Characterization of cloned ribosomal DNA from *Drosophila hydei*

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ABSTRACT

The structure of ribosomal genes from the fly *Drosophila hydei* has been analyzed. EcoRI fragments, cloned in a plasmid vector, were mapped by restriction enzyme analysis. The lengths of the regions coding for 18S and 28S rRNA were defined by R-loop formation. From these data a physical map of the rRNA genes was constructed. There are two major types of rDNA units in *D. hydei*, one having a size of 11 kb and the other a size of 17 kb. The 17 kb unit results from an intervening sequence (ivs) of 6.0 kb, interrupting the β -28S rRNA coding region. Some homology between the *D. hydei* ivs and *D. melanogaster* type 1 ivs has been described previously (1). However, the restriction sites within these ivs show considerable divergence. Whereas *D. hydei* rDNA sequences coding for mature rRNAs share restriction sites with *D. melanogaster* rDNA, the nontranscribed spacer has little, if any, sequence homology. Despite difference in sequence, *D. hydei* and *D. melanogaster* spacers show structural similarities in that both contain repeated sequence elements of similar size and location.

INTRODUCTION

Drosophila hydei has three rRNA-gene (rDNA) clusters (nucleolus organizers, NOs); one has been localized in the heterochromatic arm of the X chromosome, the other two are located on the Y (2, 3). The X chromosomal NO contains about 560 rRNA genes, whereas both Y chromosomal NOs each contain about 200 rRNA genes (4). It has been shown that each Y chromosomal NO but not the X chromosomal NO of this species can increase its rDNA content disproportionately (4). Furthermore, it has been reported for this species that tissue specific regulation of rDNA replication occurs (5, 6, 7). As a prerequisite to comparison of the three rDNA clusters, it was necessary to investigate the rDNA repeat unit structure. Structural data derived from analysis of cloned

D. hydei rDNA fragments will be described. Most rDNA repeating units have a length of 11 kb. In addition to the 11 kb unit, a 17 kb unit, which is caused by an intervening sequence interrupting the 28S rRNA coding region, will be described.

MATERIAL AND METHODS

Preparation and Purification of D. hydei DNA

Nuclei from frozen adult flies of different genotypes were isolated as described by Renkawitz (8) and lysed for 15 min at 65°C in Marmur buffer (9) (0.15 M NaCl, 0.1 M EDTA), containing 2% Sarkosyl (Geigy). The Sarkosyl concentration was lowered to 0.5% by addition of Marmur buffer. Proteinase K (Merck) was added to a final concentration of 200 µg/ml. After 1h incubation at 65°C, the sample was extracted with chloroform: isoamylalcohol (24:1), and then dialyzed against 10 mM Tris, 1 mM EDTA, pH 8.0. Further purification was achieved by Cs₂SO₄ gradient centrifugation in a type 75 rotor ($\rho=1.470$ g/cm³; 38,000 rpm; 48h; 20°C). DNA of the peak fraction was dialyzed against 10 mM Tris-HCl, pH 8.0.

Labeling of DNA by "Nick-Translation"

Nick-translation was carried out according to Maniatis et al. (10) with DNA polymerase I (Boehringer) and α -³²P dATP (400-600 Ci/mmol, New England Nuclear); DNase I treatment was omitted. Separation of labeled DNA from free dNTPs was accomplished by selective precipitation with ammoniumacetate and ethanol (11). Specific activity obtained usually was in the range of 2-5 x 10⁶ cpm/µg DNA.

Cloning of D. hydei DNA with pBR322 as Vector and Screening for rDNA Clones

rDNA genes of D. hydei contain recognition sites for the restriction endonuclease EcoRI (Fig. 1A). D. hydei DNA from wild-type flies was partially digested with EcoRI, and a fragment size class from 4-20 kb was isolated by sucrose gradient centrifugation (5-20% w/w sucrose in 10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 8.0; type SW 40 rotor; 8h; 40,000 rpm; 5°C). The plasmid pBR322 (12) was used as vector; restriction sites are known

from the sequence (13).

