age. This DNA damage is not seen in a thy$^+$ background, but the isogenic experiment has not been done. This DNA damage is reflected in the distribution of plasmid forms. The predominant plasmid band is the linear form, which has a mobility between that of the supercoil and nicked circle forms.

This procedure is a contained way of measuring plasmid size, since no large volumes or aerosols need be risked by preparing significant amounts of DNA. If care is taken that all bacteria contact the lysing sodium dodecyl sulfate, all cells are killed in the immediate heat step—that is, there were no detectable survivors out of 10^9 cells (*10*). Telford *et al.* (*10a*) have independently developed a similar screening procedure.

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9. Plasmid sizes used were taken from the literature assuming 1510 base pairs per 10^6 daltons. For example, Col E1, 6300 base pairs (*1*); mini Col E1, 3300 base pairs (*1–3*); pMB9, 5300 base pairs (*13*).
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13 December 1976

Isolation of Eukaryotic DNA Fragments Containing Structural Genes and the Adjacent Sequences

Abstract. *In* Drosophila melanogaster *structural genes are located close to moderately reiterared sequences. One of the clones obtained contains the DNA related to intercalary heterochromatin of* D. melanogaster. *These are individual differences in the distribution of genetic material in polytenic chromosomes of different stocks of* D. melanogaster. *The techniques that allow isolation of DNA fragments containing structural genes at the beginning, in the middle, or the end of the coding strand have been elaborated.*

One of the main areas where the genetic engineering (*1–3*) could be studied is the investigation of the organization of genetic material in eukaryotic cells. Working only with amplified homogeneous DNA, one can obtain information concerning the structure of eukaryotic genes. For this purpose, it is very important to have fragments containing both the structural gene and the adjacent DNA sequences.

Two general strategies could be used to isolate such structures. First is the amplification of random DNA fragments in "shotgun" experiments (cloning with unselected fragments from the total genome) followed by detection of the interesting clones by hybridization with mRNA. The second includes the preliminary enrichment of DNA used for amplification with the fragments containing structural genes.

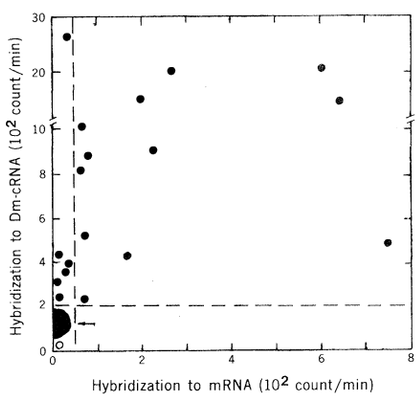


Fig. 1 Hybridization of poly(A)$^+$ mRNA and Dm cRNA with DNA samples obtained from 62 clones. The arrow indicates the position of 44 points. (•) DNA of clones; (○) *E. coli* DNA. The vertical dotted line separates clones, whose DNA does not hybridize to mRNA; the horizontal dotted line cuts the clones containing only unique DNA sequences.

The first approach was used in studies on the *Drosophila melanogaster* genome both by Hogness and co-workers (*3, 4*) and by ourselves (*5*).

For amplification of the Eco RI fragment of *D. melanogaster* DNA, λgt–λC bacteriophage (*1*) was used. The C fragment was replaced by *Drosophila* DNA and, after transfection of *Escherichia coli* strain 802 rk$^-$mk$^+$ with this recombinant DNA, a number of clones were obtained.

The DNA was isolated from groups containing ten clones each and those groups containing DNA hybridizable with messenger RNA (mRNA) of *Drosophila* were selected. Then each clone of the group was studied separately. In this way seven clones were obtained, the DNA of which efficiently hybridized to mRNA prepared from cultured *Drosophila* cells. In other words, these clones contained structural genes that were expressed in the cultured cells. Among them, three clones (NN 118m, 225, and 234) bind the highest percentage of mRNA (about 0.1 percent).

