence of Repeated DNA Sequences. In the chicken genome, there are different families of repeated DNA sequences that seem to have a definite structural relationship. These are families whose members are adjacent or nearly adjacent at many different locations in the genome. This phenomenon of concurrence is related to the concept of genetic linkage but apparently operates on the much smaller scale of kilobase pairs of DNA. Selection by cloning of any 20-kbp genomic segment containing a particular repeated sequence (probe 1 in our case) led to co-selection of certain other repeated DNA sequences. This coincident selection occurred much more often than chance would predict had these sequences been randomly dispersed throughout the genome. However, whatever maintains linkage is apparently not restrictive of local variability in sequence juxtaposition. The order of sequences seems unconstrained because in selecting just five different clusters at random we found a highly scrambled order of repeated sequences, with no two cloned segments having exactly the same sequential order of the homologous sequence elements.

The Southern transfer experiments with genomic DNA provide additional but more indirect evidence for concurrence. The hybridization of these cloned probes to a limited array of genomic restriction fragments suggests a limited repertoire of sequences flanking them. Many of the fragments detected by hybridization clearly extend beyond the probe into other repeated sequences, confirming the adjacency of different repeated sequences in the genome.

There was little indication of any association of these cloned sequences with single-copy DNA. However, a quantitatively significant interspersed component could be overlooked in the Southern transfer format, which tends to focus attention on strong bands rather than faint smears. To address this point more directly, we selected other recombinant phages from the chicken genomic library by using a clustered repeated sequence probe. Of 15 additional phages, none were found to contain significant amounts of a single-copy DNA sequence (R. Cuevo, unpublished experiments). From this, it is clear that most copies of these sequences occur within repeated sequence clusters. The overall distribution of these sequences appears to be under some kind of control, although the functional significance of controlling the extent of clustering or dispersal remains elusive.

As knowledge of repeated DNA structure increases, particular modes of organization are becoming more easily recognizable. It may be that there are ubiquitous functional or structural requirements that lead to similar modes of organization in diverse organisms. The evolutionary constraints may relate more to the mode of organization of repeated DNA than to the nucleotide sequences that comprise the repetitive genomic component.

Appendix

Restriction Mapping of Recombinant Phages. Restriction endonuclease cleavage patterns were determined by digestion with several enzymes, individually or pairwise, and measurement of the lengths of the fragments by gel electrophoresis. The linear order of the fragments was then determined by hybridization. Purified fragments isolated from agarose gels and labeled with ^{32}P in vitro by nick translation were used as probes against Southern blots of phage DNA. Within each restriction digest, all the fragments hybridizing to a given probe were identified. Additional probes were tested until all of the fragments could be ordered without ambiguity. The method is illustrated here by using a phage designated λGg233 as the example. Restriction enzyme cleavage of this phage generates

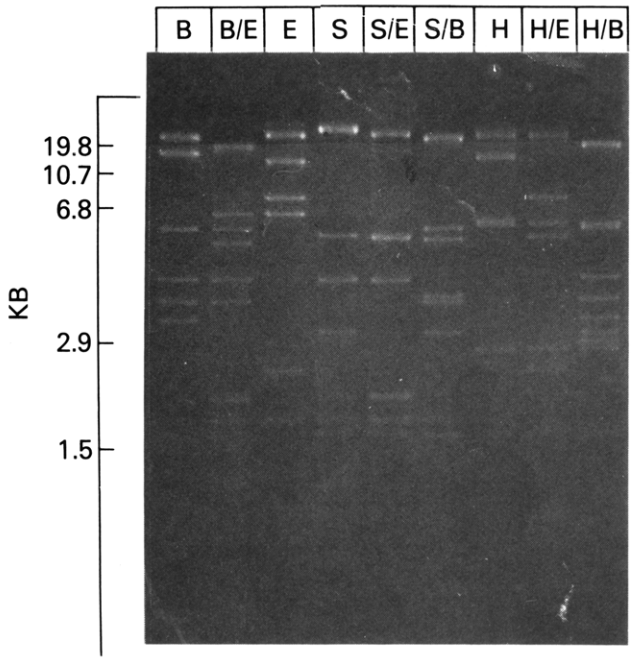

FIGURE A1: Restriction fragments of λGg233. Aliquots of λGg233 DNA were digested with restriction endonucleases (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I). The fragments were separated by electrophoresis through 1% agarose gels and visualized by ethidium bromide staining. A length scale is indicated at the left.

the fragments shown in Figure A1. The order of fragments from each digest is shown in Figure A2. Figure A3 shows the hybridization data used to establish these maps.

