Selection and some properties of recombinant clones of lambda bacteriophage containing genes of *Drosophila melanogaster*

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ABSTRACT

The λ gt clones containing fragments of the *Drosophila melanogaster* genome were prepared and characterized by hybridization of their DNA with (1) λ gt-crRNA; (2) λ C-crRNA; (3) Dm-crRNA; (4) the mRNA of *D. melanogaster* culture cells and (5) the stable cytoplasmic poly(A)⁺RNA from the same source. The technique for a simple selection of hybrid clones is described. The hybridization with mRNA allows one to select the clones containing structural genes of *D. melanogaster*. It was found that in all cases when the clone contains the structural gene it also contains the reiterated base sequences of the *D. melanogaster* genome. Several clones containing *D. melanogaster* DNA fragments with a size of $(2-4) \times 10^6$ daltons hybridizing with a relatively large portion of mRNA were selected for further analysis.

INTRODUCTION

The development of the recombinant molecule techniques¹⁻⁵ opened the possibility to study the structural organization of individual eukaryotic genes and to check some predictions from the models based on the results of studies of total nuclear pre-mRNA (hnRNA)^{6,7}. With the λ gt- λ C phage kindly presented by Prof. R.W. Davis, we obtained lambda clones containing *D. melanogaster* DNA fragments. The first step of further study was to select the clones containing the structural genes actively expressed in the cell culture

Abbreviations: λ gt-crRNA, complementary RNA synthesized on the λ gt DNA template; λ C-crRNA, the same but transcribed from the C fragment of λ gt; Dm-crRNA, the same but transcribed from the total *Drosophila melanogaster* DNA; SSC - 0.15 M NaCl - 0.015 M sodium citrate, pH 7.2; pre-mRNA - precursor of messenger RNA; hn-RNA - heterogeneous nuclear RNA; poly(A)⁺mRNA - poly(A)-containing cytoplasmic RNA; poly(A)⁻RNA - cytoplasmic RNA which does not contain poly(A).

of D.melanogaster. In this paper several techniques for the selection of such clones are described. As a result the clones containing "active structural genes" were selected from the 300 tested and some of their properties were determined. It was found that all the clones which contain DNA sequences hybridizing with mRNA also contain reiterated base sequences hybridizing with Dm-cRNA. The majority of clones lacking structural genes do not contain reiterated base sequences either.

MATERIALS AND METHODS

Phage, media. λ gt- λ C phage was grown on the E.coli strain KI2 802 rK⁻mK⁺ on LB-broth⁸ containing 1% bactotrypton (Difco), 0.5% yeast extract (Difco), 0.5% NaCl. For growing the phage on the plates, the 1.8% bottom agar and 0.5% top agar were prepared on medium containing 0.5% nutrient broth (Difco), 1% peptone, 0.5% NaCl.

Phage preparation. The lysate was clarified by a 30 min centrifugation at 7000 g and then the phage particles were pelleted by centrifugation at 80000 g for 2 hours. The pellet was suspended in 0.01 M tris-HCl, pH 7.5, 0.001 M MgCl₂, 0.1 mM EDTA with a Dounce homogenizer and layered on a discontinuous CsCl gradient. After a 1.5 hour centrifugation at 150000 g the phage band was collected and taken for DNA preparation by the phenol-detergent method.

D.melanogaster DNA. DNA was prepared from culture cells of the diploid line 67J25D grown in a monolayer⁹. About 10⁹ cells were suspended in 15 ml of 0.14 M NaCl, 0.01 M EDTA. An equal volume of 10% sodium p-aminosalicylate, 1% SDS was added and the protein was removed by extraction with phenol and chloroform under mild shaking. The DNA was collected, treated with RNase and additionally deproteinized.

