on a molar basis, ENU was found to be 3.5-fold more effective at producing this modification. If indeed DNA chemical modification is responsible at least in part for the observed biological effects of these compounds we would expect, to a first approximation, that the degree of biological response be proportional to the degree of one or a few kinds of critical DNA lesions. The ENU-MNU comparison suggests that MNU is more efficient, on a molar basis, at inducing a biological effect and at cellular DNA alkylation, while ENU is more effective at O^2-thymine site modification. This, then, may be the first indication that O^2-thymine alkylation is not, in itself, a critical DNA lesion.

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Homology of Single Copy and Repeated Sequences in Chicken, Duck, Japanese Quail, and Ostrich DNA†

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ABSTRACT: The extent of reassociation of ^{3}H-labeled repetitive or single copy DNA sequences from the chicken with excess unlabeled DNA from the duck, the Japanese quail, and the ostrich, respectively, was measured by hydroxylapatite chromatography. Chicken repetitive DNA reassociated to an equal or greater extent than chicken single copy DNA with the DNA of each of the other birds. Using an isolated subfraction of chicken repetitive DNA representing those DNA sequences common to the chicken and ostrich genomes, we determined that many repetitive DNA sequences that occur at high repetition frequency in the chicken genome have a much lower repetition frequency in ostrich DNA. The data indicate that there has been a striking change in the number of copies of many repetitive DNA sequences during avian evolution.

The repeated DNA of eukaryotes contains families of related sequences that are not identical but are sufficiently similar to reassociate with one another under standard conditions of salt and temperature (Britten et al., 1974). Repeated DNA usually contains families of related sequences with widely varied repetition frequencies, ranging from a few copies to many thousands of copies per genome (Britten & Kohne, 1968; Britten & Davidson, 1971; Davidson et al., 1975). It is not yet clear whether these repetition frequencies reflect a functional requirement for different numbers of copies of different sequences, or whether the number of copies present is the result of other processes related to the origin and evolution of repeated DNA (Kohne, 1970; Britten & Davidson, 1971).

Studies of DNA homology between species can contribute greatly to our understanding of the process of evolution as it relates to the structural organization of the genome. It should be especially informative to compare the rates of nucleotide substitution within different frequency classes of repetitive DNA, and to compare these rates with the rate of divergence of single copy DNA. It is also important to determine whether the repetition frequency of a sequence in DNA remains constant in the DNA of related species, or whether the repetition frequency of DNA sequences changes during evolution.

It has recently been determined that the chicken genome contains an unusually small fraction of repetitive DNA (Eden, in press). However, the repetitive DNA does conform to the typical pattern of containing more than one frequency class; two-thirds of the chicken repeated DNA consists of sequences repeated about 1500-fold, and one-third of the repeated DNA has a repetition frequency of about 15-fold. Thus, studies that compare the extent of homology of chicken repeated and single copy DNA fractions with the DNA of other birds should be

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especially informative when compared with other, similar studies using animals with much larger fractions of repetitive DNA (Angerer et al., 1976; Whiteley et al., 1970; Galau et al., 1976).

We have compared the extent of reassociation of chicken repeated or single copy DNA, respectively, with the DNA of three other avian species: the duck, the Japanese quail, and the ostrich. In addition, the repetition frequency of those repeated sequences that occur in both chicken and ostrich DNA was determined in the DNA of each species. The results indicate that the number of copies of most of the repetitive sequences that cross-react has been strikingly altered during avian evolution. About 3% of the chicken genome consists of sequences with a repetition frequency of 3000-fold. Most of the homologous sequences in ostrich DNA are repeated only 30-fold.

Experimental Procedures

Chickens (White Leghorn), Japanese quail, and the duck were obtained locally from Truslow Farms, Inc., Chestertown, Md. DNA from chickens and from Japanese quail was isolated from the combined livers of many individuals. Duck DNA was isolated from the liver of an adult Mallard duck. Ostrich DNA was obtained from heparinized blood drawn from the wing vein of a single individual currently inhabiting the Baltimore Zoo.

DNA Isolation. DNA from chicken, duck, and Japanese quail was isolated by grinding frozen liver in a Waring blender with dry ice, as described previously (Britten et al., 1974). Ostrich DNA was isolated from blood cells collected by centrifugation at 1000*g* for 10 min at 4 °C. Cells were lysed in 0.1 M Tris, pH 8, 50 mM NaCl, 50 mM EDTA,[1] 0.1% $NaDodSO_4$. The lysate was incubated with 50 μg/mL proteinase K (Sigma) at 37 °C for 3 h. The DNA was purified by extraction with redistilled phenol (pH 7), followed by two extractions with chloroform–isoamyl alcohol (24/1). DNA was precipitated by addition of 2 volumes of cold, 95% ethanol and wound out on a stirring rod. The DNA was redissolved in 5 mM NaCl, 1 mM EDTA and dialyzed to remove residual organic solvents and $NaDodSO_4$. RNA was removed by digestion with RNase A (Sigma) (50 μg/mL, heated for 30 min at 80 °C to inactivate DNase), and ribonuclease T1 (Sigma) (2 μg/mL) in 50 mM Tris, pH 8, 50 mM NaCl, 10 mM EDTA at 37 °C for 45 min. The DNA was further digested with proteinase K (50 μg/mL) for 1 h at 37 °C, and extracted once again with chloroform–isoamyl alcohol (24/1). DNA was reprecipitated twice from 0.1 M sodium acetate by addition of 2 volumes of cold 95% ethanol and wound out on a rod after each precipitation.

Radioisotope Labeling of DNA. Labeled total chicken and ostrich DNA were prepared by nick translation of nicked, native DNA as previously described (Crain et al., 1976).

Fragmentation of DNA and Measurement of Fragment Lengths. DNA was fragmented by shearing in a Virtis 60 or a Virtis 45 homogenizer, and DNA fragment lengths were determined by isokinetic alkaline sucrose gradient centrifugation, as detailed previously (Britten et al., 1974).

DNA Reassociation and Hydroxylapatite Chromatography. DNA was reassociated in 0.12 M PB at 60 °C, or 0.4 M PB at 68 °C. These conditions provide the same criterion for fidelity of base pairing in the reassociated DNA, but DNA reassociates 4.9 × faster at the 0.4 M PB, 68 °C criterion (Britten et al., 1974). The rates of reassociation observed using the 0.4 M PB, 68 °C criterion were corrected for comparison with those measured in 0.12 M PB at 60 °C by multiplying C_0t by 4.9.

The fraction of DNA reassociated was determined by hydroxylapatite chromatography (according to Britten et al. 1974). Radioactivity in the hydroxylapatite column fractions was measured in 0.08 M PB and INSTAGEL (Packard) in a refrigerated Packard Tri-Carb liquid scintillation spectrometer.

