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Intragenomic DNA Sequence Homologies in the Chicken and Other Members of the Class *Aves*: DNA Re-association Under Reduced Stringency Conditions

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Summary. We have investigated the intragenomic DNA sequence homologies of twelve species of birds representing five orders, and emphasizing Galliformes. This study differs in two important ways from the classical approaches taken in constructing and evaluating phylogenies based on DNA sequence similarities. Comparisons are made on the basis of sequence homologies *within* genomes of related birds, rather than between genomes. DNA is reassociated at 50° C in 0.5M phosphate buffer; these conditions allow formation and detection of duplexes containing more mismatch than would normally be permitted using more stringent conditions, affording an opportunity to observe more ancient sequence homologies. Thermal stability profiles of DNA duplexes formed under these conditions are the basis of comparison; three general patterns were observed. This approach emphasizes differences in sequence composition between genomes while the more traditional method of intergenomic tracer DNA hybridization at higher stringency emphasizes sequence similarities.

No correlation was found between taxonomic position and intragenomic sequence composition, either within or between lineages. The thermal stability profiles of DNA duplexes formed within avian genomes did not reflect the biological similarities inferred from morphology, karyotype, and studies of interspecific hybridization. While all of the differences observed could have occurred over geological time, it was surprising that the genomes of the domestic chicken and the Red Jungle Fowl (*Gallus gallus*) differ in their sequence compositions. It appears that amplification/reduction events and/or positional changes occur rather often during evolution of a lineage.

Abbreviations: SDS, sodium dodecyl sulphate; PB, equimolar sodium phosphate buffer pH 6.8; Cot, concentration of DNA in moles of nucleotide per liter times the incubation time in seconds; Equiv. or Equivalent Cot, Cot corrected for the monovalent cation concentration effect on re-association rate; HAP, hydroxylapatite; $Te_{1/2}$, temperature at which one-half the DNA has eluted from HAP; SSC, 0.15M sodium chloride-0.015M sodium citrate

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Introduction

A genome contains the record of amplification/reduction events that have occurred during the evolution of a species. If the *intragenomic* DNA sequence homologies of related species are compared, it might be possible to assess the frequency of occurrence of such events, and to correlate them with the taxonomic positions of the animals, especially if such events occur over a geological time scale. If this were the case, lineages might be characterized and perhaps even identifiable on the basis of similarities in the relatedness of sequences within their genomes. Blocks of sequences representing ancestral amplifications might constitute a family of similar but mismatched sequences in genomes of modern members of a lineage; in this case, the mismatch would be introduced by nucleotide substitution over evolutionary time. However, if the event were recent, amplification of a pre-existing family of related sequences could also result in mismatched intragenomic duplexes, but in this case, if amplification events occur frequently a lineage might not be characterized by intragenomic sequence homologies. In either case, the extent of mismatch cannot be predicted accurately.

Thermal stability profiles of DNA duplexes formed during re-association experiments are the most common and convenient means of estimating sequence mismatch and divergence. Traditionally, lineages have been compared on the basis of sequence similarities *between* genomes; trace amounts of radioactively labeled DNA are hybridized with a large mass excess of DNA from a related species. The degree of relatedness is estimated from the amount of tracer reacted and the thermal stability of the tracer duplexes; this approach has been quite useful, but because no information can be obtained about the unreacted tracer, and because the mismatched tracer reaction is kinetically disfavored, these experiments tend to emphasize similarities; also, the heterologous duplexes formed are quite dependent upon the reassociation conditions (Kohne et al., 1972).

These thoughts prompted the investigation reported here. DNA from five orders of birds was reassociated under reduced stringency conditions to allow formation of duplexes containing more mismatch. Thermal stability profiles of such intragenomic duplexes were compared within and between lineages. Three general types of melting profiles were obtained, and there was no correlation between the profile and taxonomic position. Amplification/reduction events and positional changes may occur often enough in evolution as to obscure the ancestry.

Experimental

Preparation and Shearing of DNA. DNA was purified from tissues using the procedure of Flamm et al. (1966), with one additional step. After the RNase and α-amylase treatment, the preparation was made to 0.5% SDS and incubated overnight at 37°C with Proteinase K (Beckmann) at 50 μg/ml. The final ethanol precipitate was redissolved in 0.01M Tris-Cl, 0.1M sodium acetate, pH8. When blood was used as the source of DNA, the procedure was modified as follows: blood was collected in 3.8% sodium citrate

(about 0.1 ml per 5–10 ml blood) and the cells were collected at 5,000 rpm for 5 min. The cells were washed several times in 0.01M Tris-Cl, 3mM magnesium chloride, 0.9% sodium chloride, pH 7.4. The cells were resuspended in 0.1M sodium chloride, 0.1M EDTA, 1M sodium perchlorate, 1.5% SDS and homogenized vigorously in a Dounce homogenizer. The ultraviolet ratios of the resulting DNAs were routinely 260/280 = 2 and 260/230 = 2.3 to 2.4.

DNA was sheared to a single stranded fragment length of 400–600 bases, estimated using alkaline agarose gels (McDonell et al., 1977). DNA concentration was adjusted to 0.5–1 mg/ml before passing it through a French press at 20,000 psi. Divalent cations were removed by passing the sheared solution over a Chelex-100 (Bio-Rad) column equilibrated with 0.01M Tris-Cl, 0.1M sodium acetate, pH 8, the same solution in which the DNA was dissolved for shearing. Two volumes of cold ethanol were mixed with the DNA and the precipitate was allowed to form overnight at -20°C. The precipitate was pelleted at 10,000 x g for 10 min and redissolved at about 10 mg/ml in 0.01M Tris-Cl, pH 8.

