THE GENOME ORGANIZATION OF THE BOBWHITE QUAIL (COLINUS VIRGINIANUS)

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ABSTRACT

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The Genome Organization of the Bobwhite Quail (Colinus virginianus)

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The DNA of the bobwhite quail (Colinus virginianus) was examined using isopycnic centrifugation in neutral CsCl, alkaline CsCl, Hg^{++}-Cs_2SO_4 and Ag^{+}-Cs_2SO^4. When quail DNA was centrifuged in neutral CsCl, two low density minor bands (satellite DNA) were detected in addition to the main band DNA. Main band DNA had a buoyant density of 1.701 g/ml. The larger of the two minor bands had a buoyant density of 1.681 g/ml (I) while the smaller of the two minor bands had a buoyant density of 1.674 g/ml (II). The main band has a G + C content of 41.8% while the satellites, I and II, have G + C contents of 21.4% and 14.2%, respectively. Thermal denaturation studies were used to confirm the base composition data obtained from the buoyant density data.

Restriction endonucleases (Hind 111, EcoR 1, EcoR 11, Hpa 1, Hpa 11, Bam 1, Bgl 11, Alu 1, Hae 111, Hha 1 and Mbo 1) were used to investigate the heterogeneity of repetitive DNA's in the total bobwhite quail genome. Bobwhite quail DNA consists of a heterogeneous population which is indicative of a genome in which most of the

restriction endonuclease sites are randomly distributed. However, the Hind III sites are non-randomly distributed as indicated by the seven discrete fragments generated.

The arrangement of repetitive and non-repetitive sequences was studied in the genomic DNA of the bobwhite quail (Colinus virginianus) and the Pharoah quail (Coturnix coturnix). The reassociation kinetics of short (450-500 nucleotide) and long (2000 nucleotide) DNA fragments were studied by the hydroxylapatite method. The data indicate that the bobwhite genome consists of three kinetic components; non-repetitive ($Cot_{1/2}$ pure = 70; 70%), moderately repetitive ($Cot_{1/2}$ pure = 0.50; 10%) and highly repetitve ($Cot_{1/2}$ pure = 0.01; 20%). The genome of the Pharoah quail is similar to the bobwhite with the non-repetitve sequences accounting for 68% of the genome ($Cot_{1/2}$ pure = 66.6); moderately repetitive sequences constitute 12% of the genome ($Cot_{1/2}$ pure = 0.65); and the highly repetitve sequences constitute about 20% of the genome ($Cot_{1/2}$ pure = 0.012).

The lengths of the repetitve sequences obtained by reassociating total DNA to Cot 40 and digesting with S1 nuclease were estimated by agarose gel electrophoresis and alkaline sucrose gradients. The repetitve sequences in both species of birds are approximately 1000 nucleotides long.

The interspersion of repetitive and non-repetitive or single copy sequences have been examined. The results indicate that about 20-23% of the non-repetitve sequences in fragments 2000 nucleotides long are interspersed with long repetitive sequences in the bobwhite quail. In the Pharoah quail genome approximately 25-27% of the non-repetitive sequences are interspersed.

These data indicate that the bobwhite and Pharoah quail genomes contain some highly repetitive DNA sequences which are clustered and band as distinct satellites in neutral CsCl and some repetitive sequences which are interspersed

repetitive sequences. The data also indicate that the bobwhite quail DNA consists of a heterogeneous population of molecules with most of the restriction endonuclease sites randomly distributed in the bobwhite quail genome.

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CHAPTER I

INTRODUCTION

Repetitive DNA occurs widely, if not universally, among higher organisms. A variety of procedures has been developed or adapted to examine its characteristics and a body of concepts and language has grown to deal with its complexities (Britten et al., 1974). Highly repetitive, simple sequence DNAs, can usually be resolved from bulk DNA as satellite components in appropriate density gradients. Neutral cesium chloride (CsCl) density gradients demonstrate the existence of minor satellite DNA components from the total DNA. Gradients of cesium sulfate (Cs_2SO_4) containing either silver (Ag^+) or mercury (Hg^{++}) ions have a greater ability to separate minor DNA components. Satellite DNAs may constitute anywhere from less than one percent to over fifty percent of total nuclear DNA (Beauchamp et al., 1979). Although satellite DNAs were first reported in the early 1960s (Kit, 1961), their function is still unclear.

The fact that satellite DNAs can be purified relatively easily by cesium salt centrifugation, and that in some cases the complementary strands can be purified separately, has enabled detailed examination of their properties. Apart from the fact that the satellite DNAs must be relatively homogeneous in order to form a discrete fraction of DNA, the sharp nature of satellite DNA bands also suggests a high degree of base sequence homongeneity.

Satellite DNAs are composed of simple (oligomeric) sequences tandemly repeated, with variable accuracy, hundreds or thousands of times without

interruption (Waring and Britten, 1966; Bond et al., 1967; Southern, 1970; Gall and Atherton, 1974; Goldring et al., 1975; Walker, 1971 (review)). Main band DNA usually contains highly repeated as well as moderately repeated sequences. A highly repeated DNA may have a density similar to other repeated species (Skinner et al., 1970; Skinner and Beattie, 1973; Gall and Polan, 1971; Endow et al., 1975).

DNA sequences which are highly repeated are largely confined to the heterochromatic regions of chromosomes. Definitive data linking them to specific cellular functions are lacking. Studies suggest that during evolution these sequences have been conserved relative to other sequences of the genome (Salser et al., 1976; Peacock et al., 1977; Fry and Salser, 1977; Manuelides and Wu, 1978; Mullins and Blumenfeld, 1979).

The nuclei of eukaryotic cells contain much more DNA than is accounted for by current knowledge of the requirements for coding and metabolic regulation. Satellite DNA is relatively easy to isolate and there are not many clues that explain its function in the cell.

Several hypotheses have been proposed for the function of satellite DNA or highly repetitious DNA in general. These are:

1. Satellite DNAs located in the constitutive heterochromatin in the chromosomes may function as sterility barriers between diverging incipient species in eukaryotes. Satellite DNAs seem not to be adaptive and to be a means of speciation independent of phylogenetic evolution. Here, it is suggested that satellite DNAs not only allow the pairing of homologous chromosomes in meiosis, but also hinder the pairing of homologous chromosomes in hybrids of species having differing satellite sequences (Corneo, 1976).

2. Subspeciation and speciation are progressive steps in a continuum based upon changes in the macrostructure of the genome. It is proposed that variability in the generation of satellite DNAs and possibly in the deletion of intermediate density DNA, is a fundamental molecular aspect of this mode of evolutionary behaviour (Hatch and Mazrimas, 1977).

3. Since the first discovery of satellite DNAs, one of the popular hypotheses has been that they have a role in meiotic pairing. When this role was considered in the light of the question posed above, it was obvious that a sudden change in the amount of localization of the satellite sequences in the progeny of a single individual could provide a very crude "species" barrier in which matings within the group were fertile, but matings with the general population were more or less infertile and such events could serve as a first crucial step towards sympatric speciation (Fry and Salser, 1977).

Recent considerations on the molecular basis of gene regulation in higher organisms have focused attention on the organization of the eukaryotic genome. Discussions of gene regulation and chromosome structure have resulted in specific predictions regarding the arrangement of repetitive and non-repetitive sequences (Callan, 1967; Thomas et al., 1970; Britten and Davidson, 1969, 1971). Evidence that some repetitive and non-repetitive sequences are interspersed in eukaryotic DNA have been shown in reports on Drosphila DNA (Wu et al., 1972; Kram et al., 1972), and calf and sea urchin DNA (Britten and Smith, 1970; Britten, 1972).

The present study was undertaken to further characterize the DNA of bobwhite quail by using density gradient centrifugation, restriction endonucleases and DNA analysis via reassociation kinetics.

CHAPTER II

REVIEW OF LITERATURE

The presence of satellite DNAs has been shown in several animal species (Kit, 1961; Schildkraut et al., 1962). Mouse satellite DNA has been extensively studied (Bond et al., 1967; Chun and Littlefield, 1963; Corneo et al., 1968; Flamm et al., 1967; Flamm et al., 1969; Kit, 1961; Maio and Schildkraut, 1967; Schildkraut and Maio, 1968; Walker and McLaren, 1965; Waring and Britten, 1966). In 1961 Kit reported that mouse nuclear DNA could be separated into two fractions by centrifugation to equilibrium in a neutral CsCl density gradient. Approximately 90% of the DNA will form a single main band with a density of 1.70 g/ml and the remaining 10% forms a satellite band with a density of 1.69 g/ml. Reassociation kinetics suggested that mouse satellite DNA consists of about 350 base pairs in length, per haploid set of chromosomes (Waring and Britten, 1966). Human DNA has been shown to contain at least four satellite DNAs in isopycnic Cs_2SO_4 gradients. Human satellite DNA I is concentrated, mainly in centromeric constitutive heterochromatin, on many chromosomes but is especially obvious in the flourescent distal segment of Y chromosome (Jones et al., 1974).

Mitchell et al. in 1979 did a study of sequence homologies in four satellite DNAs of man. They found that each of the four human satellite DNAs studied showed varying degrees of homology to at least one or more of the other three satellite DNAs. Although each one could be distinguished on the basis of buoyant density criteria, some degree of relatedness could be detected using other parameters. Melting profiles of native satellite DNAs II, III and IV were

indistinguishable. Reassociated duplexes of these three satellites had in common the biphasic nature of the melting curves, with the melting point (Tm) values of the phases essentially the same for all the satellites. Satellite II could be distinguished from the other two by a greater proportion of its reassociated duplexes melting during the second transition.

Mitchell et al. in 1979 also digested the satellites found in man with restriction endonucleases. Digestion of satellite III and Hae III gave rise to a series of fragments whose sizes were 2, 3, 4, 5, 6, 7, 8 and 11 times the size of the smallest 0.17 x 10^3 basepair (bp) fragment, in addition to a 3.4 x 10^3 bp male-specific fragment and high molecular weight material. The sequences contained in the fragments of the Hae III ladder were diverged from each other as well as being non-homologous with those of the 3.4 x 10^3 bp and high molecular weight fragments. The latter contained EcoR 1 recognition sites. Satellite II had fragment patterns similar to satellite III on the basis of the products of EcoR 1 digestion. Satellite I contained neither Hae III nor EcoR 1 recognition sites.

Marx et al. in 1979 studied primate repetitive DNA's. They isolated repetitious DNA sequences from a number of the primates in both Suborders Anthropoidea and Prosimii by hydroxyapatite chromatography at a Cot of 10. In addition to them finding, previously unreported, AT-rich satellite DNA's in Orangutan, Gibbon, and Slow Loris, a clear similarity to human DNA was found in the non-satellite repetitious DNA sequence properties of the primates in the Suborder Anthropoidea.

Chimpanzee DNA was found to contain two satellite fractions when centrifuged in Ag^+-Cs_2SO_4 density gradients: satellite A with a buoyant density of

1.69 g/cm^3 and satellite B with a buoyant density of 1.683 g/cm^3 (Prosser et al., 1973). Mayfield et al. in 1979 found that in the bovine DNA satellite I has a density in CsCl of 1.715, satellite II and III have densities of 1.721 and 1.723, respectively, and satellite IV and V have densities of 1.706 and 1.709, respectively.

In studies of small mammals, it was discovered that Tetraogallus himalayensis exhibited a great deal of satellite DNA. The satellites of this species comprise more than one-half of the total cell DNA.

Satellite DNA has also been reported in several species of Drosophila. In D. virilis, four major peaks are distinguishable. These correspond to the main peak sequences (ρ = 1.700) and three large satellites numbered, respectively, I (ρ = 1.692), II (ρ = 1.688) and III (ρ = 1.671) (Gall et al., 1973). A major fraction (~ 80%) of the highly repeated DNA sequences from D. melanogaster has been isolated as well-defined satellites by buoyant-density-centrifugation in CsCl gradients containing DNA binding antibiotics. Four major satellites of densities 1.672 g/cm^3, 1.686 g/cm^3, 1.688 g/cm^3 and 1.705 g/cm^3 were observed. Several minor satellites were found with one (1.600 g/cm^3) appartently representing linkage molecules resulting from covalent joining of large blocks of the major satellites.

Carlson and Brutlag in 1979 did restriction endonuclease studies on D. melanogaster DNA. The 1.688 g/cm^3 satellite DNA of D. melanogaster is composed primarily of 359 base-pair units repeated in tandem. Most of these units contain a single cleavage site for both Hae III and Hinf I restriction endonucleases; however, some units lack one or both sites. Carlson and Brutlag showed that the distribution of Hae III and Hinf I endonuclease sites varied widely between different regions of 1.688 g/cm^3 satellite DNA. These investigators cloned

molecules of 1.688 g/cm^3 satellite DNA which lacked Hae III sites and showed that the absence of sites was caused by sequence variation rather than base modification. This result indicated that regions of 1.688 g/cm^3 satellite DNA with different distributions of restriction sites differed in the sequence of their repeating units. These investigators also showed that a large fraction of the satellite DNA which was cleaved by Hae III endonuclease still contained Hinf I endonuclease sites (and Alu I sites) spaced about 359 base-pairs apart.

Renaturation analysis showed that the haploid D. melanogaster genome contains 12% rapidly reassociating, highly reiterated DNA, 12% middle repetitive DNA with an average reiteration frequency of 70, and 70% single-copy DNA. The distribution of the middle repetitive sequences in the genome has been studied by an examination in the electron microscope of the structures obtained when middle repetitive sequences present on large DNA strands reassociate and by the hydroxyapatite binding methods developed by Davidson et al. (1973). At least one third by weight of the middle repetitive sequences were found to be interspersed with single-copy sequences. These interspersed middle repetitive sequences have a fairly uniform distribution of lengths from less than 0.5 to 13 kilobases (kb), with a number average value of 5.6 kb. The average distance between middle repetitive sequences is greater than 13 kb (Manning et al., 1975).

In a study of several species of the D. hydei group, Hennig et al. (1970) demonstrated that certain repetitive satellite DNA species are located in the centromeric heterochromatin, while others may be more widely spread in the genome. In the species D. novamexicana, D. americana americana and D. americana texana exist a striking common feature. Like D. virilis itself each has a satellite at ρ = 1.691 g/ml to 1.692 g/ml which constitutes 20 to 25% of the total

DNA which separates in alkaline CsCl into light and heavy strands whose buoyant densities are approximately ρ = 1.798 g/ml and ρ = 1.687 g/ml (Gall and Atherton, 1974).

The arrangement of repetitive and non-repetitive sequences was studied in the DNA of Xenopus. Labeled DNA sheared to various fragment lengths was reassociated to Cot 50 with excess 450 nucleotide fragments of unlabeled DNA, and binding of the labeled DNA to hydroxyapatite was measured. Repetitive sequences monitored in this way were found present on about 45% of the 450 nucleotide fragments. As DNA fragment length increased, larger fractions of the DNA were found to contain repetitive elements. Up to 80% of the DNA was found at an average fragment length of 3700 nucleotides. Analysis of the data showed that a little more than 50% of the genome consisted of closely interspersed repetitive and non-repetitive sequences. The average length of the repetitive sequence elements is 300 ± 100 nucleotides, while the non-repetitive sequences separating adjacent repetitive sequence elements average 800 ± 200 nucleotides. The remainder of the DNA is mainly non-repetitive, though most of it contained rare interspersed repetitive elements spaced at a minimum of 4000 nucleotides apart. It was concluded that a high degree of order exists in the arrangement of DNA sequences in the Xenopus genome (Davidson et al., 1973).

The cytological distribution of satellite DNA in Rhynchosciara is similar to that of mouse satellite DNA (Jones, 1970; Pardue and Gall, 1969, 1970). The DNA of R. hollaenderi was examined using isopycnic centrifugation in neutral CsCl. Two low density minor bands (collectively termed satellite DNA) were detected in addition to the main band DNA. Main band DNA has a buoyant density of 1.695

g/cm^3. The larger of the two minor bands has a buoyant density of 1.680 g/cm^3 while the smaller of the two minor bands has a buoyant density of about 1.675 g/cm^3.

Nuclear DNA from liver cells of the Kangaroo rat species Dipodomys ordii was fractionated and characterized with the aid of buoyant density gradients in neutral and alkaline CsCl and in Ag^+-Cs_2SO_4. More than one-half of the DNA was present in these density satellites. All satellite fractions revealed sharp isopycnic bands and narrow denaturation profiles. Two had identical buoyant densities but differed substantially in Tm, base composition, and reassociation kinetics. In alkaline CsCl all three satellites, as well as a shoulder of intermediate repetitive DNA on the heavy side of the principal band, revealed unique strand densities (Hatch and Mazrimas, 1974).

Kurnit et al. (1973) isolated four distinct nuclear satellite DNAs from calf (Bos taurus) and buoyant density gradient centrifugation was used to study the physical properties of native, single-stranded and renatured duplex molecules of each of the four satellite DNAs. Calf satellite I has a ρ = 1.716 g/ml, satellite II has a ρ = 1.722 g/ml, satellite III has a ρ = 1.706 g/ml, and satellite IV had a ρ = 1.709 g/ml.

DNA isolated from several tissues of the land crab, Gecarcinus lateralis, contains three components (Skinner, 1967): a main band (78% of the total DNA; density of 1.701 g/cm^3 and a satellite rich in guanylate and cytidylate residues [d(G+C)]- rich satellite; 4% of the total: ρ = 1.721 g/cm^3 and a satellite composed of more than 90% alternating adenylate and thymidylate residues [d(A-T)] satellite; 18% of the total: ρ = 1.677 g/cm^3 (Skinner, 1967). Two double

tissues of the hermit crab, Pagurus pollicaris. Base compositional analyses indicate that satellite DNA I has one strand containing 50% deoxyguanylate residues but is essentially free of deoxycytidylate ($\lesssim$ 3%); compositional analyses of the other strand give 47% deoxycytidylate and less than 2% deoxyguanylate. The DNA of the edible blue crab (Callinectes sapidus) has an (A+T) rich staellite with the same density in CsCl as poly[d(A-T)] = 1.677 g/cm^3. Another (A+T) - rich satellite (ρ = 1.684 g/cm^3), comprising approximately 0.5% of the DNA of the spider crab (Maja squinado), has interstrand bias composition and consequently separates into two distinct bands (ρ = 1.738 g/cm^3 and ρ = 1.752 g/cm^3) in alkaline CsCl. When reneutralized, the two strands formed a single peak 0.015 g/cm^3 more dense than the native DNA, which is the commonly observed difference between native and denatured DNA in neutral CsCl. The crayfish (Procambarus blandingii blandingii) has an (A+T)- rich satellite with a density of 1.682 g/cm^3 (Beattie and Skinner, 1972).

DNA from the blue crab, Callinectes sapidus, was studied both by isopycnic centrifugation in cesium salt-density gradients and by reassociation analyses using hydroxyapatite chromatography. Centrifugation of C. sapidus somatic and germ cell DNA in neutral CsCl revealed a main band DNA (ρ = 1.698 g/ml) and two satellite bands (ρ = 1.677 and 1.688 g/ml), comprising 21% of the total DNA. The (A+T)-rich satellite was purified by three sequential centrifugations in preparative CsCl gradients. This satellite (ρ = 1.677 g/ml) melted sharply at 62 C in 0.1 X SSC, which corresponds to a 26% G+C content. However, the buoyant density of this satellite indicates an 8% G+C content, and behaves in neutral CsCl like the poly[d(A-T)] satellite (ρ = 1.677 g/ml) in the crabs, Cancer borealis and Gecarcinus lateralis. The complementary strands of this satellite formed a single

band in alkaline CsCl, thus exhibiting no bias in their G+T content. In an attempt to purify the satellite in a single centrifugation, heavy metal cesium sulfate gradients were used. In Hg^{++}-Cs_2SO_4 gradients, the AT- rich satellites were hidden under the main band, as are "cryptic satellies," and did not form distinct bands as expected. The genome of C. sapidus was further examined by DNA/DNA reassociation kinetics. Crab DNA Cot curves were generated by means of hydroxyapatite chromatography using DNA fragments 300 NTL, in comparison with E. coli DNA fragments of similar length and plotted as equivalent Cot versus % DNA reassociated. The preliminary results indicate that at least 26% of C. sapidus main band DNA is repetitive, and 40% of the repetitive fraction is "zero time binding" DNA, about 12% is intermediate repetitive and 60% non-repetitive DNA (Edwards, 1980a).

Somatic and germ cell DNA from Cardisoma guanhumi separated in neutral CsCl gradients into a main component (ρ = 1.699 g/ml) and a satellite band (ρ = 1.677 g/ml), accounting for about 15% of the total DNA. This (A+T)- rich satellite (ρ = 1.677 g/ml) was purified by a single centrifugation in Hg^{++}-Cs_2SO_4 gradients at a Hg^{++}/DNA-PO_4 ratio of 0.1. In Hg^{++}-Cs_2SO_4 gradients, three satellite bands were revealed, the major A+T- rich component and two minor satellites. In neutral CsCl the minor satellites have buoyant densities of 1.675 and 1.681 g/ml. These minor satellites melted over a narrow range of temperatures with a Tm of 50 C and as a single component in 0.1 x SSC. Thermal denaturation studies indicated that the (A+T)-rich satellite (ρ = 1.677 g/ml) melted sharply at 51 C in 0.1 x SSC and 65.5 C in 0.12 MPB, while the main band DNA melted over a broad range of temperatures with a Tm of 71 C. The satellite DNA reassociated rapidly with a Cot½ of 1×10^{-4} in 0.12 M PB, and remelted with a Tm of 63.5 C in 0.12 M PB. The buoyant density, solubility in phenol property and melting temperature of this

satellite (ρ = 1.677 g/ml) indicate that it is a poly [d(A-T)] satellite like those found in C. borealis and G. lateralis. The complementary strands of the (A+T)- rich satellite formed a single band in alkaline CsCl. The Cot curve generated with 300 NTL fragments of C. guanhumi main band DNA indicates that 30% of the DNA is repetitive and about 10% of the main component is "zero time binding" DNA, and with the non-repetitive DNA representing 60% of the total DNA. Both approaches agree that the genomes of Callinectes and Cardisoma are strikingly rich in repetitive DNA content. This may be related to the large genome size of some crabs, relative to small genomes of chickens and other crabs, and to the presence of many small chromosomes in the crab's karyotype (Edwards, 1980b).

Angerer et al. in 1975, studied the sequence organization of the DNA of the mollusc Aplysia californica using a combination of techniques. Close-spaced interspersion of repetitive and single copy sequences occurred throughout the majority of the genome. Detailed examination of the DNA of this protostome revealed great similarities to the pattern observed in Xenopus laevis and Strongylocentrotus purpuratus. Labeled and unlabeled Aplysia DNA were prepared from developing embryos and sheared to a fragment length of 400 nucleotides. The kinetics of reassociation were studied by means of hydroxyapatite chromatography, single-strand-specific S1 nuclease, and optical methods of assay. Aplysia DNA of this fragment length contained at least five resolvable kinetic fractions. One classification of these fractions, listed with their reassociation rate constants (1 M^{-1} sec^{-1}) is: single copy (0.00057), slow (0.047), fast (2.58), very fast (4000), and foldback ($>10^5$).

Sequence arrangement was deduced from: the kinetics of reassociation of DNA fragments of length 400 or 2000 nucleotides; the hyperchromicity of reassociated fragments containing duplex regions; and the reassociation of labeled fragments of various lengths with short driver fragments.