Isolation and Radioisotope Labeling of Chicken Single Copy DNA. Chicken DNA fragments, 550 nucleotides in length, were reassociated to C_0t 500. The DNA that bound to hydroxylapatite (44%) was discarded. The fraction that did not bind to hydroxylapatite was reassociated to C_0t 12 000, and again passed over a column of hydroxylapatite. The bound fraction (85%), was eluted in 0.4 M PB at 60 °C to preserve the DNA duplexes. DNA duplexes were labeled in vitro by nick translation as previously described (Crain et al., 1976). The specific radioactivity was about 2×10^7 cpm/μg of DNA. After radioisotope labeling and removal of foldback sequences, the labeled DNA was 440 nucleotides in length.

Isolation and Radioisotope Labeling of Chicken Repetitive DNA. Chicken repetitive DNA was isolated by reassociation of chicken DNA fragments to C_0t 50, and isolation of the fraction bound to hydroxylapatite. The bound fraction was dissociated and reassociated twice more, to C_0t 20 and C_0t 10, respectively, to ensure complete removal of single copy sequences; the fraction bound to hydroxylapatite was isolated after each incubation. The repetitive DNA was labeled in vitro by nick translation. Foldback sequences were removed by reassociation to C_0t 10^{-4} and passage over a column of hydroxylapatite; the fraction containing DNA that remained single stranded at C_0t 10^{-4} was isolated. The DNA fragments were 390 nucleotides in length, and the specific radioactivity was about 1×10^7 cpm/μg of DNA.

Hydroxylapatite Thermal Chromatography. Reassociated DNA was bound to hydroxylapatite in a water-jacketed column at 60 °C in 0.12 M PB. The unreassociated single strands were removed by washing the column with 10 bed volumes of 0.12 M PB at 60 °C. The temperature was raised in 3 °C increments up to 98 °C, and the single strands resulting from dissociation of duplexes were eluted at each temperature with 8–10 bed volumes of 0.12 M PB. The radioactivity eluted at each temperature was measured by liquid scintillation counting.

Addition of Homopolymer Tracts Using Terminal Transferase. Homopolymer tracts were added to the 3′-OH termini of short, single strands of DNA by incubation with terminal transferase, under conditions similar to those used for double-stranded DNA (Roychoudhury & Wu, 1976). The reaction mixture contained 5 μg of denatured DNA fragments, 550 nucleotides in length, 100 mM sodium cacodylate, pH 6.9, 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 20 nmol of unlabeled dATP or dGTP, 20 μCi of [α-^{32}P]dATP or [α-^{32}P]dGTP (New England Nuclear, 200 Ci/mmol), and 450 units of terminal transferase (nucleosidetriphosphate:DNA deoxynucleotidylexotransferase, EC 2.7.7.31, Boehringer). The incubation was carried out for 3–4 h at 37 °C in a total volume of 0.5 ml. Unincorporated nucleoside triphosphates were removed by passage of the reaction mixture over a column of Sephadex G-100 (0.5 × 10 cm) in 0.4 M NaCl, 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$; 20–35% of the input radioactivity was incorporated. From the calculated number of 3′-OH termini and the nanomoles of radioisotope incorporated, we estimate that

[1] Abbreviations used: C_0t, DNA concentration (moles of nucleotides) × time (seconds); nt, nucleotides; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PB, equimolar mono- and disodium phosphate buffer, pH 6.8; T_m, midpoint of a thermal denaturation profile; rms, root mean square deviation.

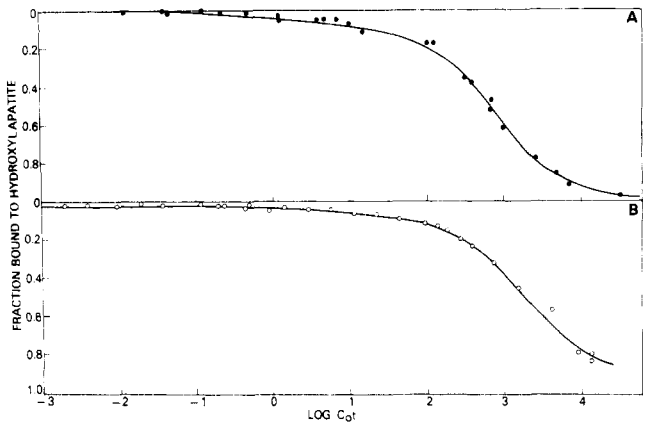

FIGURE 1: Reassociation kinetics of chicken and ostrich DNA. (A) Chicken DNA fragments were labeled in vitro by nick translation. Foldback DNA was removed by denaturation of the ^{3}H-labeled DNA, incubation to very low C_0t ($C_0t = 10^{-4}$), and removal of the reassociated foldback DNA by hydroxylapatite chromatography. The [^{3}H]DNA (440 nt) was then reassociated with excess unlabeled chicken DNA (550 nt) in 0.12 M PB, at 60 °C or an equivalent criterion. Reassociation kinetics were followed by determining the fraction of the radioactive DNA bound to hydroxylapatite as a function of the DNA concentration times the incubation time, or C_0t. The curve drawn through the data points was generated by a computer program that finds the composite of second-order components that best fits the data points (Pearson et al., 1977). Prior to computer analysis the data were normalized to exclude a small residual fraction of foldback DNA and 5% unreactable DNA. The curve represents a composite of two repetitive components and a single copy component. The parameters determined were the fraction of each component, F, and its reassociation rate constant, K (M^{-1} s^{-1}), as follows: $F = 0.075$, $K = 1.66$; $F = 0.055$, $K = 0.017$; $F = 0.87$, $K = 0.0011$. rms deviation of the curve from the data was 1.5%. (B) Ostrich DNA fragments were labeled in vitro and foldback was removed as in A. [^{3}H]DNA fragments (300 nt) were reassociated with excess unlabeled ostrich DNA (340 nt) and reassociation kinetics were followed by hydroxylapatite chromatography as described in A. Data were not normalized prior to computer analysis. The fraction of the DNA unreacted was 0.085, and three components were identified: $F = 0.026$, $K = 2.06$; $F = 0.066$, $K = 0.011$; $F = 0.803$, $K = 0.00051$. rms deviation of the curve from the data was 0.8%. Normalization of these data as described in A indicates that the fractions of total DNA in each of the two repetitive components and in the single copy component are 0.032, 0.072, and 0.870, respectively.

an average of 30 residues were added to the 3′-OH terminus of each DNA fragment.

Oligo(dT)-Cellulose Chromatography. Oligo(dT)-cellulose (T3) was obtained from Collaborative Research, Inc. Chromatography was carried out at 20 °C. Samples were applied to oligo(dT)-cellulose in 0.4 M NaCl, 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$ or 0.4 M PB, 0.1% $NaDodSO_4$. The bound fraction was eluted with 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$. An amount of 0.1 g of oligo(dT)-cellulose powder was used per mg of DNA.