The plasmid was opened at the single site for EcoRI and treated with alkaline phosphatase (BAP-F, Worthington), in order to minimize religation of pBR322 (14). For ligation equimolar amounts of D. hydei DNA and vector DNA at a concentration of 6 nmol ends/ml each were used. Ligation was performed in ligation buffer (15) at 15°C for 20h with 1.2 units T4-ligase (Bio-labs). E. coli K 12 HB101 was transformed with the ligated DNA as described by Mandel and Higa (16).

The isolated ³²P-labeled rDNA insert of a clone pBc2, which contains a complete rDNA unit from another insect, Sciara coprophila (17), was used to identify rDNA-containing clones. Colony hybridization was done according to Grunstein and Hogness (18) as modified by Gergen et al. (19). All DNA cloning was performed under L2 conditions.

Plasmid DNA Isolation

Plasmid DNA was isolated by a modification of the method of Clarke and Carbon (20) as described previously (17).

Restriction Endonuclease Digestion, Gel Electrophoresis, and Hybridization

The following restriction endonucleases were used: Bam HI, EcoRI, Hae III, Hpa II, Hind III, Pst I, Sal I, Sma I, Xba I (New England Biolabs, Bethesda Research Co., and Boehringer). Digestions were carried out in 6.6 mM Tris-HCl (pH 7.9), 6.6 mM β-mercaptoethanol, 6.6 mM MgCl₂, and 60 mM NaCl, for 6-15h at 37°C. Restriction fragments were separated either on 0.7-1.6% agarose gels or on 5% acrylamide gels. Most agarose gels were run in the electrophoresis buffer of Helling et al. (21) at 2 V/cm, 1.6% agarose gels, and acrylamide gels in TBE buffer (22) at 10 V/cm. The molecular weights of rDNA fragments were determined using either Hind III-digested λ phage (23) or Hae III-digested φX174 (24), as a standard. DNA was transferred from agarose gels to nitrocellulose filters by the method of Southern (25). Hybridization of ³²P-DNA probes to DNA on filters was done overnight in 2 x SSC (0.3 M NaCl, 0.03 M Na-citrate, pH 7.0), Denhardt's buffer (26), 0.2% SDS at 65°C. Kodak Royal X-omat X-ray

films were exposed to the filters for either 4-10 days or 1-3 days with an intensifying screen (Dupont).

Restriction Mapping of Partial Digestion

Multiple Hpa II restriction sites in the spacer of D. hydei were mapped by the method of Smith and Birnstiel (27).

The clone pDh2-D7 contains the bulk of the nontranscribed spacer and of the 18S rRNA coding region of D. hydei. pDh2-D7 was digested with EcoRI, and the fragments were separated on a 1% agarose gel. The 5.3 kb D. hydei fragment was cut out and electroeluted into a dialysis bag, purified by chromatography on benzoylated DEAE-cellulose and concentrated by ethanol precipitation. This isolated EcoRI-fragment was treated with alkaline phosphatase, then 5'-termini were labeled with T4-polynucleotide kinase and γ - ^{32}P ATP. Digestion with Hind III removed one labeled 5'-end, giving rise to a 300 base pair ^{32}P -labeled fragment (this fragment does not contain Hpa II sites). Partial digestion with restriction endonuclease Hpa II was performed. Separation of fragments on 1.6% agarose gels yielded a pattern of overlapping fragments; those with a common 5'-labeled end could be detected by autoradiography.

Electron Microscopy

R-loop formation of cloned rDNA was performed in R-loop buffer {70% formamide (purified by ion exchange on AG 501-X8, 20-50 mesh, BioRad, $\text{OD}_{270} < 0.15$), 0.1 M PIPES: piperazine-N,N'-bis(2-ethane-sulfonic acid) $\text{Na}_{1.4}$ } at pH 7.8, and 0.01 M Na_3EDTA (28). 10 μl of a solution containing 30-60 $\mu\text{g/ml}$ rDNA and 50-100 $\mu\text{g/ml}$ 18S RNA or 28S RNA, 0.1 M NaCl, 0.05 M Tris-HCl at pH 7.5, was added to 50 μl of R-loop buffer, and incubated 4h at 47°C. The temperature of strand separation was determined according to Rosbash et al. (29) to be 48°C under the above conditions.

