SITES OF THE 5S RIBOSOMAL GENES IN DROSOPHILA. I. THE MULTIPLE CLUSTERS IN THE *VIRILIS* GROUP[1]

D. E. WIMBER AND DORIS R. WIMBER

Department of Biology, University of Oregon, Eugene, Oregon 97403

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ABSTRACT

Drosophila melanogaster ^{125}I-5S RNA was annealed to salivary gland preparations of 6 species in the *virilis* group of Drosophila. Two patterns of annealing were found. *D. virilis*, *D. montana* and *D. borealis* showed three 5S gene clusters on chromosome 5; S_{d-f} and W_{c-j} were strongly labeled, but X_{a-e} was weakly labeled. *D. montana* and *D. borealis* have a greater percentage of their total 5S cistrons at S_{d-f} than does *D. virilis*. *D. americana americana*, *D. americana texana* and *D. novamexicana* showed 2 sites labeled; no label was seen at S_{d-f} while W_{c-j} was weakly labeled and X_{a-e} was strongly labeled, the reciprocal of the previous pattern in the W-X region. Hybrids between *D. a. americana* and *D. virilis* showed no difference in chromosome banding at the sites of the 5S clusters despite their pattern differences. *D. a texana* × *D. virilis*, on the other hand, did show a difference in staining the X_{a-e} region. These patterns fall squarely into the biosystematic groupings deduced by many previous workers.

IN the genus Drosophila, cytogenetic studies have shown that extensive chromosome homology exists between species. Chromosome arms may be traced from one species to another by using two criteria (STURTEVANT and NOVITSKI 1941). First, the degree of homology is reflected by the pairing of the synapsed polytene homologues in hybrids between species. Secondly, genetic studies with mutant genes in different species often show that similar mutations are linked on the same chromosome. This latter method can be extended by using *in situ* hybridization methods where it is possible to identify particular gene loci in several related species by binding radioactive gene products to the denatured DNA of cytological preparations. Perhaps the most work with *in situ* annealing methods has been done with 18S and 28S ribosomal RNA (see HSU, SPIRITO and PARDUE 1975 for a review). Generally the studies have confirmed that genes coding for this RNA are located at the secondary constrictions of the chromosomes, but in some cases unsuspected 18S and 28S gene clusters have been discovered. 5S ribosomal RNA gene clusters show no secondary constrictions in the mitotic or meiotic chromosomes. Thus, their identification has rested entirely upon *in situ* hybridization procedures (WIMBER and STEFFENSEN, 1970; PILONE *et al.* 1974; WEN, LEÓN and HAGUE 1974; ALONSO and BERENDES 1975; PUKKILA 1975; WIESLANDER, LAMBERT and EGYHÁZI 1975; LEÓN 1976; HENDERSON *et al.* 1976;

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COHEN 1976a,b). WIMBER and STEFFENSEN (1970) first identified the 5S gene cluster in *Drosophila melanogaster* on the right arm of chromosome *2* at 56F. ALONSO and BERENDES (1975) located the 5S gene cluster in *D. hydei* on chromosome *2* at $23B_{1,2}$ and a second probable cluster on chromosome 5 at 95D. Previous studies of chromosome homology within the genus (SPENCER 1949; STALKER 1972) suggest that the right arm of chromosome *2* of *D. melanogaster* is homologous to chromosome 5 of *D. hydei*. However, the main 5S cluster of *D. hydei* at $23B_{1,2}$ is on chromosome *2*, which is thought to be homologous to chromosome *3R* of *D. melanogaster* (BERENDES 1963; PATTERSON and STONE 1952). Thus, the position of the main 5S gene cluster in *D. hydei* is not consistent with the usual Chromosome relationships generally suggested for these species.

The genus Drosophila has been taxonomically divided into several subgenera, one of which is the subgenus Drosophila, a very large taxon. The *virilis* group is one of about 9 species groups placed in the subgenus and the group itself contains 9 species (see PATTERSON and STONE 1952). The *virilis* group affords an excellent opportunity for work with the 5S gene clusters because it is well-known genetically and cytologically and has a number of closely related taxa where interspecific hybridization is possible. Five chromosome maps of *D. virilis* have been published (see HSU 1952) and one of *D montana* (MOORHEAD 1954). Also in this association of the living forms, *D. virilis* is considered the most nearly primitive, with *D. americana americana*, *D. a. texana* and *D. novamexicana* forming one line of descent while *D. montana* and *D. borealis* are representatives from another line. Each taxon can produce hybrids with at least one other member of the group (STONE, GUEST and WILSON 1960).

COHEN (1976b) recently published work done with the 5S gene clusters in the *virilis* group. *D. virilis* was reported to have two 5S gene clusters on chromosome 5. The *virilis* group chromosomes are similar enough in band patterns so that the same map can be used for all of the species. *D. a. americana*, *D. a. texana* and *D. novamexicana* lacked the distal 5S cluster but retained the proximal cluster. On the other hand, *D. montana* showed only the distal cluster and lacked the proximal 5S cluster. In this paper we wish to confirm some of COHEN's observations, modify others, introduce observations on an additional species and some species hybrids, and refine and quantitate the chromosome localizations.

MATERIALS AND METHODS

The strains of flies were all obtained from the Department of Zoology, University of Texas, Austin: *D. virilis* Sturtevant, 1801.1; *D. americana americana* Spencer, 2515.3; *D. americana texana* Patterson, Stone and Griffen, 1128.1; *D. novamexicana* Patterson, 1952.2; *D. montana* Patterson and Wheeler, 3000.1; *D. borealis* Patterson, 2077.4.

