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Clustering and methylation of repeated DNA: persistence in avian development and evolution

Donna A.Sobieski and Francine C.Eden[1]

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, USA

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ABSTRACT

In the chicken genome, clusters of repeated DNA sequences occur which have alternate arrangements of the component sequence elements. Many of these clustered, repeated sequences are extensively methylated. We have established that both their arrangement and their methylation are invariant regardless of the source of chicken DNA. Comparisons included DNA from sperm, from a series of embryonic stages, from tissues of single adult individuals, and from thirty individual chickens of two strains. These same sequences are found in the DNA of some avian species related to chickens, and there they show the same clustered, methylated form. In related species, some of the arrangements found in chicken DNA are different or missing.

INTRODUCTION

Clusters of repeated DNA sequences are a substantial part of the repetitive component of the chicken genome. These clusters often have the same assortment of sequences, but there is a scrambled order of sequences from cluster to cluster (24). Here we address the question of whether this cluster scrambling is occuring to a significant extent on a contemporary time scale or whether it reflects an evolutionary history of rearrangement.

Many published reports have established that differences in organization of segments of the genome can arise during developmental specialization of the tissues of a single individual. Somatic gene scrambling is well studied in the genes encoding immunoglobulins (5,28,32), whose components have a different arrangement in embryonic and adult cells. Another case of somatic alteration is the amplification of some of the ribosomal genes that occurs in the oocytes of toads (7,16,34). Thus somatic alterations of the genome can affect both the arrangements of genes and their copy number. In either case they directly influence biological function.

Other kinds of alteration are genetically determined. Here the genome organization is the same in all the somatic cells of a given individual, but different among individuals. Some of these polymorphic differences in genome

organization involve repeated DNA. In the genomes of fruit flies (15,25,31) and yeast (8), movable repeated DNA sequences occur that resemble procaryotic transposable elements, and that are believed to have analogous properties (9,19). New juxtapositions of these segments of the genome influence gene expression (18,30,37).

The process of genome re-organization is also active on an evolutionary time scale. The DNA of related species can have sequences that are homologous but whose arrangement differs (1,2,14). In addition to these species-related alternative arrangements, changes in copy number have been detected (2,10,11,17,20,22,23). The expansion or contraction of families of repeated DNA sequences during evolution seems to be commonplace.

We have considered the stability of both the arrangement and the methylation of clustered repeated sequences in the chicken genome with respect to embryonic stages, adult tissues, contemporary populations, and evolution. The results indicate stability of both arrangment and methylation throughout development and in contemporary populations. In the DNA of related species, both clustering and methylation persist, but each species seems to have only a few of the several alternative arrangements found in chicken DNA.

MATERIALS AND METHODS

(a) Animals

Adult chickens (*Gallus gallus var. domesticus*) were obtained locally from Truslow Farms, Inc., Chestertown, Md. Individuals of White Leghorn and Rhode Island Red strains were used. Pheasants (*Lophophorus sp.*), turkeys (*Meleagris sp.*), Japanese quail (*Coturnix coturnix*) and the duck (*Anas platyrhynchous*) were obtained from the same supplier, and all were adults. The ostrich (*Struthio camelus*) from which blood was obtained was an adult female. Chicken embryos were reared from fertile eggs using a commerical poultry incubator. Many embryos from the same stage were pooled for DNA isolation.

(b) Tissue Isolation

Liver, brain, and pectoral muscle were obtained by dissection of animals freshly killed by decapitation. Blood was drawn from a wing vein, heparanized, and centrifuged at 4°C to collect packed cells. To isolate liver tissue depleted of blood, liver perfusion was carried out using normal saline. Tissues were quick frozen in dry ice and stored at -70°C

until DNA isolation.

(c) DNA Isolation

DNA was isolated from finely powdered frozen tissue by a procedure described previously (6,11).The yield of purified DNA per gram (wet weight) of tissue was constant for each tissue but varied from 0.2 mg/g from muscle to 3 mg/g from packed blood cells. All DNAs were isolated and purified identically except that muscle and liver DNAs were freed of residual glycogen by centrifugation at 30,000 x g at 4°C for 1 hour. All DNA preparations were of high molecular weight (double strand length exceeded 100,000 nucleotide pairs) and of similar purity (absorbance ratio 260/280 nm > 1.8).

