A *Drosophila* ribosomal protein gene is located near repeated sequences including rDNA sequences

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ABSTRACT

We have isolated a cloned segment of *Drosophila* genomic DNA containing a ribosomal protein gene. Hybridization analysis of the DNA in this clone indicates a complex organization of repeated elements within this cloned segment. At least one of these repeated elements is homologous to regions of rDNA. Restriction analysis of the clone shows that some of the repeated elements are present as tandem duplications and in scattered locations within the cloned DNA segment. There are also three non-ribosomal protein genes contained in this clone, each of which is expressed along with the ribosomal protein gene into RNA species present in *Drosophila* embryos.

INTRODUCTION

Recently, cloned DNA segments coding for ribosomal protein genes in various organisms have been described (1-6). These include mouse, yeast, *Xenopus* and *Drosophila*. We have recently reported the isolation of a ribosomal protein gene-containing clone from a Charon 4 (Ch4)¹ library of *Drosophila* genomic DNA. This cloned DNA contains a single ribosomal protein gene and at least one sequence present in 5 other ribosomal protein mRNAs (7). This paper details the organization and presence of a variety of repeated elements in this cloned DNA segment.

MATERIALS AND METHODS

DNA Isolation, Restriction Analysis and Hybridization Reactions.

DNA from clone 2 (C2) was isolated as previously described (7). The 17 Kb insertion of pDml03 (Dml03'), containing a ribosomal RNA repeat unit with a Type I insertion in the 28S gene (8), was cloned in pACYC184 and was generously provided by Drs. D. Fouts and J. Manning.

Restriction enzymes were obtained from Bethesda Research Labs, Inc. Restriction digests were separated on agarose gels in 40 mM Tris base, pH 8.0, 5 mM sodium acetate, 1 mM EDTA. The buffer was recirculated during electrophoresis. DNA was transferred to nitrocellulose using the procedure of Southern (9). DNA fragments were isolated from gels using the NaI procedure of Vogelstien and Gillespie (10). DNA was labelled with [^{32}P] α -dCTP using the nick translation kit available from New England Nuclear.

Hybridizations were carried out according to the procedure of Wahl et al. (11), with the following modifications. The prehybridization mixture contained 50% formamide, 0.8 M NaCl, 3X Denhardt's solution, 0.1 M Pipes, pH 6.4, 0.01% N-lauryl-sarcosine, and 500 $\mu\text{g}/\text{mL}$ denatured, sonicated calf thymus DNA. The hybridization mixture was the same as the prehybridization mixture, except that it contained, in addition to probe, 10% dextran sulfate. Following hybridization, DNA blots were washed in 2 changes of 2X SSC, 0.01% NaPPi, 0.01% N-lauryl-sarcosine, each of 500 ml, at room temperature for 10 minutes, then in 0.2X SSC, 0.01% NaPPi, 0.01% N-lauryl-sarcosine at 60°C, 4 changes each of 1 liter for 2 hours. The blots were exposed to XAR-5 X-ray film at -70°C with an intensifying screen.

Electron Microscopy.

For R-loop analysis, cloned DNA was incubated with cytoplasmic RNA or poly(A)+ RNA from Drosophila embryos (7) under conditions described by Woolford and Rosbash (12). The DNA concentration was 20 - 30 $\mu\text{g}/\text{mL}$ and the RNA concentration was 500 $\mu\text{g}/\text{mL}$. The R-looped DNA was separated from the non-hybridized RNA by Sepharose column chromatography and directly spread for electron microscopy (12). The plasmid pBR322 was used as an internal length standard.

RESULTS

Repeated Elements in Clone 2 DNA

Preliminary restriction analysis of C2 by hybridization of nick-translated restriction fragments to blots of various restriction enzyme digests of C2 indicated a complex arrangement of homologous sequences were present in this cloned DNA. Therefore,

Figure 1. Restriction Analysis of Clone 2 DNA. Only those restriction sites within the 14.4 Kb inserted DNA are shown. Not all PstI sites are shown, only those within the 4.1 Kb and 2.6 Kb BamHI fragments. Position of the EcoRI sites was determined by EcoRI digestion of isolated BamHI fragments. Positions of the SstI and BglII sites were determined by measuring fragment lengths of digested C2 DNA, and positioning these fragments using the known sites in Ch 4. Positions of some of the HindIII and BamHI sites were determined in the same way. Other sites were located by secondary digestion of isolated restriction fragments with the appropriate enzymes. The bars below the restriction may represent regions at least partially homologous to Dm 103'.

it was necessary to determine the pattern of restriction sites by both single and double digestions of isolated restriction fragments. These data were used to construct the restriction map shown in Figure 1. Not all the PstI sites were localized, only those within the 2.6 and 4.1 Kb BamHI fragments.