D.melanogaster mRNA. The D.melanogaster cells were grown on plates (about 7·10⁷ cells per plate in 7 ml of medium). ³H-uridine was added twice at the beginning (10 μ Ci) and at the 17th hour (5 μ Ci per plate) of 20 hour incubation. The poly(A)⁺mRNA was prepared according to Spradling et al.¹⁰. The procedure included cell lysis in a buffer containing 0.1 M

NaCl, 10 mM CaCl₂, 0.5% Nonidet P-40, 30 mM tris·HCl, pH 8.3, 25 µg/ml polyvinylsulfate, 35 µg/ml spermine and 0.5% diethylpyrocarbonate. The nuclei were removed by centrifugation, SDS and EDTA were added to a final concentration of 0.5% and 25 mM respectively. Cytoplasmic RNA obtained by phenol-chloroform extraction was loaded on a poly(U)-Sephadex (Pharmacia) column. After removal of non-bound material in 0.1 M NaCl, 5 mM EDTA, 0.01 M tris·HCl, pH 7.5, the polyA-containing RNA fraction was eluted by 0.1% SDS, 0.01 M tris·HCl, pH 7.5 at 50°C. Poly(U)-Sephadex chromatography was repeated once again. The specific activity of mRNA was equal to about 5×10^5 cpm/µg.

Stable poly(A)-RNA. The *D.melanogaster* cells were labeled with ³H/uridine (10 µCi/plate) during one day, then washed, put in the new cold medium and allowed to grow for one day more. The cytoplasmic RNA was prepared as described above, and the poly(A)-RNA fraction was collected. The specific activity of the stable poly(A)-RNA was about 2×10^5 cpm/µg.

cRNA. ³H-cRNA was transcribed from 2 µg of different DNA templates in 80 µl of 4 mM MgCl₂, 10 mM β-mercaptoethanol, 0.15 M KCl, 0.05 M tris·HCl, pH 7.9, containing 20 nmoles each of ATP and GTP and 40 µCi each of ³H/-UTP (47 Ci/mmol, Amersham) and ³H/-CTP (17 Ci/mmol). The mixture was incubated at 37°C for 1.5 hours with 6 µg of E.coli RNA polymerase. After incubation the tRNA was added (200 µg/ml) and mixture was treated by DNase followed by phenol extraction and purification on a Sephadex G-50 column.

Enzymatic treatment of DNA. Endonuclease EcoRI was obtained according to Green et al.¹¹. The reaction mixture contained 100 µg/ml of DNA in 0.04 M tris·HCl, pH 7.4, 0.01 M MgCl₂, 0.01 M β-mercaptoethanol. DNA ligase prepared from phage T4-infected cells of E.coli was a gift of Dr. V.Tanyashin and Dr. A.Solonin. Ligation was performed at 12°C for 16 hours in a reaction mixture containing 0.1 M NaCl, 0.05 M tris·HCl, pH 7.5, 0.08 mM ATP, 7 mM β-mercaptoethanol, 20 µg/ml albumin, λgt-ends (30 µg/ml) and EcoRI-treated *D.melanogaster* DNA (30 µg/ml). The efficiency of ligation was determined in a separate experiment where the same enzyme preparation was incubated with SV 40 DNA pretreated with EcoRI endo-

nuclease. The yield of oligomers was checked electrophoretically and was found to be equal to 70-80% indicating high efficiency of the reaction.

Electrophoresis. The method of Sharp et al.^{I2} was used. The elution of λ gt ends from 1% agarose gels was performed by the freezing-thawing method of Thuring et al.^{I3}. The gels from which the material was taken for ligation and transfection were not stained by ethidium bromide. The position of the bands to be excised was detected by comparing with other gels which were stained.

Transfection. E.coli strain 802 rK⁻mK⁺ was used. Cells for transfection were grown on L-broth (0.5% peptone, Serva, 1% yeast extract (Difco), 0.5% NaCl) to OD₆₀₀ = 0.7. CaCl₂-treated cells were prepared by the method of Mandel and Higa^{I4} in a modification of Cameron et al.^{I5}. We had about 5x10⁵ plaques/ μ g λ gt- λ C DNA under transfection.

Cloning. Each plaque obtained after transfection of E.coli cells treated with CaCl₂ was placed in a vial containing 2 ml LB-broth. Then a part of the stored material from each vial was used for propagation. λ gt-clones cannot be propagated in a pool because of a different rate of their growth. Therefore we used a separate propagation of clones on plates. Clones which have given rise to less than (3-5)·10³ plaques/plate were used for second plating after which all of them were grown in about (2-5)·10³ plaques/plate. This procedure was used in order to have about 2-10 μ g DNA of each clone. Then the top soft agar with the grown plaques of each clone was suspended in 5 ml of 0.01 M tris·HCl, pH 7.5, and one half of this suspension was mixed with the material from other clones. Thus the pools were formed containing such material from five - ten clones.

