short piece of quartz fiber 2 or 3 μm in diameter. A small tissue sample (2×10^{-8} g or less) is easily picked up with this tool and transferred to the balance tip. A similar quartz-tipped "hair point" is also used for transferring weighed samples into the oil wells for analysis.

Specificity. The specificity of the overall analysis rests largely on the first enzymatic step. As in any enzymatic analysis this specificity depends on the properties and purity of the enzymes used and on the conduct of the assay. Increasing sensitivity does not increase the specificity problem and may make it less. For example, at higher dilutions relatively less enzyme may be required with less danger from contaminants. Similarly, with amplification by cycling the "signal" is increased relative to the "noise" (tissue blank).

Performance and Applications. The microanalytical system described is not only unlimited in theory but has been shown in practice to be capable of very high sensitivity and quite satisfactory precision over a ten-million-fold range in sample size (10^{-9} to 10^{-16} mol). The following are typical examples. In the 10^{-10} mol range Karlsson and coworkers[13] have made extensive studies of muscle metabolites in small human biopsy samples, and numerous enzymes have been measured in different segments of single kidney tubules.[14,15] Metabolite levels have been measured in discrete layers of mouse cerebellum[16] (10^{-11}–10^{-12} mol), in different layers of retina (10^{-12}–10^{-13} mol),[11] and in single mouse pancreatic islets (10^{-12}–10^{-14} mol).[17] In one study[18] single nerve cell bodies from mouse spinal cord were analyzed for ATP, phosphocreatine, glucose, and glycogen under a number of different experimental conditions. The sample sizes ranged from 1×10^{-12} to 3×10^{-12} g dry weight and the amounts of the four substances measured were all in the 10^{-12} mol range. Mrs. Elizabeth Barbehenn in this laboratory is currently measuring metabolite levels ranging down to 2×10^{-15} mol in single mouse ova. Measurements have been made of NAD in single nerve cell nuclei in the 10^{-16} mol range.[19] In an unpublished study, Dr. Frank A. Welsh determined the activity of single molecules of glucose 6-phosphate dehydrogenase. This required the measurement of 10^{-17} mol of enzyme product and a 10,000,000-fold amplification by two cycling steps.

This performance exceeds that attainable at present with methods based on other analytical principles. Of these, radioactive tracer methods are perhaps the most sensitive and may be used for comparison with the above. In the case of labeled amino acids having the highest available specific activity, the limit for accurate assay is about 10^{-12} mol with ^{14}C, 10^{-14} mol with ^{3}H, and 10^{-15} mol with ^{35}S (methionine).

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Deoxyribonucleic Acid Renaturation Kinetics and Hybridization. Probes to the Structure of the Eukaryotic Chromosome

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The cell nucleus is defined as that part of a living cell containing the genetic information or, in molecular terms, the DNA. In bacteria and in blue-green algae the nucleus is not separated by a nuclear membrane from the rest of the cell, the cytoplasm. Under the microscope the nucleus appears amorphous and spread throughout the cell. Cells without nuclear membranes are called prokaryotes. Until approximately 5 years ago the major discoveries of molecular biology were made in experiments on bacteria and their viruses.

Eukaryotes are cells with nuclear membranes. Such cells are found in most of the remaining single-cell organisms not mentioned above, such as fungi, protozoa, and most algae. Eukaryotic cells are also found in all higher plants and animals. The molecular biology of the eukaryotes is only now emerging as an area of useful experimentation. There are many similarities with the prokaryote, for the central dogma of molecular biology is common to all cells: DNA codes for DNA, DNA codes for RNA, and RNA codes for protein. The differences between the two

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classes of cells, however, are the stimulus for most of the studies presently undertaken. The multicellular organism is far more complex than the single-cell organism because its cells become differentiated or committed to a particular characteristic class or function.