More than 80% of the single copy DNA sequences were found to be interspersed with repetitive sequences. The maximum spacing of the repeats in Aplysia was about 2000 nucleotides, and the average less than 1000. The very fast fraction did not show interspersion with single copy sequences or with other kinetic fractions. The foldback fraction sequences were fairly widely interspersed. The slow fraction sequences were found to be interspersed with the fast fraction, and possibly also with the single copy DNA. The fast fraction was the dominant interspersed repetitive fraction. Its sequences are adjacent to the great majority of the single copy sequences and have an average length of about 300 nucleotides (Angerer et al., 1975).

Measurements were reported which lead to the conclusion that repetitive and non-repetitive sequences are intimately interspersed in the majority of the DNA of the sea urchin, Strongylocentrotus purpuratus. Labeled DNA was sheared to various lengths, reassociated with a great excess of 450 nucleotide-long fragments to Cot 20, and the binding of the labeled DNA to hydroxyapatite was measured. Repetitive sequences measured in this way were found present on about 42% of the 450 nucleotide long fragments. As the DNA fragment length was increased, larger and larger fractions of the fragments contained repetitive sequences. Analysis of the measurements led to the following estimate of the quantitative features of the pattern of interspersion of repetitive and non-repetitive sequences. About 50% of

the genome consists of a short-period pattern with 300-400 nucleotide average length repetitive segments interspersed with about 1000 nucleotide average length non-repetitive segments. Another 20% or more consists of a longer period interspersed pattern. About 6% of the genome is made up of relatively long regions of repetitive sequences. The remaining 22% of the genome may be uninterrupted single copy DNA, or may have more widely spaced repeats interspersed. The similarity of these results to previous measurements with the DNA of an amphibian suggested that this interspersion pattern was of general occurrence and selective importance (Graham et al., 1974).

In the snake, Ptyas mucosus, male DNA has a repetitious DNA satellite (ρ = 1.700 g/cm^3) constituting 5% of the haploid genome (Singh et al., 1976). Singh et al. in 1976 showed that when total DNA of the snake, E. radiata, is centrifuged to equilibrium in Ag^+/Cs_2SO_4 gradients, four major bands were seen in the female and three in the male. In 1980, Singh et al. demonstrated by in situ hybridization that satellite III in E. radiata is mainly concentrated on the W sex chromosome. Cucumber DNA has been shown to have a large percentage of heavy satellite DNA (Ingle et al., 1973).

Satellite DNA has also been reported in the fibroblasts of Aves (Comings and Mattocia, 1970; Brown and Jones, 1972). Analytical centrifugation of Japanese quail DNA demonstrated a main band with a buoyant density of 1.702 g/ml and a satellite of about 5% of the total DNA at a density of 1.715 g/ml. Edwards and Coleman (1973) have demonstrated the presence of four satellite bands in the chicken myoblasts. The four satellite bands representing about 6% of the total DNA appeared in Hg^{++}-Cs_2SO_4 gradients at densities of 1.544, 1.473, 1.471 and 1.445 g/ml, respectively, in addition to the main band at 1.517 g/ml. In neutral

CsCl, three of the satellites (1.544, 1.445 and 1.473) had buoyant densities of 1.697, 1.710, and 1.706 g/ml, respectively. Blasingame and Edwards (1978) reported two satellite DNAs in the Pharoah quail. In the New Zealand White rabbit liver DNA, two satellites were observed in preparative neutral CsCl gradients (Howard and Edwards, 1978).

The repeated sequences in oats DNA have been used to study chromosomal repeated sequence organization in wheat. Approximately 75% of the wheat genome was found to consist of repeated sequences but only approximately 20% will form heteroduplexes with repeated sequences from oats DNA at 60 C in 0.18 M Na^+. The proportion of wheat DNA that formed heteroduplexes with oats DNA was shown to be independent of the wheat DNA fragment length. However, the proportion of wheat DNA that was retained with the heteroduplexes when fractionated on hydroxyapatite is very dependent upon the wheat fragment length up to 3500 nucleotides. This is because more non-renatured wheat DNA is attached to the heteroduplexes with longer fragments. The results indicated that the repeated sequences in the wheat genome homologous to repeated sequences in oats were not clustered in the chromosomes but distributed amongst other repeated and possibly non-repeated sequences (Smith et al., 1976).

DNA of Schizophyllum commune was isolated, both from mycelial cells and from protoplasts, by Dons et al. in 1979. Nuclear DNA was isolated after solubilization of the mitochondria with the detergent Nonidet. The G + C content of the nuclear DNA was 57%, calculated from its buoyant density (1.716 g/ml) and from the Tm (77.4 C in 15 mM NaCl/1.5 mM trisodium citrate). Analysis of CsCl profiles and melting patterns suggested that mitochondrial DNA contained interspersed A + T rich sequences. When these investigators did reassociation

analysis of sheared nuclear DNA, the genome size of S. commune was determined to be 22.8 x 10^9 daltons. A small amount of DNA (0.5 x 10^9 daltons) bound to hydroxyapatite at zero time Cot. Seven percent of the genome (1.6 x 10^9 daltons) represented repetitive DNA.

Reassociation kinetics showed that the satellite DNA of Brassica nigra contained a relatively rapidly reassociating component with the length of the repeating element about 3.3 x 10^3 nucleotide pairs (Beridze, 1979). Nucleotide sequence organization in the genome of rye, Secale cereale, has been studied using renaturation kinetics and S1 nuclease digestion of the renatured products. Approximately 25 to 30% of the genome was found to consist of very slowly renaturing, possibly single copy sequences. Most, if not all, of these sequences are interspersed between repeated sequences and are between 400 and 3500 nucleotide pairs long. Approximately 70 to 75% of the genome was found to consist of repeated sequences. The more highly diverged repeated sequences (30% of the genome) which fail to renature under highly stringent renaturation conditions ranged from 500 to more than 5000 base pairs long and were interspersed in the chromosomes with less diverged repeated sequences (40% of the genome) which were usually less than 800 nucleotide pairs long. Four percent of the genome was found to consist of very rapidly reannealing sequences which might be inverted duplications. These clusters might be in clusters distributed through at least 30% of the genome (Smith and Flavell, 1977).

The organization of repetitive DNA sequences has been investigated in all kinds of bovine DNA. Repetitive sequences of all kinds constituted 25% to 30% of the total DNA. Five density satellites were found to constitute about 20% of the genome and most of the remainder consisted of alternating repeating and non-

repeating sequences. The bulk of the renaturation of repetitive sequences in the bovine DNA occurred between Cot values of 10^{-2} and 10^{0} with approximately second order kinetics (Mayfield et al., 1979).

The reassociation kinetics of Paramecium primaurelia micronuclear DNA best fit a single-order reaction curve indicating that the majority of sequences are uniquely occurring. The $Cot_{½}$ value of P. primaurelia is 234 M x sec. Therefore, the macronuclear DNA of P. primaurelia was found to contain a sequence complexity of 5.2×10^{10} daltons of 1.6×10^{8} nucleotides (MacTavish and Sommerville, 1980).

In 1980, Miklos et al. did restriction endonuclease studies on Rattus villosissimus, Rattus sordidus and Rattus norvegicus. These investigators found that R. villosissimus and R. sordidus yielded very similar patterns, except for minor bands which constitute a tiny fraction of the genome. R. norvegicus differs from both species in a number of ways. It showed fragments of EcoR 1, Hinf 1 and Hae 111. If these fragments were present in R. villosissimus and R. sordidus, they were present in very low amounts. α-Satellite DNA from African green monkey cells was analyzed with restriction endonucleases by Graf et al. in 1979. With EcoR 1 and Hae 111 (or BsuRI isoschizomer), about 25% and 30%, respectively, of the satellite DNA were cleaved into a series of fragments of the 172 bp repeat length and multiples thereof.

Crain et al. (1976a) examined the organization of the repeated and single copy DNA sequences in the genomes of two insects, the honeybee (Apis mellifera) and the housefly (Musca domestica). Analysis of the reassociation kinetics of honeybee DNA fragments 330 and 2200 nucleotides long showed that approximately 90% of both size fragments were composed entirely of non-repeated sequences.

Thus honeybee DNA contains few or no repeated sequences interspersed with non-repeated sequences at a distance of less than a few thousand nucleotides. On the other hand, the reassociation kinetics of housefly DNA fragments 250 and 2000 nucleotides long indicated that less than 15% of the longer fragments were composed entirely of single copy sequences. A large fraction of the housefly DNA therefore contains repeated sequences spaced less than a few thousand nucleotides apart. Reassociated repetitive DNA from the housefly was treated with S1 nuclease and sized on agarose A-50. The S1 resistant sequences have a bimodal distribution of lengths. Thirty-three percent is greater than 1500 nucleotide pairs, and 67% has an average size about 300 nucleotide pairs. The genome of the housefly appears to have at least 70% of its DNA arranged as short repeats interspersed with single copy sequences in a pattern qualitatively similar to that of most eukaryotic genomes.

The genome of parsley, Petroselinum sativum, was studied by DNA/DNA reassociation to reveal its spectrum of DNA reiteration frequencies and sequence organization. The reassociation of 300 nucleotide DNA fragments indicated the presence of four classes of DNA differing in repetition frequency. These classes are: highly repetitive sequences, fast intermediate repetitive sequences, slow intermediate repetitive sequences, and unique sequences. The repeated classes were reiterated on average 136,000, 3,000, and 42 times respectively. A minor part of the genome is made up of palindromes. The organization of DNA sequences in the P. sativum genome was determined by the reassociation kinetics of DNA fragments of varying length. Further information was derived from S1 nuclease resistance and from hyperchromicity measurements on DNA fragments reassociated to define Cot values. The portion of the genome organized in a short period interspersion pattern amounted to 47%, with the unique sequences on an average

1000 nucleotides long, and most of the repetitive sequences about 300 nucleotides in length, whereas the weight average length might be up to 600 nucleotides. About 5% unique DNA and 11% slow intermediate repetitive DNA consisted of sequences from 10^3 up to 10^4 nucleotides long; these are interspersed with repetitive sequences of unknown length. Long repetitive sequences constituted 33% of the genome, 13% are satellite-like organized, and 20% in long stretches of intermediate repetitive DNA in which highly divergent sequences alternate with sequences that show only minimal divergence (Kiper and Herzfeld, 1978).

The complexity of the flax genome has been determined by reassociation kinetics. The total complexity of one constituent genome was 3.5×10^5 nucleotide pairs. The single copy sequences comprised 44% of the genome and showed a long period interspersion pattern with the repetitive sequences. The repetitive sequences occurred in clusters which stretched for at least 10,000 base pairs. Within these clusters the individual repetitive elements were about 650 basepairs. These elements themselves showed little interspersion of different frequency classes in lengths less than 3000 base pairs. The repetitive sequence duplexes formed on reassociation, except for the satellite DNA, showed a high thermal stability. The foldback DNA comprised 1% of the total genome, and was itself clustered in a small fraction of the genome (Cullis, 1981).

Dennis et al. in 1980 did a restriction enzyme analysis of the major satellite of Macropus rufogriseus, red necked wallaby consisted of a number of related families of sequences arranged in tandem arrays. Particular families were shown to be subpopulations of other families. This particular satellite in the red necked wallaby could be detected in related kangaroo species in much reduced amount, and with changes to the long order periodicity of the repeat units. In the cat, Felis

catus, a satellite comprising 0.5% of total DNA was isolated by repeated centrifugation in CsCl with netropsin. The reassociation kinetics ($Cot_{½} = 10^{-3}$ M • s) showed that the satellite in the cat is of simple sequence type and hence a candidate for centromeric heterochromatin (Matthews et al., 1980).

Epplen et al. in 1978 studied the sequence organization in the duck, chicken and pigeon DNA. Fourteen percent of the duck DNA consisting of highly repetitive and foldback sequences renatured with $Cot_{½}$ values 10^{-4} M - s. Another 13% of the genome contained middle repetitive sequences occurring with a repetition frequency of about 420 copies. The kinetically determined $Cot_{½}$ value for the slowest fraction, which made up 73% of the DNA, corresponded with single copy DNA. These results were obtained from 0.23 kb duck DNA fragments. The very fast reannealing fraction of chicken DNA (0.22 kb long fragments) amounted to about 20%. Ten percent of the fragment reannealed as moderately repeated sequences and was bound to HAP (hydroxyapatite) with an $ECot_{½}$ (equivalent $Cot_{½}$) of 1.2 M - s. The single copy fraction in the chicken DNA comprises 68% of the total DNA. When the fragment size was raised from 0.22 to 2.1 kb the zero-time binding fraction increased by 5% while the slowest kinetic fraction was reduced by about 20%. These results indicated that in the chicken DNA on 2.1 kb fragment length only some moderate repeats were interspersed with unique sequences; 72% of the unique DNA were not interrupted by repetitive elements. In the pigeon DNA (0.25 kb long fragments), foldback sequences and highly repetitive DNA constituted about 15% of the DNA, 11% of the DNA was composed of middle repetitive sequences, and 60% was single copy. The expected $ECot_{½}$ value of 5.5×10^{2} M • s for the unique DNA fraction corresponded satisfactorily with the empirically determined $ECot_{½}$ value of 6.7×10^{2} M • s.

When Jumbo Wisconsin quail DNA was fractionated in Ag^{+}-Cs_2SO_4 gradients, two distinct bands were observed. The major band has a buoyant density of 1.700 g/ml and represents 85% of the total DNA, while the minor band has a density of 1.695 g/ml and accounts for 15% of the total DNA. The main band has a G + C content of 39% while the minor band has a G + C content of 34%. Thermal denaturation studies were used to confirm the G + C content values. The main band and satellite melted to 70 and 54 C, respectively. Total, satellite and heavy shoulder DNA were further analyzed by restriction endonuclease digestion and gel electrophoresis. When total DNA was digested with EcoR 1, Alu 1, Hind 111 and Hind 11 and electrophoresed, a large heterogeneous smear was observed without any distinct minor bands. Digestion of satellite I gave the following results: (1) Hind 111 produced a heterogeneous smear, (2) EcoR 1 digests contained a heterogeneous smear of 27-2 kb in length. When heavy shoulder DNA was digested, two endonucleases produced discrete bands. EcoR 1 digests contained one distinct band of 7 kb in length, Hind 111 produced two distinct fragments of 2.4 and 7 kb in length. These fragments were transferred to nitrocellulose filters and hybridized with ^{32}P-rRNA according to the method of Southern. These data indicated that the fragments produced by Hind 111 and EcoR 1 contain the ribosomal genes. Across the gradient hybridization with ^{3}H-rRNA indicated that the ribosomal genes are not a component of the satellite and supports the restriction digestion studies (Edwards, 1979).

CHAPTER III

MATERIALS AND METHODS

DNA Extraction

Embryos were dissected from 10-12 day old bobwhite quail eggs, weighted and placed in SSC (0.15 M NaCl and 0.015 M Na Citrate, pH 7.0± 0.2). This buffer maintains ionic strength of dissolved DNA and chelates divalent ions. A small amount of SSC was used at this point because the buffer, SSC, was added according to the grams of tissue used. For every 1 g of tissue, 4 vol of SSC were added (this includes the small amount of SSC already added). The tissue was homogenized on an omni mixer at 4 C, and brought up to 1% SDS (sodium dodecyl sulfate) with 10% SDS. The homogenate was shaken for 1 hr at 25 C. Afterwards, 300 mg of solid NaCl per ml of SSC was added, and shaken for 30 min at 25 C. An equal volume of CIA (chloroform - isoamyl alcohol), 24:1 ratio, respectively, was added, shaken for 1 hr at 25 C and centrifuged at 5,000 RPM for 15 min. The aqueous phase was removed, Sevaged again for 15 min, and spun in the centrifuge at 5,000 RPM. The aqueous phase was removed and diluted with 2-2.5 vol of absolute ethyl alcohol (ETOH). Absolute ethyl alcohol precipitated the nucleic acids and contaminating proteins. The precipitated DNA was pelleted by centrifugation and redissolved in 1-3 ml of 0.1 x SSC. The DNA solution was further purified by digestion with α-amylase, pancreatic RNase, and T_1 RNase at a final concentration of 50 µg/ml at 37 C for 1 hr. The enzymes were obtained from Sigma Chemical Company. Pronase (50 µg/ml) was added to the nucleic acid and incubated for 2 hr at 37 C.

The concentration of the DNA solution was brought up to 0.5% with 10% SDS and up to 1 x SSC with 10 x SSC. An equal volume of CIA was added and the lysate was shaken for 15 min, and centrifuged at 5,000 RPM for 15 min. The aqueous phase was removed, Sevaged twice, placed in dialysis tubing and dialyzed for 75 hr against 0.1 x SSC. The DNA was dialyzed to get rid of the extracting solutions and to equilibrate the DNA in 0.1 x SSC buffer. The purity of the DNA was tested by reading the optical density at 260 nm, 240 nm, 280 nm and 320 nm. A ratio of 2 and above indicated the purity of the preparation (260/280 = 2; 260/230 = 2.3).

Preparative Neutral CsCl Gradients

CsCl gradients are used as important tools for precise and relatively simple determinations of base composition (% G + C) of DNA, providing that no unusual bases are present (Szybalski, 1968). Gradients (4.5 ml) were prepared and spun 40 hr at 25 C at 48,000 RPM in a Beckman ultracentrifuge using the Type 50 rotor. Approximately 300 µg of DNA was used for each gradient. The DNA solution (1.11 g) was placed in a cellulose nitrate tube or a polyallomer tube, with 6.52 g of saturated CsCl (7.7 M) in 0.1 x SSC, the refractive index read at 25 C (N^{D25C}), and the density of the gradient was adjusted to 1.700 g/ml. The gradients were centrifuged at 50,000 RPM for 24 hr at 4 C in a Beckman L5-75 Preparative Centrifuge. The tubes were punctured in the bottom, fractionated (four drop fractions), the absorbancy measured at 260 nm, and plotted against the fraction number. The main band and the satellite DNA were readily identified. Some fractions were pooled after each run for further study.

Preparative Alkaline CsCl Gradients

The alkaline CsCl gradients were spun for 40 hr at 23 C at 49,000 RPM in a a Type 50 rotor. In these gradients, polyallomer tubes were used to prevent the

loss of DNA. The gradients consisted of 1.21 g of saturated CsCl, and 0.35 g of 1 N NaOH which brought the total volume up to 8.1 ml. The density of the gradient was 1.740 g/ml with a N^{D25C} of 1.403. The gradients were centrifuged, fractionated (four drop fractions), the absorbancy measured at 260 nm and plotted against the fraction number.

Analytical Neutral CsCl Gradients

When doing the analytical gradients, the Model E Analytical Ultracentrifuge with ultraviolet absorption optics and a 4-hole An-F rotor with two sets of four cells were used. To prepare this gradient, 0.869 g of saturated CsCl in 0.1 x SSC and 0.147 g of DNA solution were used. The DNA solution was composed of 2 µg of marker DNA (Micrococcus luteus) and a small volume of 0.1 x SSC. The N^{D25C} was 1.3994 with a density(ρ) of 1.700 g/ml and a total volume of 0.6 ml. The gradient was centrifuged for 19 to 24 hr at 44,000 RPM at 22 C. The purpose of the Model E Analytical Ultracentrifuge was to produce high centrifugal forces in order to measure the movement or redistribution of sedimenting particles.

Analytical Alkaline CsCl Gradients

These gradients were also run in the Model E Analytical Ultracentrifuge and consisted of 3 µg of DNA, 0.08 ml of 0.1 N NaOH, 0.68 g CsCl, and 2 µl of M luteus DNA. The N^{D25C} was 1.4030 with a pH of 13.5.

Before setting up the Cs_2SO_4 gradients (preparative or analytical), DNA samples were dialyzed against 0.005 M of sodium tetraborate buffer, pH 9.2. The two types of Cs_2SO_4 gradients used were Hg^{++} and Ag^{+}-Cs_2SO_4. The Hg^{++}-Cs_2SO_4 and Ag^{+}-Cs_2SO_4 gradients were prepared in the same way except one used mercury and the other silver.

Preparative Cs_2SO_4 Gradients

These gradients were prepared by using 4.4 g of solid Cs_2SO_4, 1.73 ml of DNA (300 μg) and 1.73 ml of 5×10^{-3} borate buffer. If a Ag^+-Cs_2SO_4 gradient were set up, 0.001 M $AgNO_3$ was added to the gradient required for the desired ratio. If a Hg^{++}-Cs_2SO_4 gradient were being set up, 0.001 M $HgCl_2$ was added according to the desired ratio. The N^{D25C} of the gradient was 1.3740 with a density of 1.500 g/ml. The gradients were centrifuged at 49,000 RPM for 40 hr at 23 C, fractionated, the optical density taken, and plotted against the fraction number.

Analytical Cs_2SO_4 Gradients

These gradients were run in the Model E Analytical Ultracentrifuge. The Hg^{++} and Ag^+-Cs_2SO_4 gradients were set up in different ratios. For Hg^{++}, the ratios (RF) were 0.1, 0.2, 0.3 and 0.50 and the Ag^+ ratios were 0.26, 0.27, 0.30, and 0.35. For each gradient, 10 μg of DNA in borate buffer (0.005 M $Na_2Br_4O_7$), 0.20 ml of borate buffer (0.005 M $Na_2Br_4O_7$), 0.5 g of Cs_2SO_4 and the Hg^{++} or Ag^+ was added dropwise. The amount of Hg^{++} or Ag^+ was added according to the desired ratio.

RNA Extraction

Before beginning the RNA extraction, all glassware was heated overnight at 300 F to inactivate RNase and handled with gloves. The RNA extraction was carried out at 4 C. Twelve day chicken embryos and rabbit liver were the sources of RNA. The tissue was dissected, placed in 8 vol of extraction buffer (0.01 M sodium acetate, 4 μg/ml polyvinyl sulfate (PVS), pH 5.0) and homogenized in a Dounce homogenizer. After homogenization, 10% sodium lauryl sulfate (SLS) was

added (1% final concentration to lyse the cells). The RNA solution was shaken for 30 min in the cold and an equal volume of m-cresol, redistilled H_2O saturated-phenol, -8-hydroxyquinoline solution was added. The suspension was then shaken manually for 1 hr at 4 C. The aqueous phase was separated by centrifugation at 4 C for 10 min at 5,000 RPM and reextracted again with the phenol reagent, shaken for 1 hr at 4 C and centrifuged. The supernatant was mixed with 0.1 vol of 1 M NaCl, precipitated with 2 vol of absolute alcohol and precipitated overnight. The RNA was collected by centrifugation at 15,000 RPM for 30 min and washed by resuspending in 80% ETOH. The ethanol removed any remaining phenol. After each resuspension, the RNA was centrifuged at 15,000 RPM for about 10 min. The pellet of RNA was dried, redissolved in the 0.01 M sodium acetate, PVS buffer, pH 5.0, dialyzed against 0.01 M sodium acetate, PVS buffer for 72 hr and stored at -20 C.

Sucrose Gradients

Sucrose gradients used to fractionate RNA were prepared by using 5.5 ml of 5% sucrose and 5.5 ml of 20% sucrose. The gradients were equilibrated for 3 hr or more in the cold room. After equilibration, 750 μ g of RNA were layered onto the gradient and centrifuged for 7 hr at 40,000 RPM at 4 C in a Spinco SW41 rotor. Fractions were collected through a hole pierced in the bottom of the tube and the absorbance of each fraction determined at 260 nm. After the fractions had been collected, the 18 S and 28 S fractions were pooled while the 5 S and 4 S fractions were pooled separately. The rRNA fractions (18 S and 28 S) were pooled, brought to 0.1 M in NaCl and then precipitated with 2 vol of 95% ethanol at -20 C. The rRNA was used for DNA-RNA filter hybridization. Hybrids (DNA-RNA) were

formed by incubating the DNA-containing filters in glass scintillation vials which contained owl monkey ^{3}H-rRNA in 2 x SSC for 16 hr at 65 C. Filters were removed, rinsed briefly in 70% ethanol, dried by 45 min at 65 C, submerged in Toluene-POPOP and counted in a Beckman Scintillation Counter.