(d) Cloned DNA Fragments

The cloned chicken DNA fragments used a probes have been described previously (12,24). Briefly, they are designated probes 1,2 and 3 and consist of segments of chicken DNA 3.6, 0.68, and 7.0 KB^2 in length, respectively, inserted into the plasmid vector pBR322. Each probe represents a different repeated DNA sequence with several hundred copies in the chicken genome. They are usually neighboring elements within much larger repeated sequence clusters.

(e) Restriction endonuclease digestion, agarose gel electrophoresis, Southern (29) transfer and hybridization, and radioisotope labelling of DNA were carried out as described (12). Details appear in the Figure legends.

RESULTS

(a) Method and Scope of the Present Study

The genomic organization of clustered repeated DNA sequences can be deduced from their relatively simple pattern of hybridization in Southern transfer experiments. Within a restriction digest of total DNA, a probe representing a clustered repeat detects several prominent bands. These bands are each composed of multiple copies of a given restriction fragment. Some bands arise from restriction sites internal to the probe, while others span the border of the probe and come from sites in neighboring sequences. Because of their clustered organization, in which a given sequence may have the same neighboring sequences in many different locations in the genome, some of the fragments extending into neighboring sequences are also multiple-copy. A consequence of any major alteration in the organization of clustered sequences would be a shift in the pattern of bands, because new sequence juxtapositions create new restriction patterns. Conversely, an invariant

genome organization can be inferred from the observation of restriction site persistence. In these experiments we vaired the source of DNA, the restriction endonuclease used for DNA fragmentation, and the hybridization probe. Tissues used as sources of DNA included sperm, embryos, and tissues from adults including blood, liver, brain, muscle, and perfused liver free of blood. We included DNA isolated from thirty individual chickens. Because the domestic chicken has a history of selective breeding, we were able to use both inbred (White Leghorn) and outbred (Rhode Island Red) strains. The DNA of five other avian species has been compared to chicken DNA. They vary in relatedness, from close relatives like turkey and pheasant to a very distant species, the ostrich. DNA samples were fragmented by digestion with EcoR1, BamH1, Hindlll, or Sst1. Each of these has a different six base recognition sequence. We tested for changes in methylation as well as in organization. This was done by digestion of total DNA either with Mspl (cleaves CmeCGG or CCGG) or with Hpall (cleaves CCGG only) (33). The repeated sequences studied here are extensively methylated in most of their genomic occurances (13). We considered the extent to which methtylation is reproduced from copy to copy and the pattern of methylated and unmethylated bases within each sequence. Methylation was tested in most of the DNA samples mentioned above.

(b) Organization of Clustered Repeated Sequences is Invariant From Sperm Throughout Embryogenesis

We found no developmental alterations in the organization of clustered repeated sequences. The invariant pattern of restriction endonuclease digestion is illustrated in Figure 1, where total DNA fragmented with Hindlll was hybridized to probe 1. The bands are the same length and of about the same relative intensity in every DNA sample. Similar results were obtained with BamH1 or EcoR1 (data not shown).

(c) Organization of Repeated DNA Sequences is Maintained During Tissue Differentiation

The restriction endonuclease cleavage pattern of these sequences is identical in different adult tissues. In Figure 2, panels A and B each compare four tissues from single individuals (A, rooster; B, hen). Panels B and C compare DNAs from four tissues of the rooster after digestion with different endonucleases. Panel D shows the results with a different probe. In panel E, we show DNA isolated from blood or from a perfused liver that was free of blood hybridized to each probe. The different amounts of DNA loaded in adjacent tracks allow comparision of both strong and weak

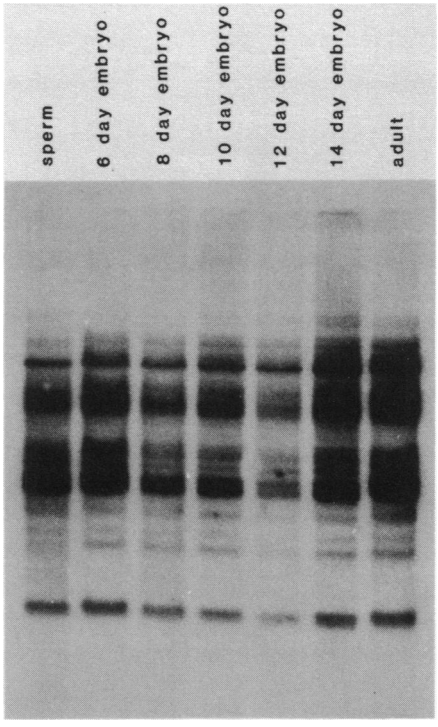

Figure 1. Genomic Organization of Repeated DNA Sequences in Sperm, Embryos, and Adults