Flies were grown on standard cornmeal, agar, molasses medium at 18°. The salivary gland preparations were made from third-instar larvae by dissecting out the glands in 45% acetic acid, squashing and removing the cover glass by the dry ice method; slides were then placed in 100% ethanol-acetic acid (3:1) for 10 min, passed through two changes of 100% ethanol, and air dried.

Preparation and labeling of 5S RNA: *Drosophila melanogaster* 5S RNA was prepared from third-instar larvae as described in SZABO (1974). Total soluble RNA was prepared by phenol extraction and passage through DEAE cellulose. The 5S RNA was separated from tRNA by

chromatographic separation on a DEAE Sephadex A25 column with a gradient from 0.15 to 0.4 M KCl in M $NaPO_4$ (pH 6.0), 5% N,N-dimethyl-formamide maintained at 34°. The 5S RNA fraction was applied to a RPC-5 column equilibrated in 0.4 M NaCl, 0.001 M imidazole (pH 7.0), 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol. The 5S RNA eluted as a single sharp Gaussian peak at 0.65 M NaCl; the peak tubes were pooled and precipitated with ethanol, collected by centrifugation, and redissolved in water for iodination.

A 5 μg aliquot was iodinated according to procedures described in PRENSKY (1976). The specific activity of the RNA was approximately 1.9×10^8 dpm/μg. Unreacted iodine was removed by passage over DEAE cellulose. The iodinated 5S RNA was eluted from the DEAE cellulose column in 1M NaCl, 0.01 Tris-Cl (pH 7.4), *E. coli* soluble RNA was added and the sample was precipitated with ethanol. The precipitate was collected by centrifugation and dissolved in the hybridization buffer.

In situ *hybridization*: Chromosome preparations were treated with bovine pancreatic ribonuclease for 1 hr at room temperature (0.2 mg/ml in 2XSSC), rinsed in 2XSSC (0.3 M NaCl, 0.03 M Na Citrate, pH 7), dehydrated and air dried. Immediately before *in situ* hybridization, the slides were placed in 0.2 N HCl for 20 min at room temperature in order to denature the DNA, dehydrated and air dried. The hybridizing solution consisted of 50% formamide in 2XSSC in which ^{125}I-5S RNA was dissolved at a concentration of about 1 μg/ml. Unlabeled, soluble RNA from *Escherichia coli* (1 mg/ml) was added to the hybridizing solution. About 0.03 ml of the annealing solution was placed on each slide, a cover glass added and the preparation was placed on glass rods in a Petri dish in an oven at 60°. The Petri dishes had been previously prepared in the following way. Bibulous paper soaked in 10 ml of the same formamide-salt solution which was used to dissolve the radioactive RNA was placed in the bottoms of the Petri dishes and glass rods were placed on the moist paper. The Petri dishes were allowed to equilibrate at 60° for at least 1 hr before the hybridization was started. The slides were kept at the 60° temperature for 1 hr and then transferred to a 40° oven for 3 additional hrs. The cover glasses were floated off in 2XSSC, the slides treated with ribonuclease for 1 hr at room temperature, washed copiously with 2XSSC, dehydrated and air dried. Kodak NTB liquid emulsion (diluted 1:1 with water and 1% glycerine) was applied and slides stored in light tight boxes over anhydrous calcium sulfate at 4° for 1 or 2 weeks. They were developed in Kodak Dektol diluted 1:1 water for 2 min and processed in the usual way. The slides were stained with 0.075% Giemsa (Gurr) in 0.01 M phosphate buffer (pH 7) for about 1 hr and cover glasses were mounted in immersion oil.

Chromosome and band identification: Altogether five chromosome maps of *D. virilis* have been published. This large number of maps probably reflects the inability of one worker to profitably use the maps prepared by others. Indeed, it is often difficult to recognize some homologous regions on these different maps in a side-by-side comparison. The most recent map is that of T. C. HSU (1952). We found this map difficult to use, but since it is the most recent, and the one to which most contemporary workers refer, we chose to use it (Figure 1F). The other species within the group are said to have very similar chromosomes, so similar in fact, that the *D. virilis* chromosome map can be used to identify sites on the chromosomes. Thus, we are using the same band nomenclature for all of the species.

RESULTS

Labeled regions of D. virilis*: D. virilis* shows a heavily labeled region about one-third of the distance from the chromocenter on chromosome 5. The label is found typically just proximal to a constricted region on the chromosome (Figure 1A–F). COHEN (1976b) identified bands S_{i-j} on HSU's map as being labeled. We are not convinced that the label covers this region. Our interpretation of HSU's map (1952) is that band S_a is at the constriction and is probably not labeled (Figure 1E). Bands S_{d-f} may all be labeled (Figure 1A-D). In some of our preparations the label seemed to be mainly concentrated over the distal band