Thermal Denaturation Studies

Melting temperature profiles for analyzing the base composition of Avian DNA were determined by the method of Mandel and Marmur (1968). DNA samples, dissolved in 0.1 x SSC, were placed in quartz cuvettes with a 1 cm light path and 1 ml capacity. All DNA samples were dialyzed against 0.1 x SSC or 0.12 M phosphate buffer for 72 hr (one change every 24 hr) at 4 C to remove contaminating traces of chloroform.

The optical density and temperature of the DNA solutions were monitored with a Beckman DU-8 spectrophotometer. The spectrophotometer was programmed to elevate at a rate of 1 C/2 min until a temperature of 60 C was obtained. Thereafter, the spectrophotometer was programmed to raise at a rate of 0.5 C/4 min. The absorbance value given for each fraction was corrected for thermal expansion and plotted against the temperature in the cuvette. In some instances, the percent hyperchromicity was plotted against the temperature of the cuvette. The G + C content of samples in 0.1 x SSC was determined from the equation: GC = [tan (70.077 - 3.32 Mg)(Tm -175.95)-260.34] (Owen et al., 1969).

Digestion of DNA with Restriction Endonucleases

In order to further analyze the DNA sequence arrangement of quail DNA, restriction enzyme digestion analysis were used. DNA was digested with several

enzymes (EcoR 1, Alu 1, Bam 1, Hind 111, Hpa 1 and others, Table 3) in different buffers according to Robinson and Landy (1977). All enzymes used in this investigation were obtained from Bethesda Research Laboratory. The buffers for each restriction endonuclease were as follows: EcoR 1 - 100 mM NaCl, 12 mM $MgCl_2$ and 200 mM Tris-HCl at pH 7.5., Alu 1 - 14 mM $MgCl_2$, 14 mM - mercaptoethanol and 20 mM Tris-HCl at pH 7.5, Bam 1 - 0.012M $MgCl_2$ and 0.2 M Tris-HCl at pH 7.5, Hind 111 - 0.006 M Tris-HCl at pH 7.5 and Hpa 1 - 10 M $MgCl_2$, 1 mM dithiothreitol, 6 mM KCl, and 20 mM Tris HCl pH 7.4. Enzyme preparations were titrated to determine the amount necessary for limited digestion. DNA concentrations were between 20-100 µg/ml and incubations were carried out at 37 C for 4.5 hr with additions of enzyme at 1.5 hr intervals or overnight. The reaction was quenched by chilling. The samples were then mixed with 5% SLS, 25% glycerol, 0.025% Bromophenol blue and heated at 65 C for 5 min before loading onto gels.

Gel Electrophoresis

The DNA fragments produced with the restriction endonucleases were separated in Seakem agarose (0.8% - 1.0%) containing 0.005 M sodium borate buffer. Ethidium bromide (1 µg/ml) was added after the agarose cooled down to 60 C. The gels were equilibrated for 30 min before loading samples. Gels were run for 5 min at 100 volts at 25 C and then 4.5 hr at 60 volts or overnight at 35 volts until the dye reached the end of the gel. Gels were removed from the apparatus, and the bands viewed with a short wavelength ultraviolet light (UV Products) and photographed on Polaroid Type 55 film (Helling et al., 1974) using a Kodak #22 Wratten Filter (orange).

Molecular Weight Determinations

Molecular weights of the DNA fragments resulting from enzyme digestion were determined by comparing them to DNA fragments of known sizes on electrophoretic gels (lambda fragments generated by EcoR I and Hind III) making use of the fact that under appropriate conditions the electrophoretic mobilities of DNA molecules approximate a linear function of the logarithms of their molecular weights (Danna and Nathans, 1971). Maniatis et al., (1975) have shown that this linear relationship is only obtained under appropriate electrophoretic conditions. Therefore, several different gel conditions were used to optimize the accuracy of molecular weight determination over the wide range of fragment sizes obtained.

For large DNA fragments agarose gels have been shown to afford better correspondence between electrophoretic mobility and fragment size than polyacrylamide gels (Thomas and Davis, 1974). The fragments obtained by the EcoR I and Hind III digestion of λ were utilized as standards, the lengths of these fragments having been determined by electron microscopy (Wellauer et al., 1974). The small fragments were sized by comparing them to Hind II and III fragments of lambda DNA on a 3% acrylamide - 0.5% agarose gel, run at high voltage to minimize diffusion.

Labelling of Total DNA

A "Nick Translation System" (New England Nuclear) was used to prepare radioactive probes for measuring DNA-DNA reassociation kinetics (Tu and Cohen, 1980; Maxam and Gilbert, 1980). Tritiated thymidine triphosphate was dried down to yield a final concentration of 18 μM. After the addition of 1.8 μl of each

unlabelled deoxynucleotide triphosphate, 10X reaction buffer and 18 µg of bobwhite quail DNA was added. After mixing together distilled water was added to bring the total volume of 97λ. Activated DNase I (100 ng/ml) was added to the reaction mixture and then incubated for 10 min at 10 C. E. coli DNA Polymerase I was added and the reaction was continued for 3 hr at 15 C before the addition of stop buffer (which terminated the reaction). The DNA was separated from residual nucleotides by chromatography on a 0.9 x 25 cm column of Sephadex G-25 medium which had been equilibrated in 10 mM Tris-HCl, 0.1 mM NaEDTA, pH 8.0. Ten drop fractions were collected and 10 µl of each fraction were placed in scintillation vials, diluted with 10 ml of Beckman Ready-Solv EP (Economy Pre-mixed) scintillation fluid and counted in the Beckman Model LS7500 scintillation counter for 10 min. A specific activity of 5×10^5 cpm/µg was obtained for ^{3}H-labelled DNA.

Preparation of DNA Fragments

DNA fragments of 500 nucleotides long (NTL) were obtained by shearing DNA, which had been dissolved in 67% glycerol-0.02 M Na acetate,with a Virtis 60 homogenizer (Virtis Company) at 50,000 RPM for 30 min in a dry ice-ethanol bath. For 2000 NTL fragments the DNA was sheared for 30 min at a speed of 15,000 RPM in an ice water bath with one blade. After shearing, the DNA solution was brought up to 0.2 M Na acetate, precipitated overnight in 95% ethyl alcohol and redissovled in 0.12 M PB.

Sizing of DNA Fragments

The size of the DNA fragments was determined by alkaline suscrose gradient centrifugation analytical centrifugation (Moving Boundary) and by agarose gel electrophoresis.

a). Alkaline Sucrose Gradient Centrifugation

The alkaline sucrose gradients were prepared by using 5.5 ml of 5% sucrose and 5.5 ml of 20% sucrose containing 0.1 N NaOH (Noll, 1967). The gradient was loaded with 50 µg of the fragmented quail DNA and with 4.3×10^3 cpm of ^{3}H-labelled DNA. The centrifugation was performed in a Beckman SW 41 rotor at 40,000 RPM and 4 C for 6 to 8 hr. The gradients were fractionated, the optical density of the fractions was measured in a spectrophotometer, and the radioactivity was counted in a Beckman liquid scintillation counter. The average molecular weight of DNA fragments was determined from their sedimentation constants relative to the marker in the same tube and the equations relating molecular weights to sedimentation rate (Studier, 1965).

b) Agarose Gel Electrophoresis

Agarose (0.85% - 1.0%) horizontal slab gels were prepared in Tris-Borate buffer. Approximately 1-30 µg of sheared bobwhite quail DNA was visualized by the flourescence of bound ethidium bromide under ultraviolent irradiation. Hind 111 and for EcoR 1 digested λ - DNA was used as a calibration marker and always run simultaneously with the sheared DNA. Unsheared DNA remained near the well of the gels.

Reassociation Kinetics

The formation of DNA-DNA reassociation "Cot curve" was carried out by the hydroxyapatite (HAP) chromatography technique. "Equivalent-Cot" values for very high points were obtained by appropriate changes in the DNA and/or phosphate

buffer (PB) concentrations. DNA in 0.12 M PB or 0.4 M PB was denatured by boiling at 100 C for 3 min. DNA samples were reassociated in 0.12 M PB or 0.4 M PB at 60 C for different times to obtain the desired Cot value. Preparative HAP chromatographic fractionation of the DNA into repetitive and non-repetitive DNA fractions was carried out with the use of a water-jacketed column. Unreassociated DNA was eluted for the column in 0.12 M PB at 60 C and double stranded fragments were eluted in 0.4 M PB in 60 C. The OD_{260nm} and OD_{320nm} of the 0.12 M PB and 0.4 M PB washes were read. The corrected OD_{260nm} for each wash was determined. The OD_{260nm} of each 0.12 M PB wash was corrected from single to double strandedness when divided by a factor of 1.2. Generally 90-95% of the input DNA was recovered in all phosphate buffer washed.

Hyperchromism Studies

The hyperchromicity of fragments separated by HAP chromatography representing reassociated DNA fragments was determined by melting in the Beckman DU-8 Spectrophotometer, recording the changes in UV - absorption between 40 C and 100 C. Hyperchromicity was calculated according to the formula

$$\frac{\text{O.D.}_{260}\text{ at 100 C} - \text{O.D.}_{260}\text{ at 40 C}}{\text{O.D.}_{260}\text{ at 100 C}} \times 100$$

The Tm was taken as that temperature at which a 50% increase in hyperchromicity was produced.

S1 Nuclease Digestion of Single Strand DNA and Sizing of the Resistant DNA Duplexes

The DNA was denatured for 3-5 min, 100 C, in PIPES buffer (0.175 M NaCl, 0.01 M PIPES, pH 6.7). After denaturation the DNA was reassociated at 60 C. The reaction was diluted with an equal volume of acetate buffer (50 mM acetic acid, 0.2 M $ZnSO_4$, pH 4.4), enough 0.5 M mercaptoethanol was added to give a final concentration of 10 l/ml and S1 nuclease (2 units/μg of DNA) was added. The reaction was incubated at 50 C for 90 min. Enzyme concentration and reaction conditions were sufficient for complete digestion of single stranded regions of reassociated DNA while mismatched repetitive duplexes are preserved. The digestion was terminated by chilling the sample and bringing the sample to 0.12 M PB with 1 M PB. The S1 nuclease resistant DNA was recovered from the exclusion peak of a Sephadex G-50 column. The size of the enzyme resistant duplexes was estimated by chromatography on Bio-Rad Bio gel A-50 (50-100 mesh) columns run in 0.12 M PB and electrophoresis through agarose, using λ DNA markers. Long native DNA and 450 nucleotide long DNA duplex standards were used to calibrate these columns.

The duplexes, in 0.12 M PB,were placed in the Beckman DU-8 Spectrophtometer and a melting profile was run on them.

CHAPTER IV

RESULTS

Fractionation of DNA by Equilibrium Density Gradient Centrifugation: Analysis of Bobwhite Quail DNA in Neutral and Alkaline CsCl Gradients

The purpose of these experiments was to examine the biophysical properties of the bobwhite quail (Colinus virginianus) DNA. The DNA used in these experiments had a 260/280 nm and 260/230 nm absorption ratio of about 2.0 (Fig. 1). The DNA from 12 day bobwhite quail embryos was banded by isopycnic centrifugation in neutral cesium chloride density gradients. Analytical centrifugation in neutral cesium chloride of the bobwhite quail total DNA revealed a single non-Gaussian band with a buoyant density of 1.701 g/ml. The absence of satellite DNA in these gradients may be attributed to the small amount of DNA used or the percent total represented by the satellite sequences.

Preparative neutral cesium chloride profiles of bobwhite quail total DNA (Fig. 2) revealed that there were two satellites present on the light shoulder. The main band DNA of the bobwhite quail had a buoyant density of 1.701 g/ml and the two satellites have a density of 1.681 g/ml and 1.674 g/ml, respectively.

Using the formula of Schildkraut, et al. (1962) for the determination of base composition from buoyant density, the average G + C content of main band DNA in the bobwhite quail would be 41.8% while the average G + C content of the two satellites would be 21.4% and 14.3%.

Fig. 1. A wavelength scan of total bobwhite quail DNA after an extraction. A 1:10 (DNA: distilled water) dilution was made and placed in the Beckman DU-8 Spectrophotometer. The DNA peaks in the 260 absorbancy region of the profile.

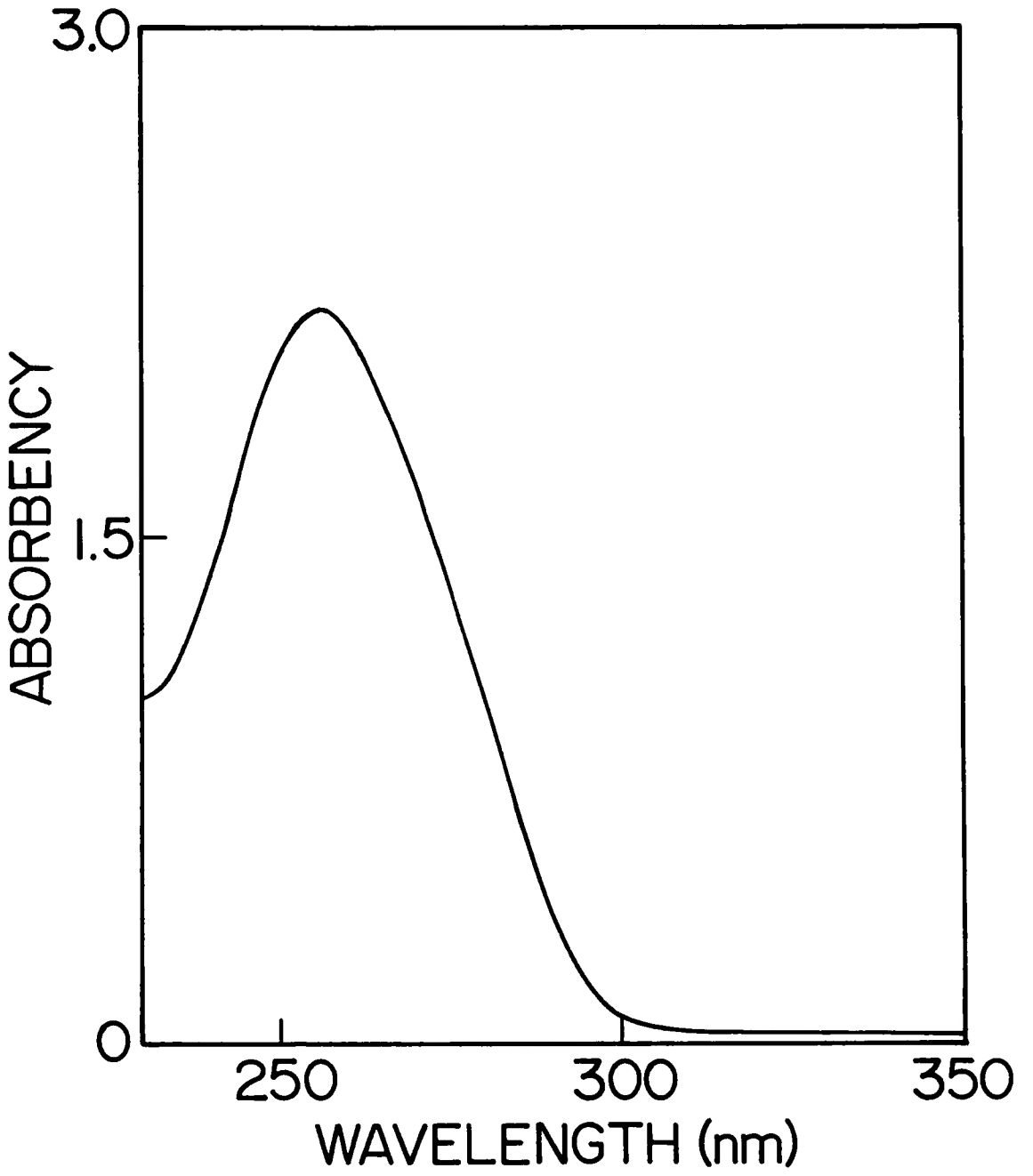
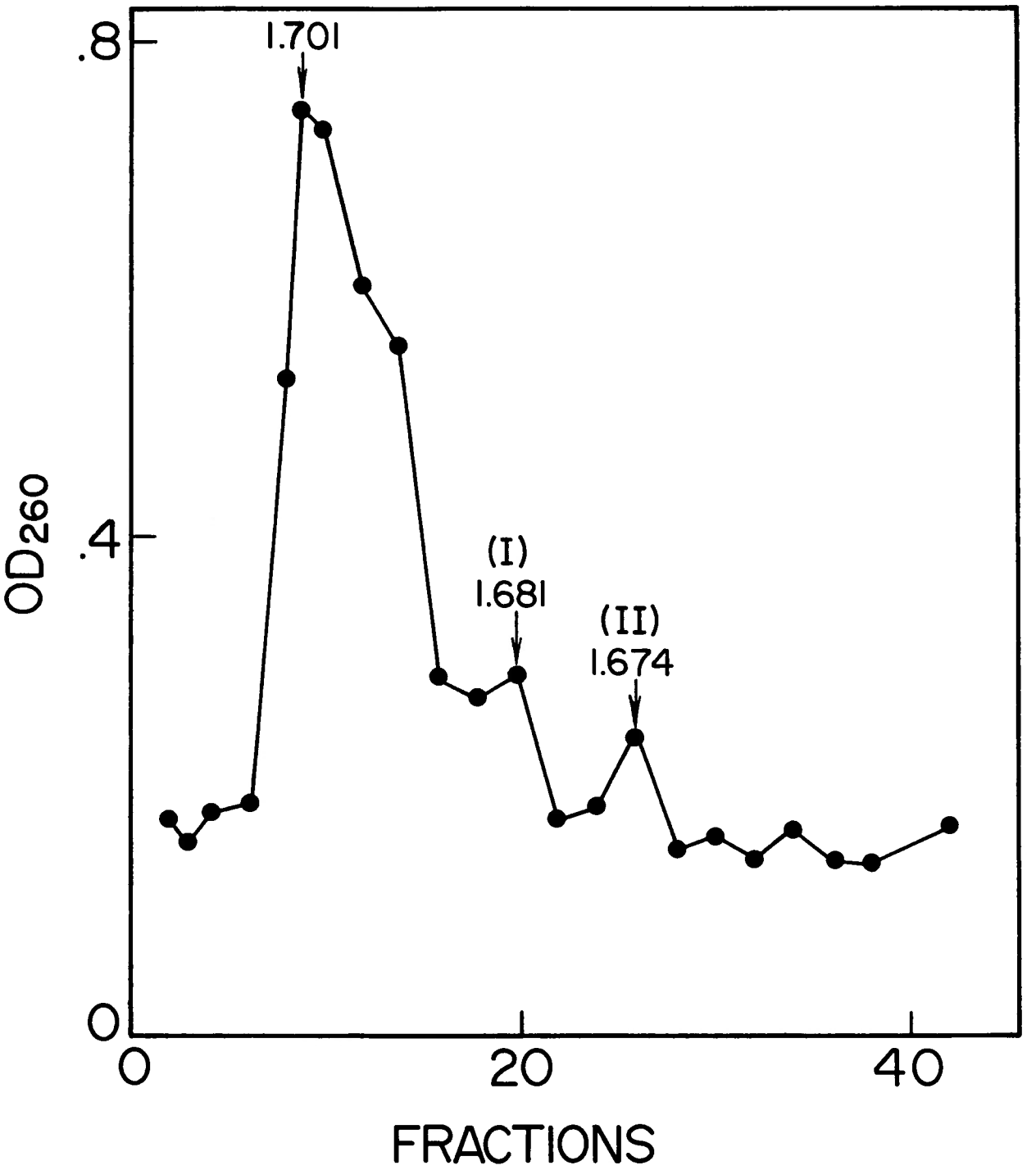

3.0
1.5
0
ABSORBENCY
250
300
350
WAVELENGTH (nm)

Fig. 2. Preparative neutral CsCl gradient of bobwhite quail total DNA. DNA was extracted from 9-12 day old quail embryos and fractionated on a 4.5 ml CsCl gradient. The gradient was spun 40 hr at 25 C, 45,000 RPM in a Type 50 aluminum rotor. Four drop fractions were collected and the fraction number plotted against the optical density (260 nm) of each fraction. The main band DNA has a buoyant density of 1.701 g/ml while the satellites, I and II, have densities of 1.681 and 1.674 g/ml, respectively.


.8
.4
0
OD260
1.701
(I)
1.681
(II)
1.674
0
20
40
FRACTIONS

DNA from bobwhite quail embryos was also preparatively centrifuged in alkaline cesium chloride at a pH of 13.5. Figure 3 of the bobwhite quail shows that there is no bias in the G + T content of the complementary strands and that a main band and two satellites were present on the light side of the gradient.

Hybridization Properties of DNA Fractionated From Preparative Neutral CsCl Gradients

To further characterize total bobwhite DNA quail filter hybridization experiments were done to determine the position in the gradient of the genes which code for ribosomal RNA. Filters, with single stranded DNA absorbed to them, were incubated with owl monkey kidney ^{3}H-rRNA (cells donated by Dr. Louise Miller) for 16.5 hr. The results of this experiment are shown in Fig. 4. The hybridization peaks were on the heavy side of the gradient as they should be because of the GC-richness of ribosomal DNA. This data indicated that the genes from ribosomal RNA were not localized in the satellite bands of bobwhite quail DNA and that all of the rDNA was not clustered in a single nucleolus organizer.

Melting Profiles of Total, Main Band and Satellite DNA

Thermal denaturation provides a convenient method of following the transition of native to denatured DNA (Mandel and Marmur, 1968). Thermal denaturation studies were used to characterize total DNA that had been centrifuged, collected, pooled and dialyzed against 0.1 x SSC. Thermal denaturation was carried out according to the method described by Schildkraut et al. [(1968) see Materials and Methods] .

Fig. 3. Preparative alkaline CsCl gradient of bobwhite quail total DNA. Total DNA extracted from 9-12 day old embryos was preparatively centrifuged in alkaline CsCl with a pH of 13.5. The fraction number was plotted against the optical density (260 nm) to see if there is a bias in the G + T content of the complementary strands.