Results

Single Copy and Repeated DNA Sequences in Birds. Very little information is available concerning both the relative amounts of single copy and repetitive DNA sequences, and the repetition frequency of repeated DNA sequences in birds. We have studied the DNA of two distantly related avian species, the chicken and the ostrich. Figure 1 shows the reassociation kinetics of short fragments of chicken and ostrich DNA measured by hydroxylapatite chromatography. The repetitive and single copy components in each genome were identified by a computer program designed to find the curve that best fits the data points (Pearson et al., 1977). The results of this computer analysis are listed in the Legend to Figure 1.

Eighty-seven percent of the DNA of both the chicken and the ostrich is single copy. By comparison of the rate of reassociation of the single copy DNA with a kinetic standard, we calculated the haploid genome size of each species. The reassociation rate constants (K, M^{-1} s^{-1}) of the single copy components were first corrected to the rate predicted for fragments 450 nucleotides in length according to the relationship $K_{corr} = (L_1/L_2)^{1/2}$ (Wetmur & Davidson, 1968). The reassociation rate constant determined for sea urchin single copy DNA, using fragments 450 nucleotides in length, was used as a standard for relating genome size to the single copy rate (Graham et al., 1974). The single copy component of the sea urchin genome (0.88 pg) has a reassociation rate constant of 0.00125 M^{-1} s^{-1}. Using the corrected rates, we calculate that the haploid genome size of the chicken is (0.0010/0.00125) × 0.88 = 1.1 pg; and the haploid genome size of the ostrich is (0.00059/0.00125) × 0.88 = 1.9 pg.

Chicken and ostrich repetitive DNA are also quite similar. Each genome contains two repetition frequency components. The repetition frequency of each component was calculated by dividing its reassociation rate constant by that of single copy DNA (Angerer et al., 1975). The two components of chicken repetitive DNA have repetition frequencies of 1500-fold and 15-fold, comprising 7.5% and 5.5% of total DNA, respectively. Similar components were identified in ostrich DNA: 3.2% of the genome has a repetition frequency of 4000-fold and 7.2% has a repetition frequency of 20-fold.

Single Copy and Repetitive DNA Sequence Divergence in Four Avian Species. We have compared the divergence of chicken (*Gallus gallus*) single copy or repetitive DNA sequences from duck (*Anas platyrhynchos*), Japanese quail (*Coturnix coturnix*), and ostrich (*Struthio camelus*) DNA. A current taxonomic classification of these species (Wetmore, 1960) indicates that the DNA of the chicken is expected to be most similar to that of the Japense quail, since both are game birds of the order Galliformes. The next closest relative of the chicken that we studied was the duck, belonging to the order Anseriformes, or water fowl. On the basis of cranial morphology, a close affinity between the game birds and water fowl has been suggested (Simonetta, 1960), and the data we describe subsequently lend some support to this proposal. The chicken is considered to be a very distant relative of the ostrich (Romer, 1966). Based primarily on the structure of the bones of the jaw, the ostrich is placed in a separate category from most other birds, and is believed to resemble ancestral forms present at the inception of avian evolution (Romer, 1966; Wetmore, 1960).

The method we used for measuring DNA homologies has been reported previously (Angerer et al., 1976) and can be described briefly as follows. Radiolabeled single copy or repetitive DNA from the chicken was prepared and reassociated with excess unlabeled DNA from each of the other birds. The reassociation kinetics and the final extent of reassociation were determined. Conditions of salt and temperature were chosen (0.12 M PB, 60 °C) that set a limit on the fraction of mispaired bases within a stable DNA duplex; only DNA sequences that share at least 80% of their nucleotides in common could reassociate under the conditions used (Britten et al., 1974). The actual fraction of bases mispaired in the heterologous DNA duplexes was determined by measuring the thermal stability of the reassociated DNA (Kohne, 1970; Laird et al., 1969; Bonner et al., 1973). Both the extent of reassociation and the percent of bases mispaired (ranging from 0 to 20%) were taken into account in estimating the divergence, or degree of dissimilarity, between the genomes of different species.

We first considered the extent of divergence of single copy sequences. The single copy fraction of chicken DNA was isolated and labeled by nick translation. The removal of repetitive

TABLE I: Divergence of Chicken Single Copy and Repetitive DNA.

labeled DNA	unlabeled DNA	max % reassociated[a]	thermal stability[b] (°C)	ΔT_m[c] (°C)	fraction of bases mispaired[d]
chicken single copy	chicken	100.0	82.0	0	0
	duck	67.3	74.5	7.5	0.075
	Japanese quail	26.3	71.0	11.0	0.110
	ostrich	0-5			
chicken repetitive	chicken	100.0	76.5	0	0.055
	duck	66.0	70.0	6.5	0.120
	Japanese quail	68.0	70.5	6.0	0.115
	ostrich	23.3	69.0	7.5	0.130

[a] The maximum extent of reassociation was determined by computer analysis of hydroxylapatite reassociation data and was normalized to the extent of reassociation with homologous DNA in each case. [b] The thermal stability of reassociated DNA was determined by hydroxylapatite thermal chromatography in 0.12 M PB. Labeled chicken single copy DNA was reassociated with unlabeled DNAs to C_0t 30 000-50 000 and applied to columns of hydroxylapatite at 60 °C. The temperature was increased in 3 °C increments and single strands eluted at each temperature. The value shown is the T_m, or midpoint of the thermal denaturation profile. The thermal stability of chicken single copy duplexes with ostrich DNA was not determined, due to the low extent of reassociation. The thermal stability of labeled chicken repetitive DNA reassociated with each unlabeled DNA was determined at C_0t 2000-3000 by the same method. [c] ΔT_m is the change in T_m relative to homologous DNA. [d] The fraction of bases mispaired was calculated from the midpoint of the thermal denaturation curve (T_m). The presence of 1% mispaired bases lowers the T_m by 1 °C (Bonner et al., 1973).