After a 20fold dilution in spreading buffer (50% formamide, 0.1 M Tris-HCl at pH 8.5, 0.01 M EDTA, and 50 μg cytochrome c), the R-loops were spread onto a distilled water hypophase (30). For internal calibration, single and double stranded $\phi\text{X } 174$ DNA was added to the spreading solution. Grids were stained with

uranyl acetate (31), and shadowed with Pt/C. Electron micrographs were taken with a Philips EM 300 at 60 kV. The negatives were projected with a 15fold enlargement, and the lengths of the molecules were measured with a Numonics Graphic Calculator.

RESULTS

Cloning of *D. hydei* rDNA

D. hydei female DNA was digested with EcoRI and fragments were separated by agarose gel electrophoresis. DNA was transferred to nitrocellulose filters according to the procedure of Southern (25), and hybridized with the *D. melanogaster* ³²P-rDNA segment Dm103 from a hybrid plasmid. The Dm103-segment contains a complete rRNA gene including the 28S intervening sequence (ivs) type 1 (32). Several bands homologous to Dm103 were observed (Fig. 1A); the 6.0 kb band is named the A-fragment, and the 5.3 kb band the B-fragment. Additional bands with lengths of 8.5 kb and 2.4 kb result from an ivs within the A-fragment (shown later); they are named A_{ivs1} and A_{ivs2}, respectively.

D. hydei DNA from wild-type females and males was subjected to partial digestion with restriction endonuclease EcoRI. After size selection by sucrose gradient centrifugation, the *D. hydei* DNA was ligated into the EcoRI site of pBR322. The ligated DNA was used to transform *E. coli* strain HB101. Several clones of different sizes containing rDNA were detected by hybridization with cloned rDNA from *Sciara coprophila* (17).

Restriction Enzyme Analysis and R-Loop Formation of pDh2-A4, a Clone Containing a 28S rRNA Coding Region (A-fragment)

The clone pDh2-A4 contains a *D. hydei* EcoRI fragment (Fig. 1B) of 6.0 kb corresponding to the A-fragment described in Fig. 1A. Restriction enzyme mapping by double digestion has revealed two cleavage sites for Hind III and one recognition site each for Pst I and Xba I (Fig. 2A). The Pst I cleavage site and Xba I site of *D. hydei* are located 0.6 kb and 0.9 kb, respectively, from the Hind III site of pBR322. The two Hind III sites in *D. hydei* DNA are located towards the other end of the insert.

Fig. 1: **A:** An autoradiograph of Southern blots of *D. hydei* with wild-type female genomic DNA. The DNA was digested with EcoRI, fractionated on a 1% agarose gel, transferred to a filter, and hybridized with 32 P-labeled rDNA of *D. melanogaster* (clone Dm103). In this and all the following figures the numbers are molecular weights, given in kb.

B: The clone pDh2-A4 contains a *D. hydei* EcoRI fragment of 6.0 kb corresponding to the A-fragment shown in Fig. 1A.

C: The clone pDh2-H8 contains three *D. hydei* EcoRI fragments. The 8.5 kb and 2.4 kb fragments have the same size as the A_{ivs1}- and A_{ivs2}-fragments in Fig. 1A. In addition, a 1.1 kb fragment is visible.

D: Restriction of pDh2-D7 with EcoRI results in two *D. hydei* EcoRI fragments of 5.3 and 0.3 kb.

EcoRI fragments of all three clones have been separated on 1% agarose and 5% acrylamide gels. In the clones pDh2-A4 and pDh2-H8 no additional fragments have been detected on 5% acrylamide gels. The vector pBR322 has a size of 4.4 kb and is marked by v.