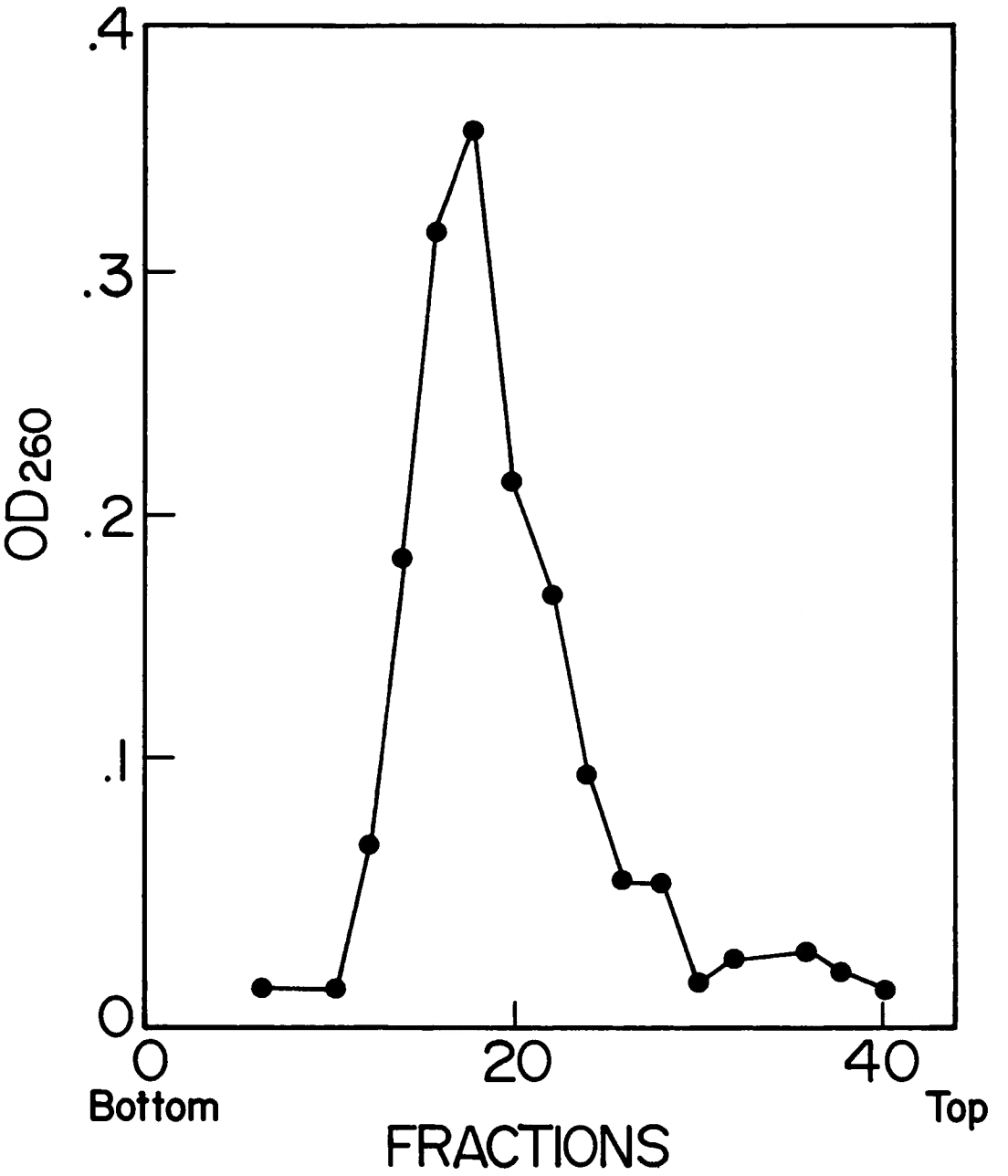
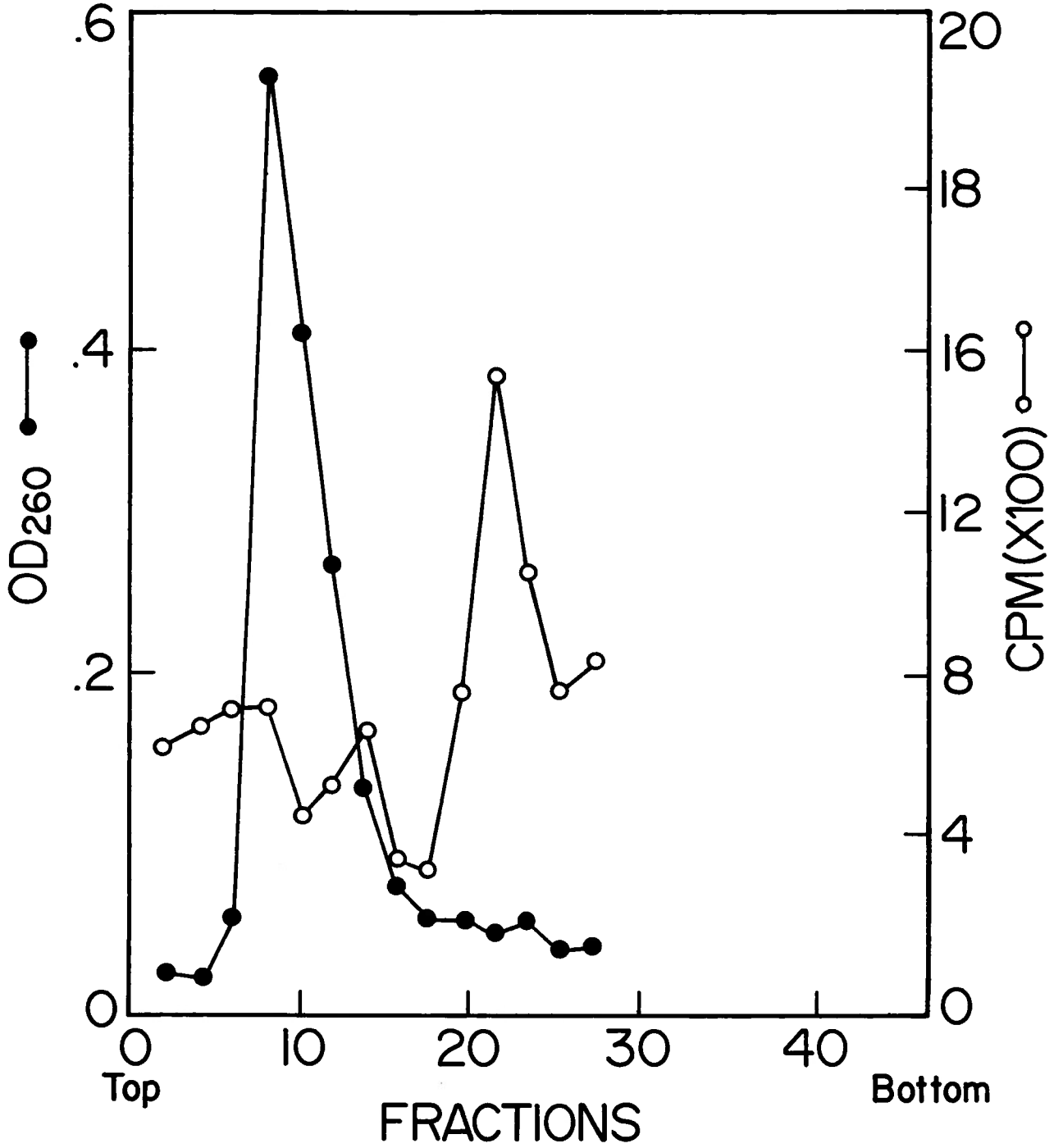

.4
.3
.2
.1
0
OD260
0
20
40
Bottom
Top
FRACTIONS

Fig. 4. Hybridization of radioactive rRNA obtained from cultured owl monkey cells to bobwhite quail (Colinus virginianus) DNA immobilized on nitrocellulose filters. The hybridization peaks are located on the heavy side of the gradient. The main band has a density of 1.701 g/ml. The rRNA was used at a specific activity of 1×10^5 CPM/ µg. The buoyant density increases from right to left.


.6
.4
.2
0
OD260
20
18
16
12
8
4
0
CPM(X100)
0
10
20
30
40
Top
Bottom
FRACTIONS

In the bobwhite quail (Fig. 5), the main band DNA was 82% of the total DNA, and satellite DNA I and II were 10% and 8% of the total DNA, respectively. The main band DNA melted at a Tm of 70 C, which represented a G + C content of 38.3%. The Tm was calculated as the temperature at the midpoint of the increase in absorbance (Owen et al., 1969).

Ultracentrifugation of DNA in Heavy Metal Cs_2SO_4 Density Gradients

Density gradients of cesium salts have long been used to separate and characterize DNAs of various organisms (Szybalski, 1968). Differences in density of two DNA species can be increased by binding mercury to AT-rich DNAs (Davidson et al., 1965) or silver to GC-rich DNAs in Cs_2SO_4 gradients. These techniques are particularly useful for separation of those satellite DNAs that have very marked base compositional biases. Silver ion and mercuric ion form complexes with the nucleic acids and with polynucleotides which undoubtedly involve covalent bond formation with the purine and pyrimidine moieties, and probably electron-pair bonds to π-electron pairs of nitrogen atoms.

Hg^{++}-Cs_2SO_4 Density Gradients

The fractionation of bobwhite quail DNA in Hg^{++}-Cs_2SO_4 density gradients was performed according to the technique of Nandi et al. (1965). The complexing of calf thymus DNA by mercuric ions was first studied by Katz (1952) and later by Thomas (1954). In the bobwhite quail (Fig. 6), the satellites were not visible at a ratio of Hg^{++}/DNA-PO_4 of 0.1. Therefore, these results indicated the higher Hg^{++}/DNA-PO_4 ratios were required to isolate the satellites in bobwhite quail.

Fig. 5. Melting profile of DNA from bobwhite quail (Colinus virginianus) in 0.1 x SSC, pH 7.0. Main band DNA melted at a Tm of 70 C which corresponds to a G + C content of 38.3%.

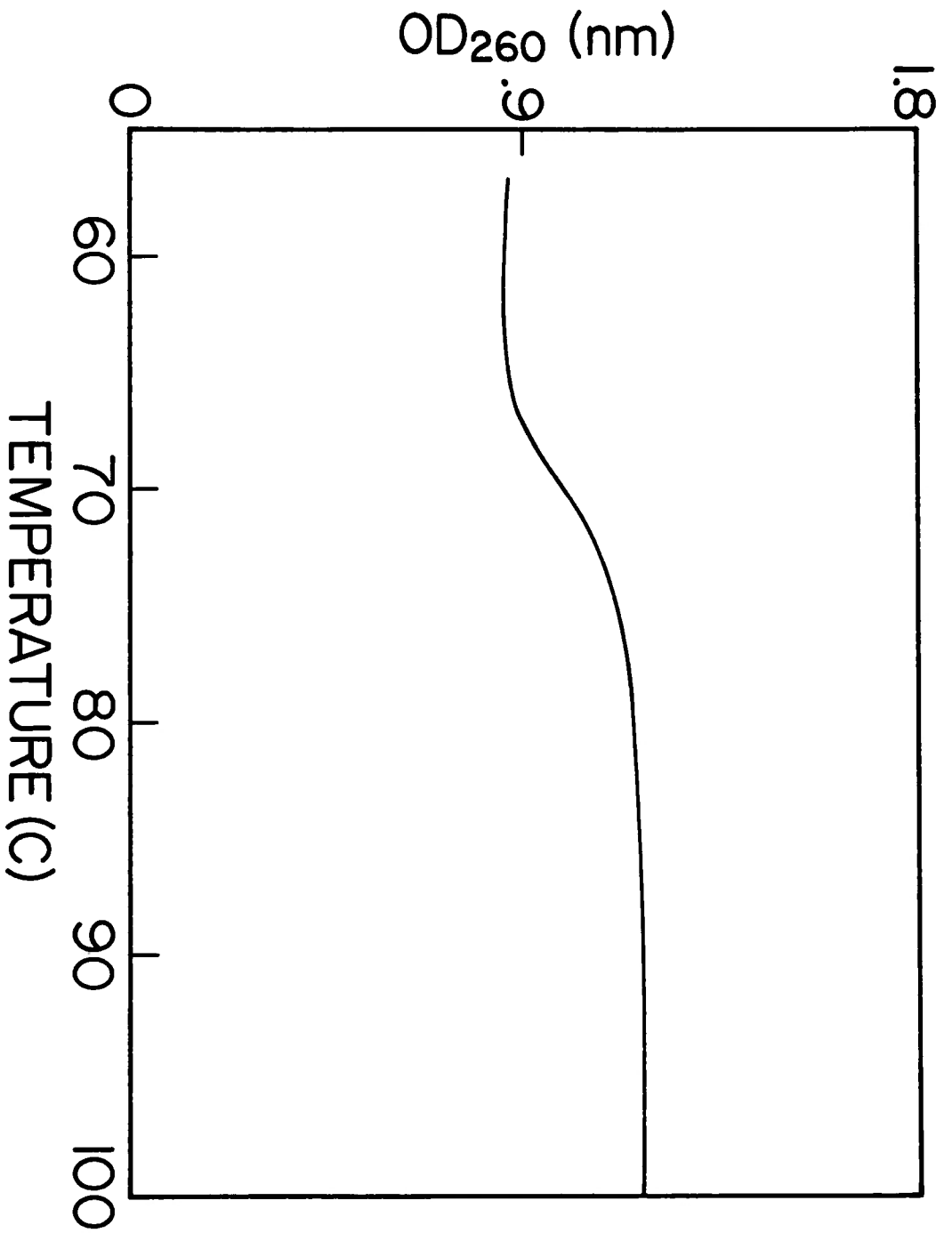
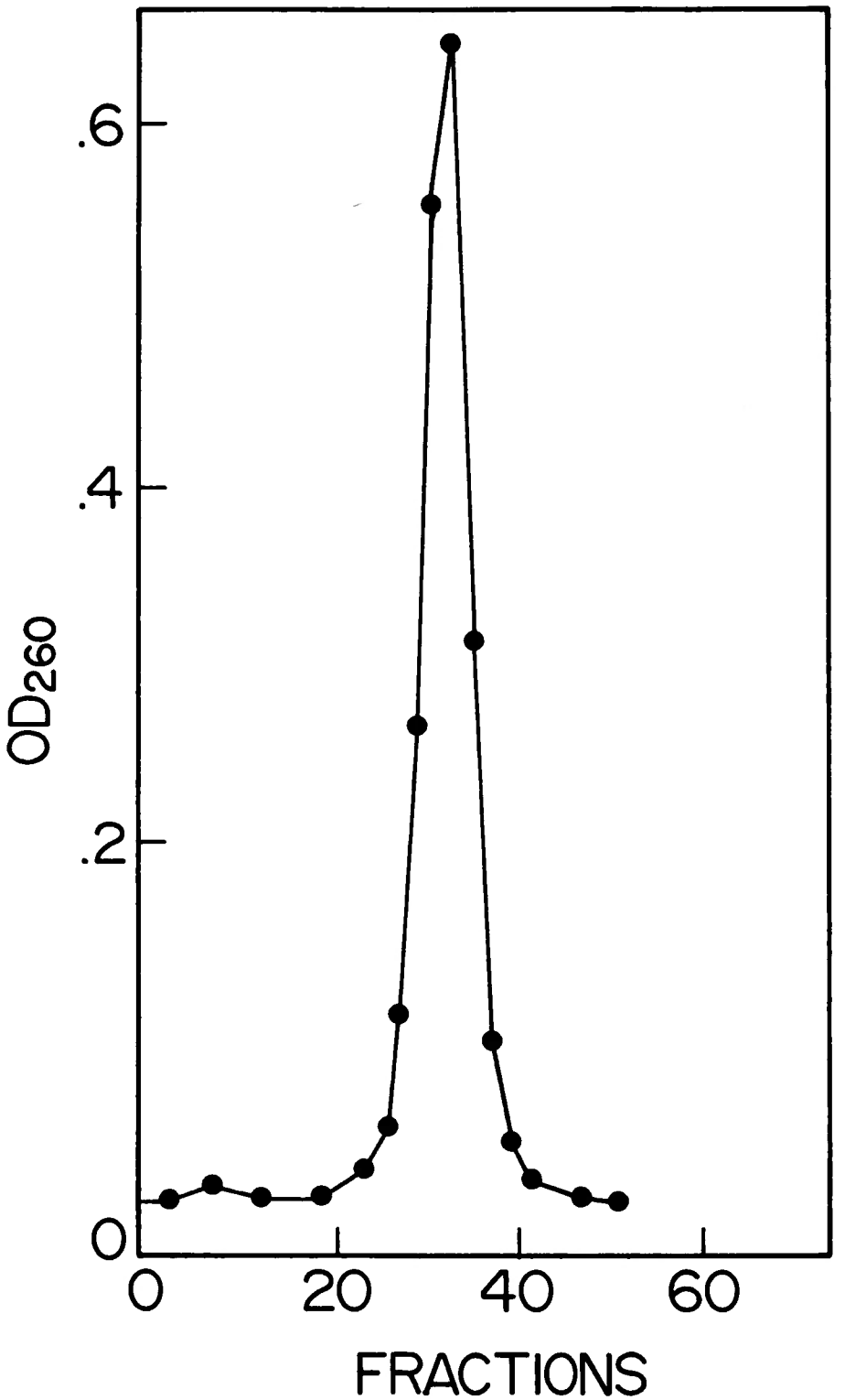

OD260 (nm)
0
.9
1.8
60
70
80
90
100
TEMPERATURE (C)

Fig. 6. Optical density profile of bobwhite quail total DNA centrifuged to equilibrium in a Hg^{++}-Cs_2SO_4 gradient in the preparative ultracentrifuge. The DNA was titrated with $HgCl_2$ to obtain a Hg^{++}/DNA-PO_4 molar ratio of 0.10 and centrifuged in Cs_2SO_4 as described in Materials and Methods.


.6
.4
.2
0
OD260
0
20
40
60
FRACTIONS

Ag^+-Cs_2SO_4 Density Gradients

Density gradient ultracentrifugation and potentiometric binding studies of Jensen and Davidson (1966) showed that GC-rich DNA's bind silver ion more strongly than AT-rich ones, and at low Ag^+/DNA-PO_4 ratios denatured DNA binds more strongly than native DNA. In view of these findings, bobwhite quail DNA was dissolved in Cs_2SO_4 buffered with 0.005 sodium borate, pH 9.1, by gently stirring on a magnetic stirrer and Ag_2NO_3 (0.001 M) was added dropwise to obtain different molar ratios of 0.10, 0.35 and 0.40.

The density of each gradient was 1.500 g/ml. After stirring for at least 1 hr, the DNA-Cs_2SO_4 sample was centrifuged for 40 hr at 50,000 RPM in an ultracentrifuge. The results are shown in Figs. 7, 8 and 9. These results reveal that Rf (ratio of Ag^+/DNA-PO_4) values other than 0.10, 0.35 and 0.40 are necessary to depict the satellites.

Moving Boundary Sedimentation

The purpose of the moving boundary sedimentation analysis was to determine the molecular weight of the DNA. The molecular weight of the total DNA in the bobwhite quail was 10×10^6 daltons. The single stranded DNA had a molecular weight of 5×10^6 daltons (data not shown).

Size Distribution of Lambda DNA after Digestion with Restriction Enzymes

The size of restriction fragments of Lambda (λ) DNA after digestion with endonucleases, EcoR 1 and Hind 111, was determined by agarose gel electrophoresis

Fig. 7. Preparative Ag^+-Cs_2SO_4 gradient of bobwhite quail total DNA. The DNA was titrated with $AgNO_3$ to obtain a Ag^+/DNA-PO_4 molar ratio of 0.1 and centrifuged in Cs_2SO_4 for 40 hr at 50,000 RPM. The density of the gradient was 1.500 g/ml. After centrifugation, six drop fractions were collected and the fraction number plotted against the optical density (260 nm) of each fraction.

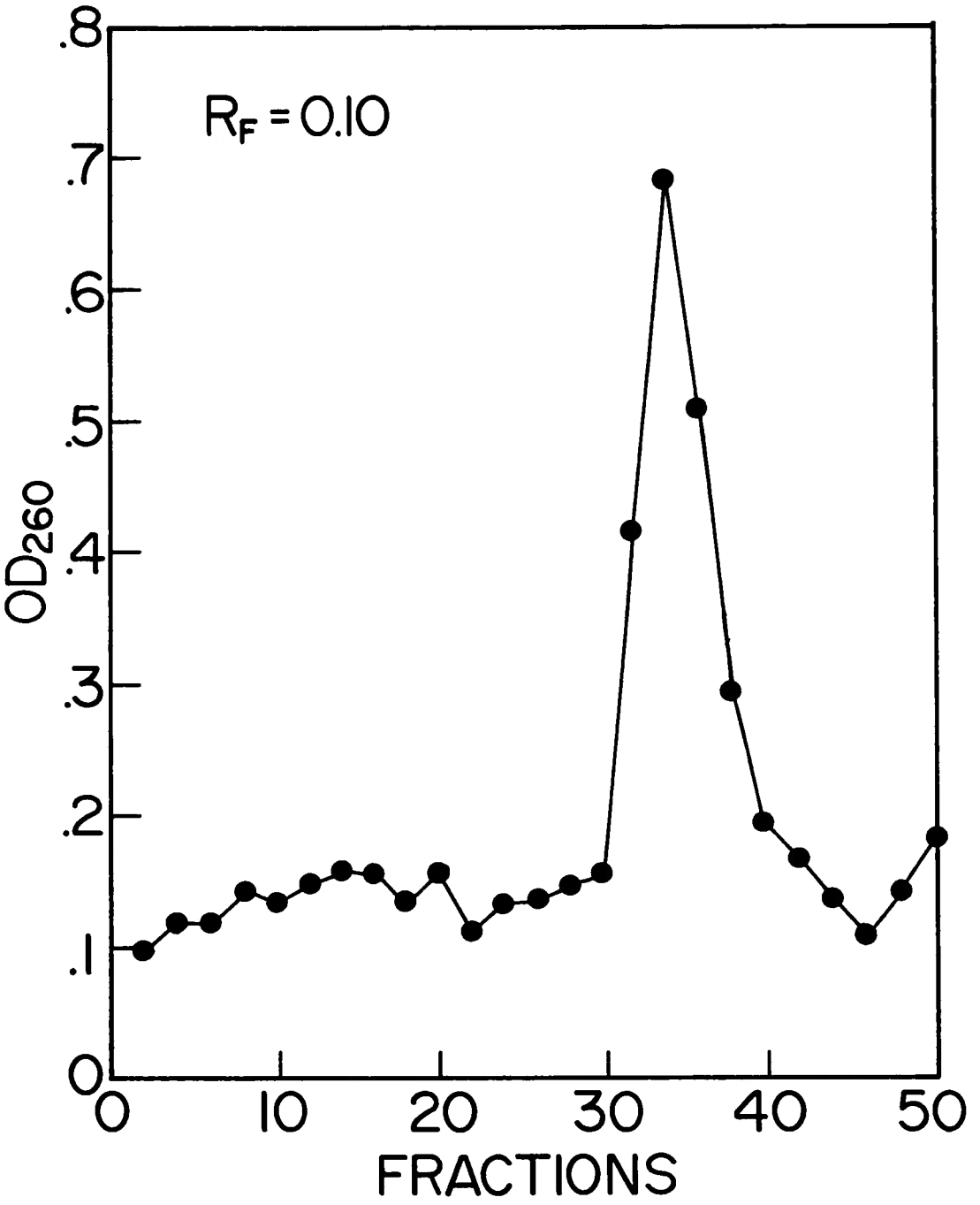

R_F = 0.10
OD_{260}
.8
.7
.6
.5
.4
.3
.2
.1
0
0
10
20
30
40
50
FRACTIONS

Fig. 8. Optical density profile of bobwhite quail total DNA centrifuged to equilibrium in a Ag^{+}-Cs_2SO_4 gradient in the preparative ultracentrifuge. The DNA was titrated with $AgNO_3$ to obtain a Ag^{+}/DNA-PO_4 molar ratio of 0.35 and centrifuged in Cs_2SO_4 as described in Materials and Methods.