There are other differences between the prokaryotes and the eukaryotes, many of which deal with the life cycles of the cells and their mechanisms of cell division. Eukaryotes undergo a clearly defined cell cycle during most of which a cell nucleus containing the DNA of the cell is discernible with a light microscope. Just preceding cell division, the nuclear membrane disappears, and the contents of the nucleus simultaneously condenses into metaphase chromosomes. Cell division continues with the split of the chromosomes into two equal halves by the spindle apparatus, the loss of the condensed state of the metaphase chromosomes, and the pinching off of the cell membrane. When division is complete, each new cell contains half of the nuclear material of the metaphase cell.

Such a division cycle is far more complicated than the cycle of prokaryotes. It is, therefore, not surprising that substantial differences can be found in the properties of eukaryotic DNA relative to prokaryotic DNA. One of the most striking differences is that eukaryotes contain DNA of a very simple repeating pattern.[1,2] A sequence of as few as six bases is repeated millions of times in the total set of chromosomes known as the genome.[3,4] These simple sequences, in many cases, have been localized in or near the centromeres of metaphase chromosomes.[5,6] The centromere is the point of attachment of spindle fibres and is the point at which the chromosomes are parted in division. It frequently appears more highly condensed in microscope spreads of metaphase chromosomes, staining darker than the remainder of the chromosome. This suggests that simple DNA sequences are important in determining the state of condensation of the DNA in chromosomes. At the molecular level the more condensed portions of the chromosomes contain more tightly packaged DNA, probably in a supercoil structure with protein.

The chromosome of the higher organism is a complex of DNA and protein (and perhaps RNA), and the molecular basis for the highly condensed state is not yet understood. What is known, however, from years of cytogenetics, is that the condensed state of portions of chromosomes is not a unique property of centromeres but is found in other regions of the genome as well.[7,8] Some regions of the genome, including sometimes whole chromosomes, remain tightly condensed in the cell nucleus. Such regions are called heterochromatic and are distinguished from the regions which do not remain condensed, which are called euchromatic. All heterochromatin is now believed to contain repetitious sequences of DNA. Cytogeneticists[9] also believe heterochromatic regions are relatively inert with respect to the expression of genes.[10,11]

Many translocation mutants in *Drosophila melanogaster* suggest that there are positional effects related to gene activity: the nearer to heterochromatin the less active the gene. We therefore have proposed a coarse genetic control mechanism[12] which depends upon the physical state of condensation of the chromatin and which acts upon relatively long stretches of the genome, 20 to 100 genes for example. We distinguish this mechanism from fine control which operates at the level of one to five genes and has been extensively studied in prokaryotes.

There are two very broad features of this proposal which are directly related to the nature of the higher organism and its evolution. First, special genes may be located in heterochromatin. Since genetic crossing-over is rarely observed in heterochromatic regions, such genes would be partially protected from evolutionary changes by virtue of their special position in heterochromatin. The genes in heterochromatin may also be expressed at a special time in the cell cycle. We suggest[13] that genes in heterochromatin will be found in proximity to simple-sequence DNA. The second proposal, made by Kenneth Jones[14] of Edinburgh, is that speciation comes about by the emergence of a new distribution of simple sequences in the genome and therefore a new control of neighboring genes by positional effects. Many others[3,4] have speculated upon the importance of simple sequences to speciation as well, because very closely related species are observed to have entirely different simple sequences.

The major interest of this laboratory[15,16] in the last three years has been the distribution of simple sequences between different chromosomes and the organization and function of these sequences within a chromosome. We have concentrated our efforts on the DNA of the fruit fly, *Drosophila melanogaster*, because of the wealth of classical genetic information available for this organism.