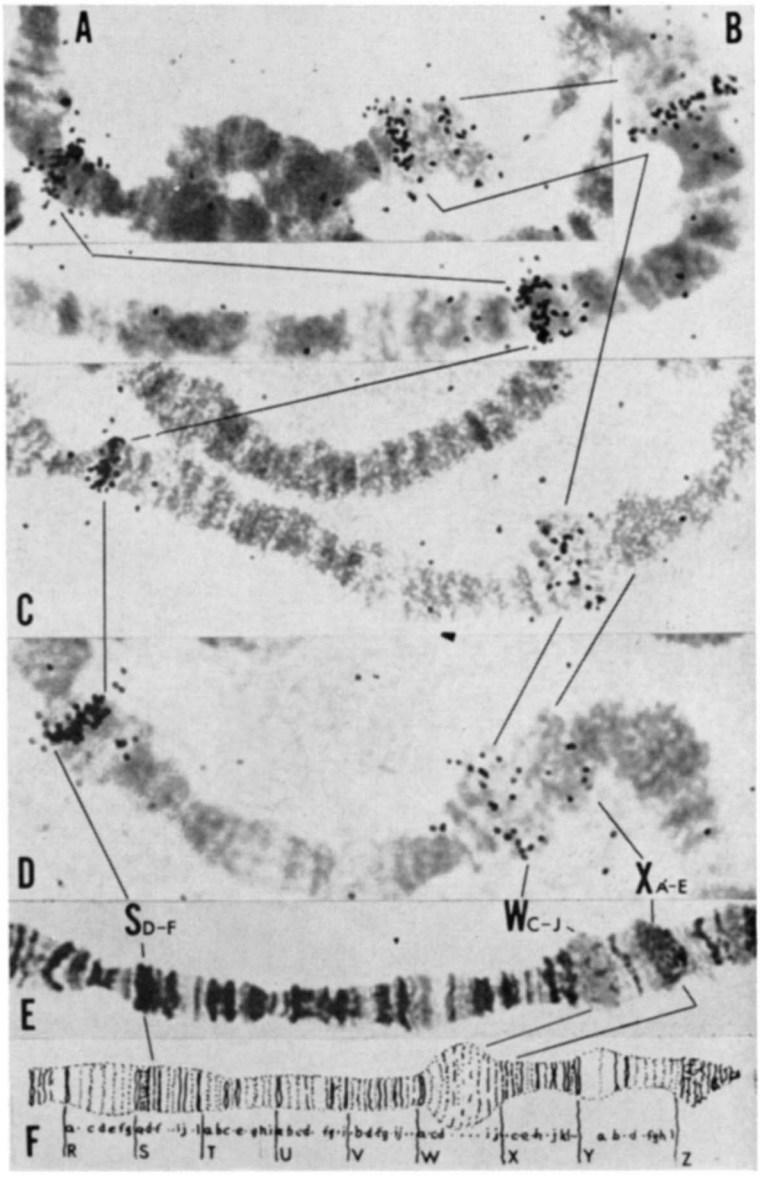

FIGURE 1.—Proximal third of chromosome 5 of *D. virilis*. (A-D) Autoradiographs after ^{125}I-5S RNA was annealed to the chromosomes. (E) Unlabeled and Giemsa stained chromosome 5. (F) Redrawing of chromosome 5 taken from HSU (1952). Z is the proximal end.

S_d (Figure 1D), but in many others the label covers the entire 3-band area. The limited autoradiographic resolution provided by ^{125}I places limits upon our conclusions.

Region W on HSU's map is a large puff (Figure 1F). HSU does not draw a second puff proximal to region W. Our interpretation is that region X_{a-e}

is also usually puffed in late third instar, giving the whole area a double-puff appearance. Our preparations show label over both halves of the double-puff, generally as two discrete regions and clearly more label over the distal than the proximal portion of the puff (Figure 1A–D). Because of the puffed nature of this region, it is difficult to place limits on the label. The heaviest label is over the distal puff (probably HSU's region W_{c-j}) and less over the proximal puff in region X_{a-e}. COHEN (1976b) records label over W_{c-j}–X_c; we feel that the label extends to X_e. This finding of the 5S gene clusters on chromosome 5 of *D. virilis* corroborates the conclusions of STURTEVANT and NOVITSKI (1941) and others (CHINO 1936; KIKKAWA 1963) that chromosome *2R* of *D. melanogaster* is homologous to chromosome 5 of *D. virilis*. *D. melanogaster* has a 5S cluster at 56F of chromosome *2R*.

In control hybridizations where the regular hybridizing medium contained 1 mg/ml of unlabeled *D. melanogaster* 5S RNA, regions S_{d-f}, W_{c-j} and X_{a-e} showed only occasional silver grains.

Labeled regions of D. montana *and* D. borealis*:* Cytologically these two species give much poorer preparations than *D. virilis*. We suspect that the polytene chromosome undergo fewer rounds of DNA replication than in *D. virilis*, since their size is usually smaller. The labeling pattern in both *D. montana* and *D. borealis* is similar to that of *D. virilis*, showing label over the double puff, W_{c-j}–X_{a-e}, and S_{d-f} (Figure 2A–C). As in *D. virilis*, the distal portion of the double puff, W_{c-j}, was more heavily labeled than A_{a-e}. COHEN (1976b) records label only over the S_{i-j} region of *D. montana* and none over the double puff; he did not examine *D. borealis*. Our findings are distinctly different from COHEN's in

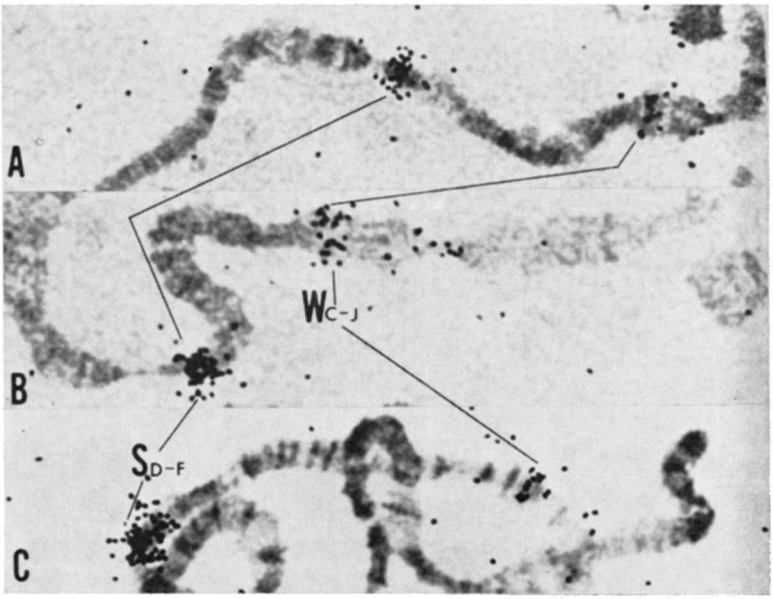

FIGURE 2.—Autoradiographs of chromosome 5 after ^{125}I-5S RNA hybridization. (A-B) *D. montana*. (C) *D. borealis*.

this respect. Our *D. montana* stock came from the University of Texas collection at Austin. COHEN did not publish the origin of his stock.