*Eco*RI digestion separates four inserted fragments, 6.8, 6.0, 2.0, and 1.6 kbp in length, respectively, from two segments of vector DNA called left and right arms. Other restriction enzymes do not completely separate vector and inserted DNA (Blattner et al., 1977). *Bam*I, *Hin*dIII, and *Sst*I digestion yields some fragments of vector DNA, some fragments from the inserted DNA, and two fragments that contain both vector and inserted DNA. In the *Bam*HI digest of λGg233, the 17.7-kbp fragment contains 14.3 kbp of the left arm of and 3.4 kbp of chicken DNA located at the left boundary of the insert. The 11.4-kbp fragment contains 4.9 kbp of the vector right arm plus 6.5 kbp of inserted DNA. The 3.5- and 3.1-kbp *Bam*HI fragments are from the inserted DNA. These four *Bam*HI fragments were recovered individually from preparative agarose gels, labeled with ^{32}P, and hybridized to duplicate Southern blots transferred from gels like the one shown in Figure A1. The four autoradiograms are shown in Figure A3. In the *Eco*RI digest, the labeled 17.7-kbp *Bam*HI fragment hybridized to the left arm of the vector (19.8 kbp) and to two inserted *Eco*RI fragments, 6.8 and 1.8 kbp in length (Figure A3, panel A), which are adjacent and at the left end of the insert. Their relative order was established by hybridization to the *Bam*HI/*Eco*RI double digest. A new fragment, 1.8 kbp in length, was detected by the probe. Thus, the 1.6-kbp *Eco*RI fragment is the left-most inserted fragment, and the 6.8-kbp *Eco*RI fragment contains a *Bam*HI site 1.8 kbp from one end, as shown in Figure A1. *Hin*dIII digestion on λGg233 yields four fragments derived from the inserted DNA, 11.0, 2.5, 0.85, and 0.75 kbp in length. Some of the inserted DNA also remains attached to the vector arms within fragments 20.7 and 5.7 kbp in length, respectively. The labeled 17.7-kbp *Bam*HI fragment hybridized to two *Hin*dIII fragments, 20.7 and 11.0 kbp in length (Figure A3, panel A), placing the 11.0-kbp *Hin*dIII fragment near the left end of the insert. In the *Hin*dIII/*Eco*RI double digest, hybridization was to the 19.8-kbp *Eco*RI vector fragment, the 6.8-kbp *Eco*RI fragment, and to two small fragments resulting from *Hin*dIII cleavage of the 1.6-kbp *Eco*RI fragment. Thus, less than 1.0 kbp of the inserted DNA remains attached to the vector left arm after *Hin*dIII digestion (Figure A2). Codigestion with *Hin*dIII and *Bam*HI confirms this, releasing a new fragment 2.5 kbp in length from the 11.0-kbp *Hin*dIII fragment.

Endo *Sst*I digestion releases many fragments from the inserted DNA. The 17.7-kbp *Bam*HI probe detected a segment of the insert attached to the left arm, which *Sst*I + *Eco*RI digestion resolves into the 1.5-kbp *Eco*RI fragment plus a 5.1-kbp fragment. The left-most *Sst*I site of the insert is within the 6.8-kbp *Eco*RI fragment as shown in Figure A2.