Total DNA was isolated from sperm, from various embryonic stages, and from adult chickens. DNA from each source was digested with endonuclease Hindlll. The resulting fragments were separated in an 0.7% agarose gel (10μg of DNA per track) and transferred to a nitrocellulose blot according to Southern (1975). A cloned, 3.6KB segment of chicken DNA representing a family of repeated sequences in the genome was labelled with ^{32}P and hybridized to the blot. The specific radioactivity of the probe was 2 x 10^7 cpm/ ug, and 5 x 10^6 cpm were used. The autoradiogram after a 3 day exposure at -70°C with intensifying screens is shown.

bands. We conclude that these sequences do not undergo substantial somatic rearrangement.

(d) Individual Chickens Have the Same Organization of Repeated DNA Sequences

Invariance of restriction enzyme cleavage was also documented among thirty individual chickens. Some examples of the results are shown in Figure 3. First, we established that the DNA of individual chickens does contain the entire pattern of restriction fragments initially detected

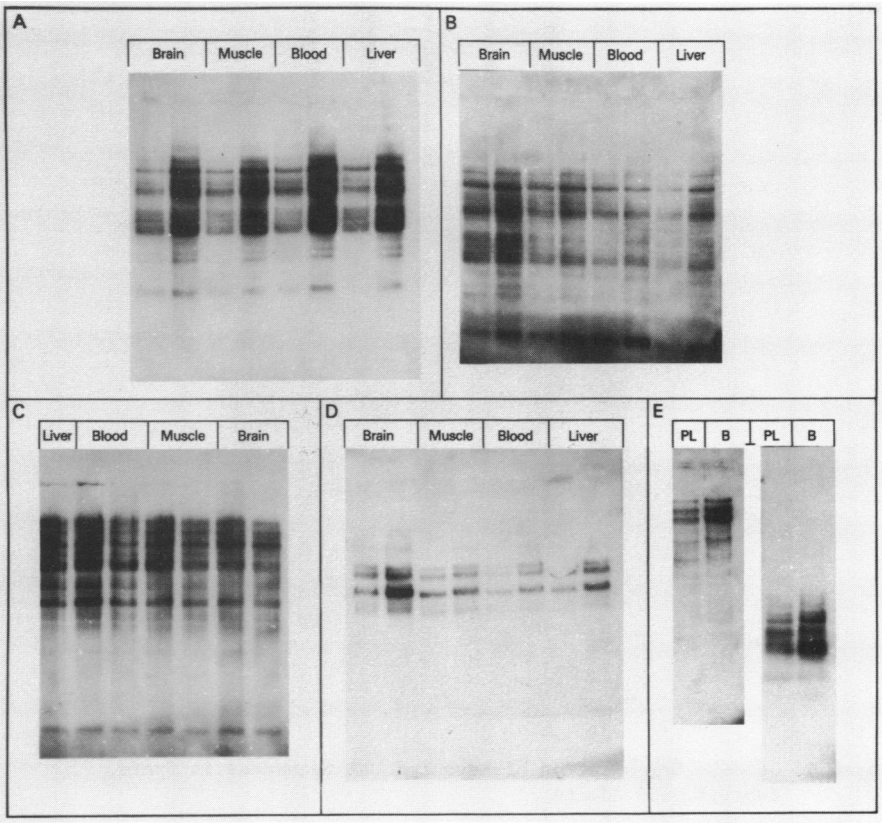

Figure 2. Comparison of Repeated DNA Sequence Organization in Different Tissues

Total DNA isolated from different tissues of a single individual was digested with restriction endonucleases. DNA fragments separated by agarose gel electrophoresis were transferred to blots and hybridized with ^{32}P-labeled repeated sequence probes. In panel A, 2.5µg and 15µg of DNA were loaded in adjacent tracks. In panels B-E, 5 and 10ug of DNA were loaded in adjacent tracks. DNA isolated from a White Leghorn female (panel A) or male (panel B) was digested with HindIII and hybridized to probe 1. In panel C, BamHI-digested DNA from the White Leghorn male was hybridized to probe 1. Panel D represents hybridization to probe 3. Here DNA was isolated from a White Leghorn female and digested with EcoRI. Panel E compares SstI digested DNAs from perfused liver (PL) or blood (B) after hybridization to probe 1 (left) or probe 3 (right).