Fractionation of Repeated and Unique DNA Sequences. Sheared chicken DNA in 0.5M sodium phosphate buffer, pH 6.8, was incubated at 50°C until an Equivalent Cot of 50 was reached. The DNA was diluted to 0.12M PB and fractionated on hydroxylapatite (HAP) (BioRad., Bio Gel HTP) columns at 50°C into bound and unbound fractions; single and double stranded DNAs were recovered using 0.12M and 0.4M PB, respectively. The double stranded fraction was readjusted to 0.5M PB, denatured, incubated to Equiv. Cot 50 a second time, and fractionated as described above. DNA bound to HAP was saved and represented the repetitive sequence portion of the chicken genome. Single stranded DNA from the first HAP fractionation was adjusted to 0.5M PB, denatured, incubated to Equiv. Cot 600, and fractionated as above. DNA not binding to HAP after the second incubation was taken as the unique sequence chicken DNA. Because the DNA fractions were in large volumes of phosphate buffer, a modification of the cetyltrimethyl ammonium bromide precipitation (CTAB) method from phosphate-containing solutions was used (Stehelin et al., 1976; Reitz et al., 1972). The DNA fractions were chilled and made to 5mM CTAB from a stock solution of 0.1M (Sigma, cat. No. H-5882). After sitting in ice for 10 min, the precipitate was collected at 10,000 x g for 10 min, redissolved in 1M sodium chloride, and precipitated with three volumes of cold ethanol; the precipitation from 1M NaCl was repeated two more times. After a final ethanol precipitation from 0.1M sodium acetate with two volumes of alcohol, the precipitate was dissolved in 0.01M Tris-Cl, pH 8.

Conditions of DNA Reassociation. Most of the DNA reassociation experiments reported here were performed under reduced-stringency conditions. Incubation temperatures ranged from 46°C to 60°C, and the phosphate buffer concentrations ranged from 0.1M to 0.5M. Incubation times and buffer concentrations were compared on the basis of the Equivalent Cot of the reaction. Incubations were carried out in sealed, siliconized glass capillaries after denaturing the DNA at 105°–110°C for 5 min in an ethylene glycol-water mixture. Incubations contained 66 to 200 μg of DNA. Specific details and conditions are given in the figure legends.

Hydroxylapatite Thermal Chromatography and Melting Curves. Incubations were terminated by expelling the capillary contents into a solution of such composition that

the final mixture was 1 ml of 0.12M PB maintained at the incubation temperature. The sample was loaded onto a thermostated, jacketed column containing 0.5g HAP (dry weight) in 0.12M PB at 50°C; the sample was mixed with the HAP and allowed to equilibrate for 10 min before opening the column outlet. Unbound DNA was washed off with 10 ml of 0.12M PB at 50°C. DNA was eluted with 5 ml of 0.14M PB as the column temperature was raised in 5 degree increments to 60°C, then in 4 degree increments to 96°C. Any DNA remaining on the column was removed with 0.4M PB at 96°C. Recovery of DNA was always 95% or greater. Three batches of Bio-Rad HAP were used; DNA Grade Bio-Gel HTP No. 9404 and No. 16399, and Bio-Gel HTP No. 9463; only minor differences were noted in the melting curves using different batches of HAP. Most of the experiments were performed using Bio-Gel No. 9463. The optical density of each fraction was determined at 320 and 260 nm; the reading at 320 nm was subtracted as a correction for any HAP fines in the fraction. Low DNA concentrations in some fractions required using cuvettes with a 5 cm path length.

Sources of Birds and DNA Used in this Study. Blood from the emu (*Dromaius novaehollandiae*) was obtained through the cooperation of M. Wickens, of this laboratory, and W. Mottram of the San Francisco Zoo. The Barbary dove (*Streptopelia risoria*) was purchased from the Monette Pet Shop, Palo Alto, CA. Ostrich DNA (*Struthio camelus*) was the generous gift of Francine Eden, National Institutes of Health. The Golden and Lady Amherst pheasants (*Chrysolophus pictus* and *Chrysolophus amherstiae*) were purchased from Ida M. Johnston of Portola Valley, CA. Turkey, Ring-necked pheasant, and chukar partridge (*Meleagris gallopavo, Phasianus colchicus*, and *Alectoris graeca*) were purchased from W. McDoulett of Castro Valley, CA. Several Red Jungle Fowl (*Gallus gallus*) were obtained from the San Diego Zoo through the generosity of K. Benirschke. White leghorn chickens (*Gallus gallus*) were purchsed at the Olivera Egg Ranch, San Jose, CA.; some experiments were done with DNA from chicken blood (Calbiochem). Japanese quail and Scrub jay (*Coturnix coturnix japonica* and *Alpheocoma coerulescens*) were provided by K. Tosney and W. Dower, respectively, both of the Department of Biological Sciences, Stanford University. T7 viral DNA was a gift from G. Kassavetis, University of California, Berkeley; tritium label was introduced into the T7 DNA using the method of Rigby et al. (1977) to a specific activity of about $2x10^6$ dpm/μg DNA.

Results

Intragenomic DNA Sequence Homologies. Total DNA from birds of five taxonomic orders was reassociated at 50°C in 0.5M phosphate buffer, and loaded onto a HAP column at 50°C in 0.12M PB. The melting curves of duplexes formed under these conditions are shown in Figs. 1 and 2. For comparison, melting curves of duplexes formed at 60°C in 0.12M PB, are shown by the lighter continuous curves in these figures.

