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Genetics

Gene localization by chromosome fractionation: Globin genes are on at least two chromosomes and three estrogen-inducible genes are on three chromosomes

(chromosome isolation/gene mapping/flow microfluorimetry/endogenous viral genes)

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ABSTRACT **Chicken metaphase chromosomes were partially purified by rate zonal centrifugation, and DNA was prepared from each of the fractions of the sucrose gradient. The DNA was digested with various restriction enzymes and subjected to electrophoresis in agarose gels. The DNA was transferred to nitrocellulose filters (as described by Southern), and the filters were hybridized with cDNA probes. Four globin genes α^A, α^D, β, and ρ or ϵ are located on at least two chromosomes, and three of the estrogen-inducible genes of the hen oviduct-ovalbumin, ovomucoid, and transferrin—are on three different chromosomes. These experiments also confirm our earlier assignment of the endogenous viral sequence related to Rous-associated virus-0 to a separate (and larger) chromosome than the cellular sequence related to the transforming gene of avian sarcoma virus (cellular sarc), although it now appears that cellular sarc is on a small macrochromosome, rather than on a microchromosome.**

The localization of genes in eukaryotic genomes has been retarded relative to mapping of bacterial genes by the complexity of the genomes, the long generation times, and the diploid status of animal cells. As a consequence, there remains considerable uncertainty about the organizational principles of the DNA of higher organisms.

A necessary step to an understanding of gene regulation in eukaryotes is a description of the positions of the genetic elements in the host genome. Chromosomal mapping of eukaryotic genes has thus far depended completely or partially upon genetic approaches, either in the intact animal, by using classical methods, or through somatic cell hybridization, generally by using a combination of functional tests for gene products and karyotypic analysis to associate products with retained chromosomes (1). Direct physical methods (e.g., molecular hybridization *in situ*) have the advantage of circumventing a requirement for genetic manipulation. However, such methods have been less widely used, in part because of the difficulty of assaying an appreciable number of specific genes and in part because of the problems of detection of single-copy genes.

We have recently shown that fractionation of avian chromosomes in sucrose gradients can be coupled with molecular hybridization in solution to allow the crude localization of genes for which functional tests or appropriate hybrids were not available (2). This approach revealed that endogenous viral DNA was not linked in the chicken genome to sequences related to the transforming gene of avian sarcoma virus. In this report we show that our earlier approach can be refined with the use of restriction endonucleases and the DNA transfer method developed by Southern (3). After fractionation of chicken chromosomes in sucrose gradients, DNA was isolated from each fraction and digested with a restriction endonuclease. The DNA samples were then subjected to electrophoresis in agarose gels and transferred to nitrocellulose filters. Such filters can be assayed with any gene-specific radioactive DNA or RNA that can be prepared with sufficient purity and in adequate quantity. By using this strategy, we have confirmed our earlier results with added precision and have localized ribosomal RNA genes to a group of small chromosomes. More importantly we have found that three genes whose expression is coregulated by estrogen are located on three different chromosomes and that four globin genes are located on at least two separate chromosomes. Implications of these findings are considered in the *Discussion*.

MATERIALS AND METHODS

Cells and Culture Conditions. MSB-1 cells are a lymphoblastic line derived from a chicken infected with Mareks disease virus (4, 5). The cells were maintained as suspensions in RPMI 1640 media supplemented with 10% fetal calf serum. The cells have a doubling time of approximately 12 hr and were maintained, by appropriate regular dilutions, between 1×10^6 and 5×10^6 cells per ml.

Chromosome Gradients and Flow Microfluorimetry (FMF). Metaphase chromosomes were prepared and fractionated as described by Padgett *et al.* (2). The distribution of chromosomes in each fraction was determined visually by microscopic analysis or by FMF (6, 7).

Isolation of RNAs. The methods described by Shank *et al.* (8) were used to prepare 35S RNA from avian sarcoma virus. Globin mRNA was prepared from chickens made anemic with phenylhydrazine (9–11). The preparation of oviduct mRNAs has been described in detail elsewhere (12–14). Ribosomal RNA was prepared by sedimenting the poly(A)-minus RNA fraction on a 15–30% sucrose gradient (15).