DNA Renaturation Kinetics

The two strands of the Watson–Crick duplex can be separated either by heating a solution of DNA to about 100° or by raising the pH of the solution to 13. This process is called denaturation. At pH 7 and at 60° or 70°, complementary regions of DNA can reform the double-stranded duplex, or be renatured. This renaturation reaction is a second-order reaction, the rate of which depends on the concentration of complementary pairs of nucleotide sequences in the solution.[2] The rate of this reaction is readily monitored in a spectrophotometer by recording the hypochromicity at 260 nm associated with the re-formation of helix. The initial native DNA is generally fragmented by sonication to single strand, mol wt 1.5

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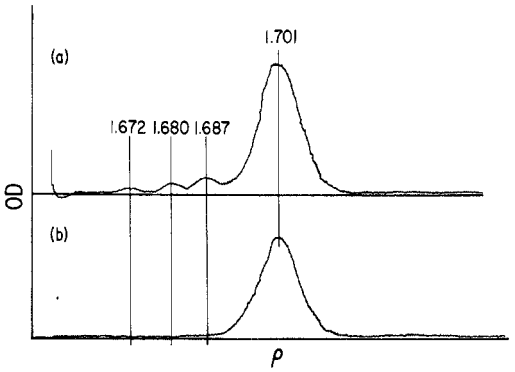

Figure 1. Neutral CsCl buoyant-density profile for DNA isolated from *Drosophila melanogaster* embryos. The double-strand molecular weight of this DNA is 30 × 10^6. (a) Total cellular DNA; (b) the main band DNA of isolated nuclei.

× 10^5, before studies of renaturation kinetics are performed, thus eliminating the variable of DNA molecular weight as a complicating parameter.[17] If all the DNA fragments contain unique sequences, the rate of renaturation at a given weight concentration of DNA is proportional to the size of the genome since the concentration of any one unique fragment is inversely proportional to the size of the genome from which it originated. Thus, T4 bacteriophage DNA renatures 20 times faster than *E. coli* DNA at some standard condition because there is 20 times as much DNA in an *E. coli* bacterium as there is in a T4 phage and all nucleotide sequences in both organisms are unique.

If the organism contains repeated DNA sequences, these sequences will renature more rapidly than the unique sequences. In the case of 10^6 copies of a repeated DNA sequence per genome, these sequences will renature 10^6 times more rapidly if the repeated sequence is longer than the length of helix required for stability at the standard renaturation conditions (about 20 base pairs). Such large spreads in rates of renaturation have been observed in the DNA of almost all eukaryotes studied. The genome has arbitrarily been divided into three classes: unique sequences, intermediate sequences renaturing at rates suggesting 10 to 1000 copies per genome, and the very rapidly renaturing or simple sequences which renature at rates suggesting 10^3 to 10^7 copies per genome. We have concentrated on simple DNA sequences in this laboratory.

Any kinetic class of DNA may be separated from the others by use of hydroxyapatite which binds double-stranded DNA but not single strands in 0.12 *M* phosphate buffer. The double-stranded DNA is readily eluted from the hydroxyapatite using a 0.45 *M* phosphate buffer. We have renatured *Drosophila melanogaster* DNA for very short times and isolated the double-stranded product on hydroxyapatite. We call this material isolated harrDNA for *h*ydroxy*a*patite-isolated *r*apidly *r*enatured DNA.

CsCl Density Profiles

Frequently, the simple-sequence DNA occurs in large enough blocks and has a base composition (GC content) different enough from the remaining DNA