It is immediately apparent that lowering the incubation and HAP column loading criteria reveals intragenomic base sequence homologies not seen when more stringent conditions are used. A qualitative examination of the curves suggests three general patterns of duplex stability. Columns six and seven of Table 1 list the percentage of DNA eluting in low and intermediate stability temperature ranges, and are the qualitative basis for distinguishing the three patterns. The first two patterns are similar in that both

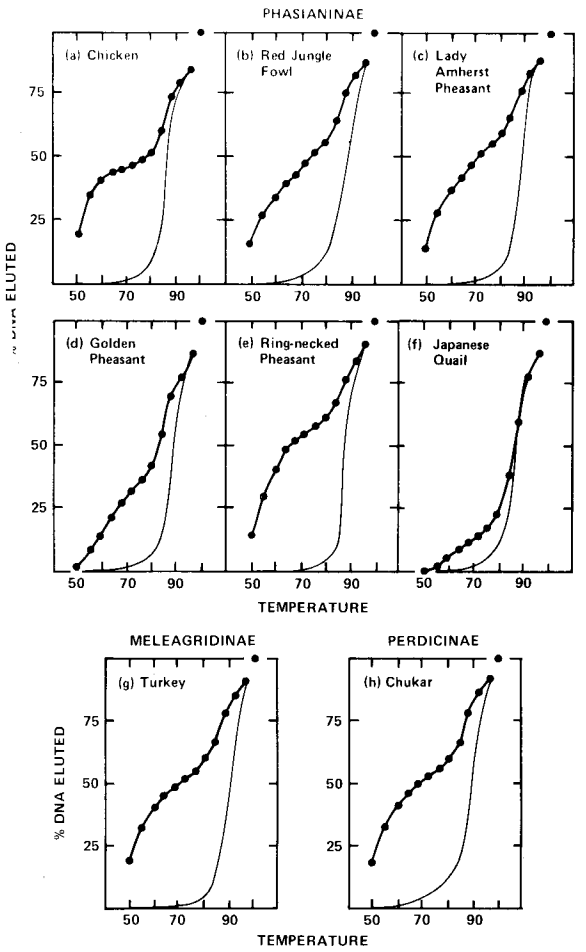

Fig. 1 a-h. Integral melting curves of intragenomic duplexes formed under reduced-stringency conditions. This figure presents the results obtained using DNA from three subfamilies of the order Galliformes: subfamily Phasianine, (**a**) chicken, (**b**) Red Jungle Fowl, (c) Lady Amherst pheasant, (**d**) Golden pheasant, (e) Ring-necked pheasant (**f**) Japanese quail, subfamily Meleagridinae, (**g**) turkey; subfamily Peadicinae, (**h**) chukar. DNA was reassociated at 50°C in 0.5M PB, and the thermal stability of the duplexes was determined as described in Experimental. The fainter, continuous curve is the melting profile of duplexes formed at 60°C in 0.12M PB

have 45–50% of the DNA eluting in the low temperature region (50–68°, inclusive), but differ in the amount of DNA eluting in the intermediate part of the curve (72–80°, inclusive); the first pattern is characterized by only 7% eluting (chicken, ostrich, ring-necked pheasant), and the second has 9–13% eluting in this area (dove, chukar, jay, turkey, Red Jungle Fowl, Lady Amherst pheasant). The third type of pattern is characterized by a significantly lower percentage of low stability duplexes (Golden pheasant, Japanese quail, emu).

The melting curves only give information about the duplexes which will bind to HAP at 50°C in 0.12M PB. The second and third columns of Table 1, listing the Equivalent Cot of the incubation and the percentage of DNA not binding to the column, show that many of the DNA fragments were not in duplexes of stability sufficient to permit binding to HAP. The single exception is the domestic chicken; only about 14% of chicken DNA failed to bind compared to 29–58% for the other birds.

Table 1. Reassociation and hydroxylapatite thermal chromatography of avian total DNAs

Source of DNA	50°C, 0.5M PB[a]		60°C, 0.12M PB[a]		% Low-Stability duplexes[b]	% Intermediate stability duplexes[b]	Profile type[b]
	Equiv. Cot x 10^{-2}	% not bound	Cot x 10^{-2}	% not bound			
Domestic chicken	200	14	39	6	46	7	1
	185	14					
Ostrich	184	40	31	4	47	7	1
	310	29					
Ring-necked pheasant	180	38	33	3	53	7	1
	236	47					
Barbary dove	212	34	39	3	45	9	2
	260	30					
Chukar	180	44	30	4	50	10	2
	238	32					
Scrub jay	197	35	34	7	50	11	2
	238	36					
Turkey	192	36	35	4	48	12	2
	192	36					
Red Jungle fowl	196	49	37	5	44	13	2
	200	49					
Lady Amherst pheasant	171	42	38	4	48	13	2
	177	30					
Golden pheasant	195	50	36	2	27	15	3
	272	45					
Japanese quail	195	50	35	2	12	11	3
	310	47					
Emu	138	55	38	2	7	18	3
	190	58					
	290	50					

[a]Incubation criteria and time, expressed as Cot or Equiv. Cot, and the percentage of DNA not binding to HAP column at 50°C in 0.12M PB are given in these columns

[b]These columns are intended as a qualitative comparison of the low and intermediate stability regions of the melting curves shown in Figs. 1 and 2

There is no easily detectable correlation between the taxonomic position of the birds studied and the thermal stability of the reassociated intragenomic homoduplexes. Melting curves of homoduplexes obtained for Galliformes, family Phasianidae, are shown in Fig. 1. The three subfamilies studied, Phasianinae, Meleagridinae, and Perdicinae, show the three general patterns of duplex stability. The same is true of the four other orders of birds, Struthioniformes, Casuariiformes, Columbiformes, and Passeriformes, shown in Fig. 2. Even between members of the same species (domestic chicken and Red Jungle Fowl) there is a detectable difference; these experiments have been repeated many times using different batches of HAP and different preparations of DNA, always obtaining results very similar to those shown in Figs. 1a and 1b. Striking differences were found between members of the sub-family Phasianinae (Fig. 1), and members of two orders, emu and ostrich (Figs. 2a and 2b). There are similarities among very distantly related birds; the profiles of the chicken (Fig. 1a) and the ostrich (Fig. 2b) are quite similar.