Preparing DNA for hybridization. Pools of agar suspension were centrifuged at 7000 g for 20 min to remove agar. The supernatant was centrifuged once more at 80000 g for 2 hr. The pellet obtained was suspended in 0.7 ml of solution containing 0.1 M NaCl, 0.2% SDS, 1 mM EDTA, pH 7.2 and after phenol and chloroform extraction the water phase was collected and 0.3 ml of hot 4% agarose was added. This mixture was heat-

ed for 3 min at 100°C to melt all the pieces of agarose gel and then poured into a syringe with a cut end. After gel formation, the agarose disc was pushed out, treated by a NaOH solution for DNA denaturation, then neutralized and used for blotting as described by Southern¹⁶. The pools containing DNA complementary to mRNA were detected by hybridization. All the individual clones present in these pools were then studied separately. For this the second portions of the phage suspensions were used. The procedure was performed in exactly the same way as the previous one, and individual clones containing structural genes were detected at this step.

DNA-RNA hybridization. The filters loaded with DNA were dissected into 6-8 sectors of equal size, marked and placed in reaction vials. They were preincubated with 4 ml of 2xSSC 0.1% SDS for 24 hours at 65°C , the washing medium was removed and labeled RNA in 0.2-0.5 ml of 2xSSC was added. Each vial contained a certain RNA and about 30 pieces from different filters. Thus every DNA sample was hybridized with λ_{gt} -cRNA ($3 \cdot 10^5$ cpm), λ_{C} -cRNA ($3 \cdot 10^5$ cpm), Dm-cRNA ($5-10$) $\times 10^6$ cpm, mRNA ($1-2$) $\times 10^6$ cpm and poly(A)⁻ stable RNA (5×10^5 cpm) or to some of them. The mixtures were annealed for 24-48 hours at 65°C . After hybridization the filters were washed in reaction vials with a large volume of 2xSSC - 0.1% SDS, then incubated for 2 hours in 7 M urea, 2xSSC, 0.1% SDS at 41°C . This procedure was repeated three times. Then filters were incubated in 2xSSC, 0.1% SDS at 65°C for 2 hours, washed by 2xSSC, treated by RNase, washed with 2xSSC once more, dried and counted in a liquid scintillation spectrometer SL-30 (Intertechnique, France).

RESULTS

The cloning and detection of clones containing DNA of D.melanogaster. For preparing recombinant molecules containing λ_{gt} and heterologous DNA fragments, the endonuclease EcoRI-DNA ligase method described by Davis^{1,2,15} was used. As shown in table I, endonuclease EcoRI treatment of $\lambda_{\text{gt}}-\lambda_{\text{C}}$ DNA dramatically decreased its infectivity while ligation of λ_{gt} -ends together with D.melanogaster DNA cleaved by endonuclease EcoRI considerably increased it. EcoRI endonuclease cleaved

λ gt- λ C had about a 0.02% infectivity of uncleaved DNA because of incomplete EcoRI cleavage. The relatively high infectivity of ligated λ gt-ends without adding of any insertion Table I.

The infectivity of different preparations of λ gt DNA

No. Exp.	D N A	plaques/ng DNA
1.	λ gt- λ C	500
2.	λ gt- λ C treated by EcoRI	0.1
3.	Ligated λ gt-ends (C-fragment was removed by electrophoresis)	1.0
4.	λ gt-ends ligated in the presence of EcoRI treated DNA of <i>D.melanogaster</i>	10.0

DNA is probably the result of the presence of some amounts of C-fragment in the λ gt-ends eluate (see Methods) because of the non-specific absorbance of this DNA fragment on agarose gel during fractionation².