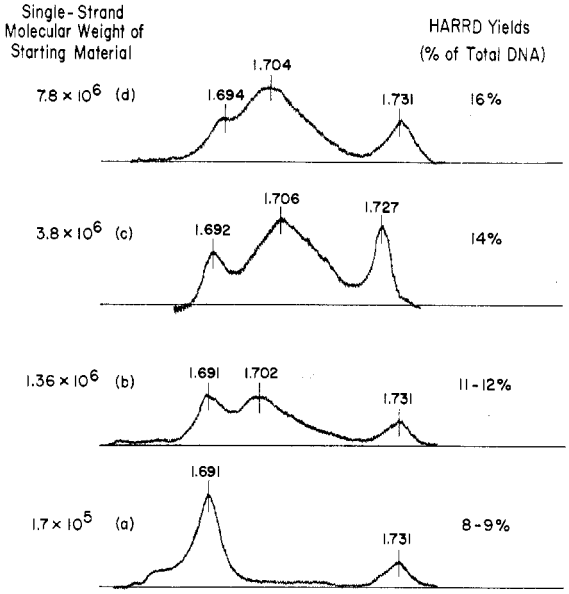

Figure 2. Buoyant-density profiles of hydroxyapatite-isolated rapidly renaturing DNA from different single-strand molecular weights of the starting DNA. Marker DNAs are *E. coli* [^{15}N]DNA (ρ = 1.727 g/ml) and *M. lysodeikticus* DNA (ρ = 1.731 g/ml). The figure also contains the yield of rapidly renaturing DNA from the hydroxyapatite.

so that banding the partially fragmented DNA in the ultracentrifuge in a CsCl density gradient results in the separation of the simple DNA from the bulk of the DNA in a density satellite. Figure 1a shows the CsCl density profile of total cellular DNA of mol wt 30 × 10^6 from *Drosophila melanogaster* embryos. The profile shows the bulk of the DNA bands at a density of 1.701. The satellite at density ρ = 1.672 represents 2–3% of the total DNA, and its origin is unknown, although it has been shown to be partially associated with the Y chromosome. The satellite at ρ = 1.680 representing 3–4% of the total is mitochondrial DNA and thus cytoplasmic in origin.[13] The satellite at ρ = 1.687 representing 7–9% of the total DNA has been localized to the centric heterochromatin by Gall, Cohen, and Polan[18] using *in situ* hybridization, a technique described below. It is thus nuclear in origin.

We have isolated harrDNA from main-band *Drosophila melanogaster* DNA with different single-strand molecular weights[16] and determined the density profile of the product. Main-band DNA, Figure 1b, is DNA from which all satellites have been removed. Figure 2 shows the results of this experiment. For sonicated DNA, the harrDNA appears as a single peak at ρ = 1.691 and a yield of 8–9%. The fact that this density would be easily resolved in Figure 1b suggests that the simple sequences are attached to a more dense DNA and that their length is shorter than mol wt 15 × 10^6 single strand. A complete analysis of the profiles obtained at higher molecular weights suggests that the simple sequences come in blocks of mol wt 0.75 to 3 million interspersed by more complex sequences of mol wt 1 to 2 million single strand.

Since renaturation is a rather complex process yielding double-stranded product with mismatching

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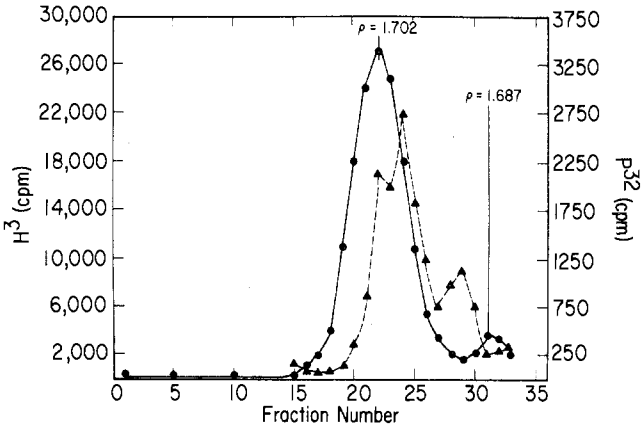

Figure 3. The location of sonicated harrDNA sequences in a native DNA CsCl gradient. [^{32}P]RNA complementary to harrDNA was incubated with filter papers containing the DNA from each fraction. (▲) Hybridized [^{32}P]DNA; (●) [^{3}H]DNA counts from starting fractions.