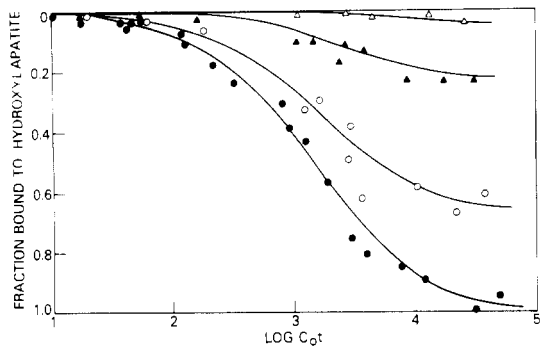

FIGURE 2: Single copy DNA divergence in four avian species. ^{3}H-labeled fragments of chicken DNA (440 nt) were reassociated with a 10^6-fold excess of unlabeled DNA fragments from chicken (550 nt), duck (500 nt), Japanese quail (540 nt), and ostrich (860 nt), respectively. DNA was reassociated in 0.12 M PB at 60 °C or an equivalent criterion. Reassociation kinetics were measured by determining the fraction of the labeled DNA bound to hydroxylapatite as a function of C_0t. Data have been normalized to the extent of reassociation of the labeled DNA with homologous DNA, which decreased from 80% to 73% during the course of the experiment. The extent of self-reassociation of the labeled DNA did not exceed 1%. The curves were generated by computer analysis of the data points and represent a single component in each case. The values determined for maximum extents of reassociation and the reassociation rate constants, respectively, were: (●) chicken DNA, 100%, K = 0.00063, rms = 3.8%; (○) duck DNA, 67.3%, K = 0.00060, rms = 3.8%; (▲) Japanese quail DNA, 26.3%, K = 0.00043, rms = 1.9%; (△) ostrich DNA, 5%, K = 0.00026, rms = 0.3%.

sequences from the isolated single copy fraction was confirmed by reassociation of the ^{3}H-labeled single copy DNA with excess unlabeled chicken DNA, as shown in Figure 2. The hybridization data were analyzed by computer, and the best fit to the data points is a curve representing a single component with a reassociation rate constant of 0.00068 M^{-1} s^{-1}, in reasonable agreement with the predicted rate constant for pure single copy DNA (Figure 1). From the absence of any detectable reassociation at low C_0t, we conclude that more than 98% of the labeled DNA is single copy sequence.

Figure 2 shows the rates and extents of reassociation of labeled chicken single copy DNA with a vast excess of unlabeled total duck, Japanese quail, and ostrich DNA, respectively. The results of computer analysis of the hydroxylapatite reassociation data are shown in the upper portion of Table I. The extent of reassociation was less than 5% with ostrich DNA, 26% with Japanese quail DNA, and 67% with duck DNA. Thus, the very distant relationship of the chicken and the ostrich is confirmed by these data, as is the close phylogenetic relationship of the chicken and the duck. However, the results with Japanese quail DNA are surprising, since this species is considered to be quite closely related to the chicken. This point will be discussed later after all the pertinent data have been presented.

Additional information can be gained by considering the rate of reassociation of the chicken single copy DNA with duck and Japanese quail DNA, respectively. In each case the results of the computer analysis (shown in the Legend to Figure 2) indicate that the data are best fit by a single copy component. The absence of a detectable component reassociating more rapidly than single copy DNA indicates that most of the chicken single copy sequences that reassociate with duck or Japanese quail DNA do not occur as moderately or highly repeated sequences in the genome of the duck or the quail.

The divergence of chicken single copy DNA from the DNA of the duck and the Japanese quail was also determined by measuring the thermal stability of the reassociated DNA. Labeled chicken single copy DNA was reassociated with excess unlabeled DNA from each bird, and the DNA duplexes were bound to columns of hydroxylapatite at 60 °C. The temperature was raised in increments, and the single strands, arising from dissociation of the duplexes, were eluted at each temperature. The results are shown in Figure 3. Panel A shows the cumulative percent of the radioactivity eluted at each temperature. This form of the data is most convenient for determining the midpoint of the thermal transition, or T_m. The T_m of the homologous duplexes, which are presumed to have no mispaired bases, was 82 °C. This value was used as a standard to determine the fraction of mispaired bases in the duplexes with duck and Japanese quail DNA. As shown in Table I, the chicken-duck and chicken-Japanese quail duplexes had a T_m 7.5 and 11 °C lower than the homologous duplexes, respectively, indicating that these duplexes have about 7.5% and 11% of their nucleotides mispaired (Bonner et al., 1973). Thus, divergence measured by the extent of reassociation between the single copy DNAs of these birds is paralleled by the fraction of bases mispaired in the DNA that does reassociate.

Presumably, the chicken single copy sequences that failed to react in each case were prevented from doing so by the reassociation criterion (conditions of salt and temperature) that

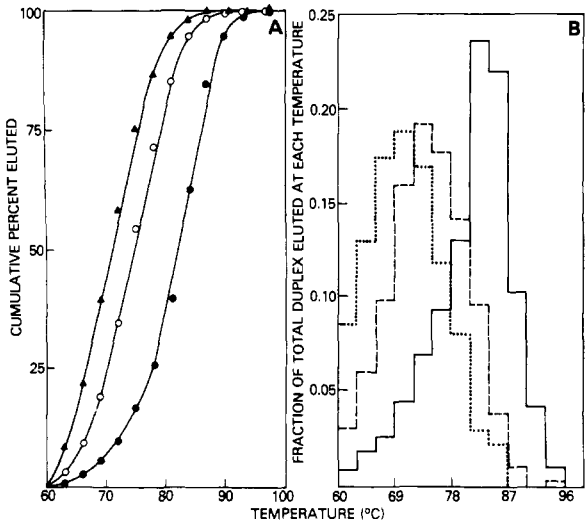

FIGURE 3: Thermal stability of chicken single copy DNA reassociated with chicken, duck, and Japanese quail DNA. (A) ^{3}H-labeled fragments of chicken single copy DNA were reassociated with unlabeled DNA from chicken (●), duck (○), and Japanese quail (▲), as described in the legend to Figure 2. After reassociation to C_0t 30 000–40 000, the DNA was loaded onto a column of hydroxylapatite in 0.12 M PB at 60 °C. The column was washed extensively at 60 °C to remove any single-stranded DNA. The temperature was then increased in 3 °C increments and the single strands, resulting from dissociation of DNA duplexes, were eluted from the column. The fraction of the total radioactivity bound at 60 °C was 69% with chicken DNA, 43% with duck DNA, and 13.5% with Japanese quail DNA, respectively. Hydroxylapatite column recoveries ranged from 97 to 100%. The cumulative percent of the radioactivity eluted at each temperature is shown. (B) These same data are presented in differential form, showing the fraction of the total duplex eluted at each temperature. Labeled chicken single copy DNA reassociated with unlabeled DNA from chicken (—), duck (- - -), and Japanese quail (···) is shown.