Many clones were obtained and first of all we need to know how many of them do really contain fragments of *D.melanogaster* DNA. To check it, a rapid hybridization procedure was developed. The clones were grown on separate Petri plates and the DNA from one plate was put on the nitrocellulose filter and used for hybridization with cRNA synthesized either on total λ gt- λ C DNA (λ gt-cRNA) or on the purified C-fragment DNA (λ C-cRNA). It is known that λ gt with a lacking C-fragment cannot grow and therefore the absence of fragment C indicates the insertion of *D.melanogaster* DNA instead of it. This technique allows one to easily detect recombinant molecules.

The DNA of all clones efficiently hybridizes with λ gt-cRNA (Figure 1). The hybridization is proportional to the amount of λ gt-DNA on a millipore filter and therefore it was used to determine the amount of DNA bound to the filter. The DNA of a few clones (approximately 10%) also hybridizes with λ C-cRNA, the ratio of the λ gt-cRNA hybridized to the λ C-

Fig. I. Hybridization of λ gt-cRNA and λ C-cRNA with clonal DNA. The filters containing 0.1-0.8 μ g DNA were placed in two vials containing $3 \cdot 10^5$ cpm of either λ gt-cRNA or λ C-cRNA in 0.2-0.3 ml of 2xSSC, 0.1% SDS. Incubation was performed for 48 hr at 65°C. $\circ$ - DNA of clones; $\square$ - λ gt- λ C-DNA; Δ - λ gt-ends (the C-fragment was removed by electrophoresis).

-cRNA hybridized being equal to about 3. These clones contain the λ C fragment and in most cases do not contain D.melanogaster DNA, as was demonstrated by hybridization with Dm-cRNA (data not shown). The DNA of the majority of clones obtained does not hybridize with λ C-cRNA (Figure I) and these clones definitely contain D.melanogaster DNA fragments. We found that if the λ gt-end is fractionated from fragment C by agarose gel electrophoresis, the yield of recombinant clones is equal to about 90%.

Selection of clones containing structural genes of D.melanogaster. As 90% of the clones obtained contain D.melanogaster DNA fragments, we did not further check the clones for the absence of the λ C-fragment, but directly used the isolation DNA for selection of clones containing structural genes hybridizing DNA with highly labeled mRNA of D.melanogaster. According to Spradling et al.¹⁰ poly(A)⁺cytoplasmic RNA was considered to be poly(A)⁺mRNA. The hybridization with λ gt-

-cRNA was nevertheless performed in all experiments to check the amount of DNA on the filters.

The background in the hybridization reaction with mRNA was extremely low and even a 0.002% binding of mRNA indicated the existence of structural gene in the DNA of clones. However, for a further study we needed clones containing structural genes which are actively expressed in the cell culture. It was found that such clones are rare. Therefore, after a check of individual 50 clones among which only one efficiently hybridizes to mRNA, we changed the plan of the experiment and started to combine DNA from ~ 10 clones and checked these tens for the presence of structural genes in some of them. The clones from pools, the DNA of which binds a relatively

Fig.2. Hybridization of poly(A)⁺mRNA and Dm-cRNA with clonal DNA. For hybridization, DNA was isolated from 62 clones obtained from eight pools, the DNA of which show hybridization with mRNA. These 62 DNA samples were hybridized with poly(A)⁺mRNA ($1 \cdot 10^6$ cpm) and Dm-cRNA ($5 \cdot 10^6$ cpm). Each filter contained 4-10 μg and 1-2 μg DNA, respectively. Hybridization data for seven clones containing λC fragment is not shown. The arrow indicates the position of 44 points. $\circ$ - DNA of clones; $\square$ - E.coli DNA. Vertical dotted line separates clones DNA of which does not hybridize to mRNA; horizontal dotted line cuts the clones containing only unique DNA sequences.

high amount of mRNA, were then studied separately and seven clones containing structural genes most efficiently transcribed in culture cells were isolated (Fig.2). All of these clones (except one) contained single EcoRI DNA fragment of D.melanogaster DNA with a molecular weight of $2-4 \times 10^6$ daltons (data not shown).