and free ends, an additional experiment on native DNA which demonstrated the bimodal distribution in density seemed advisable. Figure 3 shows the results of banding total nuclear DNA (including the satellites) in a preparative CsCl gradient.[13] The solid circles show the distribution of DNA (^{3}H labeled, mol wt 7×10^6) in the gradient. Labeled ^{32}P RNA complementary to sonicated harrDNA(the ρ = 1.691 peak in Figure 2) was synthesized using RNA polymerase, ^{32}P-labeled nucleotide triphosphates, and harrDNA as a template. The labeled RNA was hybridized to the various DNA fractions which were denatured and mounted on nitrocellulose filters.[19] This localizes the harrDNA sequences in the native DNA density profile. There are essentially no counts coincident with the ρ = 1.687 satellite. A 25% peak occurs at ρ = 1.691, the density of pure simple sequence DNA, while the major portion of the [^{32}P]RNA hybridizing ability is found in the light portion of the main band (ρ = 1.695–1.705). This shows that in this native DNA most of the simple harrDNA sequences are attached to a heavier DNA at 7×10^6 molecular weight.

Localization of harrDNA in the Salivary Chromosome

Salivary cells of *Drosophila melanogaster* are polytene; that is, each cell contains many copies of the haploid genome (more than 1000 copies). The salivary chromosome has a banded structure in which each of these 1000 copies of DNA are lined up side by side. It is partially these unique banded structures which make the fruit fly such an attractive organism for the geneticist. All of the centromeres and centric heterochromatin are connected in the salivary cell at the chromocenter. Thus localization of a sequence to the salivary chromocenter is equivalent to localizing the sequence to the centric heterochromatin of the normal diploid cell.

If ^{3}H labeled RNA complementary to harrDNA is synthesized using RNA polymerase, it can be hybridized to a partially denatured chromosome on a glass slide. A ribonuclease treatment degrades any bound RNA unprotected by its hybridization to DNA. The RNA is then located by autoradiography. The results of this experiment for harrDNA are shown in Figure 4a. Figure 4b is a control demonstrating the specificity of the technique. Clearly our harrDNA comes from centric heterochromatin. We mentioned earlier that Gall, *et al.*,[18] have shown that the 1.687 satellite also is in centric heterochromatin.

Table I
Comparison of % of DNA Hybridized to *in Vivo* RNA by Cs_2SO_4 and Hydroxyapatite Methods

[RNA], mg/ml	% hydridization Cs_2SO_4	% hydridization HAP	Type of DNA
1	0	3	A
	15	18	B
	0	8	control
4	0	2	A
	39	49	B
	0	6	control
10	0	5	A
	58	70	B
	0	8	control

In Vivo Expression of the harrDNA

The next step toward our eventual goal of demonstrating coarse control is the demonstration that there is some DNA in heterochromatin which is transcribed (used as a template for making complementary RNA).

The hybridization experiments designed[13] to demonstrate the existence of an *in vivo* RNA complementary to our harrDNA were performed in large RNA excess in solution. ^{3}H-labeled DNA of a specific activity of 1.5×10^5 cpm/μg was isolated from a *Drosophila melanogaster* tissue culture line obtained from I. Schneider. The density profile of DNA isolated from this line is identical with that isolated from embryos. The unlabeled RNA was isolated from embryos and extensively purified to eliminate any contaminating protein, polysaccharide, and DNA.

Labeled harrDNA was isolated at a single-strand molecular weight of 1.5×10^6. Figure 5 shows the resulting density profile in a CsCl preparative gradient. Fractions as indicated by the cross-hatched areas of Figure 5 were further purified by additional bandings in CsCl gradients. This yielded a sample of purified simple sequence DNA (peak A, ρ = 1.691) and a sample of simple sequence DNA with more unique sequences attached (peak B, ρ = 1.702).