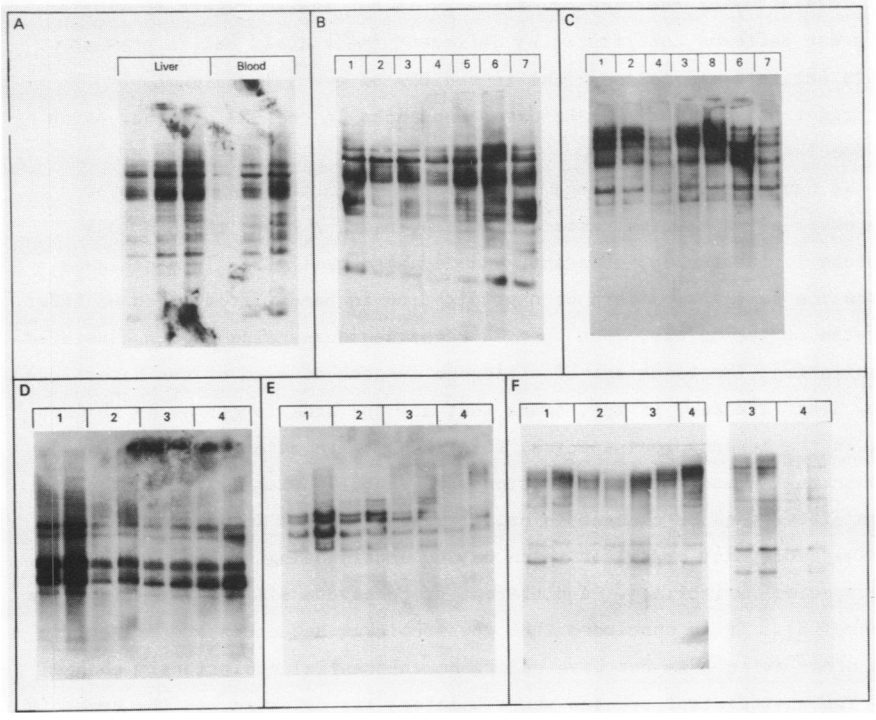

Figure 3. Comparison of Repeated DNA Sequence Organization in Different Individuals.

Total DNA ioslated from a pool of many individuals (panel A) is compared to DNA from single individuals (panels B-F). Both inbred (panels A-C) and outbred (panels D-F) strains were used. The numbers above each track designate different indivduals. The amount of DNA per track was varied in panels A and D-F. Panel A shows 5,10, and 20 μg of DNA from each of two tissues. Panels D-F have 5 and 10μg of DNA loaded in adjacent tracks. Each track of panels B and C has 10 μg of DNA. Two different probes were used. Probe 1 was hybridized to Hindlll (panels A,B) or BamHl (panel C) digests, and probe 3 was hybridized to DNA digested with Hindlll (panel D), EcoRl (panel E) or BamHl (panel F, duplicate experiments shown).

using DNA from the pooled blood of many individuals. This comparison is made in panels A and B of Figure 3. The gel was run longer in panel B, so the pattern of bands is expanded compared to panel A, but the pooled DNA and each of the individual DNAs show essentially the same pattern of bands. With a different restriction nucelase (panel C, Figure 3), the DNA of different individuals again showed the same pattern of bands. Similar results were obtained using a different probe (panels D-F, Figure 3). We conclude

that this array of restriction fragments is not the aggregate of simpler, different patterns contributed by different individuals but is present in its entirety and is identical in the DNA of every individual.