Properties of clones containing D.melanogaster structural genes. In all experiments the DNA isolated from the clones was also hybridized to cRNA transcribed from the total D.melanogaster DNA (Dm-cRNA). It was shown that Dm-cRNA is transcribed more or less randomly from the whole D.melanogaster DNA (see Discussion). Therefore only the clones containing DNA represented in genome in many copies (reiterated base sequences) could bind a significant amount of Dm-cRNA. The majority of structural genes are represented by one copy per genome¹⁷ and their DNA should not hybridize to Dm-cRNA. One can see from Fig.2 that the binding of Dm-cRNA by DNA from the majority of clones is very low (50-80 cpm above the background from a $5 \cdot 10^6$ cpm input). This corresponds to the figure one could expect for Dm-cRNA binding by a non-reiterated DNA sequence of $(2-4) \times 10^6$ daltons if the hybridization efficiency is $\sim 30\%$. One can conclude that most of the clones do not contain reiterated sequences. However, some clones bind much higher amounts of Dm-cRNA, and such clones occur more often than clones containing DNA hybridizing to mRNA. But the important feature is that in all cases when DNA from the clone hybridizes with mRNA it also hybridizes with Dm-cRNA as well (Fig.2). It is clear from the figure that there is no quantitative correlation between the binding of two types of RNA. However, even very low binding of mRNA always coexists with the increased binding of Dm-cRNA. Possibly some of the clones which bind Dm-cRNA, but not mRNA, still contain structural genes which are not expressed in the cell culture.

One could suggest that the poly(A)⁺mRNA of the cytoplasm is contaminated to some extent by ribosomal or other stable RNAs, originated from reiterated base sequences and this could give an alternative explanation for the hybridization of clonal DNA with both of them. For this reason we pre-

pared labeled stable poly(A)⁻ cytoplasmic RNA and hybridized it to 8 clones which hybridized with the mRNA preparation. All of these clones but one did not bind stable poly(A)⁻ cytoplasmic RNA at all. The DNA of the one clone (which is not presented in Fig.2) hybridized with stable RNA and this DNA was originated from the nucleolus according to in situ hybridization experiments to polytene chromosomes of D.melanogaster (unpublished data). Thus, for the other 7 clones rRNA, tRNA and other stable RNAs do not interfere with the reaction. The conclusion is that the structural genes in the D.melanogaster genome are localized very closely to the reiterated base sequences and that a considerable part of the reiterated base sequences are characterized by such a localization.

DISCUSSION

This paper describes some techniques which may be helpful in work with the recombinant clones of the λ gt phage containing fragments of eukaryotic genomes obtained according to the Davis technique ^I. These are: 1) the simplified method of clonal DNA isolation for hybridization reaction; 2) a modification of the technique for transfer of DNA samples to millipore nitrocellulose filters; 3) the use of hybridization with λ gt-cRNA and λ C-cRNA for the detection of clones definitely containing heterologous DNA; 4) the use of hybridization with mRNA to detect structural genes, in particular the modification of technique allowing one to minimize the background to zero level. These and some other methods permit to select clones containing structural genes from those obtained in shot-gun experiment.

Besides this, our results gave some information on the sequence arrangement in the genome of D.melanogaster. Namely, they indicate the close location of structural genes to the reiterated base sequences.

It is well known that the D.melanogaster genome contains less reiterated sequences than the genome of many other eukaryotes ^{18,19}. Besides satellites, reiterated structural genes (for histones, rRNA, tRNA, etc.), only few percents of total genome belong to moderately reiterated base sequences. Therefore many clones containing D.melanogaster DNA lack the re-

iterated DNA and do not hybridize with Dm-cRNA. This confirms the previous results obtained by Hogness who showed the absence of the reiterated base sequences in 6 of the 9 clones isolated ^{5,20}. In our experiments ~20 of the 62 clones investigated separately contain reiterated base sequences of D.melanogaster (Fig.2). A considerable part of these clones can also bind mRNA. The true number of clones containing structural genes is probably higher as not all genes are expressed in the D.melanogaster cell culture. It is known that the number of structural genes in D.melanogaster is about 5000-6000. Thus, assuming the average molecular weight of the fragment inserted into the λ genome to be about $3 \cdot 10^6$ daltons it could be expected to have one clone containing a structural gene for every 5 clones. Our results are in agreement with this conclusion.