These two fractions were then hybridized with the *in vivo* RNA in vast excess so that the harrDNA had insufficient time to renature with itself but ample time to react with the complementary RNA sequences if present. The product was treated with ribonuclease to destroy any single-stranded RNA and banded in a Cs_2SO_4 density gradient. Pure RNA–DNA hybrids have a density of 1.49–1.51 in Cs_2SO_4 while denatured DNA has a density of 1.445, so if an appreciable portion of any piece of DNA hybridizes to the RNA its density will be shifted to a higher value and be resolved. Figure 6 shows the result of this experiment. It is clear that peak A, or simple-sequence DNA, does not hybridize any RNA. This

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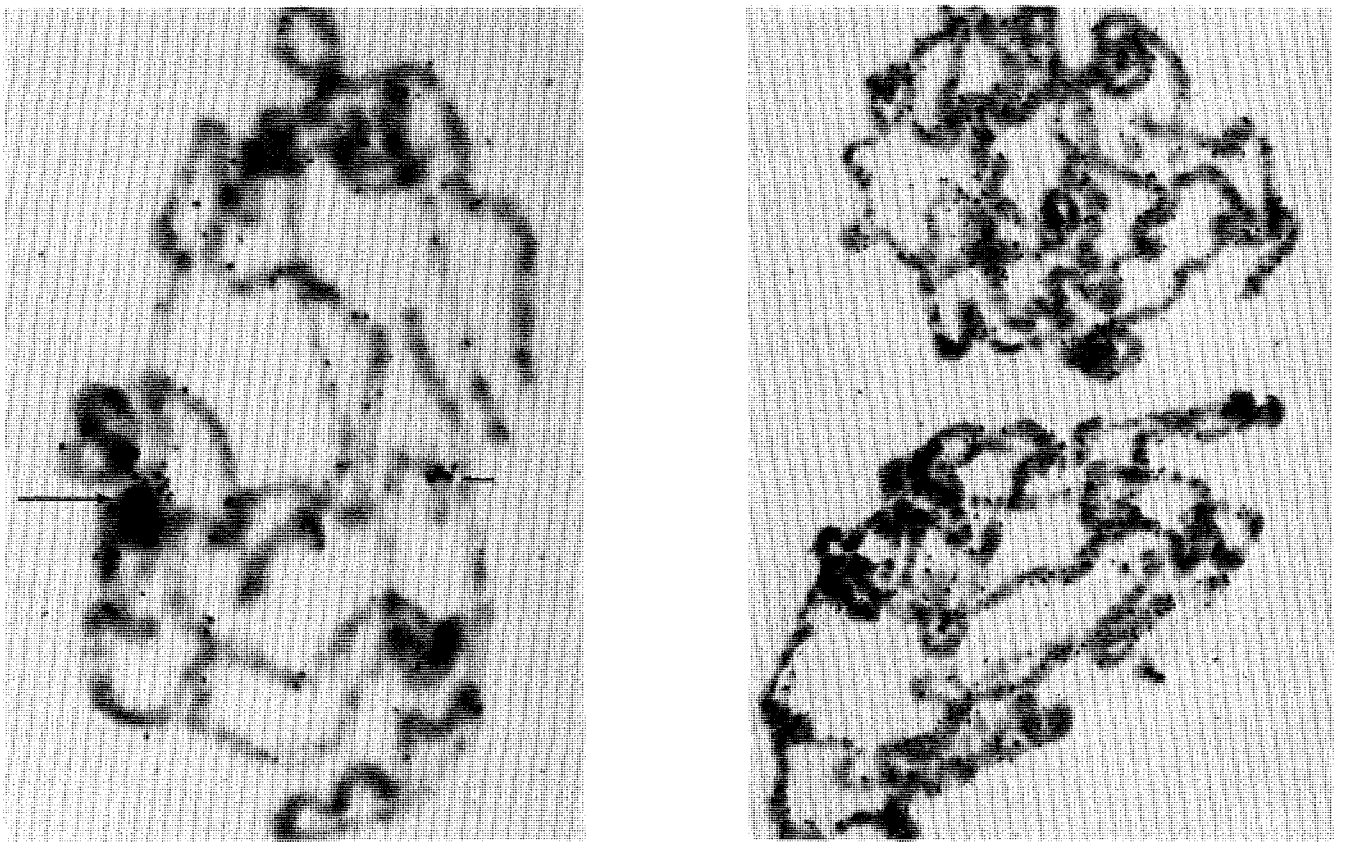