Regions coding for 28S ribosomal RNA were defined by electron microscopic analysis of hybrid molecules formed between 28S ribosomal RNA and EcoRI digested pDh2-A4 under R-loop conditions; an example of such a molecule is shown in Fig. 2A. The R-loops of 1.7 kb and 1.8 kb formed with 28S rRNA correspond to the known

sizes of the α and β sequence of 28S rRNA. These two R-loops are separated by a region of about 0.2 kb. This interruption probably corresponds to the central break within the 28S rRNA of D. melanogaster (33) which has also been observed in D. hydei (34). A similar gap region has been demonstrated in D. melanogaster rDNA (30, 35, 36), and in Sciara coprophila rDNA (17).

In addition, R-loop molecules formed between 28S rRNA and Sal I digested pDh2-A4 were measured to confirm which R-loop corresponds to which 28S RNA segment. Sal I endonuclease cuts this clone only at one position in the pBR322 segment (Fig. 2A), such that 3.7 kb of the vector are adjacent to the 1.1 kb fragment of EcoRI-Hind III double digestion. R-loop molecules show that the coding region for the β -chain is located adjacent to this 1.1 kb fragment.

Analysis of pDh2-H8, a Clone Containing a 28S rRNA Coding Region interrupted by a 6.0 kb Intervening Sequence (A_{ivs1} -, A_{ivs2} -fragments)

The EcoRI restriction pattern of the clone pDh2-H8 contains the 8.4 kb A_{ivs1} - and 2.4 kb A_{ivs2} -fragments (shown in Fig. 1A) and, in addition, a 1.1 kb fragment (Fig. 1C). After hybridization of D. hydei DNA with the D. melanogaster clone Dm103 (which also contains an *ivs* within the 28S- β -region), the 1.1 kb fragment of the *ivs* in pDh2-H8 is not detected. Double digestions of pDh2-H8 with EcoRI-Hind III, Xba I-EcoRI, Pst I-EcoRI, or Xba I-Hind III produce several fragments of the same size as similar digestions of pDh2-A4. Additional fragments are probably caused by the insertion of the *ivs* into the center of the β -28S-region close to the Hind III site. Electron microscopic analysis of R-loop molecules formed between 28S rRNA and pDh2-H8 shows the presence of a 6.0 kb loop which corresponds to the *ivs* (Fig. 2B). The *ivs* splits the β -28S rRNA into parts of 0.8 kb (β_1) and 0.9 kb (β_2).

Analysis of pDh2-D7, a Clone Containing the 18S rRNA Coding Region and Most of the Non-Transcribed Spacer (B-Fragment)

The clone pDh2-D7 includes a 0.3 kb EcoRI-fragment and the

B-fragment, which contains a large part of the 18S rRNA coding region (Fig. 1D). Restriction sites and R-loop molecules of this clone are shown in Fig. 2C. Before hybridization, the clone pDh2-D7 was digested with Sal I, so that 3.7 kb of pBR322 are adjacent to the EcoRI site of *D. hydei* DNA near the two Hind III sites in *D. hydei* DNA. 18S rRNA forms one R-loop of 1.6 kb with *D. hydei* DNA of this clone. Near the EcoRI site adjacent to 3.7 kb of pBR322, some molecules show a whisker of single stranded RNA not hybridized to the DNA of this clone, suggesting that this clone does not contain a complete 18S rRNA coding region.

The idea that an EcoRI site is located within the 18S rRNA coding region is confirmed by R-loops of the clone pDh2-B6. This clone contains besides the 28S coding region a complete 18S R-loop of 1.8 kb and a shorter one of 1.6 kb (Fig. 3) as in pDh2-D7. Again a whisker of single stranded RNA is visible at the position of the 1.8 kb R-loop. Therefore, an EcoRI site is located 200 base pairs (bp) from the 3' end of the 18S rRNA coding region. Such an EcoRI site has been observed at a similar and probably identical position in the rDNA of several other organisms so far investigated (cited in 37).

Furthermore, the clone pDh2-B6 with a complete 18S rRNA coding region allowed us to determine the size of the internal transcribed spacer to be 1.1 kb (Fig. 3). The other spacer between the 28S and 18S coding regions, which includes the nontranscribed spacer, has a length in this clone of 4.1 kb. This size,

Fig. 2: A: pDh2-A4 DNA was digested with EcoRI and hybridized under R-loop conditions with 28S rRNA.