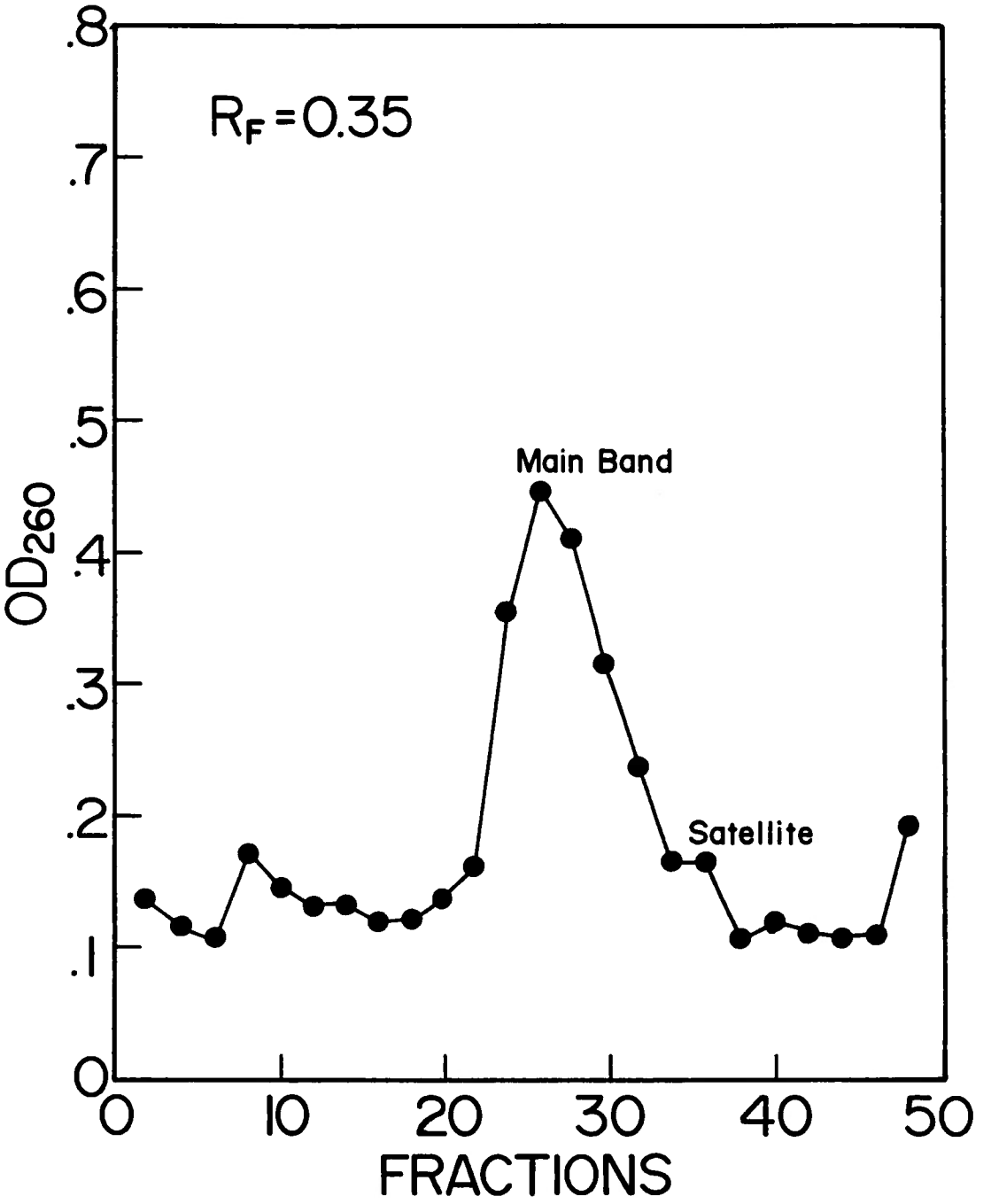
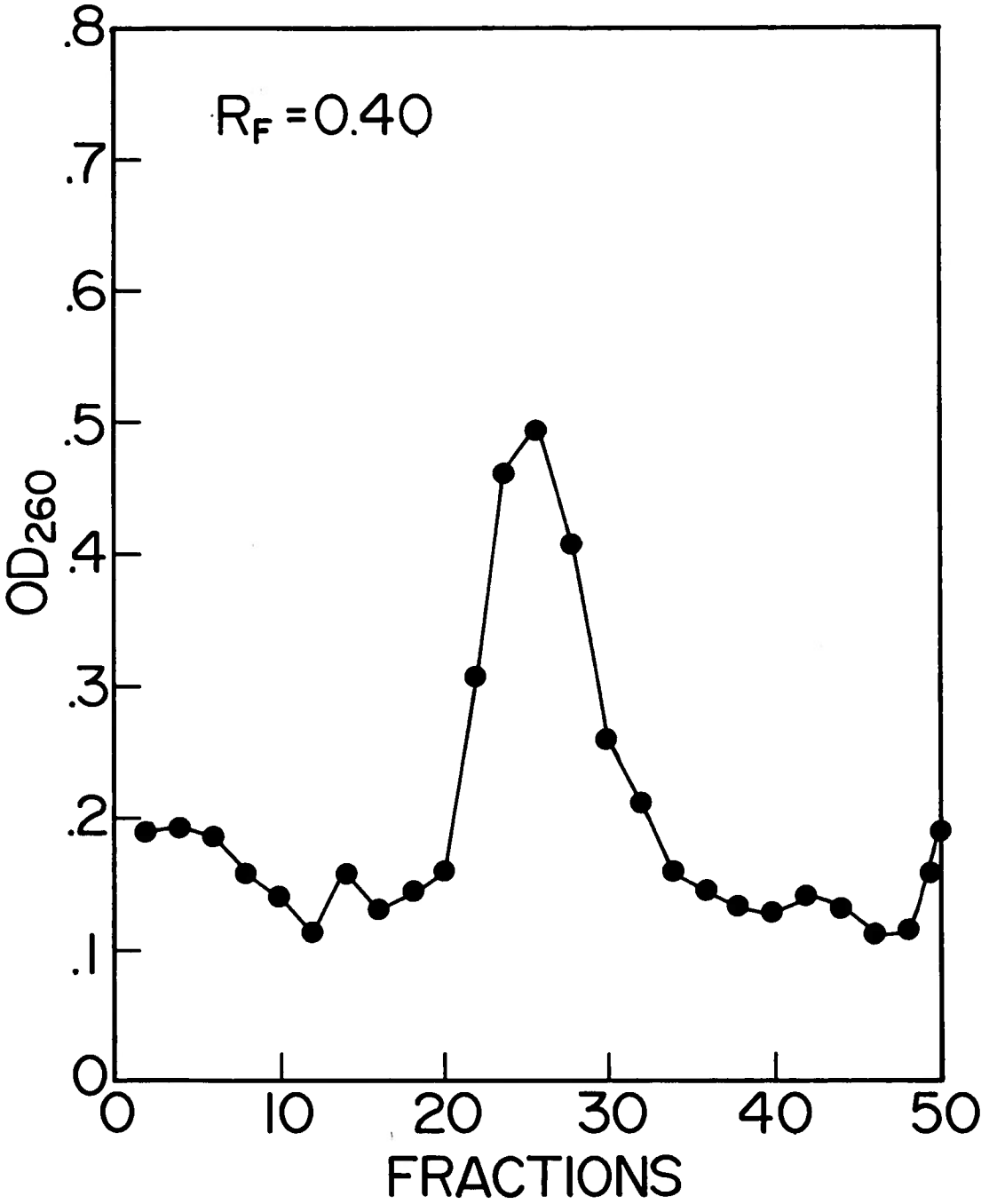

.8
.7
.6
.5
.4
.3
.2
.1
0
R_F = 0.35
Main Band
Satellite
OD_{260}
0
10
20
30
40
50
FRACTIONS

Fig. 9. Total bobwhite quail DNA centrifuged to equilibrium in a Ag^+-Cs_2SO_4 gradient. The DNA was titrated with $AgNO_3$ to obtain a Ag^+/DNA-PO_4 molar ratio of 0.40 and centrifuged in Cs_2SO_4 as described in Materials and Methods.


.8
.7
.6
.5
.4
.3
.2
.1
0
R_F = 0.40
OD_{260}
0
10
20
30
40
50
FRACTIONS

as described in the legend to Fig. 10. The results of Hind 111 on λ DNA and the addition of more endonuclease and further incubation did not alter the profile (Fig. 10). This indicated that the bands shown are terminal digests. However, there were occasions in which the digestion was not to completion. In all of these experiments (Fig. 10) the concentration of agarose was sufficiently high enough to retain all of the fragments on these gels. Mobility of the fragments was inversely related to the log of the molecular weight of the fragments. Some of the fragments were not explicitly resolved but migrated at a limiting mobility. The results obtained here are consistent with previous finding indicating that Hind 111 cleaves λ DNA six times, producing seven discrete fragments which were resolved on 0.85-1% agarose (Fig. 10). These fragments have molecular weights of 14.6, 5.84, 4.05, 2.67, 1.40, 1.21 and 0.34 x 10^6 daltons respectively and correspond to kilobase - pair lengths of 24.3, 9.0, 6.8, 4.5, 2.3, 2.0 and 0.6, respectively (Table 1, Fig. 10) Robinson and Landy, 1977 .

In contrast, Hind 111 digested quail DNA migrated in a broad distribution, ranging in molecular weights from 14.6 to 0.44 x 10^6 daltons (Fig. 11) and fragment lengths of 24.3 to 0.73 kilobase pairs (Table 2). Several regularities or bands were observed in this pattern and were detected in the low molecular weight fragment range (Fig. 11). These bands were detected better in the densitometric profile than in the ethidium bromide stained gels (Fig. 12). However, the bands are discernible in the gels and represent a small percentage of the total digest (Table 2). Some of the restricted quail DNA remained at the limiting mobility, indicating lengths greater than 24 kilobase pairs. Knowing the molecular weight of the λ fragments, it was possible to construct a calibration curve of log molecualr weight versus mobility. This curve was used to assign molecular weight values to each fragment

Fig. 10. Agarose gel electrophoresis of λ DNA digested with Hind 111. One microgram of DNA was incubated with 1 unit of Hind 111 in 50 µl buffer for 16 hr at 37 C, intercalated with ethidium bromide and electrophoresed horizontally for 4-6 hr in 0.85-1% agarose concentration as described in Materials and Methods.

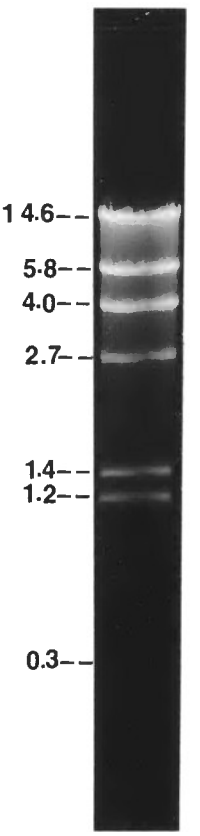

14.6
5.8
4.0
2.7
1.4
1.2
0.3

Table 1. Effect of restriction endonucleases on Lambda (λ) DNA.

Endonuclease	Fragments			Molecular Weights ($x10^6$ daltons)			Kilobase-Pairs		
EcoR 1		1	2		13.7	4.67		22.8	7.8
		3	4		3.71	3.57		6.2	6.0
		5	6		3.04	3.57		5.1	3.3
Hind 111		1	2		14.6	5.84		24.3	9.0
		3	4		4.05	2.67		6.8	4.5
		5	6		1.40	1.21		2.3	2.0
			7			0.34			0.6
Hind 111	1	2	3	21.8	5.24	5.05	36.33	8.73	8.42
+	4	5	6	4.21	3.38	1.96	7.02	5.63	3.27
	7	8	9	1.91	1.62	1.32	3.18	2.70	2.20
EcoR 1	10	11	12	0.93	0.88	0.59	1.55	1.47	0.98
			13			0.10	1.47	0.98	0.17

Fig. 11. Hind 111 digested bobwhite quail DNA. Four micrograms of quail DNA in 50 µl buffer were digested for 16 hr with 8 units of Hind 111. The fragments were separated on a 1% agarose horizontal slab gel (Tris-borate buffer) by 4-6 hr of electrophoresis, stained with ethidium bromide and photographed. (A) Lambda DNA; (B) quail DNA.

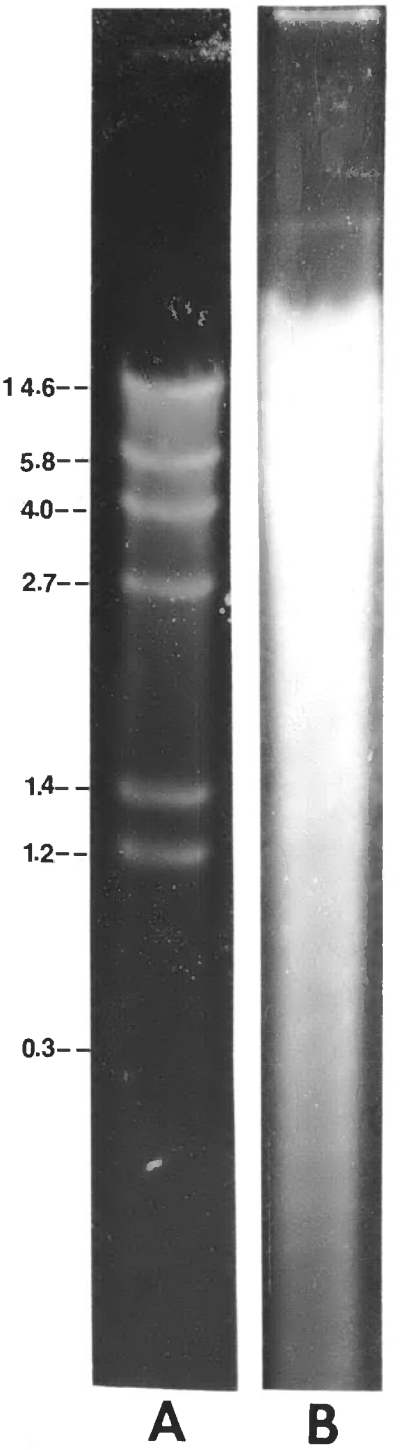
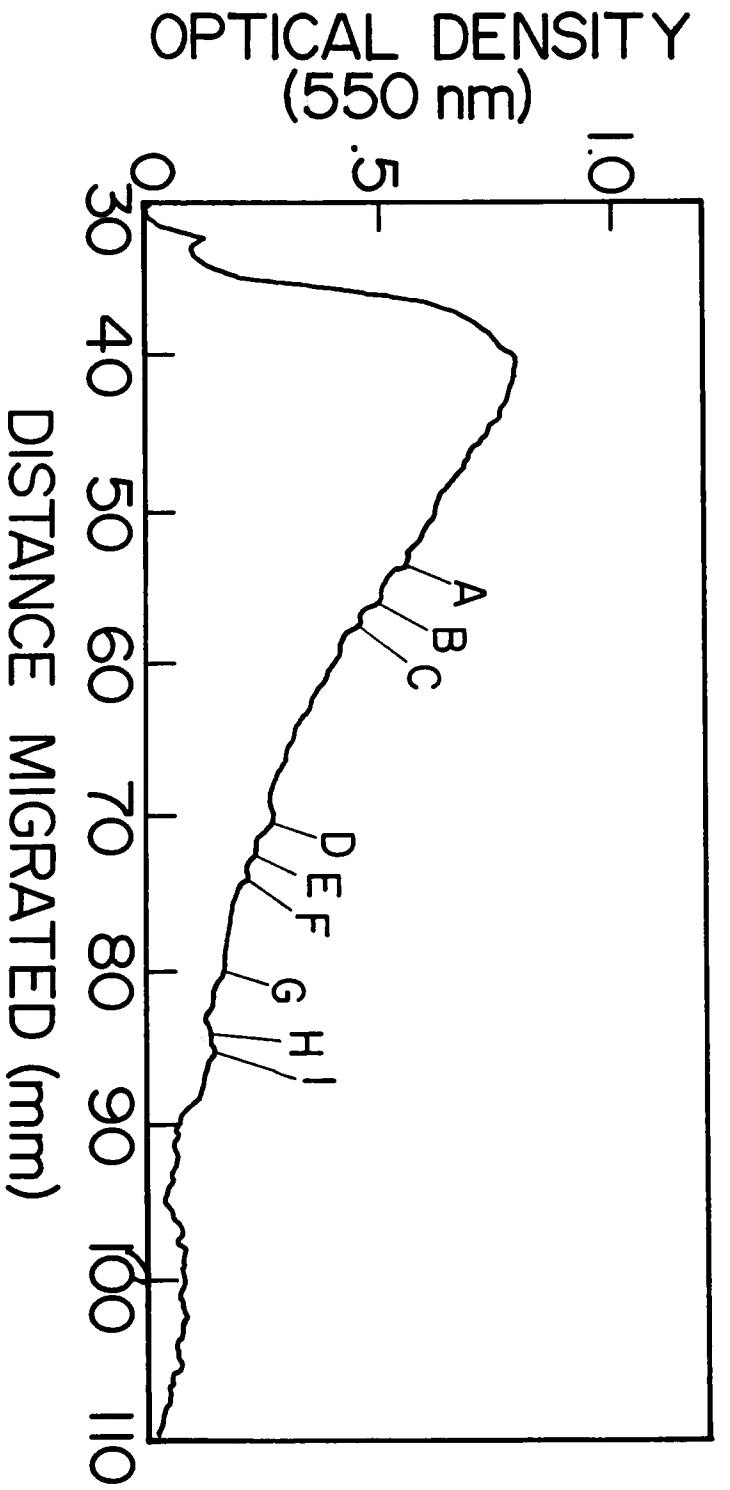

14.6
5.8
4.0
2.7
1.4
1.2
0.3
A
B

Table 2. Molecular weight, percent of DNA and buoyant density of quail DNA.

Fragment	Molecular Weight ($\times 10^6$ daltons)	Percent Total DNA	Buoyant Density[a]	Base-Pairs
A	2.87	0.014	1.680-1.718	4783
B	2.46	0.013	1.680-1.718	4100
C	1.26	0.015	1.680-1.718	2100
D	1.12	0.025	1.680-1.718	1867
E	0.94	0.023	1.680-1.718	1567
F	0.80	0.019	1.680-1.718	1333
G	0.72	0.013	1.680-1.718	1200
H	0.50	0.009	1.680-1.718	833
I	0.44	0.019	1.680-1.718	733

[a]Region of gradients pooled.

Fig. 12. Densitometric tracing of total bobwhite quail DNA. The negative from the electrophoretic profile (Fig. 11) of Hind 111 digested quail DNA was scanned with visible light (ohm) using the Beckman DU-8 Spectrophotometer. The baseline was chosen to give the minimum value of each peak.


OPTICAL DENSITY
(550 nm)
0
.5
1.0
30
40
50
60
70
80
90
100
110
DISTANCE MIGRATED (mm)
A
B
C
D
E
F
G
H
I

falling between the largest and smallest visible frament. Typical results of these experiments are shown in Fig. 13.

Some of the EcoR 1 terminal digests of bobwhite quail DNA contain a large proportion of high molecular weight fragments. Such high molecular weight fragments were not found in digests of λ DNA. The spared quail fragments were shown to be terminal digests products by three tests. First they were present in digests in which admixed DNA was completely cleaved (Fig. 14). Secondly, the amount of DNA remaining at the limiting mobility was constant over a wide range of endonuclease to DNA ratios and incubation times. In order to accurately determine segment size distributions and molecular weights, quail DNA was mixed with λ DNA, digested with a restriction enzyme and electrophoresed through the appropriate concentration of agarose (Fig. 14). The gels were stained with ethidium bromide and photographed to determine the migration rate of the λ DNA. In all cases, the pattern of λ segments or fragments was characteristic of terminal digests. These types of experiments also indicated whether the endonucleases were active. If fragments or smears were not detected, this was interpreted to mean that the restriction sites for the endonuclease were not present or that the nucleotides in the sequence may be methylated, thus inhibiting the enzyme activity. Figure 14 illustrates the restriction pattern obtained when λ and quail DNA were mixed and digested for 16 hr with EcoR 1. Most of the λ fragments were observed in the photograph (Fig.14) and in the absorption profile. Thirdly, the fragments isolated by preparative gel electrophoresis were resistant to further digestion with the same restriction enzyme, even though admixed λ DNA was completely cleaved. Restriction endonuclease, EcoR 1, in high salt, cleaved

Fig. 13. Calibration curve of bobwhite quail DNA (*—*) digested with Hind 111. A calibration curve of log molecular weight versus mobility was constructed from the molecular weight of the λ (• —•) fragments which were generated by digestion with Hind 111. Bobwhite quail DNA was digested for 24 hr.

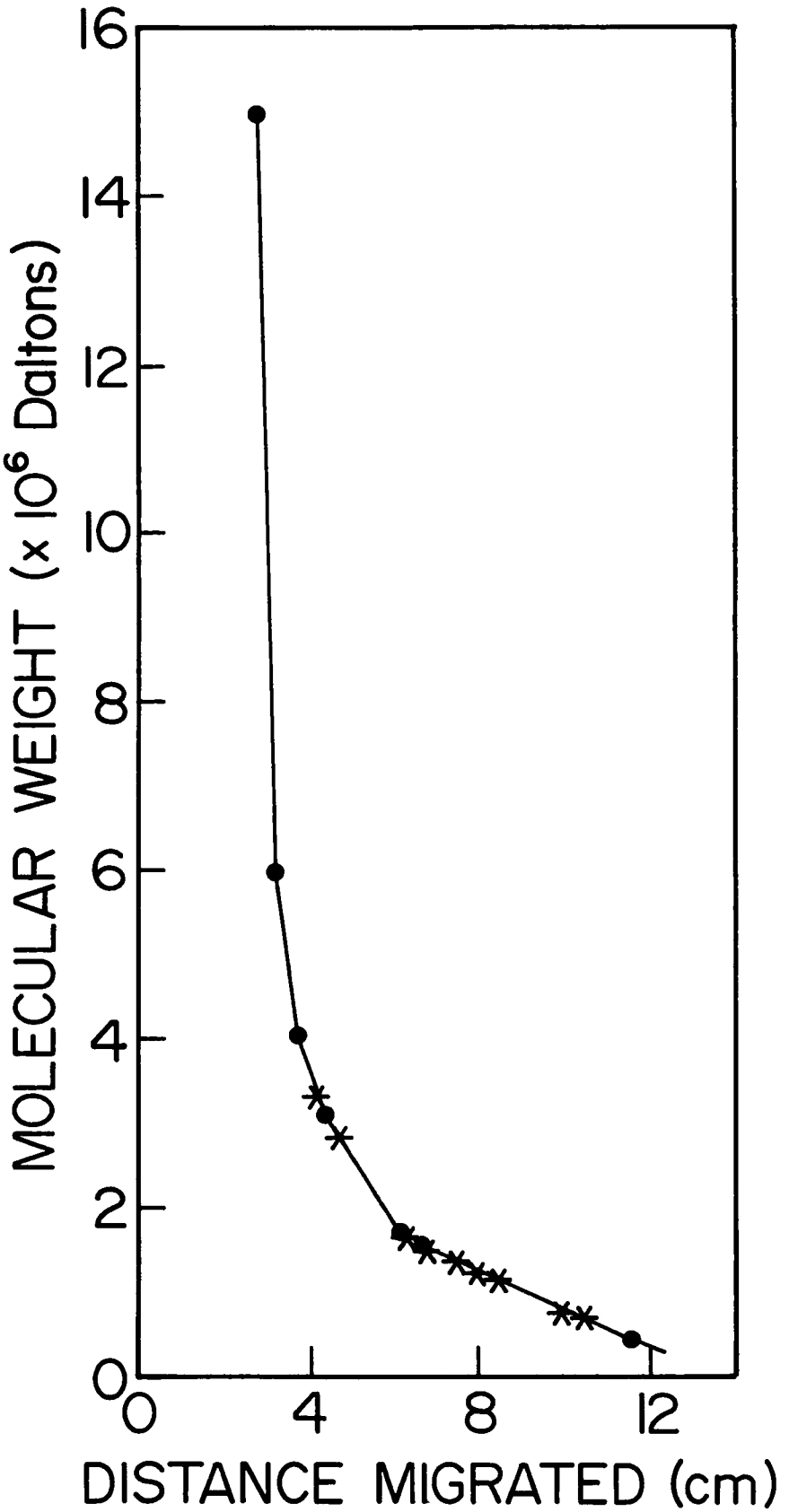
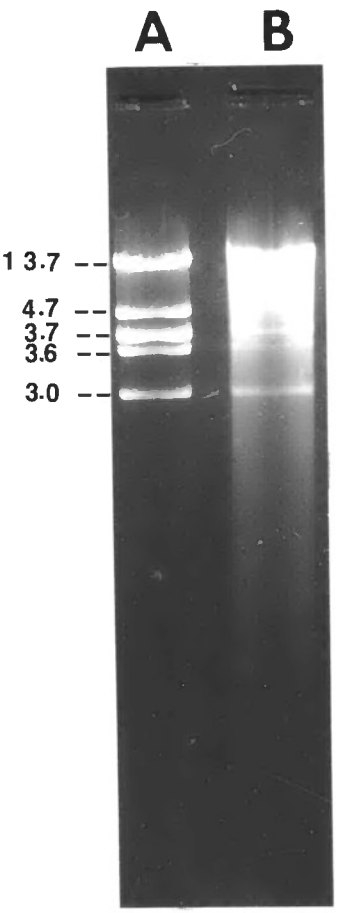

16
14
12
10
8
6
4
2
0
MOLECULAR WEIGHT (x 10⁶ Daltons)
0
4
8
12
DISTANCE MIGRATED (cm)

Fig. 14. Digestion of λ DNA with EcoR 1 in the absence (A) and presence (B) of total bobwhite quail DNA. One microgram of λ DNA was mixed with 1 μg of bobwhite quail DNA, 2 units of EcoR 1, digested for 16 hr, electrophoresed and photographed.