Figure 4. (a, left) [^{3}H]RNA made from harrDNA localizes specifically to the chromocenter in the salivary chromosome (long arrow) in an *in situ* hybridization experiment. One band also showed repeated labeling (marked by a smaller arrow). Magnified 1467×. Exposure of the film to ^{3}H decay was 10 days. (b, right) [^{3}H]RNA made from the unrenatured DNA which is in the 0.12 *M* phosphate buffer elution of the hydroxyapatite column does not localize but hybridizes all over the chromosome. This serves as a control for the previous photograph which demonstrates the specificity of the technique. Magnified 1000×. Exposure of the film to ^{3}H decay was 11 days.

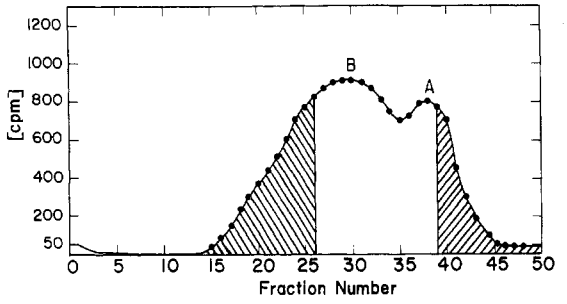

Figure 5. Preparative CsCl gradient separation of repetitive sequences. Total harrDNA isolated from a DNA of single-strand molecular weight equal to 1.5×10^6. Peak A, buoyant density in CsCl, $\rho = 1.691$; peak B, $\rho = 1.702$.

was expected since its information content is so low and no simple sequence DNA has ever been observed to be expressed *in vivo*.

On the other hand, peak B does hybridize to *in vivo* RNA, and this hybridization is extensive for the density of the product which is greater than 1.490 indicates at least 70% of the DNA in the hybrid peak is covered with RNA. In addition to the Cs_2SO_4 gradient assay, hydroxyapatite was used to determine the amount of DNA which has at least a small section of RNA hybridized to it. The summary of the results are presented in Table I. Again, the results indicate an extensive hybridization of *in vivo* RNA to the more unique sequences in harrDNA, but no hybridization to the simple sequences.

In conclusion, we have presented evidence that harrDNA contains sequences of DNA which are capable of hybridizing to *in vivo* RNA, suggesting, although not proving, that there are genes in heterochromatin. Our picture of the organization of DNA in heterochromatin of *Drosophila melanogaster* is shown in Figure 7. Part of the total DNA in heterochromatin is simple-sequence satellite DNA in very long stretches (greater than mol wt 15×10^6) and perhaps so long that each chromosome contains only one continuous piece of this DNA at or near its centromere. The remainder of the DNA in heterochromatin is half simple-sequence DNA in lengths of 0.75×10^6 to 3×10^6 mol wt, spacing transcriptively active DNA in lengths of 1×10^6 to 2×10^6 mol wt, where all molecular weights are single-strand values.

What is the relationship between the conclusions made here and other facts regarding chromosome structure? At present, the reason for the correlation between the condensation of chromatin and the presence of simple repeated sequences is not understood at the molecular level. We do, however, have a model in mind. We believe there is a condensation factor in all higher cells which cross-links double-stranded DNA sections to one another. This factor is likely to be a protein and it must have some DNA sequence specificity, but one of an unusual nature. If this factor is common to all eukaryotes, then its specificity cannot relate to a particular base sequence, but must be specific for a common property of all repeating DNA sequences. One such factor has been postulated by Professor James C. Wang in this