B: pDh2-H8 DNA partially digested with EcoRI was hybridized under R-loop conditions with 28S rRNA. This shows that the *ivs* interrupts the β -28S rRNA coding region.

C: The clone pDh2-D7 was cut by Sal I (in pBR322) and hybridized under R-loop conditions with 18S rRNA.

In each case the bar in the micrograph represents 0.5 μ m which corresponds to 1.5 kb; the values on the interpretative drawings are given in kb.

Restriction enzyme symbols:

Bam HI (V), EcoRI ($\downarrow$), Hind III ($\uparrow$), Pst I (∇), Sal I ($\mid$), Xba I ($\downarrow$).

Fig. 3: pDh2-B6 was digested with Sal I and hybridized under R-loop conditions with 18S and 28S rRNA. The bar represents 0.5 μ m.

however, varies considerably among different rDNA repeating units (38).

Digestion of pDh2-D7 with Hpa II reveals several fragments of 242 bp, suggesting that a 242 bp sequence occurs repeatedly within the B-fragment (Fig. 4A). To determine the location of this repeated sequence, the EcoRI B-fragment from clone pDh2-D7 was 5' end-labeled with 32 P, digested with Hind III, and partially digested with Hpa II, according to the method of Smith and Birnstiel (27) (Fig. 4B). Products of partial digestion yield the order of recognition sites for Hpa II. This was correlated with the fragment sizes from complete digests to give a detailed

Fig. 4: A: pDh2-D7 was double digested with EcoRI and Hpa II; restriction enzyme fragments were separated on a 5% acrylamide gel. For comparison Hpa II-digested DNA from the vector pBR322 is shown.

B: Restriction site mapping of the B-fragment from pDh2-D7 after digestion with Hind III, and partial digestion with Hpa II, according to Smith and Birnstiel (27). The resulting partials were separated on a 1.6% agarose gel. The numbers indicate the time (in minutes) of incubation with Hpa II. In addition to the partials, a fragment of 300 bp is visible in all lanes, which corresponds to the Hind III-EcoRI fragment in the 18S DNA.

map of Hpa II cut sites within the B-fragment.

The results show that nine Hpa II restriction sites follow each other in tandem in regular intervals of 242 bp starting a distance of 2.1 kb from the end of the 28S coding sequence. This regular order ends 1.0 kb before the beginning of the 18S coding region (Fig. 7).

rDNA Repeat Units in Genomic DNA

In order to investigate the question of whether A- plus B-fragments add up to a full rDNA repeating unit, we subjected genomic DNA of the genotype $\overline{XX}/Y$ (a genotype without *ivs*) to

partial digestion with EcoRI. Both the A- and B-fragments are shifted to sizes 0.3 kb larger than in complete digest (Fig. 5). This indicates that the 0.3 kb EcoRI-fragment, which has been detected in the spacer of the pDh2-D7 clone, is located contiguous to the A- as well as the B-fragment.

In addition, partial digestion produces larger rDNA bands with sizes between 10.5 and 11.5 kb. The variations within this size range are due to spacer length heterogeneity (38). Since the A- and B-fragments add up to this length, we can exclude the existence of other EcoRI-fragments besides A, B, and the short 0.3 kb fragment within the rDNA repeating unit. The sum of these fragments also is in good agreement with the length of the repeat unit in *D. hydei* rDNA as measured in "Miller spreads" (39, 40). Additional evidence that A plus B (and the 0.3 kb fragment)

Fig. 5: Genomic DNA from flies of the genotype $\overline{XX}/Y$ has been partially digested with EcoRI, separated on 0.7% agarose gels, transferred to a filter, and hybridized with a mixture of ^{32}P -labeled DNA from the clones pDh2-A4 and pDh2-D7. The DNA was digested with one unit EcoRI for 30 min (B) and 60 min (C). A- and some of the B-fragments are shifted to sizes 0.3 kb larger than in complete digests (A).

add up to a full repeat unit of about 11 kb comes from measurements of the clone pDh2-B6, which includes one and a half repeating units (Fig. 3).