There is a region of DNA in the middle of this clone which contains a series of HindIII and PstI sites separated by spacings of approximately 250 and 500 bp, respectively. These repeats are not exact, however, since the lengths of these units show some heterogeneity as judged by their gel patterns and the appearance of other non-repeated restrictions sites in this area of the DNA. The exact amount of this heterogeneity was not determined in these experiments.

In order to further investigate the amount of repeated DNA in C2, nick translated C2 DNA was used to probe an EcoRI blot of total Drosophila DNA. Large amounts of hybridization were seen to several regions of the blot (data not shown), showing that sequences in C2 are repeated in the Drosophila genome. To determine if these repeated sequences were localized to any one region of C2 DNA, restriction digests of C2 DNA were blotted to nitrocellulose and probed with nick translated Drosophila total

genomic DNA. In this type of experiment, only those C2 DNA segments which contain an element repeated in the Drosophila genome will show significant hybridization (see legend to Fig. 2). When single restriction digestions of C2 are analyzed using this technique, all inserted DNA fragments show hybridization, indicating that repeated elements are present throughout the inserted DNA.

When C2 is digested with 2 or 3 restriction enzymes and the fragments analyzed in a similar fashion, it is apparent that some of these repeated elements are small. As shown in Figure 2, of the numerous fragments produced by double and triple digestions of C2 DNA, relatively few show significant hybridization to [³²P] labelled Drosophila genomic DNA. These fragments of C2 also show different levels of hybridization to genomic DNA, while a large (> 10 Kb) BamH1/HindIII fragment (Fig. 2), running at the exclusion limit of this 1.35% agarose gel, shows strong hybridization to repetitive genomic DNA at a level 3- or 4-fold higher than that shown to the 280 bp HindIII fragment. It is likely that both of these two fragments are complementary to sequences with different repetition frequencies in genomic DNA. These results also suggest there is more than one type of repeat in C2.

Scattered locations of homologous repeat units in C2 DNA.

When nick translated individual restriction fragments of C2 DNA are used to probe total restriction digests of C2 DNA, hybridization of a single C2 fragment was observed to many other C2 fragments which are widely separated in the clone. For example, when the 4.4 Kb EcoR1 restriction fragment of C2 (rightmost insert DNA fragment) is used to probe a BamH1 digest of C2 DNA, strong hybridization is seen to the 19 Kb BamH1 fragment (from the Ch4 left arm and leftmost portion of insert DNA, see Figure 1), as well as to the 6.5 Kb BamH1 fragment (the rightmost portion of insert DNA along with a portion of the right arm of Ch4) and to the 2.6 Kb and 4.1 Kb BamH1 fragments. This is shown in Figure 3, lane 2. These results illustrate that the 4.4 Kb EcoR1 fragment has within it an element that is found in at least 2 widely separated BamH1 fragments. The hybridization of the 4.4 kb EcoR1 fragment to the 6.5 Kb and 2.6 Kb BamH1 fragments is expected, since the 4.4 Kb EcoR1 fragment completely

Figure 2. Analysis of repeated elements in C2 DNA. Each lane contains 0.75 μ g of C2 DNA digested with the indicated restriction enzymes. Panel 1 shows the ethidium bromide stain of 2 lanes digested with the enzymes indicated. Panel 2 is the autoradiogram of the blot of this gel. This blot was hybridized to [32 P]labelled *Drosophila* total genomic DNA (2.5×10^7 cpm, 3.5×10^7 cpm/ μ g). Bands which show significant hybridization are marked by a dot (•). The exposure time was 60 hours. Numerous bands that are common to more than one lane are seen, indicating that many of the repeated elements are contained in small fragments. Approximate sizes of fragments in bp are indicated on the side of the autoradiogram.

overlaps the insert DNA contained in these two fragments. The fact that an equally strong hybridization signal is observed from the 19 Kb BamH1 fragment which does not overlap the 4.4 Kb EcoR1

Figure 3. Homologous regions in C2 DNA. BamHI restricted C2 DNA was electrophoresed on 0.8% agarose, transferred to nitrocellulose and the blot probed with either the 2.6 Kb BamHI fragment or the 4.4 EcoRI fragment. Lane 1 is a photograph of the stained gel of the BamHI fragments (from the top) 19 Kb, 6.5 Kb, 5.5 Kb, 4.1 Kb, 3.9 Kb and 2.6 Kb. The 1.5 Kb fragment (which is vector-derived) is not visible. Lane 2 is the autoradiogram of this blot after hybridization versus the 4.4 Kb EcoRI fragment. Lane 3 is an autoradiogram of the BamHI blot probed with the 2.6 Kb BamHI fragments. Hybridization intensities can be compared only within lanes, not between lanes.

fragment suggests that a repeated element in the 4.4 Kb EcoRI fragment has a representation in the 19 Kb BamHI fragment.