(e) Organization of Chicken Repeated Sequences in the DNA of Other Avian Species

We have detected homology to these probes in the DNA of three of five other avian species tested. Figure 4, panel A shows the homology of probe 1 with turkey, pheasant, and Japanese quail DNA. In most cases we see the familiar pattern of hybridization to bands, from which we infer that the in the heterologous species these sequences also have a clustered organization. The bands are of different lengths in each of the heterologous DNAs. There are few, if any, bands that are the same length as in chicken DNA. In the heterologous species, fewer bands were detected than in chicken DNA and the hybridization was generally weaker. We ascribe these differences to changes in organization, changes in copy number, and to divergence, any or all of which may be contributing to the observed interspecies variability. A different probe gave a similar result (Figure 3, panel B). It is concluded that these chicken sequences are present in some other avian DNAs but have undergone substantial evolutionary change.

The most distant species where homology was detected was the duck, in whose DNA we could detect only one very faint band after a very long autoradiographic exposure. Ducks are classified in a different order than chickens, turkeys, and pheasants (35). We did not detect any homologous sequences in the DNA of the ostrich, which is believed to be even more distant from the chicken, diverging from the last common ancestor of the chicken more than 60 million years ago (27). We conclude that these elements of chicken repeated DNA clusters have considerable evolutionary persistence in avian DNA, although their organization and representation in the genome have been significantly altered in the evolutionary process.

(f) Methylation of Repeated Sequences Compared Among Tissues, Individuals, and Species

Differential cleavage of DNA with MspI or HpaII indicates alteration of restriction endonuclease cleavage site CCGG to the form CmeCGG. Figure 5, panel A compares the hybridization to MspI or HpaII cleaved DNA isolated from different chicken tissues. The MspI fragments are not present in the HpaII digests, so it is concluded that the restriction sites that generate them are methylated. This is the result for each of the adult tissues we examined. In every tissue, most, if not all of the copies of each sequence

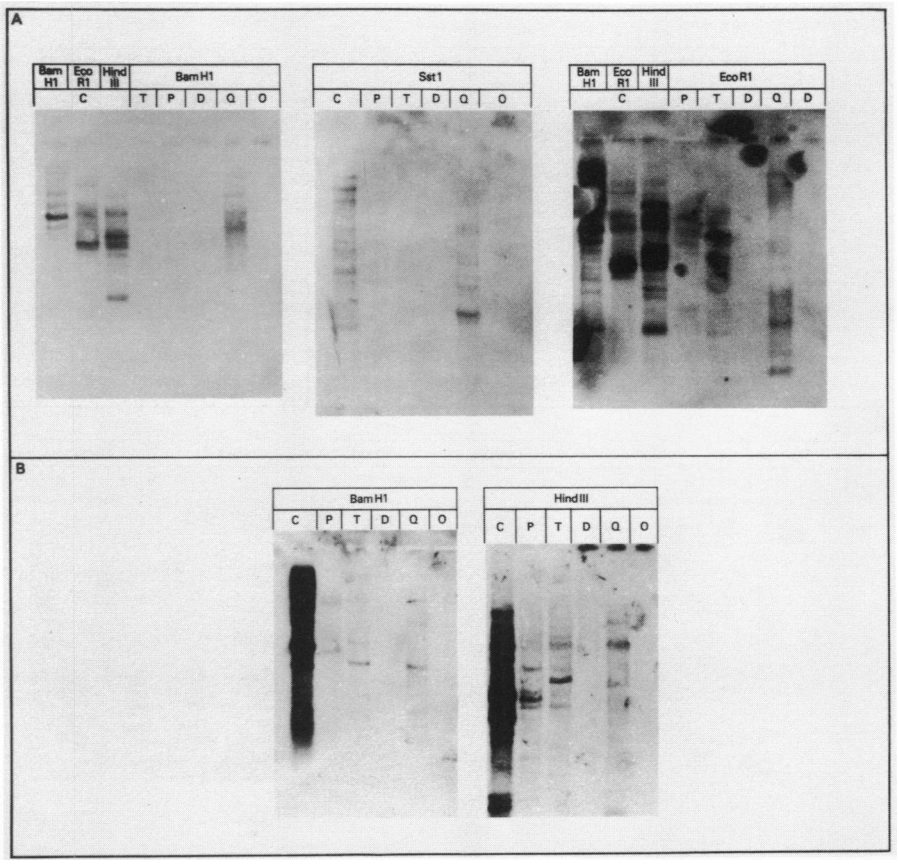

Figure 4. Comparison of Repeated DNA Sequence Organization in the DNA of Related Avian Species