We have described an intervening sequence of 6.0 kb in the clone pDh2-H8. The A_{ivs1} and the A_{ivs2} EcoRI-fragments of this clone are detected in genomic rDNA after hybridization with Dm103 (Fig. 1A). In addition to the A_{ivs1} and the A_{ivs2} , a 1.1 kb EcoRI-fragment is found in pDh2-H8 (Fig. 2B). This fragment does not hybridize with Dm103. We wanted to know if this 1.1 kb fragment also is present in the rDNA *ivs* of genomic DNA. A comparison of an EcoRI and an EcoRI-Sal I double digestion of pDh2-H8 reveals that the only *D. hydei* fragment in this clone containing a recognition site for Sal I is the 1.1 kb EcoRI-fragment (Fig. 6A). The other cloned *D. hydei* fragments do not have restriction sites for Sal I (Fig. 2). If the 1.1 kb EcoRI-fragment were linked to the A_{ivs1} - and A_{ivs2} -fragments also in geno-

Fig. 6: A: The clone pDh2-H8 has been digested with EcoRI and Sal I; fragments were separated on 1.0% agarose gels. EcoRI fragments of 4.4 kb (pBR322) and 1.1 kb contain a restriction site for Sal I as shown by double digestion.

B: DNA of *D. hydei* wild-type females (X/X) and $\overline{XX}/Y$ females was digested with Sal I, transferred to nitrocellulose and hybridized with ^{32}P -labeled 18S and 28S rRNA.

mic DNA, a 17 kb fragment would result from Sal I digestion.

We compared wild-type females containing the X chromosomal NO with an $\overline{XX}/Y$ genotype which contains only the two Y chromosomal NOs. The X-NO includes *ivs*-containing rRNA genes, whereas the two Y-NOs in the $\overline{XX}/Y$ genotype are free of *ivs*-sequences (38). DNA of these two genotypes was digested with Sal I and fragments were separated on a 0.7% agarose gel. After Southern transfer and hybridization with ^{32}P -labeled rRNA, we detected a 17 kb fragment in the XX genotype (Fig. 6B). This 17 kb fragment results from two adjacent 17 kb units. The experiment demonstrates that the 1.1 kb EcoRI-fragment included in the 6.0 kb *ivs* is present in chromosomal rDNA and does not originate from other parts of the genome ligated to rDNA fragments during the cloning procedure.

The R-loop mapping and restriction enzyme analysis of cloned and genomic rDNA are summarized in Fig. 7.

A comparison between the *ivs* of *D. hydei* and the type I *ivs* of *D. melanogaster* shows substantial divergence. None of the restriction sites known in both species are held in common. Cross hybridization between the *ivs* sequences of both species shows some degree of homology (1). We have shown that the 1.1 kb

Fig. 7: Summary of the mapping data from cloned rDNA and direct genome analysis. Restriction sites for Hpa II and Hae III were not determined for the entire rDNA unit.

Bam HI (V), EcoRI (↓), Hae III (↑), Hind III (q), Hpa II (|), Pst I (∇), Sal I (I), Xba I (J).

EcoRI fragment of the D. hydei ivs does not hybridize with type 1 ivs of the D. melanogaster clone Dm103.

We were interested in investigating whether the spacer of D. hydei is similar in sequence to the spacer of D. melanogaster. The restriction enzyme Hae III has many recognition sites in the coding regions of D. hydei rDNA but only one recognition site in the spacer (Figs. 8A, B). By utilizing this site, which is near the 18S coding region of D. hydei, together with the Hae III site close to the end of the β -28S rDNA region, fragments of about 5 kb, which include most of the spacer, are obtained. All other rDNA regions of the 11 kb unit are cut by Hae III to fragments smaller than 2.3 kb (Fig. 8B).