If repeated elements are scattered throughout the cloned DNA, one would expect any one restriction fragment of C2, when used to probe a blot of the same enzyme digest, to hybridize not only to itself but also to other fragments. This was seen in C2 DNA. When the 2.6 kb BamHI fragment of C2 is used to probe a BamHI digest of C2, hybridization was seen not only to the same size fragment, but to the other fragments of C2 DNA. This is

shown in Figure 3, lane 3. A stronger hybridization of the 2.6 Kb BamHl fragment is seen to the 4.1 Kb BamHl fragment than to itself or to any other fragment. This suggests a repeated element in the 2.6 Kb BamHl fragment is present in more than one copy on the 4.1 Kb BamHl fragment. This is reasonable because of the pattern of HindIII sites in the 4.1 Kb and 2.6 Kb BamHl fragments as shown in Figure 1. If these sites represent duplications of a repeat, there are more of these repeats in the 4.1 Kb BamHl fragment than the 2.6 Kb BamHl fragment. However, hybridization of this 2.6 Kb BamHl fragment is also seen to the 19 Kb and 6.5 Kb BamHl fragments suggesting this 2.6 Kb BamHl fragment contains in addition to this HindIII type repeat, another sequence repeated in the 19 Kb and 6.5 Kb BamHl fragments. The fact that there is no HindIII site(s) in the 19 Kb BamHl fragments supports this idea, since this rules out the possibility of an exact copy of a HindIII type repeat in the 19 Kb BamHl fragment.

One Repeated Element in C2 is Homologous to a Region of rDNA.

Preliminary Southern blot analysis of EcoRI digested Drosophila genomic DNA with C2 DNA indicated C2 was homologous to many fragments of Drosophila DNA, including a strong homology to the 12 Kb - 17 Kb region of DNA fragments. This is a region where the rDNA repeat units are found. In order to firmly establish if hybridization to rDNA occurs with C2, various restriction digests of C2 were probed with nick translated Dml03', a plasmid containing a rDNA repeat unit which has the 28S gene interrupted by the Type I intervening sequence. As seen in Figure 4, hybridization of this rDNA probe was observed to occur with a number of fragments in C2. In the BamHl digest, hybridization is seen to the 19 Kb, 6.5 Kb and 2.6 Kb fragments. This pattern is similar to that seen in Figure 2, suggesting the second, non-HindIII repeat, in C2 is the same region that is homologous to a sequence found in rDNA. Hybridization of Dml03' to the SstI fragments is also seen; these include the 26 Kb and 11 Kb SstI fragments, but not the 4.0 Kb SstI fragment, a region of C2 DNA which contains a number of the HindIII type repeats. Dml03' hybridizes to the 12 Kb insert DNA fragment and the 3 Kb BglIII insert plus vector DNA fragment of C2, but not to any other BglIII fragment. The general

Figure 4. Regions of rDNA Homology in C2. C2 DNA was digested with BamHl (lane 1), BglII (lane 2) or SstI (lane 3), electrophoresed and blotted to nitrocellulose. This blot was hybridized to nick translated Dm 103'. The autoradiogram of this blot is shown in lanes 4, 5, and 6.

regions of C2 that show homology to Dm103' DNA, as judged by this analysis, are from left to right (see the lower bars, Figure 1): (a) the DNA to the right of the first insert DNA BglII site, extending to the first insert DNA BamHl site, and (b) the DNA from the rightmost insert DNA BamHl site in C2 (approximately 32 Kb from the end of the left arm of Ch4) to the end of the insert DNA (the rightmost EcoRI site). The fact that these regions of homology to Dm103' do not overlap the region containing most of the HindIII repeats in C2 again suggests that there are at least 2 different types of repeats in C2.

To better understand the relationship between C2 DNA and rDNA, parallel Southern blots of genomic DNA were probed with either C2 or Dm103', and the patterns compared. As shown in

Figure 5. Parallel Southern Blot analysis of Dm 103' and C2 with genomic DNA. Each lane contains 2 μ g of total *Drosophila* DNA digested with the following enzymes, Lanes 1 and 4 *Sst*I, lanes 2 and 5, *Sst*I and *Bam*HI, Lanes 3 and 6, *Xba*I and *Bam*HI. Each set of lanes was hybridized versus [32 P]labelled C2 DNA or Dm 103'. (1×10^8 cpm, 5×10^8 cpm/ μ g) Exposure time was 60 hours.