DNA Extraction. DNA was extracted from the chromosome fractions as described by Padgett *et al.* (2).

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Abbreviations: FMF, flow microfluorimetry; RAV, Rous-associated virus; ASV, avian sarcoma virus.

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Digestion of the DNA Samples with Restriction Endonucleases and Agarose Gel Electrophoresis. DNA samples were digested with *Eco*RI or *Hin*dIII (8, 16), the digested DNA samples were subjected to electrophoresis in 0.8% agarose gels (Seakem) that were 0.8 cm thick (17), and the DNA was transferred to nitrocellulose filters (3).

cDNA Preparation and Annealing. cDNAs were prepared from the RNA samples with avian myeloblastosis virus RNA-dependent DNA polymerase in the presence of calf thymus primers (8, 16). cDNAs were annealed to nitrocellulose filters, and the filters were washed as described by Shank *et al.* (8). The filters were exposed to x-ray film in the presence of Du Pont Lightning Plus x-ray intensifying screens (18).

RESULTS

Chromosome Fractionation. We have depended here, as in our previous study, upon the MSB-1 line of chicken lymphoblasts as a source of chromosomes (1). The karyotype of these cells has been shown to be normal with the exception of a single translocation to chromosome 1 (2, 4). Thus, like other chicken cells, the MSB-1 cells contain 15 or 16 pairs of individually identifiable macrochromosomes, including sex chromosomes (z and w), and 30–40 pairs of indistinguishable microchromosomes.

The improved sensitivity of our analytic methods allowed us to perform all the experiments described here with the DNA obtained from only two gradients of fractionated chromosomes. In the first case (gradient I), the distribution of chicken chromosomes in each fraction was determined by counting stained chromosomes (data not shown). Fractions from the second gradient (gradient II) were analyzed by FMF (Fig. 1), in addition to staining procedures. Gradient I had a chromosome distribution similar to our published gradients (which were also analyzed with FMF; ref. 2); in contrast, gradient II lacked a significant portion of the largest macrochromosomes, particularly numbers 1 and 2. The loss was due to the partial pelleting of those chromosomes during prolonged centrifugation, but the resolution of the smaller chromosomes was improved.

Confirmation that Rous-Associated Virus (*RAV*)-0-Related and *src*-Related DNAs Are Not Linked. Gradients I and II were first assayed for the virus-related sequences previously detected by solution hybridization to assess the utility of restriction endonuclease digestion and gel electrophoresis of DNA from chromosomal fractions. *Eco*RI was used in these experiments because we had observed (unpublished observations of S. Hughes and D. Spector) that the two major *Eco*RI fragments of 2.5×10^6 and 6×10^6 M_r (and a minor fragment of 10×10^6 M_r) contained sequences related to the endogenous virus of chickens, RAV-0, whereas three *Eco*RI fragments (8, 10, and 15×10^6 M_r) contained sequences related to the transforming gene (*src*) of avian sarcoma virus (ASV). DNA from each fraction of the sucrose gradient was digested with *Eco*RI, fractionated by electrophoresis on a 0.8% agarose gel, and transferred to a nitrocellulose filter. [^{32}P]cDNA representative of the entire ASV genome was annealed to the filter (Fig. 2*A*).

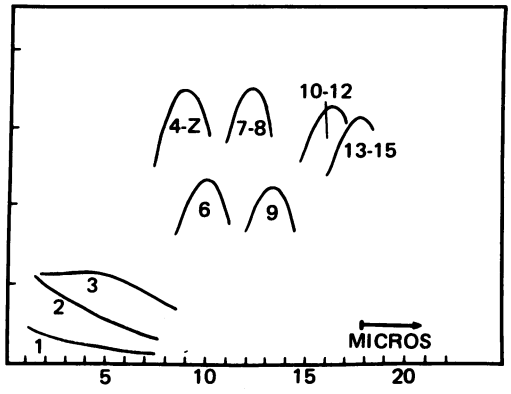

FIG. 1. Distribution of chicken chromosomes in gradient II. Metaphase chromosomes from MSB-1 cells were subjected to velocity sedimentation in a 20–40% linear sucrose gradient. Fractions from the gradient were analyzed for chromosomal composition by staining with ethidium bromide and measurement of the distribution of fluorescence by FMF. By fluorescence, nine classes of chicken macrochromosomes can be distinguished. The number of chromosomes from each class in each fraction has been plotted; the scale (on the ordinate) is arbitrary. Micros, microchromosomes.