The second probe that was used, the labeled 3.5-kbp *Bam*HI fragment, hybridized to the left-arm fragment in the *Sst*I digest and also in the *Sst*I/*Bam*HI double digest (Figure A3, panel B), placing the 3.5-kbp *Bam*HI fragment near the left end of the insert, as shown. In the *Eco*RI digest, the 6.8-kbp fragment was the most prominent band, and the *Bam*HI/*Eco*RI digest shows that this *Bam*HI fragment was not cleaved

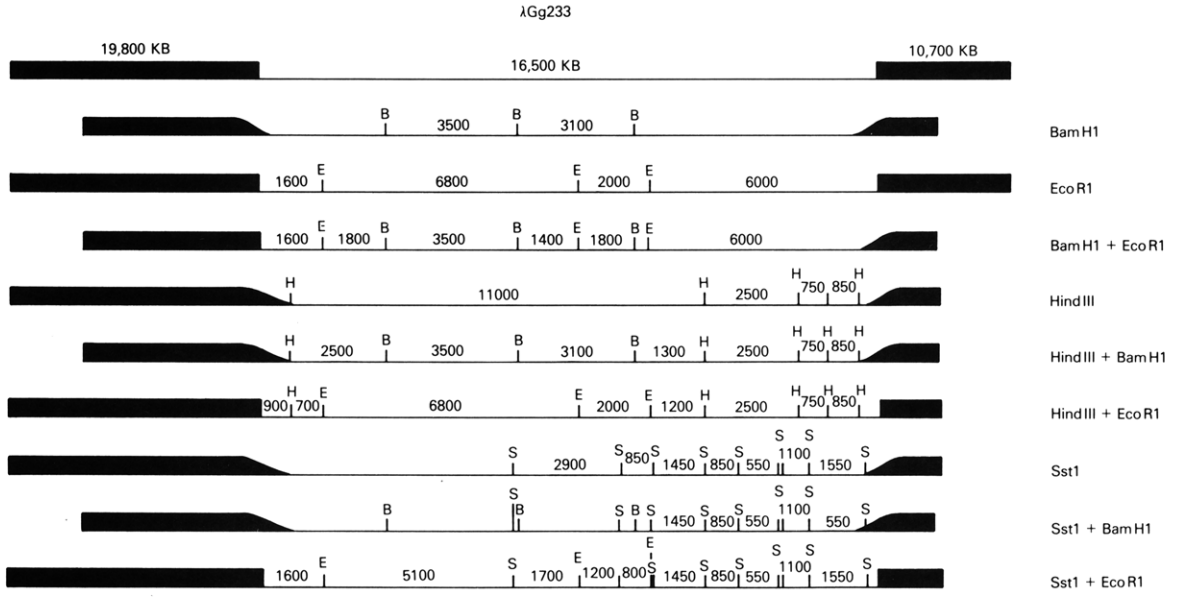

FIGURE A2: Map of restriction sites in λGg233. The positions of restriction endonuclease cleavages in the 16.5-kbp segment of chicken DNA (thin lines) inserted into the vector charon 4a (bars) are shown. Fragment lengths are indicated in nucleotide pairs.