Except in the hybridization with mRNA, the DNA of clones was tested for hybridization with complementary RNA (cRNA) transcribed from total DNA of *D. melanogaster* (Dm cRNA). It was shown in separate experiments that cRNA was transcribed more or less randomly from the whole DNA. Therefore only the clone containing DNA sequences represented in the genome in many copies could bind a significant amount of cRNA. In all cases, when DNA from the clone hybridized with mRNA, it also hybridized with Dm cRNA (see Fig. 1). Even low binding of mRNA always coexists with the increased binding of Dm cRNA. The size of DNA fragments of *Drosophila* in the clones containing structural genes varied from 2 to 4 million daltons, whereas the average size of structural gene seemed to be of about 1.5×10^6. Thus, at least in many cases, the structural gene in *D. melanogaster* may be closely linked with the repetitive DNA sequences. Another interpretation of the above results is that in all cases the structural genes themselves are repetitive. To check these two possibilities we performed experiments with one of the clones (λgt–Dm 225). The DNA prepared from it was hybridized with either mRNA or Dm cRNA in the presence of an excess of unlabeled mRNA. The addition of a competitor sig-

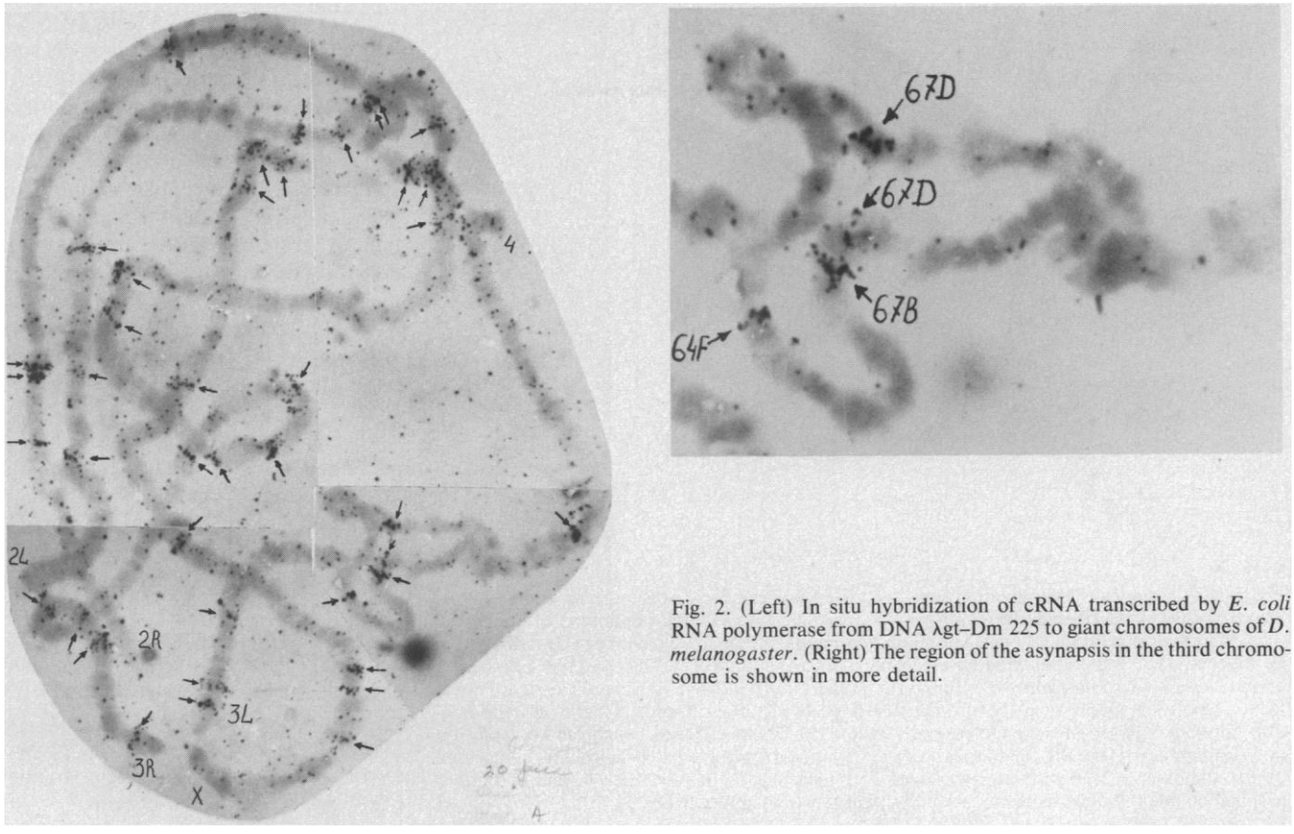


Fig. 2. (Left) In situ hybridization of cRNA transcribed by *E. coli* RNA polymerase from DNA λgt–Dm 225 to giant chromosomes of *D. melanogaster*. (Right) The region of the asynapsis in the third chromosome is shown in more detail.

nificantly decreased the binding of labeled mRNA, but was almost without effect on hybridization of Dm cRNA (Table 1). One can conclude that at least in the case of clone 225 the structural gene and reiterated sequence are physically different.