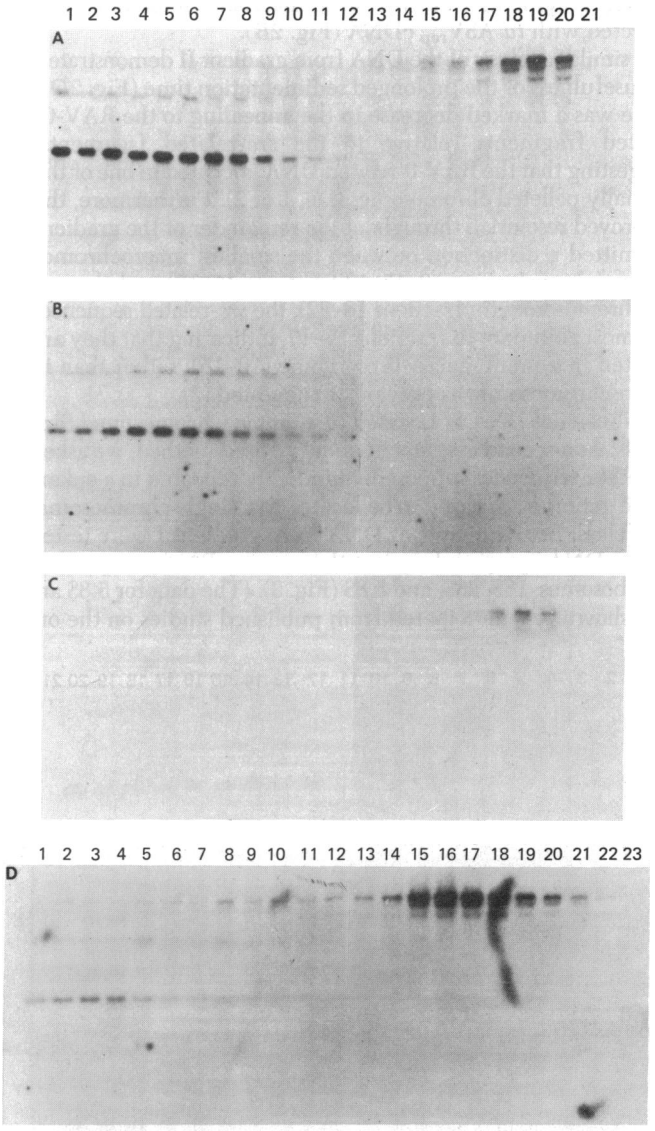

FIG. 2. Localization of ASV-related sequences in MSB-1 chromosomes. Samples (10%) of DNA from chromosomes fractionated in gradients I and II were digested with *Eco*RI and subjected to electrophoresis on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose sheet according to the procedures of Southern (5), and virus-specific sequences were detected by hybridization with ASV-specific cDNAs. (*A*) Analysis of chromosome gradient I with ASV $cDNA_{rep}$. Lane 1 is from the bottom of the gradient; lane 21, from the top. (*B*) Hybridization of *td*ASV $cDNA_{rep}$ to DNA from gradient I. *td*ASV $cDNA_{rep}$ lacks most or all of the *src* sequences (i.e., those represented in $cDNA_{sarc}$). (*C*) Hybridization of $cDNA_{sarc}$ to DNA from gradient I. $cDNA_{sarc}$ represents most or all of the ASV *src* gene. (*D*) Analysis of chromosome gradient II with ASV $cDNA_{rep}$. Lane 1 is from the bottom of the gradient; lane 23, from the top.

It is evident that the *Eco*RI fragments characteristic of the RAV-0-related provirus were derived from large macrochromosomes (the 10×10^6 M_r fragment is too faint to be clearly seen), whereas the fragments characteristic of *src*-related sequences were derived from microchromosomes or the smallest macrochromosomes. To verify the specificity of the fragments that were considered *src* related, we performed a similar analysis with [^{32}P]cDNA$_{sarc}$, which represents most or all of the ASV *src* gene (19), and with *td* ASV$_{rep}$ cDNA, which represents the genome of a transformation-defective deletion mutant (lacking most or all of the *src* gene). As expected, only the large *Eco*RI fragments obtained from fractions near the top of the gradient reacted with cDNA$_{sarc}$ (Fig. 2*C*), whereas the virus-specific fragments from the large macrochromosomes were detected with *td* ASV$_{rep}$ cDNA (Fig. 2*B*).