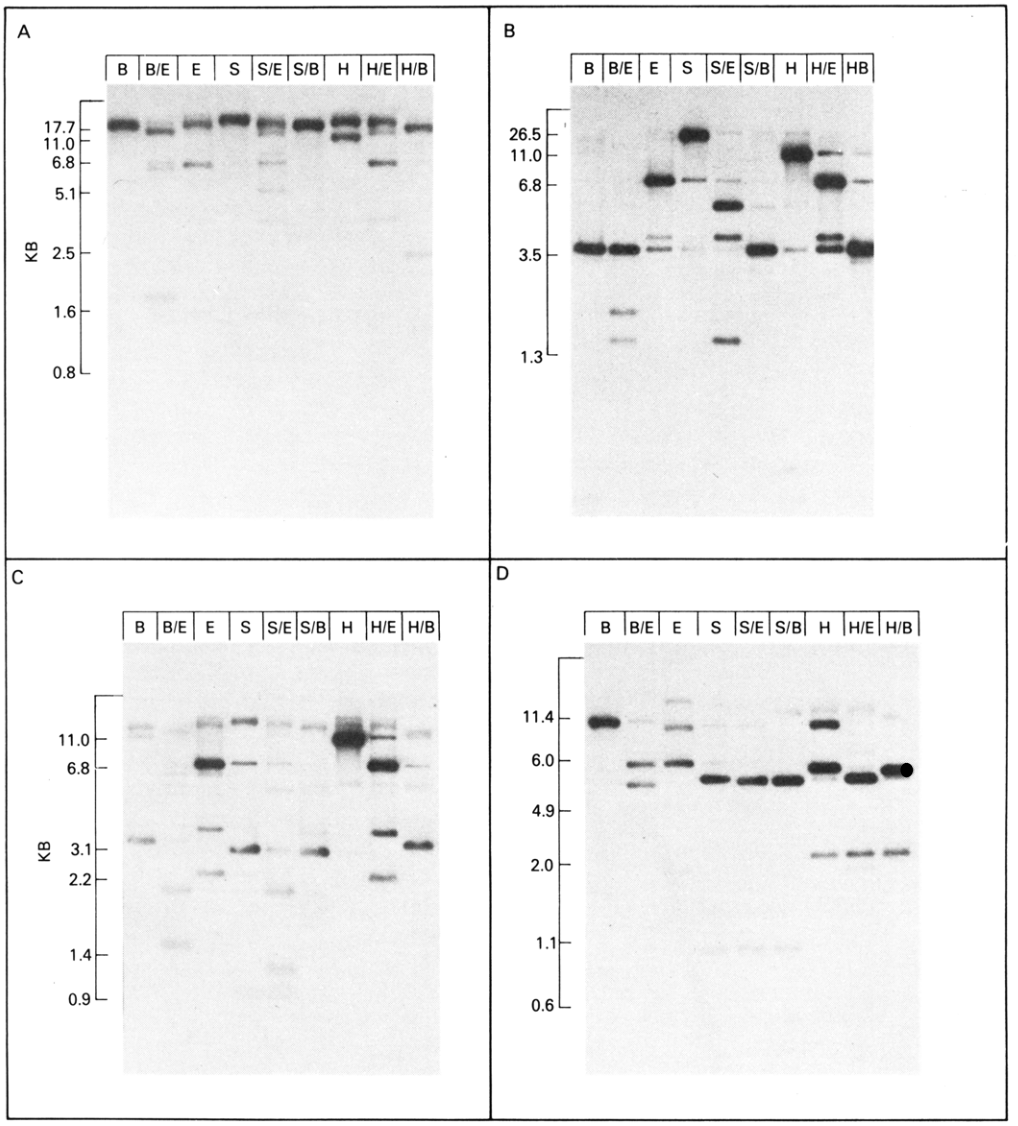

FIGURE A3: Hybridization data establishing the restriction map of λGg233. The inserted DNA in λGg233 was separated into four segments by digestion with *Bam*HI. As shown in Figure A2, this digest leaves two segments of the inserted DNA attached to the left and right arms, respectively, of the vector DNA. Two additional *Bam*HI fragments, 3.5 and 3.1 kbp in length, derive from the central region of the inserted DNA. These four segments were recovered individually from preparative agarose gels and labeled with ^{32}P in vitro. For the preparation of the blots, restriction fragments of λGg233 were separated by agarose gel electrophoresis as shown in Figure A1 and transferred according to Southern. Each of four duplicate blots was hybridized to one of the probes. (A) *Bam*HI left arm; (B) 3.5-kbp *Bam*HI fragment; (C) 3.1-kbp *Bam*HI fragment; (D) *Bam*HI right arm. A scale of fragment lengths is shown at the left.

by *Eco*RI. The faintly hybridizing bands in this track are not considered: they result from trace contaminants in the probe. In the *Hin*dIII digest, this probe recognized only the 11.0-kbp fragment. Fragments 6.8 and 3.5 kbp in length in the *Hin*dIII/*Eco*RI and *Hin*dIII/*Bam*HI double digests, respectively, were also detected, confirming the map as developed so far.