DNA isolated from chicken (C),pheasant (P), turkey (T), duck (D), Japanese quail (Q) or ostrich (O) was digested with the indicated restriction endonucleases, separated by gel electrophoresis, and transferred to blots for hybridization with probe 1 (section A) or probe 3 (section B). Ten micrograms of DNA fragments were loaded per track. Each blot was hybridized with 5 x 10^6 cpm of probe. Hybridizaiton was carried out in 6 x SSC^2,1 x Denhardt's solution at 68°C for 18-24 hours. Blots were washed in 0.1x SSC at 52°C, dried, and autoradiographed. The duration of the autoradiograaphic exposure varied from a few days to three weeks.

are at least partly methylated, because there was never a residum of bands in the HpaII digest corresponding to those generated with MspI. Both the fact of methylation and its general extent are reproduced in the DNA of each tissue.

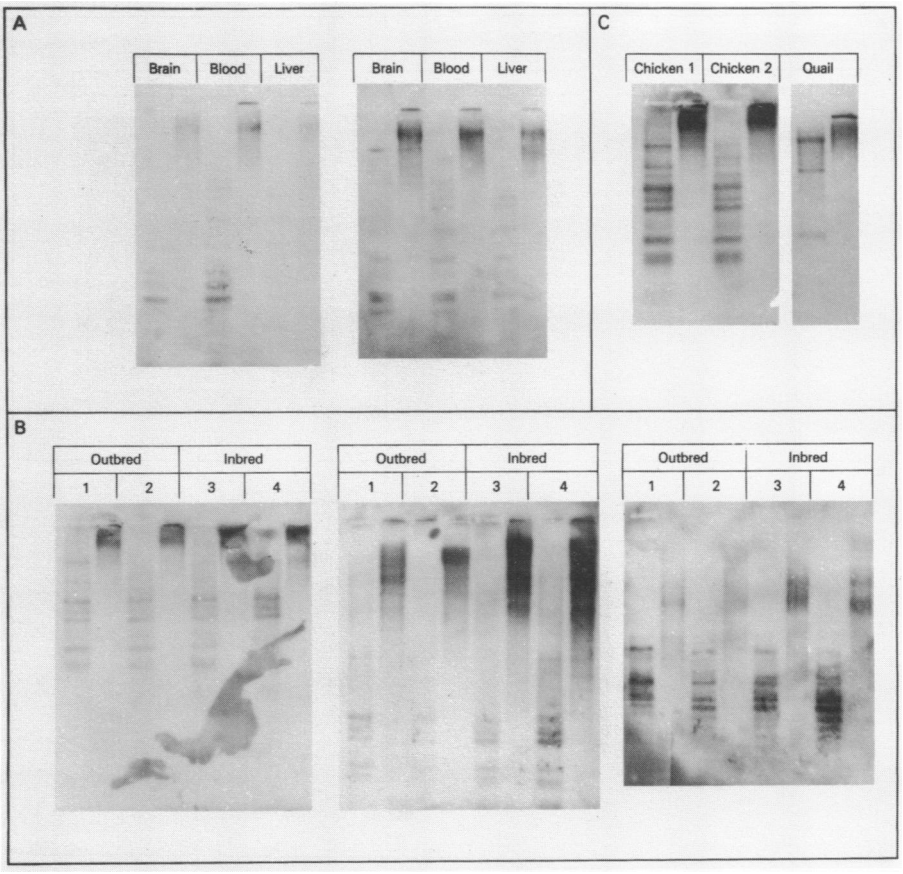

Figure 5. Methylation of Repeated DNA Sequences Persists Across Tissues, Individuals, and Species

Each pair of adjacent tracks shows MspI (left) or HpaII (right) digested total DNA. The restriction fragments were separated by gel electrophoresis and transferred to blots for hybridization. Panel A shows DNA isolated from different tissues within two White Leghorn chickens (rooster, left; hen, right) after hybridizaiton with probe 1. Panel B compares DNA from different individuals (numbered 1-4) after hybridization with probe 3 (left, 2% gel; center, 0.7% gel), or with probe 2 (right, 0.7% gel). Panel C compares DNA of two chickens with DNA of the Japanese quail; probe 1 was used here.