Labeled regions of D. a. americana, D. a. texana *and* D. novamexicana: ^{125}I-5S RNA binds to the double-puff region of *D. a. americana*; however, the proximal portion of the double puff, X_{a-e}, is more heavily labeled than the distal portion, W_{c-j}—opposite to the labeling of the double puff in *D. virilis*, *D. montana* and *D. borealis* (Figure 3A–D). In *D. a. americana*, no sign of label appears in the region S_{d-f}. This 5S gene cluster, present in *D. virilis*, *D. montana* and *D. borealis*, seems to be totally missing. A similar labeling pattern to that of *D. a. americana* is found in *D. a. texana* (Figure 4A–B) and *D. novamexicana* (Figure 5A–C). COHEN's (1976b) pictures show a labeling pattern resembling ours in these species with the proximal portion of the double puff being much more heavily labeled than the distal portion, though he does not mention this in his text.

Relative numbers of 5S cistrons: D. melanogaster has 130–200 5S cistrons in the haploid genome (PROCUNIER and TARTOF, 1975). In *D. melanogaster* the 5S cistrons are probably restricted to 56F, in 2 to 4 bands (SZABO 1974). It is clear that the distribution pattern of the 5S clusters is more extensive in the species within the *virilis* group than in *D. melanogaster*. Autoradiographic silver grain numbers were determined on two slides of each species over the labeled regions—

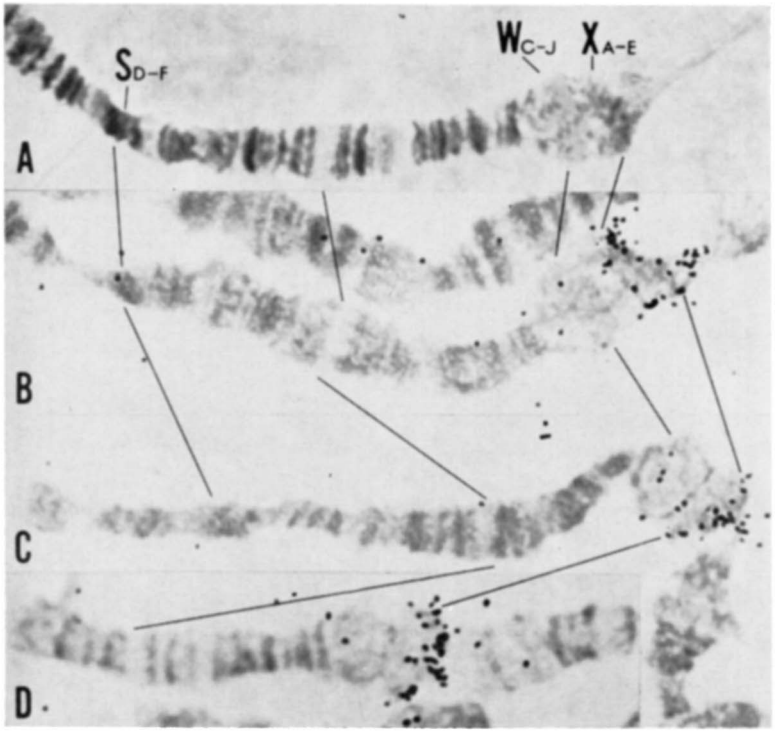

FIGURE 3.—Proximal third of chromosome 5 of *D. americana americana*. (A) Unlabeled and Giemsa stained. (B-D) Autoradiographs after hybridization with ^{125}I-5S RNA.

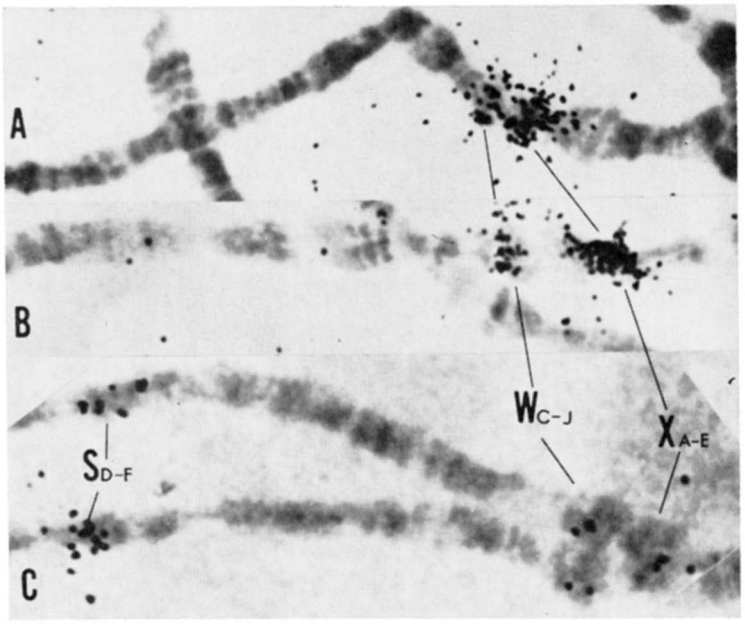

FIGURE 4.—Autoradiographs of the proximal third of chromosome 5 after annealing to ^{125}I-5S RNA. (A-B) *D. americana texana*. (C) Hybrid between *D. virilis* × *D. montana* ♂.