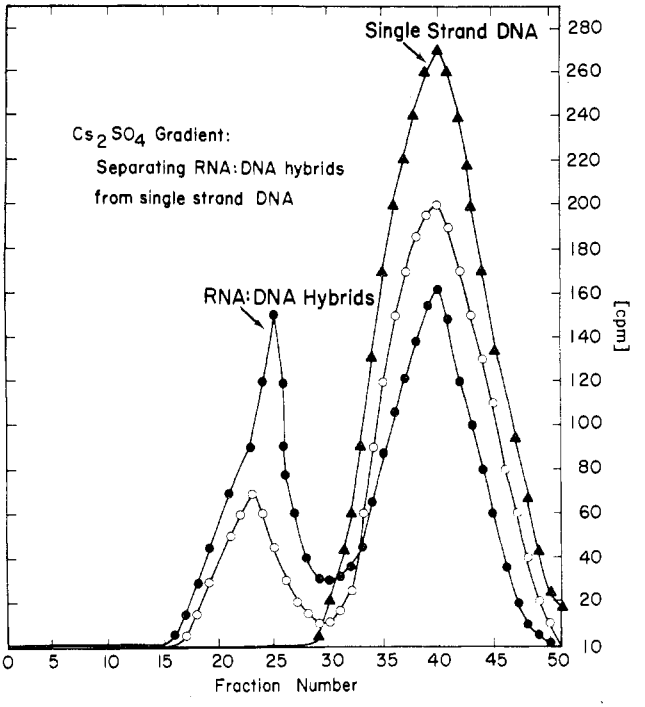

Figure 6. (▲) Peak A of Figure 5 renatured in the presence of 10 mg/ml of RNA; (○) peak B renatured in the presence of 1 mg/ml of RNA; (●) peak B renatured in the presence of 4 mg/ml of RNA.

department as a result of studies of the termini-generating enzymes of λ bacteriophage. He postulates the existence of a protein factor which aligns two double-stranded DNA sections if the purine–pyrimidine sequence in one strand of one double helix is the same as the purine–pyrimidine sequence of the strand of opposite polarity in the second double helix. All possible repeating sequences of five nucleotides and 81% of all possible repeating sequences of six nucleotides would permit such a simple DNA to loop back upon itself and satisfy such a condition, thus providing a mechanism of condensation of this DNA. Another possibility is that the simple repeating sequences have a slightly altered helical structure that would permit a factor to cross-link them. We are convinced that studying this part of the genome will provide the mechanism for chromosome condensation and centromere formation.

Figure 7. A possible model for heterochromatin in *Drosophila melanogaster*. (————) satellite sequences. Minimum length, 1.5×10^7 molecular weight, single strand. Maximum length, one piece/chromosome or 1.5×10^9 molecular weight, single strand. (|||) Simple-sequence harrDNA. (------) Transcriptively active DNA of heterochromatin with single-strand weights of 1×10^6 to 2×10^6.

The second question of major interest in studies of DNA from heterochromatin deals with the genetic activity of such DNA. We have provided evidence that there may be genetic activity in a heterochromatic part of the genome. Why should there be genes in heterochromatin? We believe the important genes common to all cells are perhaps to be found in or near heterochromatin, for genes are partially insulated from mutational changes in heterochromatin. There is some genetic evidence for this position, for the ribosomal RNA genes in *Drosophila melanogaster* map in the center of heterochromatin while the ribosomal proteins and histones map in or near heterochromatin in regions relatively inert to crossing-over. A characterization of the DNA from heterochromatin will provide a test of the goodness of this hypothesis.

Many of the students and postdoctoral fellows in this laboratory have contributed to the development of the ideas and experiments expressed here. In particular, we must single out Raphael Kram, whose experimental skills and critical understanding have been a stimulation to all of us. This work was supported in part by grants from the National Institutes of Health, GM 11180 and GM 15661, for which we are grateful.