A similar analysis of the DNA from gradient II demonstrated the usefulness of the prolonged sedimentation time (Fig. 2*D*); there was a marked decrease in the annealing to the RAV-0-related fragments relative to the *src*-related fragments, suggesting that the RAV-0-related DNA is located in one of the partially pelleted chromosomes (i.e., 1 or 2). Furthermore, the improved resolution throughout the remainder of the gradient permitted a distinction between the smallest macrochromosomes (chromosomes 10–15, in fractions 15–17) and the microchromosomes (in fractions 18–22); the *src*-related sequences are most abundant in fractions 15–17, indicating that they are located in a small macrochromosome (10–15), rather than in microchromosomes as previously suggested (2).

Ribosomal DNA Is Located in a Single Chromosomal Size Class. As a second assessment of our revised method, we asked whether sequences supposedly tandemly repeated in a eukaryotic genome appeared to be located in a single chromosomal size class. *Eco*RI digests of DNA from gradient I were tested with cDNAs prepared against three major RNA components of ribosomes: 18S, 28S, and 5.8S (Fig. 3). (The data for 5.8S are not shown.) As anticipated from published studies on the organization of ribosomal DNA in the chicken genome (20), cDNA prepared from 18S RNA detected *Eco*RI fragments of 5.5 and 12×10^6 M_r, and cDNA prepared from 28S detected only the 5.5×10^6 M_r fragment and a minor fragment of 0.7×10^6 (11). The cDNA for 5.8S RNA anneals with the 5.5×10^6 M_r fragment (not shown). In each case, however, maximal annealing was observed with DNA obtained from the region of the gradient containing the smallest macrochromosomes and microchromosomes. A similar analysis of DNA from gradient II indicated that most of the ribosomal cistrons, like *src*-related sequences, were located in chromosomes 10–15 (data not shown). The diminished annealing to DNA from other regions of the gradient probably reflects the incomplete fractionation of the chromosomes, because the fractions are not composed of pure populations (see Fig. 1) and because similar patterns are seen in analysis of single copy DNA (compare Figs. 2, 4, and 5); however, in the case of repeated genes such as ribosomal DNA, we cannot exclude the possibility that some cistrons are found on chromosomes other than 10–15.

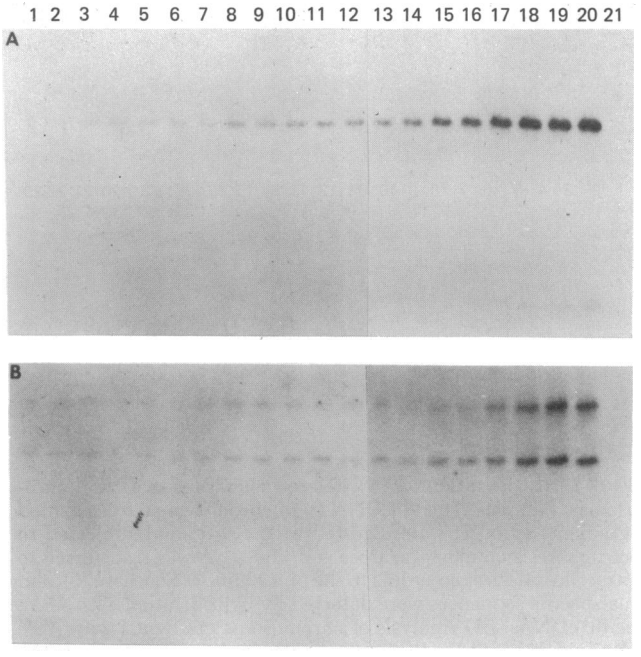

FIG. 3. Chromosomal location of ribosomal RNA genes. DNA samples from fractions of gradient I were digested with *Eco*RI, and the resulting fragments were separated on a 0.8% agarose gel. The DNA was transferred to nitrocellulose filters and hybridized with cDNA specific for either 18S or 28S ribosomal RNA. (*A*) Hybridization with cDNA$_{28S}$. (*B*) Hybridization with cDNA$_{18S}$.