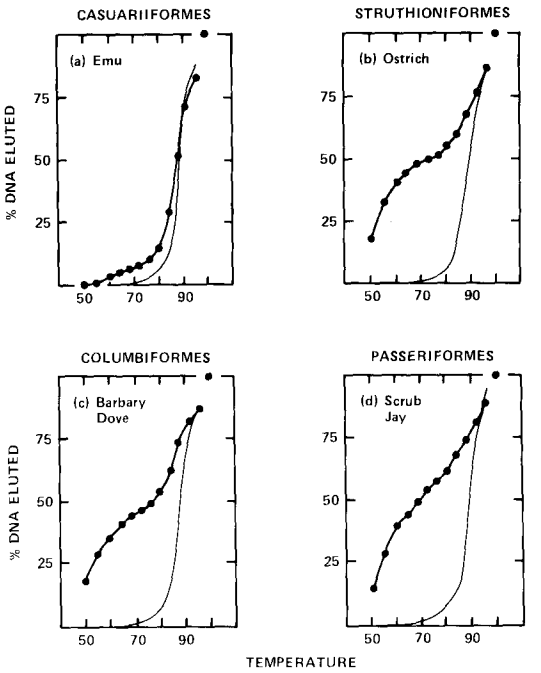

Fig. 2a-d. Integral melting curves of intragenomic duplexes formed using DNA from nongalliform birds. The incubation conditions and assay procedures were the same as those described in the legend of Fig. 1. The results shown here are for four orders: Order Casuariiformes, (a) emu; Order Struthioniformes, (b) ostrich; Order Columbiformes, (c) Barbary dove; Order Passeriformes, (d) Scrub jay

Localization of Low Thermal Stability Sequence Homologies in the Unique Sequence Part of the Chicken Genome. Sheared chicken DNA was fractionated as described in the Experimental section to obtain repetitive and unique sequence DNA fractions. These fractions were reassociated using standard and reduced-stringency conditions, 60°C in 0.12M PB and 50°C in 0.5M PB, respectively. The melting curves of duplexes formed using these conditions are shown in Fig. 3. The duplexes formed in the repetitive fraction of the chicken genome under standard conditions display a rather broad melting profile with no clear indication of any discrete components (Fig. 3a); the curve is one expected for duplexes of varying degrees of sequence mismatch. On the other hand, the duplexes formed within the unique sequence fraction melt over a narrower temperature range indicating uniformity in the precision of the base pairing.

When these fractions of the chicken genome are reassociated at lower criteria, the results are quite different. A clearly-resolved low-melting component is present among the duplexes formed in the unique sequence fraction (Fig. 3b). Approximately 35% of the DNA fragments are in duplexes which do not form under standard conditions. These duplexes also seem to be of a uniform degree of sequence mismatch, rather than a distribution, since there are no duplexes of intermediate stability and since about 85% of the DNA was retained on HAP at Equiv. Cot = 8000. By comparison of the $Te_{1/2}$s, the low-melting duplexes contain about 30–35% mismatch.

There is little change in the melting profile of repetitive sequence duplexes as the conditions are changed (Fig. 3a). A slight increase in lower-melting duplexes is observed but there is no evidence of many new sequence homologies which are not detected at the more stringent conditions.

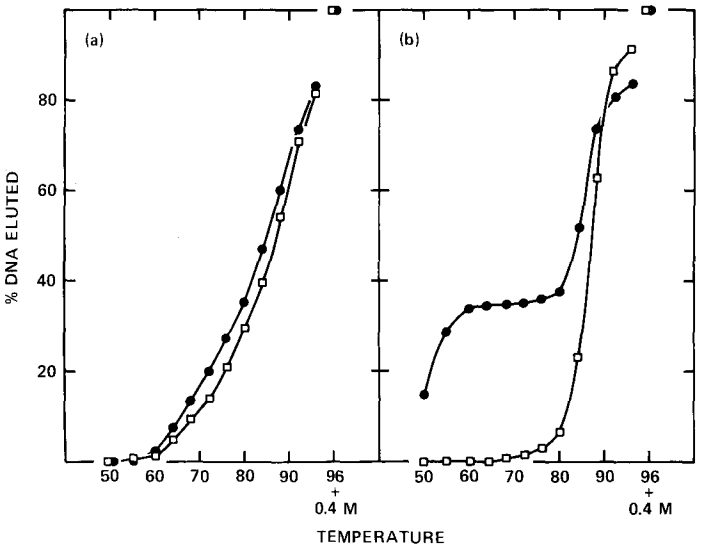

Fig. 3a,b. Integral melting curves of duplexes formed within the repetitive and unique sequence fractions of the chicken genome under standard and reduced-stringency conditions. The chicken genome was fractionated as described in Experimental. Standard and reduced-stringency conditions were 60°C in 0.12M PB (□), and 50°C in 0.5M PB (•) respectively. Panel (**a**), repetitive DNA; panel (**b**), unique sequence DNA. All experiments were incubated to Cot or Equiv. Cot 5,000 to 6,000

If the unique sequence fraction were composed of sequences which could base-pair only with their exact complement, there would be but one thermal stability class of duplexes, and the $Te_{1/2}$ of this class would not change as the reassociation criteria were changed. But this is not the result we obtained. This suggests a degree of repetition among DNA sequences which, on a kinetic basis, are considered to be unique.