Therefore, we hybridized Dm103 to a Hae III restriction digest of D. hydei (Fig. 8C). Dm103 contains the entire rDNA unit of D. melanogaster. This experiment shows that the spacer fragment of D. hydei shows very little or no homology to Dm103, in contrast to the coding regions.

DISCUSSION

Comparison between the rRNA gene structure of Drosophila hydei and D. melanogaster shows a repeat unit of similar size (11 kb). Furthermore, the coding regions exhibit cross hybridization and share restriction sites (for example the sites for EcoRI and Hind III). Studies on the rDNA of D. hydei demonstrate the existence of a 6.0 kb intervening sequence (ivs) in the 28S coding region of some of the X chromosomal rRNA genes. The position at which the ivs splits the 28S sequence is very similar to that in the rDNA of D. melanogaster (30). 28S intervening sequences have been detected in several higher dipteran insects (for review see 41). It has been shown by cross hybridization that the type 1 ivs of D. melanogaster shares some degree of homology with D. hydei and D. virilis rDNA (1). But a comparison of restriction sites between D. hydei ivs and D. melanogaster type 1 ivs shows substantial divergence. This divergence is also shown by the fact that the 1.1 kb EcoRI-fragment of the D. hydei ivs does not hybridize with Dm103.

Fig. 8: A: EcoRI-digested DNA from the clones pDh2-B6, pDh2-A4, and pDh2-D7 was compared on a 1.0% agarose gel. The clone pDh2-B6 includes one A-fragment, a B-fragment of 5.3 kb, and an additional B-fragment 4.2 kb in length.

B: The restriction enzyme Hae III has several recognition sites in the B-fragment. Hae III-digestion of pDh2-D7 produces several small fragments (< 1 kb) and a 3.6 kb fragment (data not shown). Since EcoRI-Hae III double digestion yields a 3.3 kb fragment, the 0.3 kb EcoRI fragment of the nontranscribed spacer must be part of the 3.6 kb Hae III fragment. This data places a Hae III recognition site 3.3 kb to the right of the 0.3 kb EcoRI fragment. The Hae III-EcoRI double digestion pattern of pDh2-B6 is similar to the pDh2-D7 pattern except for some additional fragments. Besides the 3.3 kb band there exists an additional 2.3 kb fragment which is due to the presence of a second spacer in this clone. Together with a 1.4 kb and an 0.3 kb fragment, this 2.3 kb band adds up to a 4.0 kb fragment, visible in the Hae III digest (left in the figure). Therefore, the Hae III cut site must be 0.3 kb left from the right end of the β -28S rRNA coding region. Additional Hae III cut sites in *D. hydei* rDNA have not been mapped. Fragments were separated on 1.2% agarose gels.

C: *D. hydei* DNA from XX/Y genotypes was digested with Hae III, transferred to nitrocellulose, and hybridized with Dm103. Fragments around 5 kb, which include almost the entire spacer and 0.3 kb of the 28S coding sequence, show only a slight cross hybridization probably only due to the 28S sequence.

But little or no sequence homology has been found between the nontranscribed spacers of the two species, either by a comparison between the positions of restriction sites or by cross hybridization between Dm103 and the nontranscribed spacer sequence of D. hydei. This result shows that the nontranscribed spacer is much less conserved during evolution than the coding regions, a situation that is known from comparison of rDNAs of other eukaryotic species (41, 42). But it is in marked contrast to the results obtained by comparison among six sibling species of the D. melanogaster subgroup (43). In this case, the spacers are at least as conserved as the transcribed rDNA sequences. A rapid divergence of spacer sequences occurs between more distantly related species such as D. hydei and D. melanogaster, which belong to different subgenera.

It is interesting to note that the spacers of both D. melanogaster and D. hydei are very similar to each other in having a regular 0.25 kb interval between sites recognized by particular restriction enzymes; this is Alu I (AGCT) in D. melanogaster (44) and Hpa II (CCGG) in D. hydei. Furthermore, the repeated sequence elements are localized at a similar position within the spacer of both species. We suggest that spacer sequences can have a function which does not depend upon conservation of the primary nucleotide sequence but rather on the presence of repeated sequence elements.

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