A MOLECULAR EXPLANATION OF THE BOBBED MUTANTS OF DROSOPHILA AS PARTIAL DEFICIENCIES OF "RIBOSOMAL" DNA¹

F. M. RITOSSA², K. C. ATWOOD, AND S. SPIEGELMAN

Department of Microbiology, University of Illinois, Urbana

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IN this paper we offer an explanation of the genetic feature known in *Drosophila* as bobbed (*bb*). Experiments will be described in which DNA preparations from flies carrying various *bb* alleles were assayed for their content of the special DNA ("ribosomal"-DNA) that forms molecular hybrids with *Drosophila* ribosomal RNA. In view of the differences found between *bb* and wild type, and the special role attributed to this DNA, rather coherent relations appear among long-known characteristics of *bb*. At the outset we review these characteristics, since each has some degree of relevance to the explanation to be developed.

Relation of bb to the X and Y chromosomes of D. melanogaster: When described in *D. melanogaster* by BRIDGES in 1915, *bb* appeared as a sex-linked recessive expressed only in females. STURTEVANT (in 1920) mapped the locus in the right (proximal) end of the X chromosome and, in order to explain the sex-limited expression, proposed that the Y chromosome carried a wild-type allele (see BRIDGES and BREHME 1944). STERN (1927) showed that XO males carrying *bb* had the phenotype, whereas XXY females with *bb* on both X chromosomes did not. Subsequently, several mutant alleles on the Y chromosome were discovered, completing the evidence that the bobbed locus in *D. melanogaster* is homologous in the X and Y chromosomes.

Hypomorphism of bobbed: Small bristles, and sometimes etching of the abdominal tergites, are the characters useful in classification of *bb* as a marker. The severity of the phenotype varies among flies bearing a given set of alleles, and varies still more among different mutant alleles. STERN (1929) in a classical quantitative study showed that the bristle length *increased* with the dosage of *bb*, and that different alleles had characteristic bristle-forming strengths which when added together predicted remarkably well the bristle lengths achieved with various combinations of alleles. Thus, the mutant alleles of *bb* can be described in the terminology of MULLER (1932), as hypomorphs; they do the same thing as the wild-type, but not so effectively.

Lability of the bb locus: The laboratory history of *bb* stocks frequently—perhaps usually—includes progressive decrease in the severity of the phenotype, or reversion to wild type. Some apparent reversions have been reported to result

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² On leave from the International Laboratory of Genetics and Biophysics, Naples, Italy.

from the presence of Y chromosomes among the females. We can find no published mention of the extent to which modifiers can attenuate the phenotype, but rather some reason to surmise that modifiers have not been the most important factor in the deterioration of *bb* stocks. Recovery of extreme alleles, after outcrossing, has not been a noteworthy feature of experiments in which *bb* has been used as a marker, although many opportunities to observe such effects of outcrossing must have presented themselves. It seems at least plausible, therefore, to suppose that less extreme alleles frequently originate and are selected. A sufficient basis for selection against *bb* is provided by another aspect of the phenotype: the time of development is prolonged, roughly in proportion to the severity of the visible manifestations.

A high frequency of *bb* among wild-type *D. hydei* was discovered by SPENCER (1944) by means of crosses to a *bb* tester stock. Alleles recovered in this way were often distinguishable from one another in the degree of their effect. The high frequency, despite adverse selection, suggests that *bb* originates from wild type, and perhaps strong alleles can originate from weak, at rates at least comparable to those of the reverse processes. The variety of alleles suggests that they arise by a process that can seldom produce exactly the same result on different occasions.

Relation of bb to the nucleolus organizer: The cytological map position of *bb* is in the neighborhood of the nucleolus organizer (NO), (COOPER 1959). In *D. melanogaster*, the published literature does not provide an unequivocal instance indicating that *bb* and NO are actually separable. In one case where a natural separation of *bb* and NO might have been observed, it was not. Thus, in *D. ananassae*, KAUFMANN (1936) found the Y and fourth chromosomes, but not the X, to be associated with the nucleolus. KIKAWA (1938) showed that *bb* in this species is similarly confined to the Y and fourth chromosomes, although the X has, with respect to other markers, substantial homology with the X in other *melanogaster* groups.

In summary, these observations give the impression that *bb* is a locus in which many hypomorphic alleles are possible and in which mutation rates in both directions are unusually high. The retardation of development in *bb* homozygotes indicates that the function of the locus is not specific to integumentary structures, but involves a necessary biosynthetic mechanism. This impression is confirmed in the case of an extreme allele, *bb^l*, which is lethal when homozygous and hence relates to an essential function, although it can be distinguished from a complete deletion of the locus by its additive interaction with other alleles. (STERN 1929; SPENCER 1944). Finally, the association of the locus with the nucleolus organizer assumes a particular significance in view of the recognized function, and especially the redundancy, of that region.