Panel C of Figure A3 shows the results after hybridization with the 3.1-kbp fragment. This probe hybridized to the 6.8-kbp *Eco*RI fragment and to the 11.0-kbp *Hin*dIII fragment, placing it to the right of the 3.5-kbp *Bam*HI fragment. It overlaps the end of the 6.8-kbp *Eco*RI fragment, because the *Bam*HI/*Eco*RI and *Hin*dIII/*Eco*RI digests show that it contains an *Eco*RI site. This allowed mapping of the next *Eco*RI fragment to the right of the 6.8-kbp fragment; it is 2.0 kbp in length, as shown in Figure A2. Two new *Sst*I fragments (2.9 and 0.85 kbp in length) hybridized to this probe. They are located next to the left-arm fragment in that digest. Their order was deduced from the *Sst*I/*Eco*RI digest, since the lengths of the products only allow one alignment (Figure A2). The 2.8-kbp *Sst*I fragment and the 3.1-kbp *Bam*HI fragment overlap almost completely, since their lengths were not visibly altered in the double digest. *Bam*HI does cleave the 0.85-kbp *Sst*I fragment, confirming its location.

The 6.0-kbp *Eco*RI fragment and the three remaining *Hin*dIII fragments (2.5, 0.85, and 0.75 kbp) are at the right end of the insert. They were all detected after hybridization with the segment of the insert attached to the *Bam*HI right arm (Figure A3, panel D). The same probe hybridized weakly to the 2.0-kbp *Eco*RI fragment, indicating a small region of overlap (Figure A2). The *Eco*RI/*Bam*HI digest confirms this, showing hybridization to the 6.0-kbp *Eco*RI fragment and to the segment of the right arm released by *Bam*HI (4.9 kbp in length). In the *Hin*dIII digest, the 11.0-kbp fragment was detected along with the three smaller fragments, which is expected because of the length of inserted DNA attached to the *Bam*HI right arm. The remaining *Sst*I fragments that hybridized to this probe were tentatively ordered according to the patterns of double digestion with *Sst*I and *Bam*HI or *Eco*RI.

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Denaturation and Renaturation of *Penicillium chrysogenum* Mycophage Double-Stranded Ribonucleic Acid in Tetraalkylammonium Salt Solutions†

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ABSTRACT: The base composition dependence of double-stranded ribonucleic acid (RNA) melting was studied by observing the structure and widths of melting transitions for *Penicillium chrysogenum* mycophage RNA as well as differences in melting temperatures of two RNAs of different base composition. Double-stranded RNA melting is independent of base compositions in 3.5 M Et_4NCl and 4.6 M Me_4NCl, where the melting temperatures are 25 and 92 °C, respectively. Double-stranded RNA renaturation rate constants are reported in Et_4NCl solutions. The nucleation rate constant is about 10 times lower than that for double-stranded deoxyribonucleic acid. Analyses of renaturation kinetics results lead to the conclusion that each of the three similar but separable RNA segments of *Penicillium chrysogenum* mycophage is unique.

Melchior & von Hippel (1973) found that aqueous solutions of small tetraalkylammonium ions eliminated base composition effects on the helix–coil transition in deoxyribonucleic acid (DNA). All DNAs tested melted at 63 °C in 2.4 M tetraethylammonium chloride (Et_4NCl) and at 94 °C in 3.2 M tetramethylammonium chloride (Me_4NCl). The width of the observed melting transition in these two solvents is generally less than or equal to 1 °C. Tetraalkylammonium salt solutions, especially Et_4NCl, have been applied to the differentiation of melting temperature effects caused by compositional differences from those attributable to base-pair mismatches (Britten et al., 1978). Thermodynamic parameters involved in DNA melting in Me_4NCl and Et_4NCl and in Et_4NCl binding to native DNA may be found in Klump (1977) and Anderson et al. (1978). Orosz & Wetmur (1977) ex-

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