Effects of Reassociation Criteria on Duplexes Formed in the Unique Sequence Chicken Genome and T7 Viral DNA. We have performed several experiments varying the incubation temperature, and the phosphate buffer concentration, and the HAP column temperature for loading the sample. These data are shown in Figs. 4, 5, and 6, and are summarized in Table 2.

Very few low-melting duplexes were formed until the buffer concentration was raised to 0.3M; the discrete component appeared, accounting for 25% of the duplexes. The less stable component increased to 32% at 0.5M PB.

The effect of temperature on formation of low-melting duplexes is more pronounced (Fig. 5). Unless the incubations were done at 50°C or lower, in 0.5M PB, no low-melting duplexes were formed, and essentially all of the DNA bound to HAP. Below 50°C, significantly more low stability duplexes formed and more DNA did not bind to HAP, even at Equiv. Cot greater than 40,000 (Fig. 5, and lower half of Table 2). At 46°C in 0.5M PB, 46% of the DNA eluted at low temperatures; however, only 60% of the DNA bound to the column at Equiv. Cot = 52,000. We point out that the column loading

criteria are more stringent than the incubation criteria, and that fragments not binding to HAP might be in duplexes not stable at the higher criteria. When the HAP column temperature was lowered to 44°C and the DNA which had been reassociated to Equiv. Cot 54,000 at 46°C in 0.5M PB was loaded, 90% of the DNA bound (last line of Table 2).

The repetition frequency of the mismatched sequences must be rather high. The difference in $Te_{1/2}$ is about 35°C; the reassociation rate at optimum temperature would be less than 0.1 that of exactly complementary sequences (Bonner et al, 1973). Even though they are kinetically disfavored, the concentration of related sequences must be substantially greater than that of exact complements since they account for many of the duplexes formed.

In contrast, the melting profiles of T7 viral DNA do not change significantly as the reassociation criteria are changed (Fig. 6).

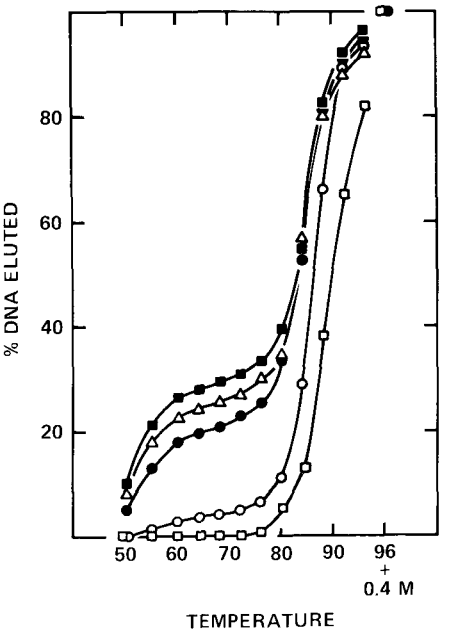

Fig. 4. The effect of phosphate buffer concentration on the integral melting curve of chicken unique sequence DNA duplexes. The incubation temperature was 50°C in all experiments. The buffer concentrations, Equiv. Cot's, and symbols are: 0.12M, 3,900 (□); 0.2M, 15,900 (○); 0.3M, 16,200 (●); 0.4M, 19,000 (△); 0.5M, 18,500 (■)

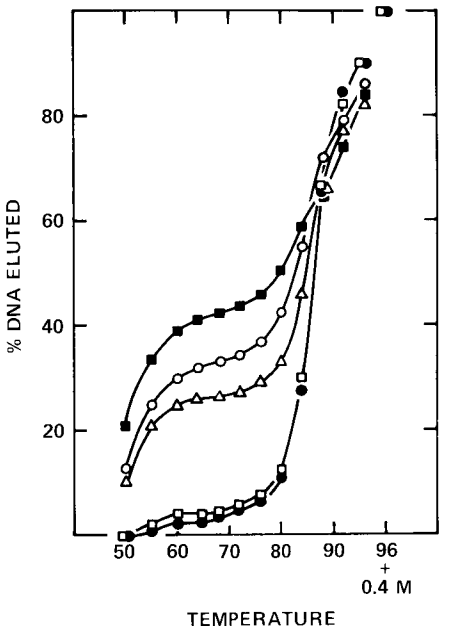

Fig. 5. The effect of incubation temperature on the integral melting curve of chicken unique sequence DNA duplexes. The phosphate buffer concentration was 0.5M in all experiments. The temperature, Equiv. Cot's and symbols are: 54°C, 21,500 (●); 52°C, 29,900 (□); 50°C, 18,600 (△); 48°C, 41,200 (○); 46°, 52,200 (■)

Table 2. Effect of reassociation criteria on formation of low thermal stability in duplexes in chicken unique sequence DNA

Incubation temperature	Buffer conc.	HAP column temperature	Equiv. Cot x 10^{-2}	% DNA not bound to HAP	% DNA eluting below 80°C
50°C	0.12M	50°C	39	8	1
50°C	0.2M	50°C	159	2	7
50°C	0.3M	50°C	162	10	25
50°C	0.4M	50°C	190	16	29
50°C	0.5M	50°C	185	14	32
54°C	0.5M	50°C	215	2	7
52°C	0.5M	50°C	299	3	8
50°C	0.5M	50°C	186	12	29
48°C	0.5M	50°C	412	18	37
46°C	0.5M	50°C	522	40	46
46°C	0.5M	44°C	540	10	70