Using the DNA of different individual chickens, males, females, inbred and outbred strains, we made the same comparisons (Figure 5, panel B). Each probe detected the same MspI fragments in every individual, and these are all absent from the HpaII digests. Thus methylation of these sequences

is the same in every individual, regardless of sex or breeding history.

The persistence of methylation extends even to the DNA of other avian species. In Figure 5, panel C, we compare chicken and Japanese quail DNA. In the heterologous DNA, the MspI bands are absent from the HpaII digest just as they are in chicken DNA. It appears that methylation is a very consistent feature of these repeated DNA sequences in the DNA of birds.

(g) Hypermethylation of Sperm DNA

DNA isolated from sperm showed a HpaII digestion pattern different from any other DNA sample considered in this study. This result is illustrated in Figure 6, where DNA from sperm is compared to that of several embryonic stages and of an adult. MspI,(data not shown) and other restriction endonucleases not sensitive to methylation (Figure 1) cleaved these DNAs identically, whereas HpaII showed differential cleavage. Several large HpaII fragments (ranging from 3-15 KB, Figure 6) were present in all the DNA samples shown except sperm. We infer that in sperm DNA the indicated HpaII fragments have become part of even larger DNA fragments appearing near the 24 KB marker on the gel in Figure 6. These two very large fragments are also detected in the DNA of somatic cells, but their relative amount seems increased in sperm DNA. We interpret this as evidence that the DNA of sperm contains some very long DNA segments where methylation affects every consecutive occurance of CCGG. Only some of these sites are free of methyation in somatic cells, giving rise to smaller HpaII fragments that contain internal methylated sites. This situation is diagrammed in panel B of Figure 6.

DISCUSSION

(a) The Stability of Cluster Organization in Chicken DNA

Using probes representing clustered repeated sequences we failed to detect variation in the pattern of restriction endonuclease cleavage of a wide variety of DNA samples. The same juxtapositions of restriction sites are present in DNA isolated from sperm, from embryos of various ages, from different tissues of single individuals, and from the DNA of many different individuals. In the chicken genome, the organization of these sequences is apparently not undergoing substantial alteration either during development or in contemporary populations.

(b) Moderate Evolutionary Conservation of Clustered Sequences

Elements of chicken repeated DNA clusters occur in the DNA of some

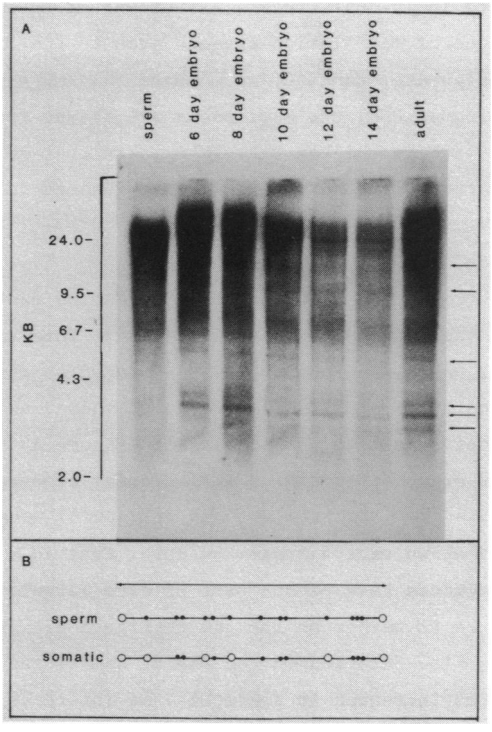

Figure 6. Hypermethylation of Sperm DNA

Panel A. DNA isolated from sperm, from a range of embryonic stages, or from adult chickens was digested with Hpa11. 10 μg of each DNA was separated in an 0.7% agarose gel and transferred to a blot. The autoradiogram after hybridization with probe 1 is shown. A scale of DNA fragment lengths is shown at the left. The arrows indicate the specific fragments discussed in the Text. Panel B. Diagrammatic interpretation of the hybridization results. Sperm DNA contains many consecutive methylated (●) sites. Somatic cell DNA contains a pattern of methylated (●) and unmethylated (○) sites.

related avian species. The clearest homology was found in the DNA of turkeys, pheasants, and the Japanese quail. The hybridization to duck DNA was very faint, and no cross hybridizing sequences were detected in ostrich DNA. These results parallel the general phylogenetic relationships of these species as they are interpreted from the fossil record. (27,35).