A
B
13.7
4.7
3.7
3.6
3.0

DNA five times producing six fragments. However in some experiments the digestion did not go to completion and only five fragments were observed when electrophoresed through agarose. The terminally digested fragments have molecular weights of 13.7, 4.67, 3.71, 3.57, 3.04 and 2 x 10^6 daltons. These molecular weights correspond to fragments of 22.8, 7.8, 6.2, 6.0, 5.1 and 3.3 kilobase pairs in length.

In low sodium salt, the digestion of λ with EcoR 1, produced approximately twenty bands which indicated the loss of specificity of the enzyme. This type of activity has been referred to as EcoR 1* (star activity) and the nucleotide sequence recognized by the enzyme is $(^{AATT}_{TTAA})$ instead of the sequence, $(^{GAATTC}_{CTTAAG})$ (Fig. 15). The refractive index of all enzyme buffers was carefully monitored during this investigation and fresh buffers were prepared when necessary. When λ DNA was digested with EcoR 1 for 16 hr ethanol precipitated, redissolved in buffer and treated with Hind 111 for an additional 16 hr, 13 discrete fragments were observed when electrophoresed through agarose (Table 1). These fragments have molecular weights of 21.8, 5.24, 5.05, 4.21, 3.38, 1.96, 1.91, 1.62, 1.32, 0.93, 0.88, 0.59 and 0.10 x 10^6 daltons, respectively. These molecular weights respectively correspond to fragment lengths of 36.33, 8.73, 8.42, 7.02, 5.63, 3.27, 3.18, 2.70, 2.20, 1.55, 1.47, 0.98, and 0.17 kilobase-pairs. The molecular weights of these 13 fragments have been confirmed by Robinson and Landy, 1977. The molecular weights of the fragments were used for confirming the molecular weight of the quail low molecular weight fragments.

High molecular weight bobwhite quail DNA was digested with other endonucleases (Table 3). Discrete fragments were not detected when the DNA was electrophoresed through agarose. However, an analysis of the restriction

Fig. 15. Lambda DNA digested with EcoR 1. Approximately twenty bands were produced which indicates the loss of specificity of the enzyme in a low sodium buffer.

Table 3. Effect of other restriction endonucleases on bobwhite quail DNA.

Enzyme	Sequence Recognized	Smear Digestion	Molecular Weight range ($\times 10^6$)
Hpa 11	C↓CGG	LMW Smear	$6 \times 10^6 - 2 \times 10^6$
Bam 1	G↓GATCC	Smear	$6 \times 10^6 - 2 \times 10^6$
Hpa 1	GTT↓AAC	Smear	$6 \times 10^6 - 2 \times 10^6$
Bgl 11	A↓GATCT	Smear	$6 \times 10^6 - 2 \times 10^6$
Alu 1	AG↓CT	Smear	$6 \times 10^6 - 1 \times 10^6$
Hae 111	GG↓CC	Smear	$6 \times 10^6 - 1 \times 10^6$
Hha 1	GGG↓C	Smear	$6 \times 10^6 - 1 \times 10^6$
Mbo 1	GATC	Smear	$6 \times 10^6 - 1 \times 10^6$
EcoR 11	CC($\frac{A}{T}$)GG	Smear	$6 \times 10^6 - 1 \times 10^6$
EcoR 1	G↓AATTC	Smear	$6 \times 10^6 - 2 \times 10^6$

fragments, for each digest, revealed a bimodal size distribution. Most of the fragments in the digest had a broad range of lengths (smear). The remainder of the fragments were extremely long and migrated at the top of the gels.

Reassociation Kinetics

An Analysis of Bobwhite and Pharoah Quail DNA Binding at "Zero Cot"

Nuclear DNA, tritium labeled, using E. coli DNA polymerase I to a specific activity of 10^5 CPM/μg, and unlabeled nuclear DNA were sheared to various lengths in a Virtis 60 homogenizer and size fractionated on isokinetic alkaline sucrose gradients (Noll, 1967; Davidson et al., 1973). Moving boundary sedimentation and gel electrophoresis were also used for size fractionation. In alkaline sucrose gradients the sheared DNA was distributed over several fragment sizes (multimodal distribution), with the size of the molecules dependent upon the speed of the homogenizing blade and length of time of shearing (Table 4). The concentration of salt also affected the size of the sheared fragments.

The Gaussian portion from each gradient was pooled and recentrifuged in an isokinetic alkaline sucrose gradient to determine the homogeneity of the fragments used for reassociation. The fractions pooled after the second centrifugation were very homogeneous in length, as determined by gel electrophoresis (Fig. 16).

In order to determine if "Zero Cot binding" DNA exist in the bobwhite quail genome and how it is arranged, tritium labeled samples were used. When the DNA at extremely low concentration (0.1 - 0.5 μg/ml) was heated to 100 C in 0.12 M phosphate buffer (PB), cooled rapidly in a dry ice ethyl alcohol bath and passed over hydroxyapatite (HAP), a fraction remained bound to the HAP. This fraction of

Table 4. Shearing conditions and size of fragments.

Shearing Conditions	Analytical Ultracentifugation		Alkaline Sucrose Gradients		Gel Electrophoresis		
	S^{o}_{20w}	Single Stranded Length[b]	S^{o}_{20w}	Single Stranded Length[a]	Mol. Wt.	Single Stranded Length[a]	Average Single Length[d]
Sonication (10 min)	4.7 4.6	260 230	4.8 4.6	280 240			250
Sonication (6 min)	6.6 6.6 6.9	520 520 590	6.7 6.4	540 500			530
Sonication (5 min with 15 sec burst)							

Table 4. Continued.

Shearing Conditions	Analytical Ultracentifugation		Alkaline Sucrose Gradients		Gel Electrophoresis		
	S^{o}_{20w}	Single Stranded Length[b]	S^{o}_{20w}	Single Stranded Length[a]	Mol. Wt.	Single Stranded Length[a]	Average Single Length[d]
Sonication (1 min)	9.9 10.4	1450 1650	11.2	1840			1650
Sonication (15 sec)	11.5 11.3	2100 2000					2050
Virtis (25 min)	13.3	2900					3000
8,000 $\frac{revs}{min}$	13.7	3100					

Table 4. Continued.

Shearing Conditions	Analytical Ultracentifugation		Alkaline Sucrose Gradients		Gel Electrophoresis		
	S^{o}_{20w}	Single Stranded Length[b]	S^{o}_{20w}	Single Stranded Length[a]	Mol. Wt.	Single Stranded Length[a]	Average Single Length[d]
Virtis (25 min)	17.1	5000		1550			4360
5,400 $\frac{revs}{min}$	14.5 15.2	3500 4000					
25 gauge syringe	22.5 19.8 22.0	11400 8270 10800	17.8	6320			9210
Virtis (30 min) 15,000 $\frac{revs}{min}$			8.5 9.2	990 1100	0.3×10^6	1000	1000

Table 4. Continued.

Shearing Conditions	Analytical Ultracentifugation		Alkaline Sucrose Gradients		Gel Electrophoresis		
	S^{o}_{20w}	Single Stranded Length[b]	S^{o}_{20w}	Single Stranded Length[a]	Mol. Wt.	Single Stranded Length[a]	Average Single Length[d]
Virtis (1 hr)			6.2	450	0.15×10^{6}	450	450
50,000 $\frac{revs}{min}$			6.2	460	0.16×10^{6}		
Virtis (25 min)			6.8	585	0.20×10^{6}	600	590
30,000 $\frac{revs}{min}$			6.9	590			
Virtis (30 min)							
5,000 $\frac{revs}{min}$[c]			11.3	2000	0.6×10^{6}	2000	2000

Table 4. Continued.

Shearing Conditions	Analytical Ultracentifugation		Alkaline Sucrose Gradients		Gel Electrophoresis		
	S^{o}_{20w}	Single Stranded Length[b]	S^{o}_{20w}	Single Stranded Length[a]	Mol. Wt.	Single Stranded Length[a]	Average Single Length[d]
Omnimixer[e]					0.58×10^{6}	1989	

[a]Markers for alkaline sucrose gradients were unlabeled 450 bobwhite DNA. Fragments determined by agarose gel electrophoresis.

[b]Fragment length in nucleotides.

[c]DNA sheared with Virtis homogenizer without glycerol.

[d]Single stranded molecular weights given.

[e]Fragments generated with the Sorvall Omnimixer were about 1660 NTL for shearing time of 5 min - 1 hr. These results are not given in text.

Fig. 16. Profile illustrating the homogeneity of sheared DNA. Total DNA was sheared for 1 hr at 50,000 rpm with the Virtis homogenizer, concentrated and electrophoresed through 1% agarose to determine its homogeneity and size.

DNA is called "Zero Cot binding" DNA. The binding of this DNA is assumed to be due to snap back or intramolecular reassociation of complementary regions within the DNA strand and not intermolecular reassociation (reassociation of complementary or homologous strands). The fraction of DNA which bound to the HAP was dependent upon the length of the fragments used. The results from the "Zero Cot binding" studies are depicted in Fig. 17 and Table 5. About 10-15% of the DNA bind to HAP when 250-450 nucleotide long fragments are reassociated. Approximately 15% was bound for fragments 450 nucleotides long. If the arrangement of the complementary regions in the "hair pin" loops is closely spaced, there should be a linear relationship between the fraction of DNA binding at Zero Cot and the length of the fragment. Figure 17 indicates that as the fragment length increases the rate and amount of DNA bound at Zero Cot also increase. The increase in bound DNA is linear from 450 nucleotide long fragments up to fragments 2000 nucleotides long. These finding indicate that in molecules longer than 450 nucleotides, the snap back sequences have a spacing greater than 400-500 nucleotides long. The fraction bound is linear up to 2000 nucleotide long fragments and in fragments greater than 2000, the amount of DNA bound is proportional to the fragment length. These results indicated that the majority of the sequences which are involved in the "Zero Cot binding" DNA are spaced in the DNA at intervals approximately 500-1000 nucleotides apart.

The results in Fig. 17 indicate that approximately 31% of the DNA was bound when fragments of 2000 nucleotides long were passed over HAP at "Zero Cot." To indicate that the "Zero Cot binding" DNA was a specific class of molecules and not just non-specifically bound fragments, the following experiments were carried out.

Fig. 17. A profile showing that the amount of DNA binding to HAP is a function of fragment length.

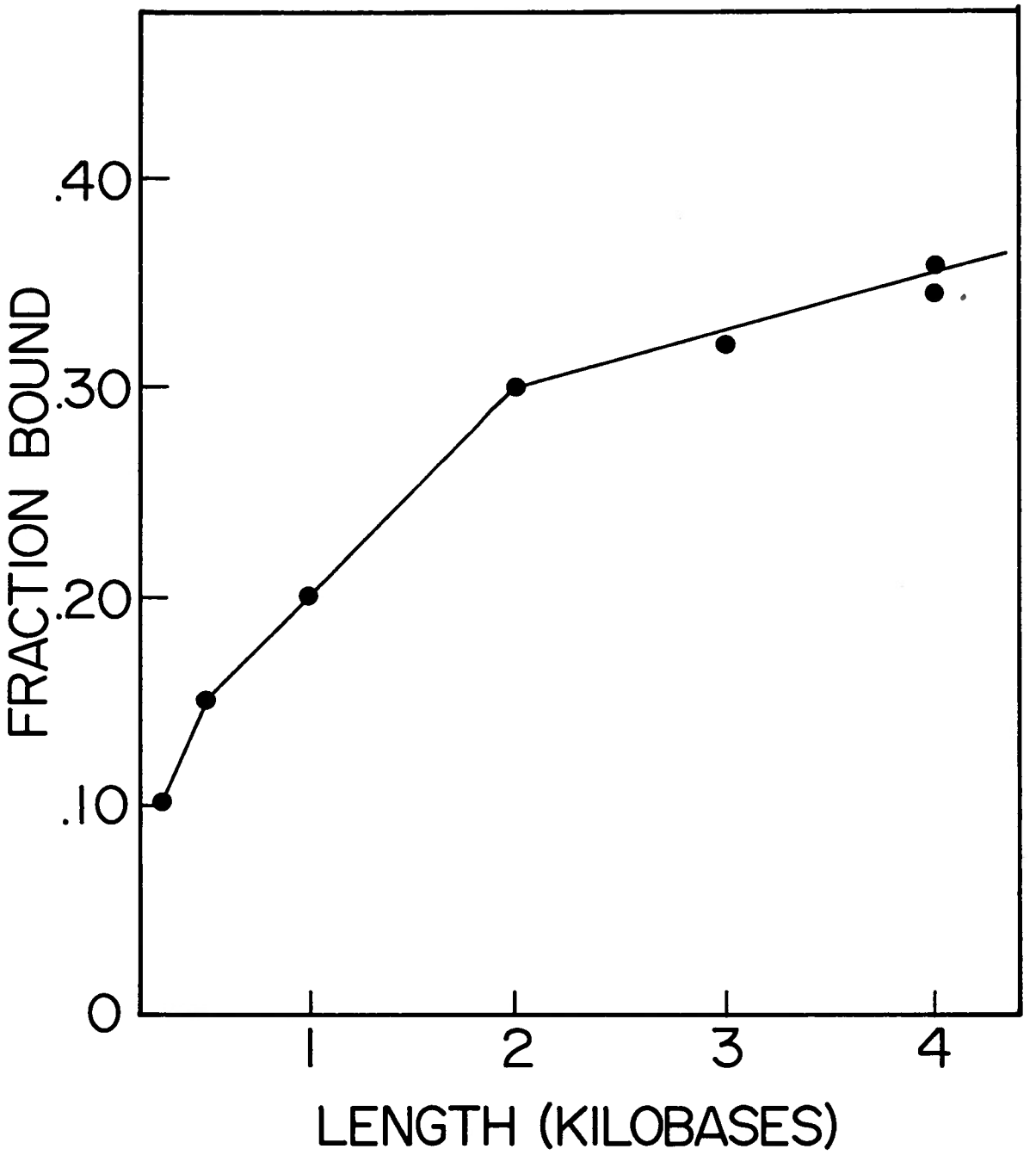

.40
.30
.20
.10
0
FRACTION BOUND
1
2
3
4
LENGTH (KILOBASES)

Table 5. Effect of length on the binding of Zero Cot binding DNA to HAP.

Fragment Length[a]	% Bound
250	0.10
450	0.15
1000	0.20
2000	0.31

[a]Fragment in nucleotides long.

Zero Cot binding fragments were passed over HAP and the bound fragments were eluted, denatured, frozen in dry ice-ethanol and passed over HAP again. Approximately 90% of the fragments 450 nucleotides long were rebound and about 80% of the fragments 2000 nucleotides long were bound by HAP. The difference in the binding and recovery of the short and long fragments was attributed to shearing during the purification and chromatographing of the molecules. It is also assumed that the stripped DNA did not contain any irreversibly binding sequences.

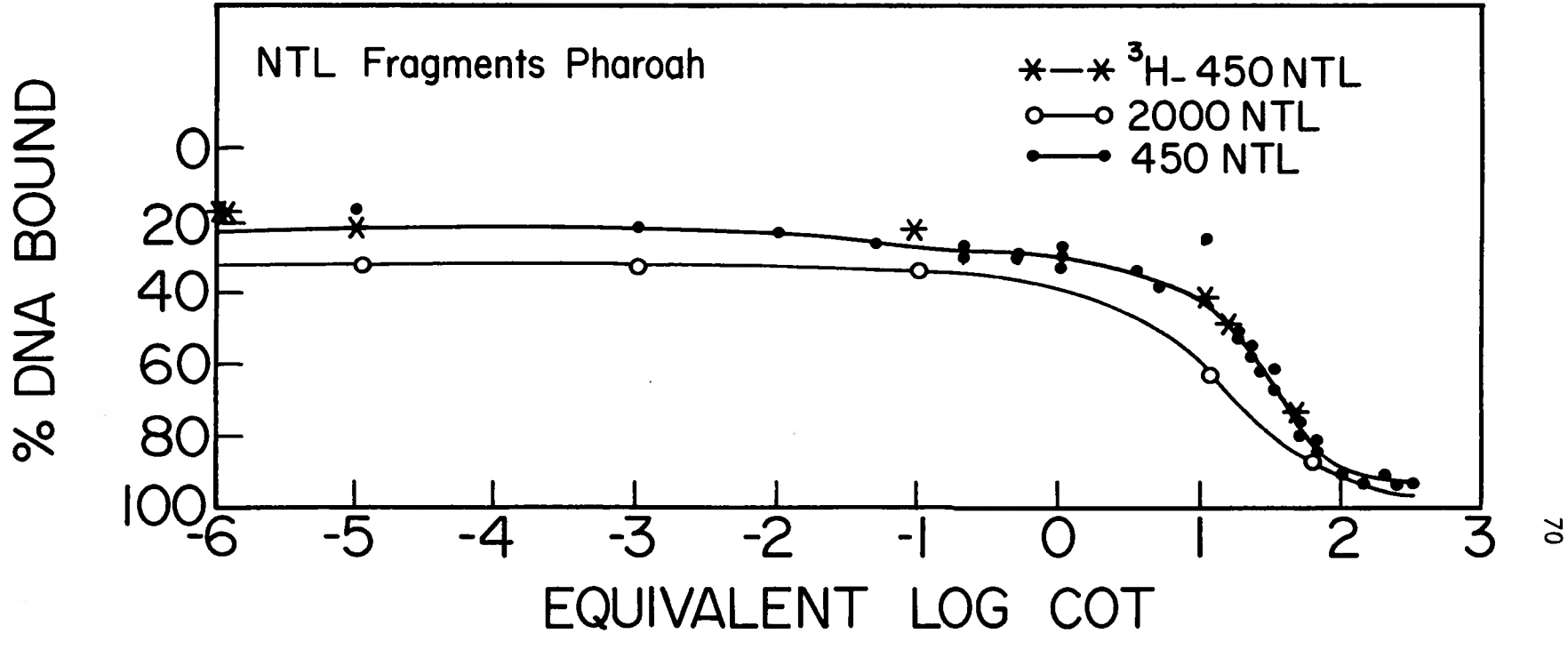
A comparative study of the Pharoah quail genome under the same experimental conditions as above, indicated that approximately 15% of the 450 nucleotide long fragments and 30-35% of the 2000 NTL fragments were bound at "Zero Cot" values (Fig. 18). These data suggest that the arrangement of the "Zero Cot binding" sequences is spaced (500-1000 NTL) very similar to those of the bobwhite quail. When the data were plotted, a linear relationship was observed between the amount of DNA bound at "Zero Cot" and the fragment length up to 2000 NTL. These data indicate that the "Zero Cot binding" fragments are precisely ordered in their spacing and are not randomly spaced throughout the genome.

DNA-DNA Reassociation Kinetics of the Bobwhite and Pharoah Quail Genomes

Reassociation Kinetics of Bobwhite Quail DNA (450 NTL)

In order to determine the arrangement of nucleotide sequences in the genomes of the bobwhite and Pharoah quails, DNA-DNA reassociation techniques

Fig. 18. Reassociation of total Pharoah quail DNA. The fraction of the DNA fragments containing duplex regions was assayed by hydroxyapatite binding. The closed circles are the 450 nucleotide long fragments and the asteriks (*) represent the tritium (^{3}H) labelled DNA. The curve is a least squares fit of second-order kinetic components to data.


NTL Fragments Pharoah
— ³H-450 NTL
o—o 2000 NTL
•—• 450 NTL
% DNA BOUND
0
20
40
60
80
100
-6
-5
-4
-3
-2
-1
0
1
2
3
EQUIVALENT LOG COT

were used. Figure 19 illustrates the data obtained when the DNA-DNA reassociation of bobwhite quail DNA (average length of 400 base pairs) was measured by hydroxyapatite chromatography and plotted as log equivalent Cot against % DNA bound to hydroxyapatite in the duplex form. The nuclear DNA was isolated from 12 day embryos by spinning the homogenates at 1,000 RPM for 10 min to pellet the nuclei. The nuclei were lysed in the DNA extraction buffer, extracted with phenol and/or chloroform-isoamyl alcohol and the DNA centrifuged in CsCl gradients. The DNA-DNA reassociation data obtained with DNA from isolated nuclei did not differ from the data obtained with whole cell DNA extracts. Therefore, most of the reassociation data were obtained using total DNA.

DNA (in 0.12 M PB) was denatured by heating for 3-5 min at 100 C, quickly cooled, reassociated at 60 C, passed over HAP in 0.12 M PB and the binding determined optically. The data representing the binding of duplex DNA to the HAP was used to generate a computer curve using three second-order kinetic components. The data in Figure 19 and Table 6 reflect the kinetic components in bobwhite quail DNA. These data were not corrected for a reduced rate of reassociation of quail DNA due to high A + T content (Wetmur and Davidson, 1968) because the satellite sequences are moderately G + C-rich, and that the A + T-rich mitochondrial DNA represents less than 0.05% of the total DNA.

As indicated in Table 6, the bobwhite quail DNA has a component of highly repetitive DNA which reassociated by Cot 0.1 and represents approximately 20% of the genome. A portion of this component may be attributed to a "Cot Zero time binding DNA" which is defined as that portion of the genome that will bind to HAP

Fig. 19. Hydroxyapatite reassociation kinetics of bobwhite quail (Colinus virginianus) DNA. Bobwhite quail DNA of fragment length 450 (closed circles) or 2000 (open circles) nucleotides were re-associated as described in Materials and Methods. The fraction of fragments containing duplex regions was measured at the indicated values of equivalent Cot by hydroxyapatite column chromatography. The profile represents values determined by optical density readings.