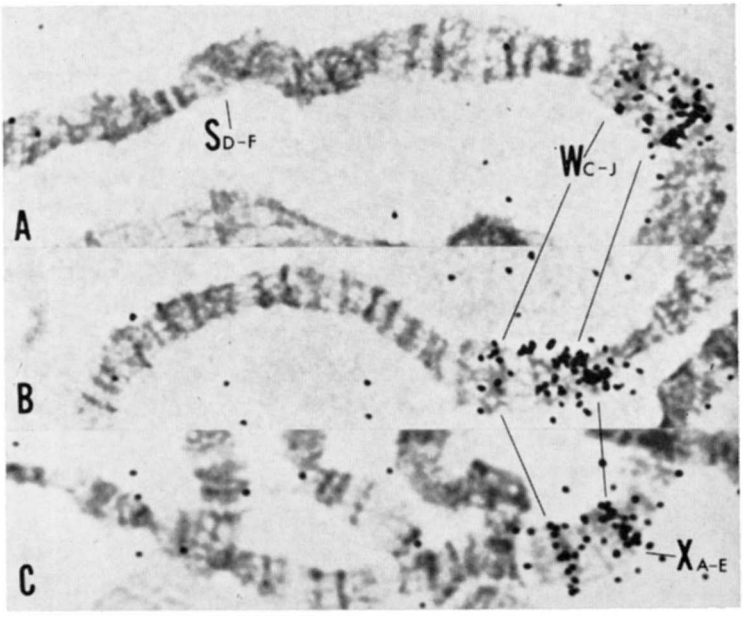

FIGURE 5.—(A-C) Autoradiographs of the proximal third of chromosome 5 of *D. novamexicana* after annealing to ^{125}I-5S RNA. No label is present at S_{d-f}.

TABLE 1

*Grain numbers over 5S gene clusters**

Slide & Species	Total grains P_p (X_{a-e})	P_d (W_{c-j})	$\frac{P_p}{P_p + P_d}$	Total grains B (S_{d-f})	$\frac{B}{B + P_p + P_d}$
A *virilis*	28	195	0.12	252	0.53
B "	77	422	0.15	517	0.51
A *montana*	45	165	0.21	375	0.64
B "	71	287	0.20	530	0.60
A *borealis*	202†		?	628	0.76
B "	(10) 463†	(51)	? (0.16)	938	0.67
A *americana*	508	75	0.87	0	
B "	758	104	0.88	0	
A *texana*	595	169	0.78	0	
B "	1068	265	0.80	0	
A *novamexicana*	756	198	0.79	0	
B "	830	155	0.84	0	

* 25 nuclei examined in each case.
† Proximal and distal puffs were not usually separable.

the proximal, X_{a-e}, and the distal portions, W_{c-j}, of the double puff, as well as the S_{d-f} band region. Table 1 is a summary of these data. If we accept the premise that the silver grain numbers reflect the numbers of 5S cistrons at each site, then we can conclude that there are clear differences between some of the species. *D. virilis* shows a pattern where there are many fewer cistrons in the proximal, X_{a-e}, than the distal portions, W_{c-j}, of the double puff. The band region S_{d-f} contains roughly the same number of cistrons as is found in the double puff. A similar pattern is found in both *D. montana* and *D. borealis*, with the distal portion of the double puff being more highly labeled than the proximal region. Because of poor preparations, we were often unable to separate the puffed region of *D. borealis* into two parts; however, in four cells where the double puff was clearly defined, 10 grains were found over the proximal puff and 51 over the distal. We thus conclude that the puff labeling probably falls into the *D. virilis* pattern. In a similar way, the band region S_{d-f} is also highly labeled in these species. Our data suggest that the relative numbers of 5S cistrons may be somewhat greater in the S_{d-f} regions of *D. montana* and *D. borealis* than in *D. virilis*.

The other clearcut pattern of 5S genes is found in *D. a. americana*, *D. a. texana* and *D. novamexicana*. In all cases, no grains were associated with the band region S_{d-f}, and the grains found over the double puff showed a reversed pattern when compared to *D. virilis*, with many more grains over the proximal puff, X_{a-e}, than over the distal puff, W_{c-j}.

Hybrids between species: In situ annealing with ^{125}I-5S RNA was carried out with three of the hybrid preparations, *D. virilis* crossed with *D. a. americana*, *D. a. texana* and *D. montana*.

D. a. americana × D. virilis ♂: Figure 6A–B shows the results of 5S RNA binding to the hybrid 5th chromosome. The *D. virilis* chromosome can be easily identified as having a labeled region at S_{d-f}, and in many instances the *D. a. americana* homologue was seen to have more label in the X_{a-e} region than did the *D. virilis* homologue. These hybrid preparations provide us with an opportunity to compare critically the band pattern of the two chromosomes, side by side. We have a pattern where we know 5S is present in the region S_{d-f} of *D. virilis* and absent from this region of the genome of *D. a. americana*. One might suspect that a band or bands would be present in *D. virilis* and not present in *D. a. amer-*