Estrogen-Responsive Genes Are Located on Multiple Chromosomes. We next asked whether three of the genes that are transcribed at increased rates in the hen oviduct in response to estrogen were located on a single chromosome. [^{32}P]cDNAs were synthesized from the mRNAs for ovalbumin, transferrin, and ovomucoid, which were purified from the oviducts of es-

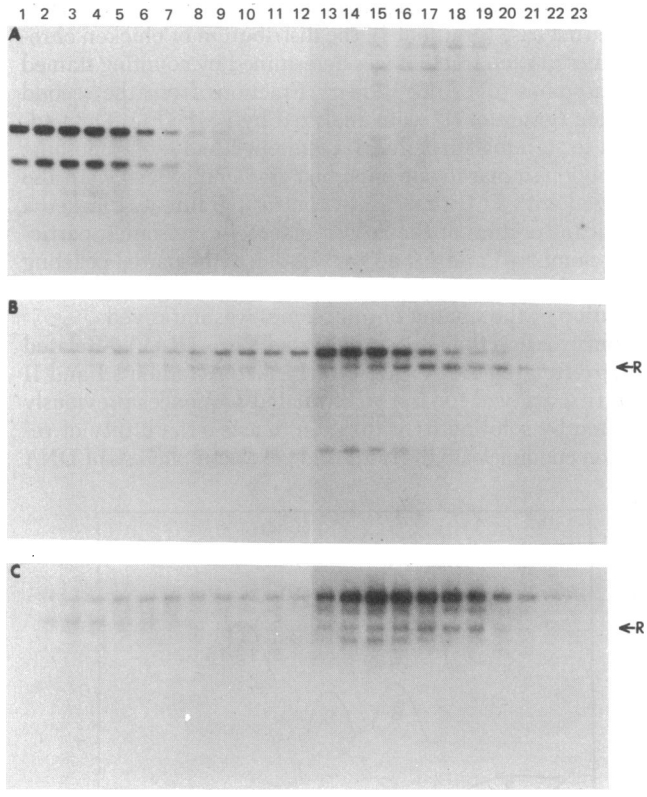

FIG. 4. Chromosomal locations of the genes for three of the estrogen-inducible proteins of the hen oviduct. DNA samples from gradient II were digested with *Hin*dIII or *Eco*RI and analyzed as in previous figures by using the indicated cDNAs as hybridization reagents. (*A*) The DNA was digested with *Hin*dIII and annealed with ovalbumin cDNA. (*B*) The DNA was digested with *Eco*RI and annealed with transferrin cDNA. The transferrin cDNA was contaminated, to a small extent, with cDNA copied from 28S ribosomal RNA. The major 28S rRNA-specific fragment is indicated by the symbol R. (*C*) As in *B*, the DNA was digested with *Eco*RI and annealed with ovomucoid cDNA. The ovomucoid cDNA was also slightly contaminated with cDNA from 28S ribosomal RNA. The major 28S rRNA-specific fragment is designated by the symbol R.

trogen-stimulated chickens (12–14). Annealing of ovalbumin cDNA to *Hin*dIII digests of DNA from gradient II (Fig. 4*A*) showed that the major fragments of 3.0 and 1.8 × 10^6 M_r were obtained from fractions 2–4, suggesting that the single ovalbumin gene (21, 22) was located in chromosome 2 or 3. Analysis of *Eco*RI digests of DNA from the same gradient with cDNAs specific for the transferrin (Fig. 4*B*) and ovomucoid genes (Fig. 4*C*) revealed that the transferrin gene was most likely to be located in chromosomes 9–12, whereas the ovomucoid gene was located in chromosomes 10–15. It is clear however, that the ovomucoid and transferrin genes are on different chromosomes, even though absolute assignments cannot be made. Thus, the three tested genetic elements under estrogen control in the oviduct reside on different chromosomes and cannot be linked. This conclusion was confirmed by additional analyses, using ovalbumin cDNA to detect *Eco*RI and *Bam*HI fragments (data not shown); DNA from gradient I also provided similar results, although the resolution was not adequate to distinguish the locations of transferrin and ovomucoid genes (data not shown).