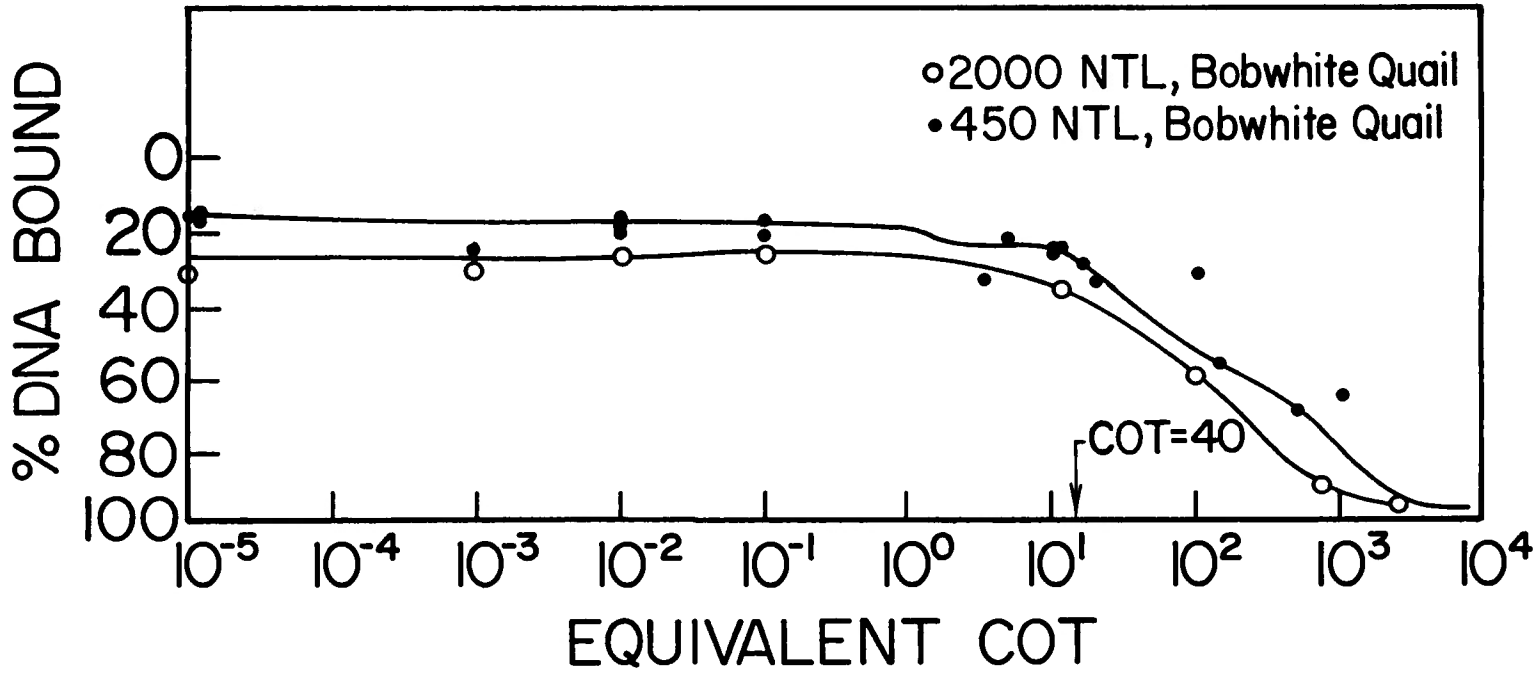

o2000 NTL, Bobwhite Quail
•450 NTL, Bobwhite Quail
% DNA BOUND
0
20
40
60
80
100
COT=40
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
EQUIVALENT COT

Table 6. Classification of bobwhite quail DNA repetition (450 NTL).

Class	Fraction of Total DNA	Observed Cot 1/2	Cot 1/2 Pure[++]	Kinetic Complexity	Mass per 1N[+++] genome (pg)	Copies per Genome
Highly Repetitive[+]	0.20	0.05	0.01	1.0×10^5	0.26	4000
Moderately Repetitive	0.10	5.0	0.50	5.4×10^5	0.13	400
Non-Repetitive	0.70	100.0	70.0	7.0×10^8	0.91	1

[++] Cot 1/2 Pure = Cot 1/2 Observed x Fraction of genome

[*] Kinetic Complexity (Base pairs) calculated by comparison of the rate of reassociation of each component of Bobwhite Quail DNA to that of E. coli (Cot 1/2 = 4.2, 2.7×10^9 daltons or 4.5×10^6 BP).

[+++] Haploid Genome size = 1.3 picograms, 1.3×10^{12} daltons, 2×10^9 Base pairs.

[+] Includes the Cot Zero time binding sequences and other palindromic sequences.

at a Cot < 10^{-4}. It is thought that the binding represents intramolecular duplex formation between palindromic sequences (Britten et al., 1974). Since there is an overlap between the Cot Zero time binding and highly repetitive sequences at Cot 10^{-4}, the results as presented are insufficient in totally separating the two kinetic components.

The second kinetic component, as demonstrated in Figure 19 and Table 6, the moderately repetitive sequences, reassociated with a Cot value of 10 and comprises 10% of the genome. From Figure 19 the moderately repetitive components are greater than 10% and this may be attributed to several factors since the kinetics are dependent upon reassociated fragments of 450 nucleotide length. As shown later in Figure 19, the moderately repetitive DNA is interspersed with the non-repetitive DNA, which means that some of the reassociated fragments in this kinetic component contain some of the non-repetitive DNA. The Cot value at which this kinetic component reassociated at a fragment length of 450 nucleotides, suggest that a very small amount of the non-repetitive DNA has reassociated and binds to HAP. The data for the moderately repetitive component have been confirmed by using the enzyme S1 nuclease. Based on the results from these experiments 10% is the most likely value for the moderately repetitive sequences and 30% representing the total repetitive component in the genome. All of the repetitive sequences in the bobwhite quail genome have reassociated by Cot 50.

The third kinetic component, the non-repetitive component, represents approximately 70% of the genome as determined by hydroxyapatite binding (Fig. 19). This component is assumed to be present only once per haploid genome. As

shown in Figure 19, some of the DNA failed to bind to hydroxyapatite even at a Cot of 10^3. The lack of binding may have been due to degradation during the long incubations or/and because the size and stability of some duplexes to remain bound to hydroxyapatite at 60 C or other criterion conditions.

Reassociation Kinetics of Pharoah Quail DNA (450 nucleotide length)

The genome of the Pharoah quail is organized similarly to that of the bobwhite quail as revealed by the binding of reassociated 450 nucleotide length fragments to hydroxyapatite. Figure 18 and Table 7 indicate the DNA sequence composition of the genome. In the genome, four distinct components were observed: (1) a zero Cot binding component; (2) a highly repetitive component; (3) a moderately repetitive component; and (4) a unique or non-repetitive component.

The highly repetitive component reassociated with a Cot of 0.12 and represents approximately 20% of the genome. As in the bobwhite quail genome, a fraction (10%) of the highly repetitive component may be attributed to a "Zero Cot binding" DNA. The technique of hydroxyapatite chromatography does not totally resolve the two second-order components.

The second kinetic component in the genome, the moderately repetitive sequences, reassociated at a Cot of 10.8 and represents 12% of the genome. DNA-DNA reassociation kinetics with 2000 nucleotide long fragments indicate that the estimation for the moderately repetitive component includes a non-repetitive fraction. However, the non-repetitive component represents only a small fraction because of the Cot value at which the moderately repetitive component

Table 7. Classification of Pharoah quail DNA repetition (450 NTL).

Class	Fraction of Total DNA	Observed Cot 1/2	Cot 1/2 Pure[++]	Kinetic Complexity*	Mass per 1N[+++] genome (pg)	Copies per Genome
Highly Repetitive[+]	0.20	0.06	0.012	1.3×10^4	0.26	30,770
Moderately Repetitive	0.12	5.4	0.65	7.0×10^5	0.16	300
Non-Repetitive	0.68	98.0	66.6	7.01×10^8	0.89	1

[++] Cot 1/2 Pure = Cot 1/2 Observed x Fraction of genome.

* Kinetic Complexity (Base Pairs) calculated by comparison of the rate of reassociation of each component of Pharoah quail DNA to that of E. coli (Cot 1/2 = 4.2, 2.7×10^9 daltons or 4.5×10^6 BP).

[+++] Haploid Genome size = 1.3 picograms, 1.3×10^{12} daltons, 2×10^9 Base pairs.

[+] Includes the Cot Zero time binding sequences and other palindromic sequences.

reassociates and the size (450 nucleotides) of the reassociated duplex DNA.

To confirm the amount of moderately repetitive DNA in the genome (Table 7) and to eliminate the contaminating fraction of non-repetitive DNA, total DNA was denatured, reassociated to Cot 50, digested with S1 nuclease and fractionated. The fraction of DNA binding to the hydroxyapatite as reassociated duplexes was 90%. Based on these results, 10-12% is the most likely value for this kinetic component, because all of the single stranded regions (supposedly consisting only of non-repetitive DNA) have been hydrolyzed by the S1 nuclease.

A third component, the non-repetitive DNA, reassociated with a Cot of 196 and represents 68% of the genome as determined by hydroxyapatite binding. This component is further characterized in Table 7. Again, as with the bobwhite quail, some of the fragments failed to bind to hydroxyapatite.

The Effect of Length on the Melting Characteristics of Reassociated Fragments Containing Duplex Regions

Figure 20 and Table 8 illustrate the optical melting profiles of sheared DNA molecules and reassociated fragments, and compare them to that of native DNA in the bobwhite quail genome. In Figure 20, fragments of 2000 base pairs displayed a very small loss of hyperchromicity and reduction of Tm. It is very clear from the thermal denaturation or melting profiles that these fragments are composed mostly of duplex regions. These data, indicated that the high speed shearing conditions did not lead to a substantial loss of secondary structure in the molecules.

Fig. 20. Melting profile of the bobwhite quail DNA which had been sheared to a fragment length of 2000 NTL.

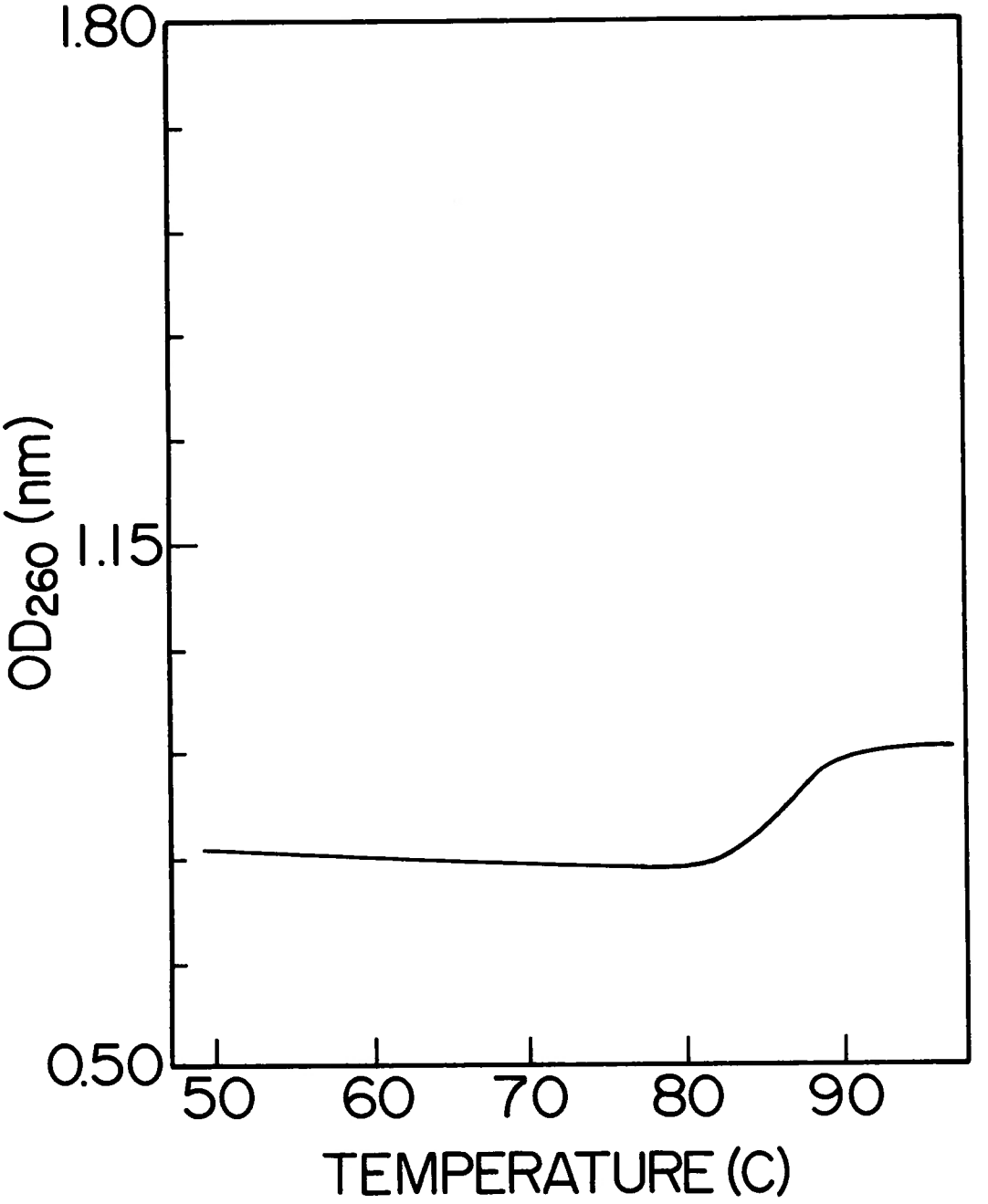

1.80
1.15
0.50
OD260 (nm)
50
60
70
80
90
TEMPERATURE (C)

Table 8. Hyperchromicity of native DNA and reassociated duplexes containing repetitive sequences in bobwhite quail DNA.

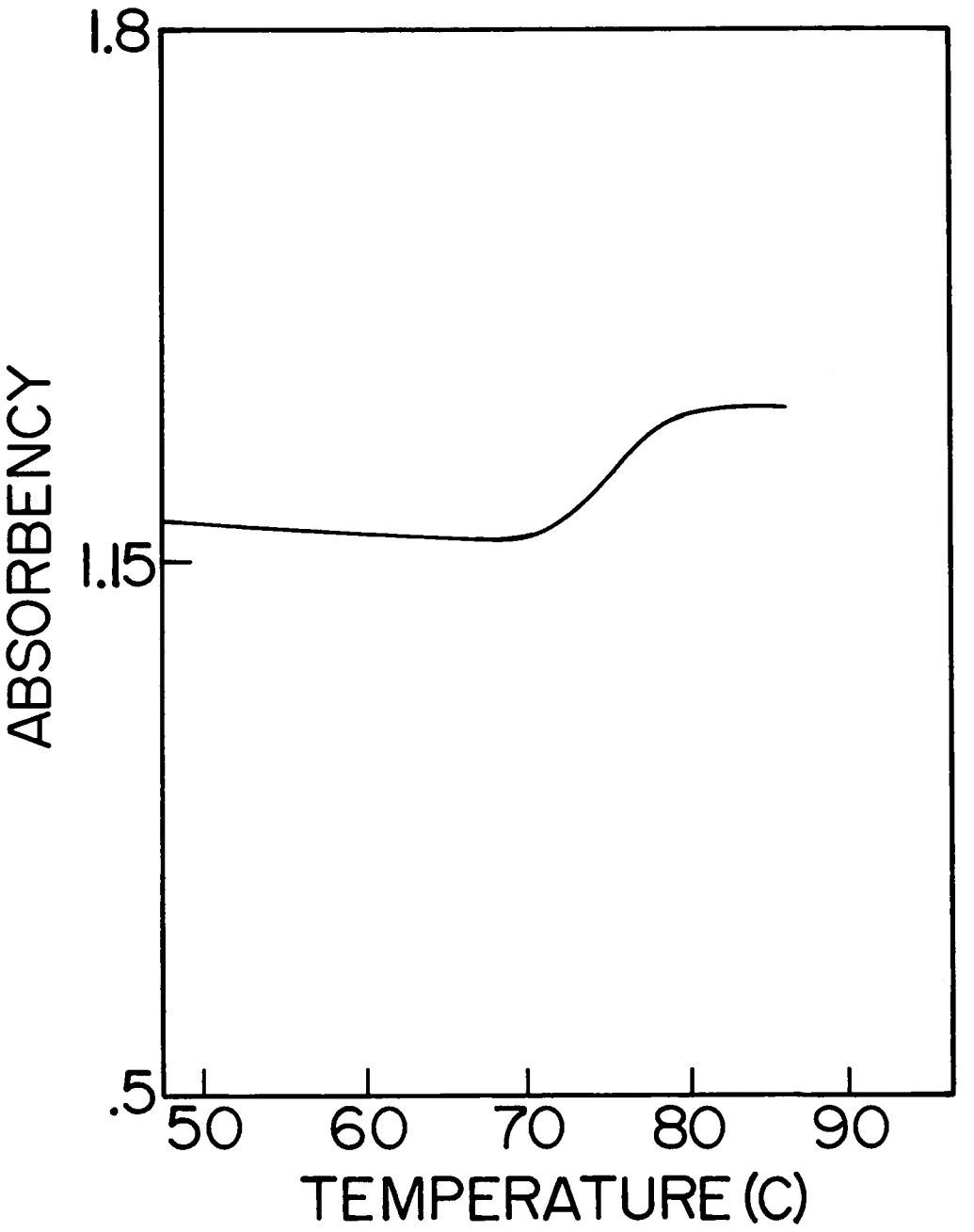
	Reassociated		Native		
Fragment Length (F)	450	2000	450	2000	unsheared
Hyperchromicity (K)	0.26	0.19	0.35	0.36	0.36
Fraction Bound to HAP (T)	0.30	0.40	-	-	-
Melting Temperature (Tm)	75.4	74.5	85.0	85.0	85.0
TXK/K (native) (TK)	0.30	0.40	-	-	-
Calculated Fraction Duplex (D)	0.90	0.66	-	-	-
Average Length of Duplex per fragment (L)	320	1000	-	-	-

In Figure 20 the non-repetitive component of bobwhite quail DNA displays a similar hyperchromic shift and Tm as that of the native DNA. Total DNA was denatured and reassociated to Cot 40, the fraction of the DNA which did not bind was then reassociated to Cot = 10,000 and the fraction which bound was used as the non-repetitive component. These data indicate that the reassociation of this component is essentially complete and represents intermolecular reassociation of homologous strands with very little, if any, mis-matched base pair regions.

In order to determine the quality of duplex formation formed by the repetitive component, DNA averaging 2000 nucleotides in length was reassociated to Cot 40 and the single stranded regions digested with Sl nuclease, and separated from duplex DNA by chromatography on Sephadex-G50. The hyperchromicity of the duplexed DNA is close to that of native DNA; however, the Tm of the duplex molecules is depressed by 10 degrees. Depression of the Tm value of reassociated duplexes of repetitive DNA relative to native DNA has been attributed to nucleotide mis-matching within families of related but not identical repetitive sequences. A one degree decrease in Tm represents approximately 1.5% base pair mis-matching (Bautz and Bautz, 1964; Laird et al., 1969). Therefore, the repetitive component in the bobwhite quail genome exhibited about 15% base mis-matching. However, much of this may be due to the Cot Zero binding component since "stripped" repetitive DNA was not used for the Sl nuclease digestion.

When DNA, reassociated to Cot 40 and the duplex molecules recovered from hydroxyapatite, was thermally denatured without digesting the single stranded regions, the Tm and hyperchromicity were reduced (Fig. 21). The reduction in Tm is due to mis-matched base pairs. The percentage of hyperchromicity was reduced

Fig. 21. Melting profile of total DNA reassociated to Cot 40.


1.8
1.15
.5
ABSORBENCY
50
60
70
80
90
TEMPERATURE (C)

to about 50% indicating that at least 50% of the length of these 2000 nucleotide fragments were still single stranded.

The effect of length on the melting characteristics of duplex DNA in the Pharoah quail is summarized in Table 9.

The Effect of Fragment Length on the Rate of Duplex DNA Formation in the Bobwhite Quail Genome

In order to determine the organization of the moderately repetitive and single copy sequence in the genome, ^{3}H-DNA molecules with an average fragment length of 250-2000 nucleotides were denatured, reassociated with 450 nucleotide long fragments and chromatographed over hydroxyapatite. Tracer amounts of ^{3}H-DNA were hybridized with an excess of unlabeled DNA. The results in Figure 19 indicate that approximately 32% of the fragments of 450 nucleotides long bind to HAP at a Cot 50, indicating that the moderately repetitive sequences have reassociated. The remainder of the DNA indicated kinetics characteristic of non-repetitive or single copy sequences. DNA sheared to 250 nucleotides long showed a slightly smaller amount of binding by Cot 50, suggesting that fragments of 450 nucleotides long contain some non-reassociated single stranded non-repetitive tails. These data indicate that only a fraction of the sequences have reassociated at Cot 50, and that the majority of the nucleotides represent single stranded regions of non-repetitive DNA bound to moderately repetitive DNA.

The data in Figure 19 indicate that when fragments 2000 nucleotides long were used, an even greater fraction (43%) of the DNA bound to HAP after reassociation to Cot 50. The rate of reassociation is approximately proportional to the length of the tracer sequence due to the linear increase in nucleation sites

Table 9. Hyperchromicity of native DNA and reassociated duplexes containing repetitive sequences in the Pharoah quail genome.

	Reassociated		Native		
Fragment Length (F)	450	2000	450	2000	unsheared
Hyperchromicity (K)	0.20	0.15	30.7	30.5	29.9
Fraction Bound to HAP (T)	0.30	0.43	-	-	-
Melting Temperature (Tm)	75.4	75.3	85.5	85.5	85.5
TXK/K (native) (TK)	0.20	0.22	-	-	-
Calculated Fraction Duplex (D)	0.71	0.50	-	-	-
Average Length of Duplex per fragment (L)	320	1000	-	-	-

available for renaturation. Therefore, one would expect approximately 12% of 2000 nucleotide long tracer molecules containing only single copy DNA to bind to HAP after reassociation with 450 nucleotide carrier DNA at Cot 50. These results clearly indicate that approximately 20% of the single copy sequences are closely interspersed with the moderately repetitive sequences in molecules up to 2000 nucleotides in length even though 43% of these molecules (2000 NTL) contain regions of repetitive sequences. About 20% of the genome is clustered repetitive sequences and 20%-23% consist of alternating repetitive and non-repetitive sequences.

Length of the Repetitive Sequences (Bobwhite and Pharoah) (Highly and Moderately Repeated)

To determine the length of the repetitive DNA, 2000 nucleotide long fragments were reassociated to Cot 40 and then digested with Sl nuclease to remove any single stranded regions. The DNA was then passed over a Sephadex G-50 column, electrophoresed through 1% agarose and the molecular weight of the fragments was determined by using lambda DNA digested with Hind III endonuclease as a molecular weight marker. Figure 22 indicates the results from these experiments. These data indicate that the repetitive elements in both species of quails are approximately 1000 nucleotides long (Fig. 23).

Fig. 22. An electrophoretic profile of repetitive units in bobwhite quail. Total DNA (10 µg) in 50 µl buffer was digested with 100 units of Hind III for 16 hr. The fragments were separated on a 1% agarose horizontal slab gel, stained and photographed. (A) quail DNA, (B) Hind III digested Lambda DNA.