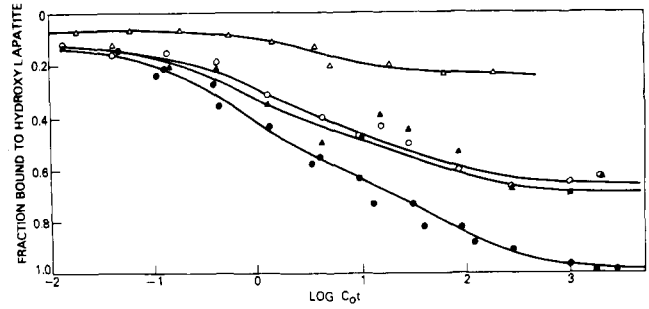

FIGURE 4: Repetitive DNA divergence in four avian species. ^{3}H-labeled fragments of chicken repetitive DNA (390 nt) were prepared as described in Experimental Procedures, and reassociated with a 5×10^5-fold excess of unlabeled DNA from chicken (550 nt), duck (500 nt), Japanese quail (540 nt), and ostrich (860 nt), respectively. Reassociation and hydroxylapatite chromatography were performed as described in the legend to Figure 2. Self-reassociation of the labeled DNA did not exceed 2%. The data have been normalized to the maximum extent of reassociation of the labeled DNA with homologous unlabeled DNA, which decreased from 90% to 60% during the course of the experiment. The curves drawn through the data points were generated by computer analysis and represent a composite of two repetition frequency components in the reassociation reactions with chicken, duck, and Japanese quail DNA, respectively, and a single component in the reaction with ostrich DNA. The final extent of reaction of the labeled DNA was 100%, 66%, 68%, and 24% with unlabeled DNA from chicken, duck, Japanese quail, and ostrich, respectively. The fraction of the reassociated DNA in each component (F) and the reassociation rate constants (K), determined by computer analysis, were: (●) chicken DNA, $F = 0.55$, $K = 0.95$; $F = 0.33$, $K = 0.01$; rms = 2.7%; (○) duck DNA, $F = 0.31$, $K = 0.98$; $F = 0.23$, $K = 0.026$; rms = 2.4%; (▲) Japanese quail DNA, $F = 0.33$, $K = 1.68$; $F = 0.24$, $K = 0.025$; rms = 4.2%; (△) ostrich DNA, $F = 0.18$, $K = 0.30$; rms = 1.4%.

set an upper limit at about 20% of the bases mispaired within a stable DNA duplex. Even with this limitation, it is sometimes possible to detect classes of more or less divergent single copy sequences as thermal stability components in the reassociated DNA, as has been described for some sea urchin species (Angerer et al., 1976). Figure 3B presents the thermal chromatograms of chicken–duck and chicken–Japanese quail single copy duplexes in a form more convenient for detection of thermal stability components. The fraction of the total duplex eluted at each temperature is shown. Only a single thermal stability component was discernible, an indication that in these avian species, all of the single copy sequences that reassociate have diverged to about the same extent.

We next determined the extent of divergence of repetitive sequences in these four birds. The method used was technically similar to that already described for single copy DNA, but there are two significant differences related to the interpretation of the results. First, the reassociated repetitive DNA from a single species has been shown to contain duplexes with a broad range of thermal stability (Britten et al., 1976; Eden et al., 1977). This finding indicates that some repeated DNA sequences are identical copies of one another (reassociate to form high thermal stability duplexes), whereas other repeated DNA sequences are not identical copies (reassociate to form duplexes with a lower T_m). The limitation of the fraction of mispaired bases within a stable DNA duplex established by the reassociation criterion may result in a failure to detect, in a second species, those DNA sequences that already form low thermal stability duplexes in the homologous reaction. Conversely, estimates of interspecies DNA divergence based on thermal stability of duplexes may lead to an underestimate if families of repeated sequences whose individual sequences are very similar dominate the heterologous reaction.

The second difference between single copy and repetitive DNA reassociation reactions derives from the fact that families of repeated DNA sequences exhibit a broad range of repetition frequencies (Britten & Kohne, 1968; Britten & Davidson, 1971). When the repetitive DNA from one species is present in trace amounts and DNA from the second species is present in excess, a few copies of a sequence in the DNA present in excess may reassociate with a much larger number of copies of the same sequence in the trace of labeled DNA, resulting in the appearance of extensive sequence homology. Thus, the extent of reassociation of repetitive DNA from two different species is a function of both the number of repetitive sequences that are similar and the repetition frequency of those sequences in the DNA of both species. When repetitive DNA sequences from two species are compared, both the extent of reassociation and the thermal stability of the reassociated DNA are influenced by many parameters whose individual effects cannot be distinguished without additional experimentation.

Our initial estimates of repetitive DNA sequence homologies in these four avian DNAs derive from measurements in which ^{3}H-labeled chicken repetitive DNA was reassociated with a vast excess of unlabeled DNA from chicken, duck, Japanese quail, and ostrich, respectively. The reassociation kinetics and the extent of reassociation were determined by hydroxylapatite chromatography, and the results are shown in Figure 4. Parameters determined by computer analysis of the data points are listed in the lower portion of Table 1. The extent of reassociation of chicken repetitive DNA with the DNA of the duck, the Japanese quail, and the ostrich was 66%, 68%, and 23% of the homologous reaction, respectively. Thus, the chicken repetitive DNA reassociated to an extent equal to or greater than chicken single copy DNA with the DNA of each of the other birds.

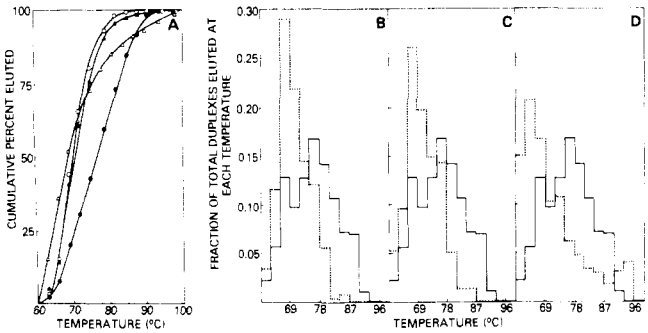

FIGURE 5: Thermal stability of chicken repetitive DNA reassociated with chicken, duck, Japanese quail, and ostrich DNA. (A) ^{3}H-labeled fragments of chicken repetitive DNA (390 nt) were reassociated with a 5×10^5-fold excess of unlabeled DNA from chicken (●), duck (○), Japanese quail (▲), and ostrich (△), as described in the legend to Figure 4. After reassociation to C_0t 2000–3000, the reassociated DNAs were applied to columns of hydroxylapatite at 60 °C and washed extensively to remove unreassociated single strands. The temperature was then raised in increments of 3 °C and the single strands resulting from the dissociation of duplexes were eluted at each temperature. The fractions of the labeled DNA bound at 60 °C after reassociation with chicken, duck, Japanese quail, and ostrich DNA were 56%, 37%, and 22%, respectively. The recovery of total radioactivity applied to hydroxylapatite columns ranged from 98% to 115%. The cumulative percent of the bound radioactivity eluted at each temperature is shown. (B–D) The data are presented in differential form to emphasize thermal stability components. The fraction of the total duplex eluted at each temperature is shown. ^{3}H-labeled chicken repetitive DNA reassociated with unlabeled chicken DNA is represented by the solid histogram in panels B–D. The dotted histograms represent chicken repetitive duplexes with duck (B), Japanese quail (C), and ostrich (D) DNA.