There is a possibility that for some reason structural genes are transcribed more efficiently in vitro, and therefore mRNA sequences are reiterated in Dm-cRNA. In other words, the binding of cRNA reflects the binding of mRNA sequences. To check this, the ³H/-cRNA was transcribed from the D.melanogaster fragment isolated from four clones containing structural genes and hybridized in situ to polytene chromosomes of D.melanogaster. It was found that each clone contained DNA complementary to the DNA of a number of bands (from 20 to 100 with different clones), and that the hybridization patterns were very specific (paper in preparation). Thus the hybridization of clonal DNA to cRNA is not a result of enhanced in vitro transcription of structural genes, but really reflects the existence of the reiterated base sequences presented in many bands. The possibility of reiteration of the structural gene which could be present in many different bands is not excluded, but it seems unlikely if we consider that the same result was obtained with all clones containing structural genes. Moreover, in the case of one clone, a competition experiment demonstrated that unlabeled mRNA inhibited the hybridization of labeled mRNA to DNA and did not interfere with the hybridization of cRNA to DNA. This again supports the assumption that the structural gene and the reiterated sequence are different parts of DNA at least in one of clones.

Considering the above mentioned reservations one could conclude that at least in many cases the structural genes of D.melanogaster are localized closely to the repetitive DNA sequence, the distance between them being no longer than $(1-2) \times 10^6$ daltons or shorter. Work is in progress to map the DNA from some of the clones obtained to get information on the sequence arrangement and transcription polarity in them.

The experiments developed after January 1976 were performed in P2-conditions.

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REFERENCES

1. Mertz, J.E. and Davis, R.W. (1972) Proc. Nat. Acad. Sci. USA 69, 3370-3374.
2. Thomas, M., Cameron, J.R. and Davis, R.W. (1974) Proc. Nat. Acad. Sci. USA 71, 4579-4583.
3. Murray, N.E. and Murray, K. (1974) Nature 251, 476-481.
4. Morrow, J.E., Cohen, S.N., Chang, A.C.Y., Boyer, H.W., Goodman, H.M. and Helling, R.B. (1974) Proc. Nat. Acad. Sci. USA 71, 1743-1747.
5. Wensink, P.C., Finnegan, D.J., Donelson, J.E. and Hogness, D.S. (1974) Cell 3, 315-325.
6. Georgiev, G.P. (1969) J. Theoret.Biol. 25, 473-487.
7. Georgiev, G.P., Varshavsky, A.J., Ryskov, A.P. and Church, R.B. (1973) Cold Spring Harbor Symposia Quant. Biol. 38, 869-884.
8. Revel, H.R. and Georgopoulos, C.P. (1969) Virology 39, 1-17.
9. Kakpakov, V.T., Gvozdev, V.A., Platonova, T.P., Polukarova, L.G. (1969) Genetics (USSR) 5, 12, 67-76.
10. Spradling, A., Hui, H., Penman, S. (1975) Cell 4, 131-138.
11. Greene, P.J., Betlach, M.C. and Boyer, H. (1974) In: Methods in Mol. Biol., vol.7, pp.87-105, R.B.Wickner, Ed. (New York: Marcel Dekker).

- I2. Sharp, P.A., Sugden, B. and Sambrook, J. (I973) Biochemistry I2, 3055-3063.
- I3. Thuring, R.W.J., Sanders, J.P.M. and Borst, P. (I975) Anal. Biochem. 66, 213-220.
- I4. Mandel, M. and Higa, A. (I970) J. Mol. Biol. 53, I59-I62.
- I5. Cameron, J.R., Panasenکو, S.M., Lehman, I.R. and Davis, R.W. (I975) Proc. Nat. Acad. Sci. USA 72, 3416-3420.
- I6. Southern, E.M. (I975) J. Mol. Biol. 98, 503-517.
- I7. Levy, W.B. and McCarthy, B.J. (I975) Biochemistry I4, 2440-2446.
- I8. Laird, C.D. (I973) Ann. Rev. Genet. 7, I77-204.
- I9. Manning, J.E., Schmid, C.W., Davidson, N. (I975) Cell 4, I4I-I56.
20. Glover, D.M., White, R.L., Finnegan, D.J., Hogness, D.S. (I975) Cell 5, I49-I58.