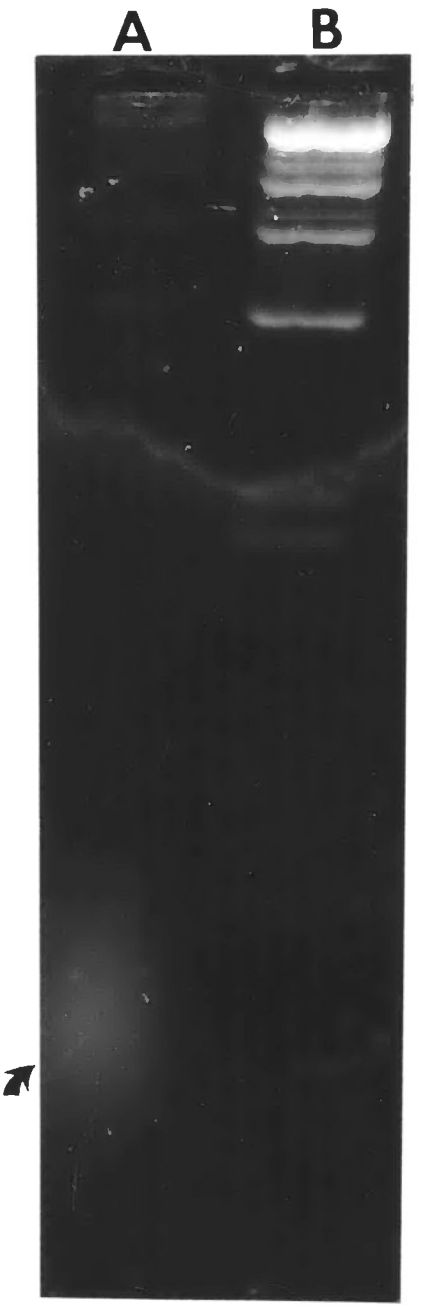
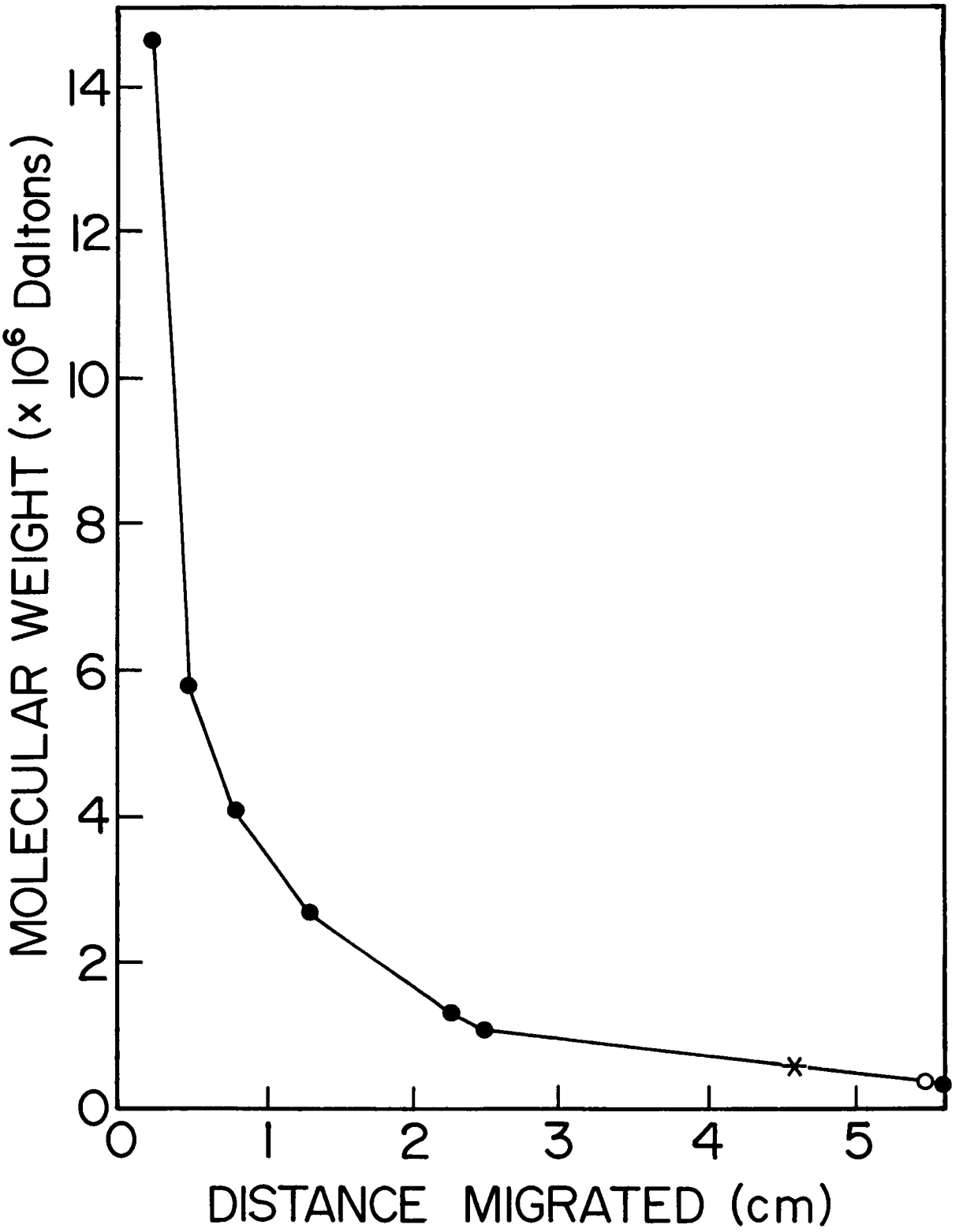

A
B

Fig. 23. Calibration curve of bobwhite and Pharoah quail DNA re-associated to Cot 40. A calibration curve of log molecular weight versus mobility was constructed from the molecular weight of the λ fragments which were generated by digestion with Hind III.


MOLECULAR WEIGHT (x 10⁶ Daltons)
14
12
10
8
6
4
2
0
0
1
2
3
4
5
DISTANCE MIGRATED (cm)

CHAPTER V

DISCUSSION

Among the vertebrates very little information on genome organization has been brought forward in the case of avians and their closest relatives, the reptiles. The DNA of bobwhite quail was isolated by isopycnic centrifugation in order to determine if satellite DNAs were present and to examine some biophysical characteristics of the quail genome.

The technique, CsCl equilibrium density gradient centrifugation, is an equilibrium method which determines the buoyant density of the macromolecular species. The gradient is self-generating in the centrifugal field, and its density range is so adjusted as to be more dense at the bottom of the tube and less dense near the meniscus than the macromolecular species (Szybalski and Erikson, 1964). Equilibrium density gradient centrifugation is now a well established technique, practically indispensable in modern biological laboratories. It permits the determination of many physical and chemical parameters for biological macromolecules, and thus, helps to characterize these entities. Equilibrium density gradient centrifugation can be and is applied to small and large scale fractionation of various kinds of RNA and DNA molecules.

Analytical centrifugation of bobwhite quail DNA in neutral CsCl revealed a non-Gaussian profile. The absence of satellite DNA in these gradients may be attributed to the small amount of DNA used or the percent total represented by the satellite sequences. Therefore, preparative neutral CsCl centrifugation was performed to investigate whether or not satellites were present.

The satellites in the bobwhite quail (Colinus virginianus) were first observed by preparative neutral CsCl centrifugation. Preparative centrifugation of bobwhite quail revealed a major band and two minor bands (satellites) located on the light side of the gradient. The main band DNA had a buoyant density of 1.701 g/ml and the two satellites have a density of 1.681 g/ml and 1.674 g/ml, respectively.

In certain organisms the existence of satellite DNA's has been demonstrated by isopycnic centrifugation of DNA in alkaline CsCl gradients (Walker et al., 1971). These workers showed that DNA from rat and Peromyscus (canyon mouse) fails to exhibit a satellite in neutral CsCl. However, in alkaline gradients the complementary strands of the highly repetitive sequences separate to produce two distinct bands which flank either side of the main band.

Alkaline CsCl gradients of bobwhite quail DNA showed that there is no bias in the G + T content of their complementary strands. The fact that total quail DNA does not show interstrand composition bias in alkaline CsCl is indicative of its heterogeneity. Additionally, the broadness of the band in alkaline cesium chloride density gradients is significant since it suggests a high degree of heterogeneity in G + T content among the high molecular weight DNA molecules comprising the band.

The selective binding of mercury (Hg^{++}) to DNA has been used for the fractionation of several DNA's in Cs_2SO_4 density gradients. The purines and pyrimidines present in nucleic acids are not very strong bases and would not be expected to bind metal ions strongly. However, mercury forms extremely strong bonds with nitrogenous bases.

Several laboratories (Katz, 1952; Thomas, 1954; and Yamane and Davidson, 1962a; Davidson et al., 1965) have reported a decrease in the viscosity and a spectral shift when mercury is added to DNA. The binding is selective in that AT-rich DNA's bind more strongly than GC-rich ones.

The selective binding of mercury to DNA was used in these studies to create buoyant density differences between DNA's with different base composition in the form of satellite DNA's.

This study shows that the optimal Hg^{++}/DNA-PO_4 ratio for isolating bobwhite quail satellites in Cs_2SO_4 gradients is higher than 0.1.

When correct Ag^{+}/DNA-PO_4 molar ratios were used, centrifugation through gradients of Cs_2SO_4 which contain silver ions improves the resolution of satellite DNA's. Heavy metal cesium sulfate density gradients have been used to isolate satellite DNA's from several species: yeast (Retel and Planta, 1972); Drosophila virilis (Gall and Atherton, 1974); Xenopus laevis and X. mulleri (Brown et al., 1971; Brown et al., 1973); mouse (Corneo et al., 1972; Fillipski et al., 1973; Kurnit and Maio, 1973); ox (Corneo et al., 1968, 1970); African green monkey (Maio, 1971; Kurnit and Maio, 1973) and human (Corneo et al., 1968, 1970, 1971, 1972; Jones et al., 1973; Chuang and Saunders, 1974).

The complexing reactions of silver ions with DNA involve primarily the purine and pyrimidine residues, and not the phosphate groups. The fact that there are changes in the ultraviolet spectrum when silver is added to DNA suggests nucleotide binding (Yamane and Davidson, 1962 b). The release of protons on adding silver would not be expected for silver binding to the phosphate groups, and because the phosphate groups are not significantly protonated at the high pH and

low salt concentration (Yamane and Davidson, 1962 a).

The binding of silver to nucleotides has been studied more extensively by Jensen and Davidson (1966). They report at least three types of binding (I, II and III) in which the first two have been intensively studied. The sum of type I and type II binding saturates at one silver atom per two nucleotide residues and zero and one proton, respectively, are displaced per silver ion bound. Their data, compiled from spectrophotometer, potentiometric and density gradient ultracentrifugation studies, suggest that GC-rich DNA's bind silver ion more strongly than AT-rich ones, and at low Ag^+/DNA-PO_4 molar ratios, denatured DNA binds more strongly than native.

The nature of the binding of silver to DNA is still unclear, but several reports have been presented relevant to the composition of the binding sites. Several lines of evidence suggest that the binding sites for silver and changes in buoyant density of the DNA may involve both nucleotide composition and the sequence of the DNA. When using Ag^+-Cs_2SO_4 gradients to study bobwhite quail DNA, Rf (ratio of Ag^+/DNA-PO_4) values other than 0.1, 0.35 and 0.40 are necessary to clearly show the satellites.

When a solution of DNA is heated, very striking changes occur in many of its physical properties, such as viscosity, light scattering and optical density. The denaturation or melting of DNA is usually characterized by the temperature of the mid-point of transition (Tm) sometimes referred to as the melting temperature. The process represents the disruption of the double helix of DNA into separated single strands (Lewin, 1974). The buoyant density data for the quail DNA was in agreement with the melting profile data.

A powerful method for investigating the heterogeneity of repetitive DNA's has been provided by restriction endonucleases, which have revealed two forms of sequence changes. Changes in particular restriction enzyme sites which show a long order periodicity are reflected in the presence of dimer and higher order length segments in addition to unit length repeat segments. The disposition and frequency of the different segment lengths are consistent with them being formed from an array of monomers in which random mutational changes of the recognition sequence have caused inactivation of some sites (Southern, 1975). A second type of sequence heterogeneity is detected when a restriction enzyme cleaves only portions of a repetitive DNA into regular repeating units. This result shows that a part of the array of repeats has no recognition sites for the particular restriction enzyme, although in the remaining repeat arrays recognition sites are regularly arranged.

When bobwhite quail DNA was digested with Hind III, a broad smear was obtained with nine low molecular weight bands (Table 2). The smear is indicative of a heterogeneous population; therefore, the restriction sites are distributed throughout the bobwhite quail genome. Hind III digested λ DNA produced seven fragments ranging in molecular weights from 14.6 to 0.34 x 10^6 daltons and fragment lengths of 24.3 to 0.6 kilobase pairs. Lambda DNA, digested with Hind III, was used as a marker to calculate the molecular weight range of the smear produced when bobwhite quail DNA was digested with Hind III. The molecular weights ranged from 14.6 to 0.44 x 10^6 daltons. A densitometric tracing was done on the bobwhite quail DNA. The densitometric profile detected bands better than the ethidium bromide stained gels. Faint bands were discernible in the gels.

When unfractionated DNA was digested with Hind III, a broad smear was obtained with seven distinct low molecular weight fragments. These bands represent repetitive DNA (satellite) which has been digested by the endonuclease. These are sequences with a non-random distribution of restriction sites. These fragments possibly fall into two classes of molecules with monomeric repeat units of 700 and 800 nucleotides. The data imply that the bands are derived from the satellite DNA's seen in preparative CsCl gradients because main band DNA, stripped of the satellite sequences and digested with Hind III, migrates as a broad smear without any discrete bands when electrophoresed through agarose. Digestion of the total DNA with other restriction enzymes indicates that the Hind III restriction sites are not interdigitated with other sites and/or the monomeric units of the sequences are too small for detection on these gels. However, the former hypothesis is better supported by the results obtained.

When bobwhite quail DNA was terminally digested with EcoR 1, a large proportion of high molecular weight fragments was obtained. High molecular weight bobwhite quail DNA was digested with Hpa I, Hpa II, Bam I, Bgl II, Alu I, Hae III, Hha I, Mbo I and EcoR II. When the quail DNA was digested with these restriction endonucleases a smear was obtained. Again this is indicative of a heterogeneous population. It was also concluded that these restriction endonuclease sites are randomly distributed in the genome and that the sites are not interdigitated. The heterogeneity in the bobwhite quail genome might be due to the existence of a number of conserved, related, alternative sequences regularly arranged.

It is clear from these experimental results on quail DNA that specific radioactive probes are not needed to determine if distinct bands are produced when the DNA is digested with enzymes other than Hind III (Table 3). Further studies, using the techniques of "Blot Transfer" and autoradiography, are in progress (Shelton and Edwards, unpublished) to determine if the ribosomal genes are digested. In the Jumbo Wisconsin quail, Hind III cleaves the r-DNA at two sites (in the 18S and 28S genes) and generates three fragments when electrophoresed through agarose. The low molecular weight fragement is complementary to 28S rRNA while the high molecular weight fragments hybridize with 18S rRNA. These data indicate that heterogeneity exists in the ribosomal genes, and most likely within the nontranscribed spacer region (Edwards, 1979). Data of this nature, may shed light on the relatedness and conservativeness in families of related, but not identical, moderately repetitive sequences and/or simple sequences.

The analysis of sequence arrangement in the DNA of bobwhite quail is a useful tool in increasing our knowledge on DNA sequence interspersion patterns. DNA reassociation kinetics are an excellent assay to determine the manner in which genes are organized, a useful technique for determining the dispersion of genes and determining the kinds of DNA or sequences. Reassociated kinetics was carried out via hydroxyapatite (HAP) chromatography. The several advantages for using HAP are: (1) an experimental system where virtually complete reaction of the DNA is routine and easily detectable; (2) the ability to easily separate and recover both the reassociated fraction and the non-reassociated fraction; (3) the easy characterization and fractionation of the DNA by thermal chromatography; (4)

the potential for easily handling widely varying amounts of DNA in a routine manner (0.0001ug to 2000ug); (5) the ability to easily maintain specific conditions of salt concentration and temperature during the fractionation of the DNA.

The rate at which nucleic acids reassociate is easy to measure. Bases in single strands are much freer to move around than in base paired double helices, so single stranded nucleic acids absorb much more ultraviolet light than double stranded nucleic acids. The analysis of reassociation speed can be done by checking the UV absorbance of a nucleic acid solution at various times during reassociation since the absorbance decreases as the bases lose mobility by forming base pairs. Alternatively, hydroxyapatite can be used to bind the double stranded DNA, and the percent which is bound can be recorded at intervals.

Several studies have been shown that the genome of many eukaryotes contain a variety of repeated sequences. These sequences have been identified by DNA-DNA hybridization and fractionated on a hydroxyapatite column, as described by Britten and Kohne (1968). In mammals these sequences account for 10-30% of the total DNA, 20% in sea urchin, 30% in Xenopus laevis, about 24% in Drosophila melanogaster and only 10-15% of the DNA in chickens (Colbertet al., 1976).

The most often encountered type of interspersion of single copy and repetitive sequences was first discovered in the DNA of Xenopus laevis. Here 50% of the genome consist of a "short period interspersion pattern" called single copy sequences (Davidson et al., 1975). A contrasting pattern of sequence interspersion was first observed in the DNA of Drosophila melanogaster (Manning et al., 1975). This form of sequence organization is characterized by approximately 5.6 kb long

repetitive sequences interspersed with longer than 13 kb unique sequences (Crain et al., 1976b).

The chromosomal organization of birds differs from that of other animals in several ways: (1) The genome is about three times smaller than that of several other eukaryotes; (2) Most of the repetitive DNA is in the micro-chromosomes (Stefos and Arrighi, 1971; Edwards, 1974); (3) Most of the constitutive heterochromatin is in the microchromosomes. These characteristics make the avian genome an interesting system for further studies on the size and arrangement of the repetitive sequences and their interspersion with single copy sequences.

The experiments on reassociation kinetics of DNA in two species of quails were carried out to determine the sequence content (kinetic components) and the sequence interspersion pattern.

The reassociation kinetics for the bobwhite quail (Fig. 19, Table 6) suggest that there are three classes of repetitive DNA ranging in repetition frequency from 6 to 4,000 copies per haploid genome. The shape of the curve for the highly repetitive fraction indicates that it is made up of more than one component. A careful analysis of the highly repetitive component indicates that it consists of a "Cot Zero Time" binding component and a simple or satellite component. The satellite component has been confirmed with the cesium salt data, and represents about 5% of the total DNA in the nucleus and may be clustered in the nucleus as opposed to being interspersed like the "Cot Zero time" binding component. The reassociation data indicate that the satellite component is of low repetition frequency as compared to the calf or mouse satellite (repetition frequency of 1 x 10^6 copies) however the complexity of the component is about 1000 base pairs. This kind of complexity and the clustering of the sequences could very easily cause

the DNA to band as a separate component in cesium salt gradients.

The configuration of the reassociation curve for the moderately repetitive component indicates that it may contain several classes of repetitive components. The shape of the curve is as expected since the repetition frequency of the ribosomal genes (200 copies) is different from that of the 5S and tRNA genes. If the histone genes are present in the moderately repetitive component, they are obviously of a low repetition frequency as compared to the sea urchin (24,000 copies). The kinetics of reassociation of the moderately repetitive component indicate a complexity of 5.4×10^5. However components within these families of repetitive molecules may reassociate slower than the average and the total complexity may be greater than indicated in the data. Since these sequences are divergent, any numerical estimate of the complexity is merely a simpliciation. The different sequences, which reassociate with each other and differ by 10 to 15% in their nucleotides, could be functionally distinct within the cell if mechanisms were present that would act on the basis of the small sequence differences. If that is the case, the functional complexity of the family of repetitive sequences could be orders of magnitude greater than the estimate of kinetic complexity.

The comparative reassociation kinetics for the Pharoah quail (Fig. 18, Table 7) suggest that three kinetic components are present and within the repetitive components is a "Zero Cot" binding fraction. In this genome, most of the repetitive components appear to be dispersed throughout the genome and are not localized in large blocks of sequences, as indicated by the isopycnic centrifugation data. However, satellite DNA has been isolated in the Pharoah quail genome and represents about 2 to 3% of the total DNA (Blasingame and Edwards, 1978). This satellite, at the most, only accounts for 10 to 12% of the total repetitive component and fits,

well with the interspersion data, in which 20 to 25% of the single copy or non-repetitive sequences are interspersed with the repetitive DNA. The reassociation kinetics of the Pharoah quail DNA are very similar to that of the bobwhite in repetition frequency and complexity. However, the degree of similarity can be further indicated from reassociation kinetics of the heterologous moderately repetitive sequences.

In order to gain a better understanding of the structural and functional organizations of the avian genome, the interspersion of repetitive and non-repetitive sequences in the bobwhite and Pharoah quail have been studied. The results indicate that about 20 to 25% of the single copy or non-repetitive sequences are interspersed with repetitive sequences of approximately 1000 nucleotides long. The average length of the interspersed non-repetitive sequences appears to be about 1000 nucleotides long. Fragments longer than 2000 nucleotides indicate a minor increase in the extent of interspersion. These findings indicate that the structural organization of the bobwhite and Pharoah quail genomes are similar to that of other avians. In comparsion with the duck and chick genomes, only 12% and 28%, repectively, of the single copy DNA are interspersed with repetitive elements on 2000 to 3000 nucleotide fragments (Epplen et al., 1978). These results show that the bobwhite and Pharoah quails, duck and chicken genomes are not similar to the short period interspersion pattern of genome organization, characteristic of most eukaryotic genomes studied. However, even though it is not of the short period interspersion pattern, it is not as complex as the sequence interspersion pattern observed in the DNA of Drosophila melanogaster (Manning et al., 1975; Crain et al., 1976b).

CHAPTER VI

SUMMARY

1. In bobwhite quail (Colinus virginianus), a major peak (main band DNA) and two minor peaks (satellite DNA) were isolated in preparative and overloaded analytical gradients.
2. In preparative alkaline cesium chloride gradients of bobwhite quail (Colinus virginianus), there was no bias in the G + T content of the complementary strands as indicated by a single Gaussian band.
3. Hybridization experiments revealed that the genes for ribosomal RNA were not localized in the satellite bands of bobwhite quail DNA and not contained in a single nucleolus organizer.
4. Melting temperature experiments on bobwhite quail revealed that the main band melted at a Tm of 70 C.
5. In bobwhite quail total DNA, restriction endonuclease sites are not clustered in the genome showing that there is heterogeneity throughout most of the genome.
6. "Zero Cot binding" DNA exist in the bobwhite and Pharoah quail genomes. In the bobwhite quail, about 10-15% of the DNA bind to HAP when 250-450 nucleotide long fragments were reassociated. Approximately 15% was bound for fragments 450 nucleotides long. Approximately 31% of the DNA was bound when fragments of 2000 nucleotides long were passed over HAP at "Zero Cot". In the Pharoah quail genome, approximately 15% of the 450 nucleotide long fragments and 30-35% of the 2000 NTL fragments were a "Zero Cot" value.

7. A linear relationship was observed in bobwhite and Pharoah quail DNA between the amount of DNA bound at "Zero Cot" and the fragment length up to 2000 NTL. This indicates that the "Zero Cot" binding fragments are precisely ordered in their spacing and are not randomly spaced throughout the genome.
8. Bobwhite quail DNA has a component of highly repetitive DNA which reassociates by Cot 0.1 and represents approximately 20% of the genome.
9. The moderately repetitive sequences in bobwhite quail DNA reassociate with a Cot value of 10 and comprises 10% of the genome.
10. The non-repetitive component in bobwhite quail represnets approximately 70% of the genome.
11. In the Pharoah quail, highly repetitive components reassociated with a Cot of 0.12 and represents approximately 20% of the genome.
12. Pharoah quail moderately repetitive sequences reassociate at Cot 10.8 and represent 12% of the genome.
13. The non-repetitive DNA in Pharoah quail reassociates with a Cot of 196.0 and represents 68% of the genome.
14. High speed shearing conditions did not lead to a substantial loss of secondary structure in the molecules.
15. In the bobwhite quail genome about 20% of the genome is clustered repetitive sequences and 20-23% consist of alternating repetitive sequences.

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