TABLE II: Specificity of Oligo(dT)-Cellulose Chromatography for DNA Fragments Terminally Labeled with Deoxyadenosine Homopolymer Tracts.

description of DNA fragments[a]			
length (nt)	radioisotope label in the DNA strands[b]	^{32}P-labeled terminal homopolymer tract[c]	% of radioact. bound to oligo(dT)-cellulose[d]
680	^{3}H	none	0.7
550	none	deoxyguanosine	2.6
550	none	deoxyadenosine	98.0
570	^{3}H	deoxyadenosine	97.0

[a] Sheared fragments of total chicken DNA. [b] Radioactivity in the DNA strands was introduced by nick translation. [c] Fragments bearing homopolymer tracts were prepared by incubation with terminal transferase and the deoxyribonucleoside triphosphate specified, as described in Experimental Procedures. The average length of homopolymer tracts, estimated from the calculated number of 3′OH termini and picomoles of radioisotope incorporated, ranged from 20 to 40 nucleotides. [d] Binding to a column of oligo(dT)-cellulose was tested in 0.4 M NaCl, 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$ at 22 °C. The bound fraction was eluted with 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$ at 22 °C. Recovery of radioactivity applied to the columns ranged from 63 to 93%. The value reported for the fragments labeled with both, ^{3}H and ^{32}P refers to ^{3}H.

The thermal stability of chicken repetitive DNA reassociated with unlabeled DNA from chicken, duck, Japanese quail, and ostrich, respectively, is shown in Figure 5A. The reassociated DNAs were bound to columns of hydroxylapatite at 60 °C, and thermal chromatography was performed as described above for single copy DNA. The T_m of the labeled chicken repetitive DNA reassociated with homologous DNA was 76.5 °C. As shown in Table I, this value is 5.5 °C lower than the T_m of chicken single copy duplexes, an indication that 5.5% of the bases of the reassociated chicken repetitive DNA are mispaired, on the average. Chicken repetitive DNA reassociated with total DNA from the duck, Japanese quail, and the ostrich, respectively, had a lower T_m than the corresponding homologous repetitive duplexes (Figure 5A and Table I). The fraction of the bases mispaired in the heterologous repetitive duplexes ranged from 11.5% with Japanese quail DNA to 13% with ostrich DNA.

Figures 5B–D show these data plotted to emphasize thermal stability components. The solid histogram reproduced in each panel represents the fraction of the reassociated chicken repetitive DNA eluted at each temperature. A minor component with low thermal stability is distinguishable from the major component with high thermal stability (with a mode at 75–78 °C). Since the thermal stability component of chicken repetitive DNA which has reassociated with the DNA of the other birds has not been identified, the heterologous thermal chromatograms are more difficult to interpret. However, in each case some heterologous repetitive duplexes had a T_m as high as the high thermal stability component in chicken repetitive DNA. Thus, in contrast to the situation with single copy DNA described above, it appears that some chicken repetitive DNA sequences that reassociate with DNA from the other birds have diverged more than others.

Isolation and Characterization of the Common Repetitive Sequences in Chicken and Ostrich DNA. We have observed that, although chicken single copy DNA shares very few sequences in common with ostrich DNA, about 25% of chicken repetitive DNA reassociates with DNA from the ostrich. To further characterize the common repetitive sequences, we used a method that allows direct isolation of those chicken repetitive sequences that are also found in ostrich DNA. A similar procedure has been described previously (Lee et al., 1977). Before the chicken repetitive DNA was reassociated, about 30 residues of deoxyadenosine were added to the 3′-OH termini of the DNA fragments, using the enzyme terminal transferase (Roychoudhury & Wu, 1976). The 3′-OH terminal deoxyadenosine tracts added in this way will be referred to as "dA tails".

The presence of homopolymer tracts made it possible to separate the "tailed" fragments from fragments without dA tails by using a column of oligo(dT)-cellulose (Aviv & Leder, 1972). The chicken repetitive sequences that are also found in ostrich DNA were isolated in two steps. First, [^{3}H]-labeled chicken repetitive DNA fragments with dA tails were reassociated with excess unlabeled DNA from the ostrich, and the duplexes were isolated on a column of hydroxylapatite. Then the duplexes were dissociated by heating, and the radioactive single strands of chicken DNA with dA tails were separated from the ostrich DNA on a column of oligo(dT)-cellulose.

To establish the usefulness of this method, two criteria had to be met. During the addition of homopolymer tracts and the oligo(dT)-cellulose chromatography steps, there must be no artificial selection for any particular repeated sequences in chicken DNA. Second, the oligo(dT)-cellulose column must be highly selective for retention of dA tailed fragments, since the fragments without dA tails (the unlabeled DNA) will be present in excess. The data presented in Table II and Figure 6 establish that these criteria were fulfilled.

Table II shows that, as expected, 98% of the fragments bearing deoxyadenosine tracts bound to oligo(dT)-cellulose. This observed binding was not due to some contaminant (such as protein) introduced during incubation with terminal transferase, since fragments to which deoxyguanosine homopolymer tracts had been added, using terminal transferase, failed to bind to the column. From the fact that chicken

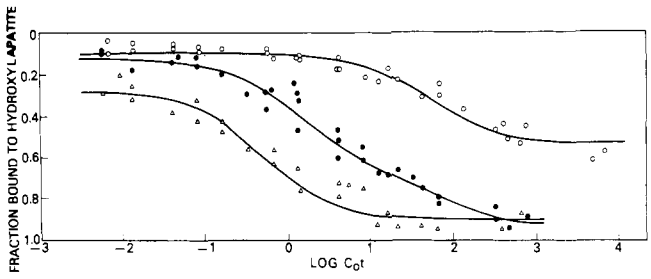

FIGURE 6: Repetition frequency of common repeated sequences in chicken and ostrich DNA. (●) ^{3}H-labeled chicken total repetitive DNA, 440 nucleotides in length, with short, 3′-OH-terminal deoxyadenosine homopolymer tracts, was reassociated with a 5×10^5-fold excess of unlabeled chicken DNA (280 nt in length) in 0.12 M PB at 60 °C. The fraction of the labeled DNA bound to hydroxylapatite was determined as a function of C_0t. The curve drawn was generated by computer analysis of the data points. Rms deviation of the curve from the data points was 4.8%. Two repetition frequency components are represented: $F = 0.52$, $K = 0.77$; $F = 0.30$, $K = 0.02$. The final percent of the labeled DNA unreacted was 7%. ^{3}H-labeled chicken selected repetitive DNA, 550 nucleotides in length with short, 3′-OH-terminal deoxyadenosine tracts, was reassociated with a 5×10^5-fold excess of unlabeled DNA from chicken (△) or ostrich (○). The unlabeled chicken DNA fragments were 280 nt in length, and the unlabeled ostrich DNA fragments were 480 nt in length. Hydroxylapatite reassociation kinetics were analyzed by computer. The curve representing the reassociation with chicken DNA represents a single component, $F = 0.614$, $K = 1.91$. The final fraction of the labeled DNA unreacted was 0.116, and the rms deviation of the curve from the data points was 7.1%. The curve representing the reassociation with ostrich DNA is also a single component, $F = 0.43$, $K = 0.017$. The final fraction of the labeled DNA unreacted was 0.47, and the rms deviation of the curve from the data points was 5.1%.