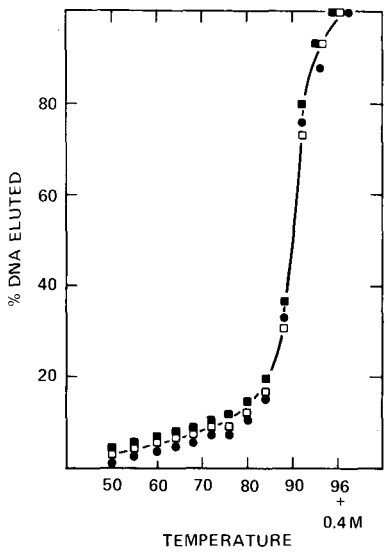

Fig. 6. Integral melting curves of T7 viral DNA reassociation products formed at 50°C in different phosphate buffer concentrations. The experiments were incubated to Cot or Equiv. Cot 1–2; this is about one Cot log beyond the $Cot_{1/2}$ for T7 DNA in 0.18M sodium cation. The symbos are (●) 0.12M, (□) 0.3M, (■) 0.5M

Discussion

Reduced-Stringency DNA Reassociation and HAP Thermal Chromatography. The reassociation conditions used in this study represent a signficant departure from those usually employed. In view of the unexpected results, several aspects of DNA preparation, hydroxylapatite performance, and specificity of base pairing should be discussed.

Variable shearing efficiency could have produced DNA samples of substantially different average fragment lengths; a sample having much shorter average length could yield a higher proportion of shorter, and therefore, lower-melting duplexes. All of the DNAs used in this study, including the T7 viral DNA, had the same average single stranded fragment length, 400–600 bases, as estimated from ethidium bromide fluorescence after alkaline agarose gel electrophoresis. Differential contamination of the DNA preparations with metal ions or protein could also influence duplex formation and melting profiles. Divalent cations can have stabilizing and destabilizing effects on helical DNA (Eichhorn 1962; Dove and Davidson 1962). All of the DNA samples were passed over a Chelex 100 column, as the last step before reassociation, to remove metal ions. No metal ion effects are observed in the melting profiles of duplexes formed under standard conditions (Figs. 1 and 2). Also, the melting profiles of fractionated chicken DNA are different and depend upon the reassociation conditions (Fig. 3). Complete removal of protein from DNA can be difficult. In the past, no serious problems have been encountered using purification methods similar to the one used here (Flamm et al. 1966; Marmur and Doty 1962); in addition, Proteinase K digestion was added to our procedure. Use of this enzyme has been shown to give DNA preparations with less than 0.2% protein contamination (Gross-Bellard et al. 1973). The results shown in Figs. 1 and 2 were the same whether the DNA was isolated from blood or liver; ostrich and emu DNA were from blood only, and chicken DNA was from liver, oviduct, and blood. It is unlikely that protein contamination from different tissues would be identical so as to affect duplex formation and melting profiles the same way.

Differences in genome size and base composition most likely are not significant factors in our experiments. All bird DNAs reassociated to about the same extent at similar Cot values and standard criteria indicating that no genomes were larger than that of chicken. The G+C content of chicken and T7 viral DNAs are 41% and 50%, respectively (Szybalski 1968). The melting profiles of T7 DNA duplexes formed at various criteria (Fig. 6) illustrate that G+C richness, per se, does not result in unusual melting profiles as might be expected if G+C rich regions were stabilizing random DNA strand associations. Similarities in $Te_{1/2}$ of bird DNAs reassociated at standard conditions do not indicate any large differences in base composition among the duplexes (Figs. 1 and 2).

Hydroxylapatite performance is an important factor in considering our results. Either the inability of HAP to bind duplexes at 50°C or the retention of DNA fragments associated through interactions other than sequence homology could be responsible for some of the results presented here. When chicken unique sequence DNA was denatured in 0.5M PB, cooled to 50°C, diluted to 0.12M PB, and loaded to a HAP column, only 4% of the DNA was retained on the column. Also, the melting curve of emu DNA shows no low-melting duplexes even though the incubations were carried to Equiv. Cot 29,000 and there was still 50% of the DNA not bound to HAP (Table 1). These results indicate that operation of HAP columns at 50°C in 0.12M PB does not result in retention of non-specifically associated DNA fragments.

Failure of DNA to bind to HAP could be the consequence of two technical problems: (1) the incubation times might have been too short to allow complete reassociation and, (2) the DNA was incapable of complete reassociation. Columns four and five of Table 1 show that all of the DNA preparations were able to reassociate essentially completely. When chicken DNA is reassociated under standard conditions, the observed $Cot_{1/2}$ is about 400 (data not shown); an ideal second order reaction with that $Cot_{1/2}$ would be approximately 90% completed at Cot = 4000. The effect of increased buffer concentration on the reassociation rate (factor = 5.8, 0.5M PB) (Britten et al. 1974) more than compensates for the effect of temperature on rate. Consequently, we believe that incubations to high Equivalent Cot, 17,000 to 31,000 , are sufficient to allow complete reassociation, and that incubation times are not greatly affecting the percentage of DNA failing to bind to HAP. Two other possibilities must also be considered. As the percentage of base sequence mismatch between two related sequences increases, the reassociation rate decreases; an eight-fold decrease in rate is expected for sequences containing 30% mismatch when reassociated at optimum temperature (Bonner et al 1973). The low-melting components seen in Figs. 1 and 2 have a $Te_{1/2}$ reduction of about 30°C; the reassociation rate of such sequences would be insignificant at standard criteria, and still sub-optimal at 50°C. Thus, we feel that at least some of the DNA not binding to HAP belongs to the low stability class. The second possibility is that because the column loading criteria are somewhat more stringent than the incubation criteria, some duplexes of marginal stability are dissociated when the reaction is diluted to 0.12M PB. If this were occuring, then lowering the column loading criteria should preserve some of these duplexes. This will be discussed further.