Figure 5, there are many sequences in common between the two blots, but differences also exist. In the case of Dm103', a large number of bands appear, presumably due to the fact that this clone contains the Type I intervening sequence in the 28S gene, a sequence also found in regions of *Drosophila* DNA other than rDNA (8). Although C2 hybridizes to many of the same bands as Dm103', there are also some bands which are unique to C2. These bands show both moderate and weak hybridizations which can be taken as an indication that some of the bands unique to C2 hybridization are the result of hybridization to single copy as

Figure 6. R-loop Analysis of C2. C2 DNA was R-looped and prepared for electron microscopy as described. A total of 67 full length molecules were measured. The size and frequencies of the R-loops observed are shown.

well as repeated Drosophila genomic DNA sequences. These results again point to the fact that C2 contains a number of different classes of repeated elements.

R-Loop Analysis of C2 DNA.

In order to determine the arrangement of coding sequences in C2 DNA, R-loop analysis was performed. DNA from C2 was R-looped with embryo RNA under conditions described by Woolford and Rosbash (12). As shown in Figure 6, there are 4 R-loops in this cloned DNA, all occurring at different frequencies. This indicates that the RNA species coded for by these genes are present at different cellular levels. This could be ascribed to differential lifetimes of the RNAs or different transcriptional levels, or a combination of both. These data show that within C2 DNA, there are at least 4 genes that are expressed as RNA species in Drosophila embryos. It is interesting to note that none of the 4 genes are large, all being less than 1 Kb in length (Figure 6). The RNA used to form these R-loops contained 18 and 28S rRNA, but no rRNA-sized genes were detected, indicating that the homology of C2 and rDNA is not due to a large segment of a rRNA gene in this clone. In fact, since rRNA molecules did not form large R-loops or small R-loops with large tails, the homology between C2 and rRNA is likely small. The R-loop which represents the ribosomal protein gene is the second from the right, since it is positioned in the 2.6 Kb BamH1 fragment, the fragment that hybrid selects ribosomal protein mRNA (7).

DISCUSSION

Repeated Elements in C2 DNA.

We have shown that there are at least two repeated elements in C2. It is clear that these elements are present in a complex

arrangement within the cloned DNA. One of these repeats is in part homologous to rDNA. The other is not homologous to rDNA. The homology to rDNA exists in two regions of the cloned DNA, separated by a distance of about 6 Kb. These two regions flank the ribosomal protein gene present on this clone. However, we do not know if both of these regions of homology are identical or homologous to the same region of rDNA. One of these regions may be homologous to the Type I intervening sequence in Dml03', but Northern blot analysis has shown that at least part of this cloned DNA is homologous to a precursor form of rRNA which does not contain the Type I intervening sequence (Fabijanski and Pellegrini, in preparation).

The other repeat present in this clone, the HindIII repeat, is not homologous to rDNA, but is homologous to other regions of DNA in the Drosophila genome. Its position, close to the ribosomal protein gene, may indicate that it has some functional role in the expression of this gene. Another repeat which is present in this clone but was not investigated is a small segment of DNA in or near the ribosomal protein gene present on C2. This segment of DNA is homologous to a region in at least 5 other ribosomal protein in RNAs (7). These findings together point out the very complex arrangement of repeated sequences in C2. It is also of interest to note that this clone exhibits clustering of small, expressed genes.

The arrangement of repeats within Drosophila DNA has been studied by a number of investigators (for a recent review, see 13). Wensink et al. showed that within some long repeat units there was a series of smaller repeats in tandem, scattered and scrambled arrangements (14). A similar arrangement of repeated units is seen in C2.

Repeated elements are frequently found to be located near expressed genes. As one example, the rRNA gene set in Drosophila contains a series of 250 bp AluI repeats found upstream from the site of initiation of transcription of these genes (15). Whether the repeats detailed in this report have functional significance has yet to be determined. The homology of rDNA to this ribosomal protein gene-containing clone suggests the possibility of a functional coordination of these two types of genes.

One other ribosomal protein gene has been isolated from Drosophila (3). The gene is single copy and has no repeated elements in the cloned segment containing it. This raises the possibility that not all ribosomal protein genes in Drosophila are organized or regulated in the same fashion. There is support for this idea in data obtained from other organisms. For example, production of some, but not all, the ribosomal proteins in yeast are under the control of *rna2*, a ts RNA processing mutant. In 3T6 cells (16) and regenerating rat liver (17) one set of ribosomal protein mRNAs show similar kinetics of induction, while a second subset exhibits different behavior. It is interesting to speculate that the coordination of ribosomal protein biosynthesis and rRNA biosynthesis in Drosophila may be accomplished by directly linking rRNA production to the control of production of at least a few ribosomal proteins. We are currently using C2 to isolate other ribosomal protein genes to investigate this possibility.

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¹Abbreviations Used Ch4, the lambda vector Charon 4. bp, base pairs. Kb, kilobases or one thousand base pairs. Dml03', the 17 Kb *EcoRI* fragment of pDM103 (19) cloned in p184. Denhardt's solution, 0.01% Polyvinyl pyrrolidone, 0.01% Ficoll, 0.01% Bovine Serum Albumin. SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.0.

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