DNA without homopolymer tracts also failed to bind to oligo(dT)-cellulose, we conclude that the chicken genome itself does not contain poly(A) tracts of sufficient length to bind to oligo(dT)-cellulose under the conditions used here. To determine whether our conditions for labeling with terminal transferase result in addition of homopolymer tracts to all of the DNA fragments, we measured the fraction of ^{3}H bound to oligo(dT)-cellulose using dA tailed fragments labeled with ^{3}H in the DNA strand. We found that 97% of the ^{3}H was bound, indicating that the vast majority of the individual DNA fragments received homopolymer tracts of sufficient length to ensure retention on oligo(dT)-cellulose.

The discrimination between dA tailed DNA and DNA without deoxyadenosine tracts by oligo(dT)-cellulose chromatography was (98/0.7) = 140-fold. Subsequent experiments demonstrated an even better purification, with up to 500-fold enrichment for dA tailed fragments in a single chromatography step.

To establish that addition of dA tails and binding to oligo(dT)-cellulose do not, in themselves, artificially enrich for any fraction of chicken repetitive DNA, we prepared ^{3}H-labeled chicken repetitive DNA fragments bearing dA tails. The dA tailed fragments were reassociated with an excess of unlabeled chicken DNA to C_0t 500; this C_0t was chosen to ensure complete recovery of both the high and low repetition frequency classes of chicken repetitive DNA (Figure 1). The reassociated DNA was loaded onto a column of hydroxylapatite, and 70–80% of the labeled DNA was bound to the column. The DNA duplexes were eluted from hydroxylapatite, denatured by heating, and loaded onto an oligo(dT)-cellulose column. About 50% of the dA tailed single strands bound to oligo(dT)-cellulose, whereas only 0.5% of the unlabeled DNA without dA tails was retained by the column. The incomplete binding of the labeled DNA probably resulted from breakage of the DNA fragments during the heat denaturation steps. The fragments bound to oligo(dT)-cellulose were still of sufficient length (440 nt) for reassociation measurements.

TABLE III: Repetition Frequency of Selected Chicken Repetitive Sequences in Chicken and Ostrich DNA.

unlabeled DNA[a]	fraction[b]	K	av repetition frequency[c]
chicken	0.69	1.91	3000
ostrich	0.81	0.017	30

[a] The ^{3}H-labeled, selected repetitive fraction of chicken DNA was reassociated with excess unlabeled DNA from chicken or ostrich as described in the legend to Figure 6. Hydroxylapatite reassociation data were analyzed by computer to determine the fraction of repetition frequency components and their reassociation rate constants, K (M^{-1} s^{-1}). [b] The fraction of each component was normalized to the final extent of reassociation of the labeled DNA. [c] The average repetition frequency was calculated by dividing the reassociation rate constant by the rate constant of single copy DNA of each species.

To establish that all of the repetition frequency classes in chicken repetitive DNA were recovered in the oligo(dT)-cellulose bound fraction, this fraction was reassociated with excess unlabeled chicken DNA. The reassociation kinetics, measured by hydroxylapatite chromatography, are shown in Figure 6. The repetition frequency components present in the dA tailed, ^{3}H-labeled repetitive DNA were identified by computer analysis as follows: $F = 0.52$, $K = 0.77$; $F = 0.30$, $K = 0.02$, corresponding to repetition frequencies of 1300- and 30-fold, respectively. These components are comparable to those present in chicken repetitive DNA without homopolymer tracts (Figure 4, $F = 0.55$, $K = 0.95$; $F = 0.33$, $K = 0.01$, corresponding to average repetition frequencies of 1500- and 50-fold, respectively). The relative proportions of the high and low repetition frequency components in chicken repetitive DNA, and the rate of reassociation of each of these components, have not been altered by the addition of dA tails and oligo(dT)-cellulose chromatography. The DNA fraction prepared by this procedure will be called "dA tailed, total chicken repetitive DNA".

We then used this same procedure to isolate those chicken repetitive sequences that are also found in ostrich DNA. ^{3}H-labeled chicken repetitive DNA with dA tails was reassociated with excess unlabeled DNA from the ostrich. After reassociation to C_0t 500, 27% of the labeled DNA bound to hydroxylapatite (average of two experiments). The duplexes were dissociated, and the labeled dA tailed chicken repetitive DNA was recovered by oligo(dT)-cellulose chromatography. The labeled DNA fragments that bound to oligo(dT)-cellulose were 500 nucleotides in length, and will be referred to as the "selected fraction" of chicken repetitive DNA. The total and selected repetitive fractions represented 13% and 3.5% of the chicken genome, respectively.

The repetition frequency of the selected repetitive sequences in the chicken genome was determined by reassociation of the labeled, selected fraction with excess unlabeled chicken DNA. The reassociation kinetics are shown in Figure 6, and the results of computer analysis of the hydroxylapatite data are shown in Table III. The data presented in Figure 6 indicate that the selected repetitive sequences reassociated with unlabeled chicken repetitive DNA more rapidly than did total chicken repetitive DNA. The best fit to this reassociation data, determined by computer analysis, was a single component with a reassociation rate constant indicating an average repetition frequency of 3000-fold. The low repetition frequency component of chicken repetitive DNA was not detected in the selected fraction. Thus, most of the chicken repeated sequences that

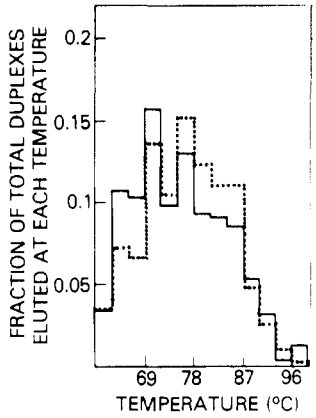

FIGURE 7: Thermal stability of selected chicken repeated DNA sequences. ^{3}H-labeled total (—) and selected (- - -) chicken repeated DNA fractions prepared as described in the text were reassociated with a 2400-fold excess of unlabeled chicken DNA to C_0t 100. The reassociated DNAs were applied to columns of hydroxylapatite at 60 °C in 0.12 M PB. More than 95% of each radioactive DNA bound to hydroxylapatite at 60 °C. The temperature was increased in increments of 3 °C and the dissociated single strands were eluted at each temperature with 0.12 M PB. The fraction of the total duplex eluted at each temperature is shown.

are also found in ostrich DNA are those with the highest repetition frequency in the chicken genome.