Hybridization in situ confirmed the results of biochemical experiments. ^{3}H-labeled cRNA prepared on the template of DNA from clones λgt–Dm 225, λgt–Dm 235, and λgt–Dm 118 hybridized with 40, ~100, and 30 sites, respectively, of the *D. melanogaster* chromosomes, an indication of an intermediate repeated sequence in their DNA.

Especially interesting hybridization patterns were observed on investigation of clone λgt–Dm 225 (Fig. 2). The ^{3}H-labeled RNA complementary to DNA of this clone hybridizes with the following regions: 3C, 4CD, 4F-5A, 9A, 13B, 18A, 19B and 20A of the X chromosome; 24F, 27A, 30B, 36AB, 36D, 37B, 37C, 39CD, 41ABC, 42A, 49E, 56F, 57E of the second chromosome; 61B, 61D, 62CD, 64F, 67B, 67D, 73C, 80-81, 87A, 87F, 89EF, 91A, 92E, 94AB, 97AB, 97EF, 98CD, 100CD of the third chromosome (Fig. 2). At least 27 of them (especially the regions 3C, 4CD, 4F-5A, 24F, 39D, 56F, 67D, 80–81, 87A, 92E, 94A, 97A, 98C, 100CD) coincide with the location of the so-called intercalary heterochromatin, which is responsible for the ectopic pairing of chromosomes (*6*). The coincidence is very significant, indicating that the repetitive sequence present in the clone λgt–Dm 225 is related to the intercalary heterochromatin (*6*).

The sites of hybridization in situ corresponding to intercalary heterochromatin are not constant in the strains of *Drosophila* studied. For example, in the asynapsis of the polytenic chromosome shown in Fig. 2, the label is localized only in one of homologs. The chromosomes were obtained from the cross ♀gtw^a × ♂gtI3^z. The hybridization of cRNA to the chromosomes of the parents showed that the same differences are part of the genetic constitution of the parent strains (*7*).

The repetition fragment of clone λgt–Dm 225 is present in the regions where the 5*S* RNA genes (56EF), the histone genes (39DE), and some other repetitive

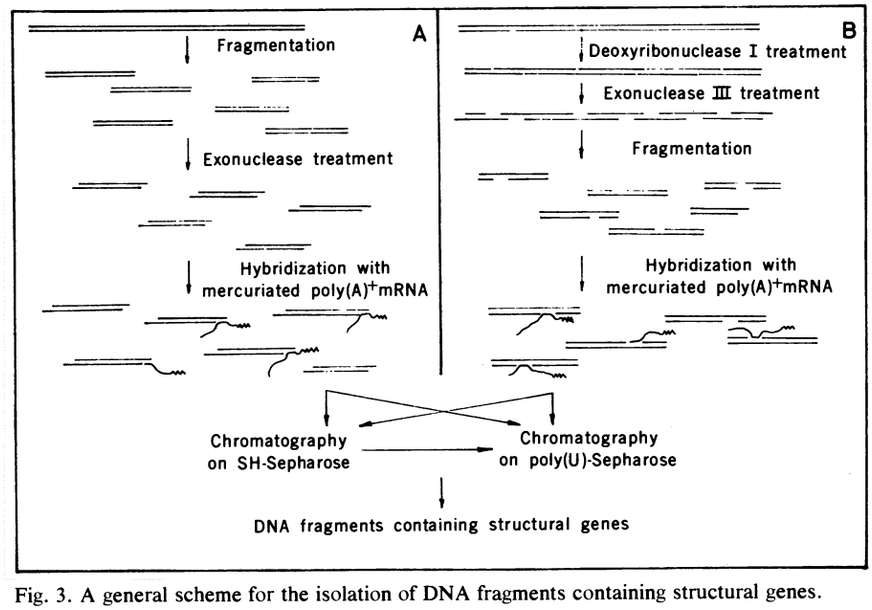
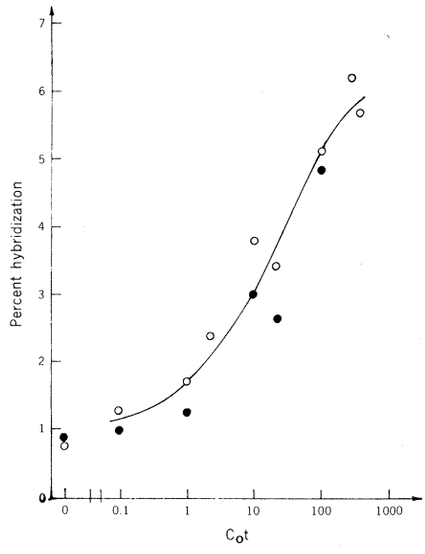