The relation of the nucleolus organizer to the redundant segment of r-DNA: Molecular hybridizations between *Drosophila* DNA and ribosomal RNA (r-RNA) have shown that about 0.27% of the DNA is complementary to r-RNA (VERMEULEN and ATWOOD 1965; RITOSSA and SPIEGELMAN 1965). This special DNA is referred to as r-DNA. From the total DNA per haploid genome, estimated by

RUDKIN (1963) to the 2×10^{11} daltons, and the mol wt of the combined 18S and 28S r-RNA, it can be calculated that the amount of r-DNA is sufficient to provide 130 templates for the transcription of each of the two kinds (28S and 18S) of r-RNA. In other words, the r-DNA is highly redundant. By the use of DNA from flies with different numbers of NO regions, RITOSSA and SPIEGELMAN (1965) obtained strong evidence that all the r-DNA is closely associated with the nucleolar organizer.

A unifying hypothesis relating bb to the nucleolus organizer: In view of the foregoing evidence on the redundancy and localization of r-DNA, let us consider the properties that might be expected of NO as a genetic locus. A complete deletion corresponds to the anucleolate mutant of *Xenopus* or the *scⁱ-sc^s* deletion in *Drosophila*, and is lethal when homozygous. Partial deletions would be expected to form a large class of alleles with different degrees of hypomorphism, affecting the rate of development, and ranging from lethal to wild type in phenotypic effect. Because a tandem redundancy affords the opportunity for unequal crossing over, the locus may appear to be unusually labile. These characteristics can now be seen to correspond remarkably well to the reported properties of the *bb* locus. To account for this, and the close association of *bb* and NO, we propose a unifying hypothesis: the *bb* locus and the nucleolus organizer are different names for the same redundant segment of DNA containing the complements of ribosomal RNA.

According to this hypothesis, the principal genetic basis of *bb* is *partial deletion* of NO. The lability of *bb* is attributed to processes equivalent to unequal crossing over, and the phenotype to a limitation of ribosomal RNA synthesis. A prediction essential to the hypothesis is that DNA from *bb* flies will usually contain a subnormal proportion of r-DNA. Our experimental results verify this prediction.

MATERIALS AND METHODS

Growth medium: The various stocks were raised on a medium made by adding the following in grams to 1 liter of H₂O; cornmeal (100); sucrose (100), or 100 ml of molasses (Brer Rabbit, Gold Label); fresh baker's yeast (100) (heat-killed during preparation); agar (10); methyl-*p*-hydroxybenzoate (2.7). The flies were collected at the adult stage and stored at -14°C for use as a source of DNA.

Isotopic labeling: To achieve a high level of labeling, the standard medium was modified to contain 0.5 g of yeast per 10 ml, an amount calculated to be the minimum necessary to support the growth of 2 to 3 g of larvae, corresponding to approximately 2 to 3 mg of RNA. To 10 ml of the modified medium 7 mc of H³-uridine (21 c/mmole, Nuclear Chicago) were added. In labeling experiments the eggs were collected with a brush, washed, and located on wet strips of blotting paper which were laid on the surface of the labeled medium. After 7 to 8 days growth, larvae were harvested by MEADE's (1964) method for extraction of the RNA.

Final preparations of pure ribosomal RNA had a specific activity ranging from 60,000 to 110,000 count/min per μg . All counting was done in a liquid scintillation counter on nitrocellulose membranes permitting assay of P³² and H³ in the same samples.

RNA extraction and purification: After repeated washing with water, the larvae were suspended in 10 volumes (w/v) of an extraction fluid which contained NaCl (0.1 M), 1% sodium dodecyl sulphate (SDS), and heparin (0.001%). The suspension was homogenized in a glass homogenizer kept at 0°C, following which one volume of phenol saturated with 0.1 M NaCl and containing heparin (0.001%) was added and the mixture shaken for 20 min at 0 to 4°C. This

was then followed by a centrifugation and removal of the aqueous layer. The phenol treatment was repeated three more times, the first for 20 min and the others for 10 min each. The phenol was then removed from the aqueous layer with two ether extractions and the ether eliminated by bubbling air through the solution. The RNA was then precipitated by the addition of two volumes of a cold 80% ethanol containing Na acetate (.02 M), taken up in a buffer (0.1 M NaCl; .05 M phosphate, pH 6.8) and passed through a methylated albumin column as detailed by YANKOVSKY and SPIEGELMAN (1963). The ribosomal fractions were pooled and dialyzed 24 hr at 4°C against 100 volumes of $2 \times \text{SSC}$ (SSC is 0.15 M NaCl, .015 M Na citrate pH 7.4).