Several minor bands were noted in the autoradiograms presented in Fig. 4. Transferrin cDNA appears to have been contaminated with cDNA transcribed from 28S RNA present in the mRNA preparation, because an *Eco*RI fragment of 5.5 × 10^6 M_r originating from chromosomes 10–15, was observed in addition to the fragments of 6.5 and 1.6 × 10^6 M_r derived from chromosomes 9–12 (Fig. 4*B*). The ovomucoid cDNA appeared on similar grounds to be contaminated with cDNA transcribed from 28S RNA; in addition, it may have been contaminated with ovalbumin cDNA because it detected *Eco*RI fragments of 6.5 × 10^6 M_r from the DNA from fractions 2–4 (Fig. 4*C*). We assume that the *Eco*RI fragments of 12, 7.2, and 6.5 × 10^6 M_r contain portions of the ovomucoid gene (a minor band of 4.7 × 10^6 is too faint to be visible). The ovalbumin cDNA detected *Hin*dIII fragments (Fig. 4A) and *Eco*RI fragments (data not shown) in regions of the gradient containing transferrin and ovomucoid genes; because these fragments have molecular weights corresponding to ovomucoid and transferrin *Hin*dIII fragments (unpublished observations), these bands may reflect minor impurities in the cDNA or limited sequence homology between the genes encoding the egg-white proteins.

Globin Genes Are on More Than One Chromosome. To determine whether members of a class of genes that encode functionally related products were located on a single chromosome, we annealed [^{32}P]cDNA transcribed from purified adult globin mRNA to *Eco*RI digests of the DNA from gradient I (Fig. 5*A*). cDNA species copied from adult chicken α and β globin mRNA have been shown to anneal with four *Eco*RI fragments from unfractionated chicken DNA; these fragments—8.5, 6.5, 4, and 2.7 × 10^6 M_r—contain the genes for α^A, either ρ or ϵ, β, and α^D globins, respectively (12). The analysis shown in Fig. 5 reveals that two of these fragments (6.5 and 4 × 10^6 M_r) are derived from DNA in fractions 1–8, whereas the others (8.5 and 2.7 × 10^6 M_r) are derived from DNA in fractions 17–20. Thus, the genes for α^A and α^D globins are positioned in small macrochromosomes or microchromosomes, whereas the gene for either ρ or ϵ (embryonic β-like globins) and β globin are located in the largest macrochromosomes. Analysis of globin genes on chromosome gradient II places the β globin gene and the embryonic β-like gene ρ or ϵ in the same chromosomal size class as the RAV-0-related sequences (i.e., in one of the largest macrochromosomes, probably chromosome 1 or 2), whereas the α globin genes are most abundant in the same fractions of the gradient as the endogenous sarc gene and the ovomucoid gene. This localizes the α genes to chromosomes 10–15 (Fig. 5*B*).

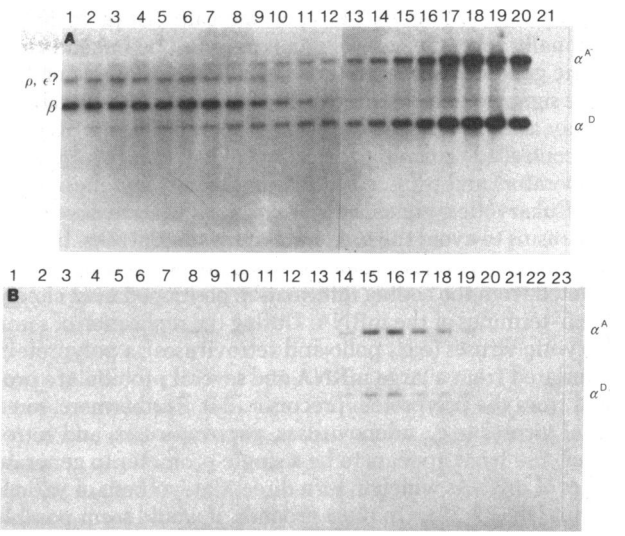

FIG. 5. Chromosomal location of globin genes. (*A*) DNA samples from fractions of gradient I were digested with *Eco*RI and analyzed with cDNA prepared from adult globin mRNA. The adult α major (α^A) the adult α minor (α^D) and the adult β(β) and an embryonic β-type (ρ or ϵ) globin which shows sequence homology to the adult β globin gene are detected with cDNA copied from adult globin RNA. (*B*) DNA samples from the fractions of gradient II were digested with *Eco*RI and analysed with cDNA from adult globin. The fragments from the α^A and α^D genes are indicated.