Fig. 3. A general scheme for the isolation of DNA fragments containing structural genes.



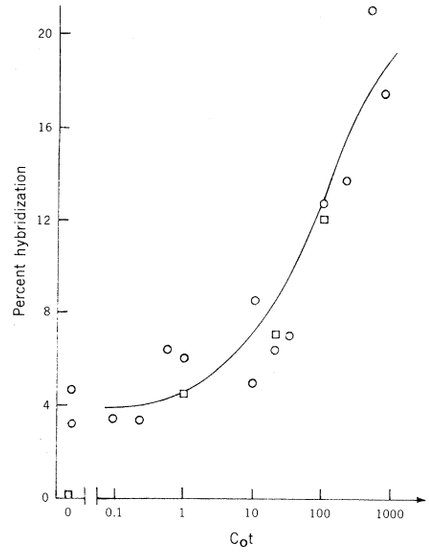


Fig. 4 (left). Kinetics of hybridization between mRNA and exonuclease III–treated mouse DNA. The isolation of ^{3}H-labeled DNA, mRNA, and exonuclease III, the conditions of enzymatic digestion, hybridization, and chromatography on poly(U)-Sepharose have been described (*15*). ^{3}H-labeled DNA was fragmented by mechanical shearing to 10×10^6 daltons and digested by exonuclease III to 7 percent (•) cytoplasmic polyA$^+$ mRNA from Ehrlich ascites carcinoma cells (○); polysomal polyA$^+$ mRNA from mouse liver. (•) Fig. 5 (right). Kinetics of hybridization between mRNA and exonuclease III–treated ^{3}H-DNA, checked by chromatography on poly(U)-Sepharose and SH-Sepharose. Native ^{3}H-labeled DNA (40×10^6 daltons) was digested by exonuclease III to 5 percent, hybridized with mercuriated polyA$^+$ mRNA, and chromatographed on poly(U)-Sepharose (○), or on SH-Sepharose (□), as described (*20*).

genes of unknown nature (*8*) are located. The explanation of this observation is not yet clear. It should be remembered, however, that, except for repeated sequences the clone, λgt–Dm 225 also contains the fragments hybridizing to poly(A)$^+$ mRNA in a manner different from the above-mentioned RNA's.

The unstable localization of the repeated sequences in polytenic chromosomes may be related to the phenomenon of gene migration, as was demonstrated in corn (*9*) and *Drosophila* (*10*). The isolation of the repetitive DNA fragment in the clone λgt–Dm 225 opens up new ways to study the nature of the "inert" heterochromatic regions of chromosomes whose function is still unknown. The physical and transcriptional mapping of DNA from the three isolated clones is in progress.

Finally, we would like to point out that our observations on recombinant clones of λgt phage confirmed the observation of Cameron *et al.* (*11*), that their viability is lower than λgt– λC. If λgt– λC phage contaminated the culture during one growth cycle, then recombinants practically disappear from the population. Therefore, we used a separate propagation of clones.