It is difficult to reach any firm conclusions about the nature of low stability duplexes formed at 50°C in 0.5M PB. The task is further complicated by the use of HAP in the experimental design; interesting sequence interactions can be missed if the HAP column conditions do not preserve them, or if binding is not quantitative. The lower

limit of length required for stable nucleation is in the range of 10–20 bases; a number of studies using DNA-DNA and DNA-RNA reactions under a variety of conditions are in general agreement on these figures (Lipsett et al. 1961; Cassani and Bollum 1967; Niyogi and Thomas 1967; Rünger and Bautz 1968). The requirements for quantitative binding to HAP are more demanding. When the HAP column is operated at 25°C, 28–37 base pairs are necessary, while 43–54 contiguous base pairs are needed at 60°C (Wilson and Thomas 1973). Indeed, lowering the column temperature to 44°C resulted in a large increase in the chicken duplexes binding to HAP (Table 2).

Another important aspect of HAP column operation as it relates to thermal stability studies deserves mention. DNA duplexes can be washed from HAP without first undergoing strand separation in certain ranges of phosphate buffer concentration (Martinson 1973). The buffer concentration range over which strand separation occurs before elution, becomes smaller as the pairing mismatch of the duplexes increases, (Fox G., Umeda J., Lee R., Schmid C., personal communication, 1979), raising the possibility that if the HAP-duplex interaction is less stable than the DNA-DNA interaction, the actual thermal stability of the base pairing could be underestimated.

A control experiment indicates that most of the chicken DNA eluting from the column at 64°C and below has undergone strand separation before dissociation from HAP (Table 3). The low-melting fractions of reassociated chicken unique sequence DNA were obtained as usual. After dilution to 0.12M PB at 50°C, the fractions were loaded onto individual HAP columns at the same temperature and buffer concentration. If the DNA had eluted from the first column without strand separation occuring at the elution temperature, the DNA should bind to the second column as it did originally; the fractions used were taken at 50°, 55°, 60°, and 64°C. None of the DNA in the last three fractions bound to the second HAP column, indicating that the DNA was single stranded as it eluted from the first column. About 50% of the DNA in the 50°C fraction did not bind to the second column; this DNA could be a few single strands not completely removed by the 0.12M PB wash. However, as mentioned earlier, these conditions were quite effective in washing single stranded DNA from the column. The 50% of the 50°C fraction remaining on the second column was melted off as usual; most of the DNA eluted at 55°C. Thus, only a small fraction (0.5 x 0.13 = 0.065) of the HAP-bound duplexes seems to be eluting from the column before strand separation.

Specificity of Base-Pairing. The influence of temperature and monovalent cation concentration on the rate of DNA duplex formation is rather well understood; their effect on the precision and extent of base-pairing permitted and excluded under various conditions is not well understood. Most of the experiments reported here were performed

Table 3. Re-binding to HAP of low-melting chicken duplexes

Sample from first HAP column	% bound DNA per sample	% sample bound to second HAP column	% bound to second column eluting at 50°, 55°, 60°, 64°C respectively
50°C	13	50	24, 57, 19, 0
55°C	9	0	–
60°C	5	0	–
64°C	2	0	–

at 50°C in 0.5M PB. There is no a priori reason to expect that these conditions will preserve sequence and species specificity. Many of the low-melting duplexes formed in the genomes examined could be artifacts of the reassociation criteria. Other recent studies have made use of low criteria but all have been somewhat more stringent than ours (Klein et al. 1978; Moore et al. 1978; Galau et al. 1976; Deininger and Schmid 1979). Experience has indicated that new interactions are detectable as the criteria are lowered. Kohne (1970) observed that more DNA behaved as it were repetitive when the incubation temperature was lowered; more extreme conditions of 53°C in 1M PB (Rice and Paul 1972), yielded such large amounts of low-melting duplexes it was concluded that species specificity was not preserved at these criteria. Somewhat more systematic studies by McCarthy and McConaughy (1968) indicated that incubation temperatures of about 40°–50°C in 1xSSC would preserve species specific reassociation of *B. subtilis* and mouse DNA. The minimum information content required to form species specific duplexes is contained in fragments of about 15 nucleotides (McConaughy and McCarthy 1967), and when fragments 40 nucleotides long were used, interspecies sequence homologies could be detected (McConaughy and McCarthy 1970). No quantitative boundaries for DNA reassociation criteria can be set which will apply to all situations and purposes.

Britten and Roberts have calcuated the probability of sequence homologies occuring by chance (1970). Their curves indicate that the probabilitiy of chance homology occurring over a distance of 60 bases with a 30% mismatch is about 10^{-12}; as the distance over which the homology extends decreases, the probability increases. If we take 50 contiguous base pairs as the lower limit for quantitative HAP binding, (Wilson and Thomas 1973), then 30% mismatched duplexes would encompass at least 70 bases. However, as Britten and Roberts suggest, duplexes containing 30% mismatch over 70 nucleotides would most likely melt much lower than 30°C below the $Te_{1/2}$ of well paired duplexes. Obviously these are only estimates. The data shown in Figs. 3 and 6 illustrate that the low stability component found in the chicken genome is not the result of interactions in which *any* DNA fragment may participate. DNA fragments from the repetitive fraction of the chicken genome all form duplexes which bind to HAP, and no duplexes of lower stability are formed at the most permissive criteria. Thus, the ability to form low-melting duplexes can be fractionated on the basis of sequence content. The thermal stability of duplexes formed within the T7 genome does not change with changes in criteria. Furthermore, the occurence and amount of low stability duplexes is a function of the genome examined, not just of DNA in general; the emu and Japanese quail genomes form no duplexes of this type.