The relatedness of these particular avian species has been measured previously using DNA/DNA hybridization reactions in solution (11). Earlier results using total DNA fractions can now be compared to this data collect-

ed using specific cloned probes. In the earlier study, single copy and repeated DNA sequence homologies were measured separately. Single copy chicken DNA sequences react 75% and 70% with the DNA of pheasants and turkeys, respectively (Eden, unpublished). This indicates a close relationship between these species. Japanese quail is more remote from the chicken by this criterion, with only 26% of chicken single copy sequences represented there. All these species are grouped with chickens in the order Galliformes, or game birds (35). Using total repetitive DNA, cross hybridization was detected not only within the order Galliformes but also in other orders of birds (11). Using the cloned repeated DNA sequences, we detected significant homology in all the game birds but not in other orders. In avian evolution,the cloned sequences have not been as highly conserved as some other repeated sequences, but have been moderately conserved within a single order of birds.

In those avian species where significant hybridization to chicken clustered repeated sequences was found, the pattern of bands detected was different from that seen in chicken DNA. Each of the heterologous species showed its own characterstic, simpler subset of the chicken pattern. The related species also showed reduced extents of hybridization, probably resulting both from reduced copy number and from divergence. Our main conclusion from these evolutionary comparisons is that the clustered repeated sequences are present in the DNA of some other species but have undergone substantial re-organization there.

(c) Methylation and De-methylation

A number of recent reports document that methylation within and around structural gene sequences in DNA can be variable from tissue to tissue and that the extent of methylation correlates with transcriptional inactivity of genes. The same correlation seems to hold for integrated viral genomes, which are often both methylated and not transcribed. (Both topics have been recently reviewed; see references 26,36). Using probes which presumably do not have a protein-coding function in DNA, we have failed to detect significant de-methylation in any of several embryonic or adult tissues. We also have preliminary evidence (Eden, Sobieski, unpublished data) that these sequences are rarely if ever transcribed in chicken cells. Thus the same correlation would appear to hold for both coding and some non-coding sequences: extensive methylation accompanies transcriptional inactivity.

The only variablity in methylation of repeated sequences we were able to detect was the hypermethylation found in sperm. We were unable to deter-

mine when the methylation pattern typical of sperm DNA changes to the somatic pattern. The earliest developmental stage we could conveniently obtain was a 3-day embryo, where the somatic methyation pattern was already found (data not shown). Probably the removal of the hypermethyation occurs early in embryogenesis, perhaps as early as the zygote or early cleavage stage. After the three day stage we detected no furthur change in methylation, so the reversion of the sperm pattern is not a gradual one during embryogenesis but seems to occur abruptly with the onset of development.

(d) The Evolutionary Persistence of Methylation

Clustered repeated sequence elements are extensively methylated both in the chicken genome and in the DNA of other avian species. This persistence of methylation contrasts with the variability of the genomic organization of these sequences among species. The maintenance of methylation throughout evolution suggests that it has a specific association with these particluar sequences. We have also determined that methylation is faithfully reproduced among most, if not all, of the many hundreds of copies of these sequences within the chicken genome itself (13), which also suggests that methylation is specifically directed towards them. Similar correlations of methylation with repeated sequences have been made by others (3,21), and have been interpreted as possible evidence of sequence-specific methylation. However, attempts to make a direct correlation between the occurance of 5-methyl cytosine and specific oligonucleotide sequences in DNA have been unsuccessful so far (summarized in reference 4). It remains an open question whether the type of methylation we observe here is sequence-specific, region specific, or is correlated with other structural features of the genome that invlove DNA-protein interactions. In any case its presence can be a conserved characteristic of certain repeated sequences through long periods of evolution and change.

FOOTNOTES

1. To whom correspondence should be addressed. Present address:
Laboratory of Molecular Carcinogenesis, National Cancer Institute
Bldg 37,Rm 3C19,National Institutes of Health, Bethesda, Md.20205
2. The abbreviations used are: SSC, 0.15M NaCl, 0.015M trisodium citrate; KB, kilobase pair.

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