Another strategy is based on the prior isolation of DNA fragments containing structural genes with adjacent sequences from total cellular DNA, which was fragmented either by mechanical shearing or by digestion with a restriction endonuclease. Several investigators have hybridized denatured DNA with mRNA or with cDNA transcribed from mRNA for the isolation of genes (*12*). However, it was not possible to achieve high purification of a long fragment of DNA when denatured DNA was used. For this reason, we worked out a technique for the isolation of large DNA fragments containing structural genes, in which the denaturation step was excluded. This technique makes it possible to prepare fragments containing structural genes either at the beginning, or in the middle, or at the end of the coding strand of DNA.

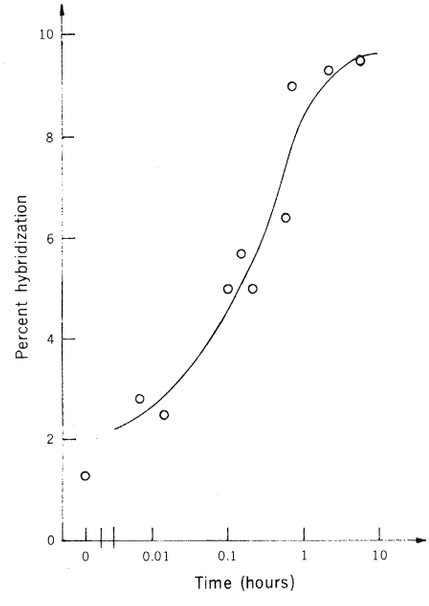


Fig. 6. Kinetics of hybridization between mRNA and ^{3}H-DNA by R-loop method. Mouse ^{3}H-DNA was treated with Eco RI and the material with the size 3×10^6 to 5×10^6 daltons was hybridized with polyA$^+$ mRNA from mouse liver under the conditions of R-loop formation and chromatographed on poly(U)-Sepharose.

The general scheme of procedure is presented in Fig. 3. We used total mouse DNA fractions of cytoplasmic RNA containing polyadenylic acid [poly(A)$^+$]. The DNA was fragmented to a desirable size by mechanical shearing or by treatment with a restriction endonuclease, and then relatively short single-stranded ends were produced by treatment with exonuclease. Exonuclease III of *E. coli* (*13*) or λ-induced exonuclease (*14*) were used to digest 3′ ends or 5′ ends, respectively. Such partially single-stranded DNA molecules were hybridized with poly(A)$^+$ mRNA (which had been previously mercuriated), and the hybrids were isolated by chromatography on sulfhydryl-Sepharose (SH-Sepharose) (*15, 16*) or on polyuridylic acid [poly(U)]-Sepharose (or both). Two-step chromatography resulted in greater purification of hybrids. By this technique we obtained DNA fragments containing a structural gene at the beginning (if λ-exonuclease was used) or at the end (if exonuclease III was used) of the coding strand (Fig. 3A). For the preparation of fragments in the interior of the sequence with a structural gene located, very long DNA (40×10^6 to 50×10^6 daltons) was prepared. The defined number of nicks (the distance between them being several thousand base pairs) was produced by treatment with pancreatic deoxyribonuclease. These nicks were converted into gaps by exonuclease III. The long fragments were then cut into shorter pieces by restriction endonucleases. As a result of this procedure, about 80 percent of the fragments contained a single-stranded region in the mid-section of the chain and only 20 percent of them contained a single-stranded region at the end of the molecule. The material was hybridized to mRNA, and the hybrids were isolated as described above (Fig. 3B).

Mouse fragmented DNA treated with exonuclease III was hybridized with mRNA (excess) and assayed by absorption of the hybrids on a poly(U)-Sepharose column (Fig. 4). The hybridization

occurred when the RNA-driven C_0t (C_0t is the nucleotide concentration in moles per liter and t is time in seconds) of 0.1 to 200 mole liter^{-1} sec. Below a C_0t of 1.0, only a small amount of DNA was bound. Most of the DNA hybridized with mRNA at C_0t values between 1 and 200. Thus the hybridization reaction follows the course expected (*17*).

Messenger RNA's isolated from different sources (such as Ehrlich ascites carcinoma cells, mouse liver) give similar hybridization curves (Fig. 4) as they have similar complexity. An increase of the DNA molecular weight and an increase of the exonuclease digestion led to an increase of DNA binding (*18*). However, the background (nonspecific) DNA binding to poly(U)-Sepharose in the absence of mRNA also increased in these cases (*18*).