A demonstration of helical content in the low-melting fraction would further strengthen the argument for specific base pairing; preliminary optical melting profiles have shown a peak of hyperchromicity between 50° and 60°C. It is doubtful that standard S_1 nuclease sensitivity experiments would be useful to prove base pairing; however, it might be possible to demonstrate different rates of S_1 digestion for well paired and mismatched duplexes and single stranded DNA.

Effect of Reassociation Criteria on DNA Duplex Formation. We have examined the effect of incubation temperature and buffer concentration on the melting profiles of reassociated chicken unique sequence DNA and T7 viral DNA. Above a buffer concentration of 0.3M, new sequence interactions are stable and their proportion increases

moderately as the buffer concentration is raised. The effect of incubation temperature is more pronounced; this is expected, since the apparent $Te_{1/2}$ of the duplexes formed at 50°C in 0.5M PB is only slightly higher than the incubation temperature. The rate of reassociation will increase as the incubation temperature is lowered away from the melting temperature of the mismatched duplexes. Interestingly, when the incubation temperature is reduced from 48° to 46°C much of the DNA is in structures not able to bind to HAP at the column loading criteria; the two degree change resulted in 40% of the DNA not binding (Table 2). Although many of these duplexes will bind to HAP if the column temperature is lowered to 44°C for sample loading, these conditions may be approaching the limits of specificity suggested from studies mentioned earlier. Reassociation criteria had no effect on the thermal stability of duplexes formed within the T7 genome (Fig. 6).

Stringency-Dependent, Low-Melting Duplexes Observed in Other Systems. Low stability duplexes have been observed to form in genomes of prokaryotes, plants, fish, mammals, and primates as a consequence of lowering the reassociation criteria. McCarthy and McConaughy (1968) noted a distinct shift to lower stability in *B. subtilis* homologous duplexes formed at 50°C in 1xSSC; the melting profile was biphasic. In the same report, they obtained a similar result with mouse DNA reassociated at 50°C in 4xSSC (equivalent to 0.44M PB in sodium ion concentration), melted from filters in 1xSSC. Homologous DNA-pulse labeled RNA hybrids formed at 50°C in 4xSSC, also in the mouse and *B. subtilis* systems (Church and McCarthy 1968) shifted to significantly lower stability; again, the prokaryotic profile was biphasic. The repetitive fraction of salmon DNA formed duplexes melting over a broad temperature range with a biphasic shape when reassociated at 50°C in 0.14M PB; the high and low melting fractions were isolated, reassociated separately, and melted. There was a clear fractionation of the ability to form low-melting duplexes (Britten and Kohne 1967).

Biphasic melting profiles have been reported for mouse DNA reassociated at 50°C in 0.14M PB; both the optical and HAP melting profiles were biphasic (Ivanov and Markov 1974). Deininger and Schmid (1979) reported that human DNA reassociated at 50°C in 0.24M PB also displayed a biphasic lower melting profile. Recently we learned that unique sequence DNA from pea and mung bean forms lower stability duplexes when reassociated at lower criteria (Thompson et al. 1979). It is important to emphasize that the last three studies described above have identified the unique sequence part of the genome as the source of sequences forming low stability duplexes at reduced stringency, as we have for the chicken.

Evolutionary Implications. Evolutionary relationships derived from *inter*genomic DNA sequence homology studies are consistent with those developed using classical organismal methods (Kohne 1970; Rice 1974; Sibley and Ahlquist 1979). In contrast, our study of *intra*genomic sequence homologies in closely and distantly related birds shows none of the expected taxonomic trends. Galliformes is considered to be an evolutionarily conservative order because chromosome number and morphology are quite similar (Chaudhuri-Ray 1973), and examples of interspecific hybridization are numerous, particularly within Phasianinae (Gray 1958). Despite these biological similarities, the sequence compositions of phasianid genomes are different; differences were reproducibly observed between the subspecies chicken and Red Jungle Fowl.

Three mechanisms could produce the variability apparent in our studies: (1) changes in size of a repeated sequence family, (2) positional changes of DNA sequences in members of a family, (3) base substitution over geological time following ancestral amplification events. Family size changes have been noted using cloned repeated sequence probes (Moore et al. 1978) and with fractionated avian DNA (Eden et al. 1978); tandem amplification, followed by translocation, or amplification of an already dispersed family could produce these changes. Dispersal of members of a clustered repeat family would result in more DNA fragments behaving with properties conferred by the repeated sequence, under the appropriate conditions. Conversely, coalescence of an already dispersed family would produce the opposite effect. Different chromosomal locations of specific repeated sequences has been demonstrated in two strains of *D. melanogaster* (Young 1979); positional changes have also been observed between cell cycle stages of *Caulobacter crescentus* (Nisen et al. 1979) and in different clones of *Trypanosoma brucei* (Williams et al. 1979). Our results cannot be easily explained in terms of base substitution following an ancestral amplification unless the substitution rates were quite different among closely related lineages.

There is an interesting parallel between our results and those of Prager and Wilson (1975); biological similarity inferred from interspecific hybridization is not reflected in the rate of avian albumin and transferrin evolution. Many studies by Wilson and his colleagues point to the disparity between peptide and organismal evolution (Wilson et al. 1977). The results of this study suggest a similar disparity between genome sequence composition/arrangement and biological relatedness.

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