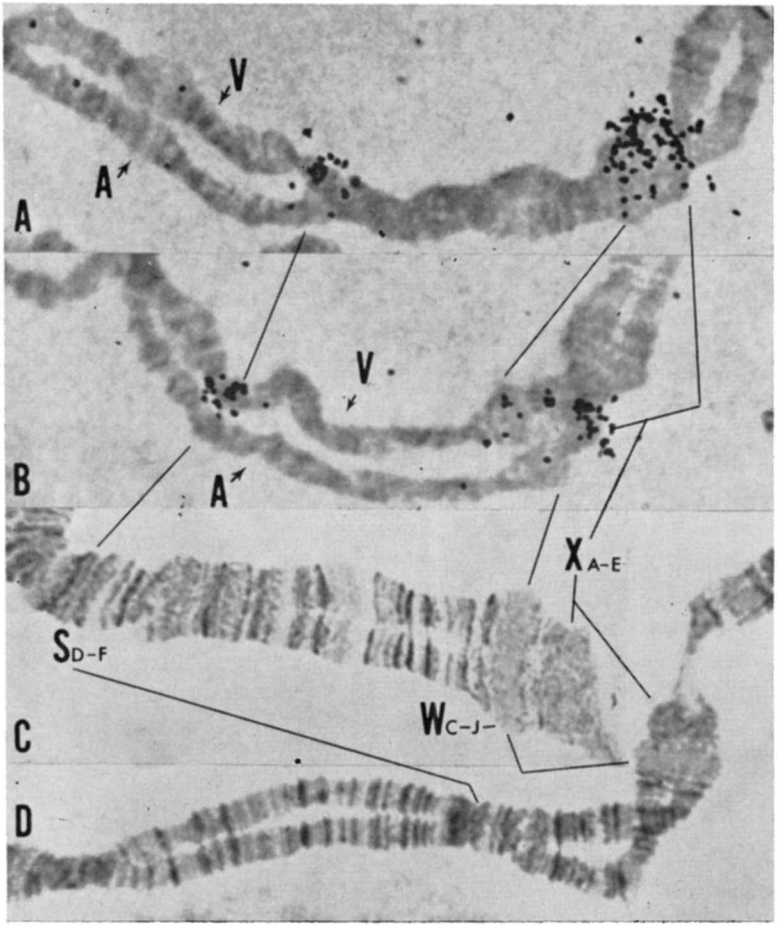

FIGURE 6.—Proximal third of chromosome 5 of the hybrid *D. americana americana* × *D. virilis* ♂. (A-B) Autoradiographs after annealing with ^{125}I-5S RNA. **A** indicates the *D. a. americana* chromosome and **V** the *D. virilis* chromosome. Region S_{d-f} is unlabeled on the *D. a. americana* chromosome. (C-D) Unlabeled Giemsa stained chromosomes. No band differences in the regions of the 5S gene clusters are detectable.

icana. No clear differences could be detected, nor could any visual differences be noted in the double-puff region where *D. virilis* apparently has more 5S cistrons in the distal portion of the double puff and *D. a. americana* has the reciprocal pattern (Figure 6C–D).

D. a. texana × D. virilis ♂: Our stock of *D. a. texana* carried a large homozygous inversion on the 5th chromosome of region K_{b-c} to V_{f-g} (see Hsu 1952). In hybrids between these species, the inversion loop is a prominent feature of that chromosome (Figure 7A–B). Figure 7A clearly shows that the labeled

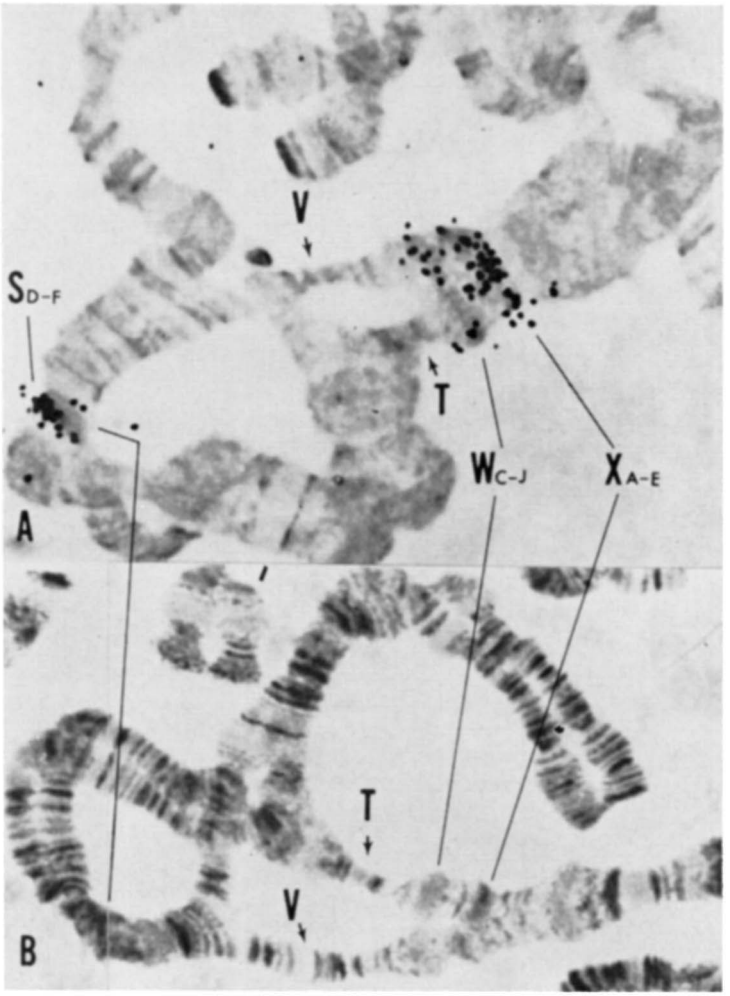

FIGURE 7.—Chromosome 5 of the hybrid between *D. americana texana* × *D. virilis* ♂. The chromosome of *D. a. texana* contains a large inverted region (K_{b-c}–V_{f-g}) when compared to *D. virilis*. (A) Autoradiograph after annealing to ^{125}I-5S RNA. Region S_{d-f} is labeled on the *D. virilis* chromosome and unlabeled on the *D. a. texana* chromosome. The chromosomes are twisted in the W–X region so that the heavily labeled X_{a-e} portion of the *D. a. texana* chromosome overlies and masks the *D. virilis* chromosome in this region. (B) Unlabeled and Giemsa stained preparation. The W–X region stains differently in the two chromosomes. **T** indicates the *D. a. texana* chromosome while **V** points to the *D. virilis* chromosome.