This background binding is due to the interaction between oligodeoxyadenylate (dA) regions of DNA with poly(U)-Sepharose. For this reason, it appears that a simple repetition of the hybridization and fractionation on poly(U)-Sepharose does not lead to a further purification of the material. Indeed, it was confirmed in rehybridization experiments. About 25 percent of the DNA that was absorbed on poly(U)-Sepharose after the first cycle of the hybridization with poly(A)$^+$ mRNA was involved in the hybridization during the second cycle. However, the background was also increased and reached about 3 to 4 percent (*18*). The use of mercuriated mRNA and purification of hybrids on SH-Sepharose resulted in significant purification because of the lower background binding (< 0.1 percent). The proportions of the hybridized material on SH-Sepharose and on the poly(U)-Sepharose were similar (Fig. 5). Thus, the SH-Sepharose technique may be used efficiently for the second cycle of purification or for both cycles.

This technique is being used for the isolation of fragments carrying the individual gene for mouse globin. Two-step hybridization-chromatography gives about 10,000-fold purification of the gene.

Another technique for the enrichment of native DNA with fragments containing some specific sequences, is the so-called "R-loop" method described by Thomas *et al.* (*19*). It depends on the formation of a hybrid between RNA and one strand of native DNA, under conditions favoring the stability of the RNA-DNA complex.

We checked the possibility of combining this hybridization procedure with the isolation of hybrids on poly(U)-Sepharose. Total mRNA was hybridized to fragments of native DNA with a molecular weight of $3 \times 10^{6\text{ to }6} \times 10^6$ under conditions for R-loop formation, and then the material was passed through a poly(U)-Sepharose column (Fig. 6). Some of the DNA was specifically retained on the column and could be eluted by heating at low ionic strength or by treatment with ribonuclease. Although it is difficult to calculate C_0t values accurately, the curve has essentially the same shape as in experiments described above.

Table 1. Competitive hybridization experiment with DNA of λgt-Dm 225. The mixture contained 0.4 μg of immobilized DNA; Dm poly(A)$^+$ mRNA, 1×10^6 count/min (5×10^5 count/min μg); or Dm cRNA, 5×10^6 count/min; and in some samples unlabeled Dm ploy(A)$^+$ mRNA, 20 μg (as competitor). The mixture was made up in 0.3 ml of double strength saline–sodium citrate and 0.1 percent sodium dodecyl sulfate; it was incubated for 20 hours at 65°C, and treated subsequently as described (*5*).

DNA	Incorporation (count/min)			
	^{3}H-labeled polyA$^+$ mRNA hybridized		^{3}H-labeled Dm cRNA hybridized	
	No competitor	Competitor	No competitor	Competitor
λgt-Dm225	483	82	848	820
λgt-λC	10	8	72	59
E. coli	5	5	32	52

The R-loop technique has some advantages in that it does not require any enzymatic treatment of DNA; the method does have some limitations, such as dependence of R-loop formation on the G · C (guanosine · cytosine) content of DNA and on the size of RNA.

Our exonuclease technique can also be used for obtaining the fragments with definite localization of structural gene. Now that several methods for the isolation of large pieces of native DNA containing structural genes are available, we believe that, after the containment problems are solved, such fragments of DNA can be studied in prokaryotic vectors.

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Nonnutritive Sweeteners: Taste-Structure Relationships for Some New Simple Dihydrochalcones

Abstract. *Six sweet and five nonsweet simple nonglycosidic dihydrochalcones were prepared, two of which have properties comparable to those of the intensely sweet neohesperidin dihydrochalcone.*

The discovery of a new class of intensely sweet compounds, obtained by a simple chemical modification of some naturally occurring flavonoids, was first reported by Horowitz and Gentili (*1*). For example, hydrogenation of the flavanones naringin (**1**) and neohesperidin (**2**), the predominant bitter constituents in grapefruit and Seville orange rind, respectively, gave the intensely sweet dihydrochalcones (DHC's) **3** and **4** in excellent yield (see Fig. 1). Dihydrochalcones seem an attractive class of sweeteners from a safety point of view since neohesperidin DHC (**4**) has not been reported to show any ill effects in either multi-