The reassociation kinetics of the selected chicken repetitive fraction with ostrich DNA are shown in Figure 6. The results are dramatically different from those obtained when this same fraction was reassociated with chicken DNA. The sequences that were found to be repeated about 3000-fold in chicken DNA are repeated only 30-fold in ostrich DNA (Table III). From these data we conclude that there has been a substantial change in the repetition frequency of many repeated sequences during the course of avian evolution.

We next determined whether the selected repetitive fraction of chicken DNA contains repetitive sequences that are precise copies of one another, or, alternatively, contains the more divergent groups of repeated sequences within the chicken genome. These two classes of repeated sequences can be identified by their thermal stability, as described above. Total and selected chicken repetitive DNA were reassociated with unlabeled chicken DNA. The thermal chromatograms of the reassociated DNAs are shown in Figure 7. Each repetitive DNA fraction was found to contain both high and low thermal stability components. The component of chicken repetitive DNA with the higher thermal stability was slightly enriched in the selected repetitive fraction, but it appears that both the relatively precisely repeated sequences and the more highly divergent groups of repeated sequences in chicken repetitive DNA are represented in the selected fraction.

Discussion

Most eukaryotic genomes that have been studied contain both single copy and repetitive DNA sequences (Davidson et al., 1975), and the DNA of the chicken and the ostrich conform to this pattern. However, these avian genomes are distinguished by an unusually small fraction of repetitive DNA, 13% and 10% for chicken and ostrich DNA, respectively. Only a few of the many animal genomes studied so far have such a small fraction of repetitive DNA, most notably two Dipteran insects, *Drosophila* and *Chironomus*, and the honeybee *Apis mellifera* (Manning et al., 1975; Wells et al., 1976; Crain et al., 1976). In these insects the genome is also unusually small, ranging from 0.1 to 0.35 pg. To our knowledge, the chicken and the ostrich are the first examples of large genomes (1–2 pg) possessing small fractions of repeated DNA. It is also of interest that this uncommon feature of genome organization is shared by two avian species that probably diverged from one another at least 60 million years ago (Brodkorb, 1964).

The taxonomic position of the four avian species studied here is based on incomplete evidence and will need to be refined as new information becomes available. The phylogeny of birds has been especially difficult to determine because of the incomplete fossil record and the great morphological similarity of most modern species (Romer, 1966). Without reliable estimates of the time since divergence from a common ancestor for any of these species, it is difficult to specify phylogenetic relationships, and it is expected that the study of DNA sequence divergence between species will contribute to our knowledge in this area. However, studies of other groups of animals have shown that conclusions about the extent of interspecies divergence can depend somewhat on whether only repetitive or only single copy sequence homologies are emphasized (Galau et al., 1976). Some apparent disparities in the relatedness between species using these different frequency classes of DNA make it more difficult to directly relate DNA sequence homology to evolutionary divergence, but raise other interesting questions about the relative degree of evolutionary conservation of repeated and single copy sequences.

The measurements of the extent of homology of single copy and repeated DNA among these four avian species, as shown in Table I, also contain some apparent disparities. For example, the fraction of the chicken single copy sequences shared with the ostrich is very small (5% or less), but almost 25% of the labeled chicken repetitive sequences reassociated with ostrich DNA. Similarly, an increase was seen when the extent of reassociation of chicken repetitive DNA with Japanese quail DNA was measured. The unexpectedly small fraction of the single copy sequences (26%) that are similar in these two birds that, from the point of view of current taxonomy, are closely related, increased to the expected 68% when repetitive sequences were considered. On the basis of the extent of reassociation of repetitive sequences, the Japanese quail would be considered to be as close a relative of the chicken as is the duck, whereas a more distant relationship would seem to be indicated if only single copy sequences were considered.

It is important to determine whether these apparent disparities result from the technical difficulties encountered in measuring DNA homology using repeated DNA fractions, as described above, or these measured differences result from actual conservation of some repetition frequency classes of DNA relative to others during evolution. Information on this point was obtained by isolation and characterization of a DNA fraction representing those repetitive sequences that are common to both the chicken and the ostrich genome.

Table III summarizes the pertinent information. The increased extent of reassociation of chicken repeated DNA relative to single copy DNA with the DNA of the ostrich is explained by the higher repetition frequency of the common sequences in chicken relative to ostrich DNA.

We calculated the fraction of the ostrich genome homologous to chicken repetitive DNA as follows. The selected repetitive fraction comprises about 3% of the chicken genome, and most of the sequences in that fraction have a repetition frequency of about 3000-fold. Those sequences are represented in ostrich DNA by sequences repeated about 30-fold, and the fraction of ostrich DNA homologous to chicken repetitive DNA is about 3%/100 = 0.03%. Although this computation includes several assumptions (including that the length of the sequences is similar in chicken and ostrich DNA and the genome sizes are similar), it is reliable within an order of magnitude, and indicates that the fraction of ostrich repetitive

DNA homologous to chicken DNA does not exceed 0.3%. Thus, the fraction of the ostrich genome homologous to chicken repetitive sequences, and to chicken single copy sequences, is similar.

The presence of mispaired bases has a small effect on the rate of DNA reassociation (Bonner et al., 1973). The data shown in Table I indicate that the common repetitive sequences have 13% and 5.5% of their bases mispaired when reassociated with ostrich or chicken DNA, respectively. Thus the common repetitive sequences would be expected to reassociate 10–20% more slowly with ostrich DNA than with chicken DNA. Since this effect is small compared with the observed 100-fold difference in the rates reassociation, we have neglected the influence of mispaired bases in the computations presented above.

The mechanism whereby the repetitive sequences common to chicken and ostrich repetitive DNA have undergone a 100-fold change in repetition frequency has not been determined. Some precedent for rapid alterations in the repetition frequency of sequences in DNA during evolution is known from studies of highly repetitive satellite DNAs. Because of the aberrant buoyant density of these DNAs, they can be directly isolated and their repetition frequency in related species can be determined. The results of such analyses (Rice, 1971; Gillespie, 1977) seem to indicate that some highly repetitive sequences in DNA of one species may be entirely absent from the DNA of other, related species. The change in repetition frequency of the common repetitive sequences in chicken and ostrich DNA is reminiscent of these descriptions of satellite DNAs, but on a much more limited scale. It is clear that many different repetitive sequences in chicken DNA have undergone alteration in repetition frequency of about 100-fold, unlike the many thousand-fold changes in repetition frequency of a single sequence as described for satellite DNAs.

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