region, S_{d-f}, of the *D. virilis* chromosome is included in the rearrangement. In this hybrid we have seen the only evidence that thicker bands or more bands or both may be present in one and not the other species in the labeled regions. The proximal region of the double puff, X_{a-e} seems to show a more densely staining region in *D. a. texana* than in *D. virilis* (Figure 7B). This would be in keeping with greater numbers of 5S cistrons in the proximal than distal portion of the double puff of *D. a. texana* as deduced from the grain counts (Table 1). However, *D. a. americana* shows the same double-puff labeling pattern as *D. a. texana* and cytological preparations of hybrids with *D. virilis* do not show obvious differences (Figure 6C–D) between the two species. Perhaps very detailed, high resolution studies will reveal differences between many of these species in the areas binding 5S RNA, but so far our preparations show demonstrable band differences only between *D. virilis* and *D. a. texana*.

D. virilis × D. montana ♂ : Relatively poor cytological preparations resulted from this hybridization (Figure 4C). However, it was clear that the 5S gene clusters were very similar in the two species.

DISCUSSION

Taxonomy: According to PATTERSON and STONE (1952), the species in the *virilis* group fall into three subdivisions based on cytological and genetic tests. *D. virilis* is thought to be the most nearly primitive species and is evolving very slowly. It is considered to stand by itself as a subdivision. A second subdivision, consisting of *D. a. americana*, *D. a. texana* and *D. novamexicana*, has an intermediate rate of cytogenetic change. A third subdivision consists of a more rapidly evolving complex made up of *D. montana*, *D. borealis* and four other species. The patterns shown by the 5S gene clusters fall squarely into this biosystematic arrangement. *D. virilis* has three 5S gene clusters. *D. montana* and *D. borealis* also have three clusters but probably have a greater percentage of their 5S cistrons in region S_{d-f} than is found in *D. virilis*. Finally, *D. a. americana*, *D. a. texana* and *D. novamexicana* fall together with only two clusters in the double puff region.

Chromosome puffing: In *D. virilis* and its close relatives, the 5S gene clusters at bands W_{c-j} and X_{a-e} are seen to be in a well-developed double puff at late third instar. By contrast, the other 5S cluster at bands S_{d-f} in *D. virilis*, *D. montana* and *D. borealis* is usually not puffed. This implies that the double-puff region may be actively transcribing, while the other cluster is turned off or functioning at a very low level in these species. KRESS (1972) also notes that in *D. virilis* the region W_{c-j}–X_c is puffed before or after ecdysone injection, while the S region is not puffed. The 5S gene clusters in *D. melanogaster* (SORSA 1973) and in *D. hydei* (ALONSO and BERENDES 1975) are found in regions that are not puffed or only slightly in mid or late third instar. Furthermore, ultrastructural studies do not show evidence that they are actively transcribing. These results suggest that in some species active transcription of some of the 5S gene clusters may continue through third instar, while in others the clusters are shut down or have only marginal activity. In cases where there are multiple 5S clusters in other genera, the evidence shows that control of individual clusters may occur.

Glyptotendipes barbipes in particular, has three 5S gene clusters on chromosome *3* (WEN, LEÓN and HAGUE 1974; WEN unpublished). Studies with the incorporation of ^{3}H-uridine into salivary glands from fourth-instar larvae show that the main 5S cluster does not seem to be transcribing, while the other two are. Fine control of the 5S gene clusters is very likely in *Xenopus laevis* also, where there are multiple 5S sites at the ends of most of the chromosomes (PARDUE, BROWN and BIRNSTIEL 1973). In Xenopus kidneys, one major type of 5S RNA is produced which originates from about 3% of the total 5S genes (BROWNLEE, CARTRIGHT and BROWN 1974). In the ovaries, on the other hand, most of the 5S RNA has a slightly different base sequence from that in the kidneys (FORD and SOUTHERN 1973). It seems likely that the two or more types of 5S genes are in separate clusters.

Function of multiple clusters: Why have multiple 5S clusters when one will suffice in some organisms? It seems likely that there is some evolutionary advantage to having multiple clusters that may be turned on or off individually when more, less, or a different kind of 5S RNA is necessary for efficient cell metabolism. In *D. melanogaster* there is one 5S cluster and roughly 200 copies each of 5S and 18S and 28S cistrons (PROCUNIER and TARTOF 1975). These numbers seem consistent with efficient genetic function, since one molecule of each is necessary for a working ribosome (MOHAN 1975). We have no information on the numbers of ribosomal DNA cistrons found in *D. virilis* and its relatives. Another relationship of 5S cistrons to the 18S and 28S genes has evolved in amphibians where 18S and 28S gene amplification occurs in the oocytes. In *Xenopus laevis* approximately 24,000 5S cistrons are found in the haploid genome compared to about 400 for the 18S and 28S genes (BROWN and SUGIMOTO 1973). In a similar way, the salamander, *Notopthalmus viridescens*, has a very large number of 5S genes —about 300,000 per genome (PUKKILA 1975). In Xenopus the 15 or more 5S gene clusters are well documented (PARDUE, BROWN and BIRNSTIEL 1973), and in *N. viridescens* at least five 5S gene clusters are known (HUTCHISON and PARDUE 1975). Presumably these numerous copies of the 5S cistrons function during oogenesis when massive amplification of the 18S and 28S genes occurs. In contrast to amphibians, the oocyte of *Drosophila hydei* derives a large part of its ribosome population from the nurse cells that accompany each oocyte (RENKAWITZ and KUNZ 1975); no amplification beyond the usual polytenization is known to occur. The two or more 5S gene clusters found in the *D. virilis* group suggest that at some time during the ontogeny of the organisms a large amount of ribosomal RNA is necessary that cannot be generated from the 5S DNA present in one cluster; whether there is a concomitant amplification of the 18S and 28S ribosomal DNA at some time is not known.