DISCUSSION

Fractionation of chromosomes in sucrose gradients affords adequate separation to permit linkage tests without genetic manipulation (2, 6, 7, 23). With the use of restriction endonucleases and the Southern DNA transfer technique, we can analyze the DNA from a single gradient for the distribution of many genetic elements among the chromosome fractions. A total of less than 50 μg of DNA was required for the experiments shown here, and DNA immobilized to a single filter can be annealed successively with as many as five different hybridization reagents.

One of the major limitations of the method we have employed is the availability of hybridization reagents specific for known genes. However, new techniques for partial purification of messenger RNAs and for cloning of cDNAs and genes are likely to generate a wide array of suitable annealing reagents in the near future. A second limitation is the incomplete resolution of the chromosomes. Although the fractionation of chromosomes in the gradients studied here has been adequate to draw conclusions about the linkages of several groups of genes, we have not been able to make specific chromosomal assignments for any of the genes examined. The resolution could no doubt be improved by resedimentation of chromosomes from the relevant fractions, but the sorting of chromosomes from gradient fractions by a fluorescence-activated device would probably afford essentially complete separation. The small quantities of DNA now required for the subsequent analysis makes this approach feasible with existing technology (8, 9, 24).

Even with the present degree of resolution, however, the physical methods we have used offer some major advantages over methods that depend upon classical or somatic cell genetics. A single successful fractionation of chromosomes can form the basis for experiments to localize a large number of genes. Although more precise assignments can be made with genetically based techniques, the efforts involved in the genetic crosses or in the preparation and characterization of appropriate somatic cell hybrids are considerable.

The central findings in this report indicate that members of functionally related classes of genes need not be linked in eukaryotic genomes. In bacterial systems, genes that respond to a single signal or encode enzymes involved in a single metabolic pathway are often linked to form an operon; this unit is generally controlled by centralized control elements (e.g., promoter and operator) and transcribed into a polycistronic messenger RNA. Eukaryotic systems, however, appear to have developed mechanisms to avoid the use of polycistronic mRNAs. In general, single polypeptides, some of which are polyfunctional, are translated from the coding information positioned most closely to the 5′ terminus of the mRNA. During the replication of some eukaryotic viruses (e.g., polio and retroviruses), a polyprotein is translated from a large mRNA and several proteins are processed from the polyprotein precursor (24). Furthermore, some animal viruses (e.g., adenoviruses, papovaviruses, and retroviruses) use what appears to be a single promoter to generate a series of mRNAs which in turn direct the synthesis of various proteins (15, 25–32). On these grounds, it would seem possible for some closely related or coordinately regulated cellular genes to benefit from contiguity in the host genome.

We have found, however, that at least three of the four (or more) genes that demonstrate heightened expression in the chick oviduct after estrogen administration are completely unlinked in the chicken genome. The presence of these three genes on separate chromosomes implies that separate regulatory sites must be present upstream from each of them.

We have also found that the multiple genes encoding chicken globin chains are located on at least two chromosomes; this result conforms with recent studies of human chromosomes in which β and γ globin genes were assigned to chromosome 11 and α globin genes to chromosome 16 (33, 34). The multiple copies of ribosomal DNA, on the other hand, appear to be located on a single chromosome. A general rule which may be emerging from such studies is that multiple genes that are closely related or identical in sequence (e.g., ribosomal cistrons, histocompatibility genes, α globin genes, etc.) are situated on a single chromosome, perhaps as a reflection of the mechanisms of duplication and recombination by which they have arisen. Certainly mapping studies of other classes of functionally related genes whose members do or do not exhibit homologous nucleotide sequences will be important to an understanding of how such classes arose and of how they are now regulated.

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