Origin of the multiple clusters: WEN (unpublished) suggested that the multiple 5S gene clusters in chromosome *3* of *Glyptotendipes barbipes* may have originated from one ancestral cluster through inversions. Indeed, the breakpoints of a naturally occurring inversion found in several populations of these midges are close to or at two of the 5S gene clusters. In contrast to most species of Drosophila, *D. virilis* does not show inversions in the natural populations. HSU

(1952) notes that by 1952 over 4000 individuals from many different strains had been tested and no inversions had been found. However, the other species in the *virilis* group do commonly have inversions. Only one, in *D. a. americana*, comes close to the 5S gene clusters at one end; HUGHES (1939) notes that some strains carry region 88A-97B inverted. HUGHES' region 97–98 includes the W–X area of HSU (1952) and 97B would come close to the distal end of the 5S gene cluster in the double puff. No known inversion breakpoints in any of the *virilis* group of species falls close to the S region. Nevertheless, staying within traditional cytogenetic thinking, it would seem likely that the multiple 5S gene clusters were generated by inversions, translocations or both.

The 5S clusters within the double puff could very well have arisen through a gross tandem duplication *via* unequal crossing over. However, our grain count data show that the number of cistrons within the two parts of the double puff are probably not equivalent in any of the species. The pattern in one complex is that the distal portion of the double puff is more heavily labeled, while in the other subdivision the proximal portion is heavily labeled. Additional rounds of unequal crossing over within this double-puff region could very well have produced the current patterns after the initial event produced the two clusters.

Following the initial production of the two clusters, the two reiteration patterns could have been generated by a short inversion in the W–X region. We examined this possibility by cytologically inspecting hybrids between taxa having differences in 5S cistron redundancy in the double-puff region, *e.g.*, *D. a. americana* × *D. virilis*. Such hybrids were usually well paired in the puff region, which suggests that an inversion was not responsible for the two patterns. Our selection of photographs for this paper emphasized the rare cases where pairing in the hybrids is not intimate (Figures 6 and 7).

Another possible mechanism for increasing or decreasing the reiteration of the 5S genes is that of magnification-reduction, as reported by PROCUNIER and TARTOF (1975). They discovered that, when a chromosome carrying the normal number of 5S genes in *D. melanogaster* was placed opposite a 5S deleted chromosome, a magnification of the 5S genes occurred in the total DNA. The molecular basis of the magnification phenomenon is probably unequal sister-chromatid exchange, if we can extrapolate from the events that occur with 18S and 28S genes of *D. melanogaster* (TARTOF 1974).

Band differences between species: We were surprised that more obvious band differences did not show up between species that had major differences in 5S DNA clusters. The haploid equivalent of an average band in *D. melanogaster*, as LAIRD (1973) points out, contains about 20,000 base pairs. Fifty copies of the 5S genes in a cluster which code directly for the 5S RNA (120 nucleotides) would account for 6000 base pairs, or about 30% of the total band DNA. The length of the DNA spacers is not known, but they may be quite long. For example, in 5S DNA from *Xenopus laevis* only about 14% of the base pairs are in 5S RNA coding regions and the rest are spacers (BROWN and SUGIMOTO 1973). Thus, a band or chromomere could very well be devoted to a moderately large 5S gene cluster and the associated spacer DNA. The hybrid between *D. virilis*

and *D. a. americana* showed no differences that we could detect between the chromosome bands in the regions of the 5S gene clusters (Figure 6C-D). The S_{d-f} region appeared to be homologous between *D. virilis* and *D. a. americana*, yet this 5S cluster is apparently present in *D. virilis* and absent in *D. a. americana*. There remains the remote possibility that 5S genes are present in the *D. a. americana* chromosome at S_{d-f}, but below the limit of resolution of our system. The double puff (W-X region), too, is very similar despite the fact that the species have a different labeling pattern in this area. However, precise matching in puffed regions can be exceedingly difficult. HUGHES (1939) prepared hybrids of *D. virilis* × *D. virilis americana* (the latter species was reclassified as *D. a. americana* later) and carefully examined preparations of all the chromosomes. His photographs and camera lucida drawings of hybrid chromosome 5 do not show any band differences in the 5S regions. Although Hughes was using a different strain of *D. a. americana* from the one we used, our results corroborate his observations.

These observations on the chromosomes of *D. virilis* and *D. a. americana* in the 5S regions proved to be an enigma. Differences must be there, but the extent of the differences are below the limit of our cytological resolution. On the other hand, the hybrids between *D. a. texana* and *D. virilis* did show different cytological detail within the W–X double puff region. The *D. a. texana* chromosome clearly stained more heavily in the proximal portion of the double puff than the *D. virilis* chromosome. It seems likely that this is a reflection of differences in the 5S gene cluster.

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Corresponding editor: M. L. PARDUE