Preparation of DNA: The DNA employed was extracted exclusively from adults suspended in 10 volumes (w/v) of the following buffer at pH 7.6: Tris (.05 M), KCl (0.025 M), Mg acetate (.005 M), sucrose (0.35 M). Homogenization was carried out in a mortar and the homogenate filtered through 8 layers of gauze. The resulting filtrate was centrifuged for 10 min at 1,000 rpm in the International Centrifuge (Rotor 253). The pellet was resuspended in 0.15 M NaCl, 2% SDS, and 0.1 M EDTA, and adjusted to pH 8. For each 10 g of flies, about 35 ml of this medium were used and the resulting suspension was shaken for 10 min at 60°C. All subsequent steps followed the procedure detailed by MARMUR (1961) with the exception that at the first precipitation one volume (rather than two) of cold ethanol was added. This avoided interference by salt precipitation and increased the yield. Fibers were collected on a glass rod and any flocculent DNA precipitate remaining behind was centrifuged, resuspended in SSC to which 1 volume of ethanol was added yielding fibrous precipitates. The fibrous DNA, dissolved in SSC, was subjected to a 2 hr digestion at 37°C with heated (100°C for 10 min) RNAase (Sigma $5 \times$ crystallized) at 150 $\mu\text{g}/\text{ml}$.

At this stage there was consistent contamination with a polysaccharide detected by Model E Schlieren optics in CsCl equilibrium gradients as a hyperfine band with no absorption at 260 $m\mu$. To remove this material, the preparation was digested for 45 min at 37°C with 250 $\mu\text{g}/\text{ml}$ of alphaamylase ($2 \times$ crystallized, Worthington). This step effectively avoided any subsequent difficulties in the final purification of DNA. The enzymes were removed by adding SDS (1%) and two phenol treatments at room temperature, followed by two successive deproteinizations with chloroform-isoamyl alcohol for 10 min each. The DNA was precipitated by the addition of two volumes of ethyl alcohol and the fibers collected and dissolved in $1/100 \text{ SSC}$.

Purity of the final DNA preparations was monitored by optical density measurements at 230, 260, 280 $m\mu$, and analysis for ribose and deoxyribose. It yielded a single peak in a CsCl gradient at a position corresponding to 38% GC.

Alkaline denaturation of the DNA was carried out in $1/100 \times \text{SSC}$ at a concentration of 150 $\mu\text{g}/\text{ml}$. The pH was adjusted to 12.2 with freshly prepared 1 N NaOH, the solution left to stand at room temperature for 10 min, followed by readjustment of the pH to 7. Denaturation was complete as measured by either hyperchromicity or adherence to nitrocellulose membrane filters.

Hybridization and detection of hybrid structures: The method of GILLESPIE and SPIEGELMAN (1965) was used. It involves three steps: *Step 1—Irreversible fixation of DNA to filters:* The denatured DNA is dissolved in a solution of $6.6 \times \text{SSC}$ at a concentration of between 5 and 10 $\mu\text{g}/\text{ml}$. Nitrocellulose filters (SCHLEICHER and SCHUELL, B-6, 27 mm) are first soaked in $6.6 \times \text{SSC}$ and 10 ml of the same buffer is then passed through the filter, following which the appropriate amount of the DNA solution is filtered through. The amount of DNA actually retained is always monitored by measuring the OD of the DNA solution before and after the filtration. The loaded filters are allowed to dry at room temperature and then incubated in a vacuum dessicator at 80° for approximately 4 hr. When monitored with radioactive DNA, no detectable DNA (less than 1%) is lost during any of the subsequent steps involving washing, hybridization, or enzyme treatment. *Step 2—Hybridization:* The hybridization is carried out by immersing the loaded filter into a $2 \times \text{SSC}$ solution (3ml) of the labeled RNA at the desired concentration and incubated in a stoppered vial at 65°. The incubation period was 12 hours. *Step 3—Removal of the unpaired RNA:* The filters are removed from the hybridizing mixture and washed with $2 \times \text{SSC}$ on both sides. They are then placed in a solution containing RNAase (free of DNAase) at a level of 20 $\mu\text{g}/\text{ml}$ in $2 \times \text{SSC}$ and allowed to digest at 30° for one hour. After

the digestion, the filters are again removed and washed with 60 ml of $2 \times \text{SSC}$ on *both* sides, dried and counted.

It is of obvious importance to monitor the degree of contaminating "noise" and this was accomplished by including in the hybridizing mixture a heterologous RNA (*E. coli*) carrying a different identifying isotopic label (P^{32}). The amount of P^{32} found on the filter after Step 3 provides a measure of the noise level. In the experiments to be described, this noise level is negligible.

Sources of DNA: The following stocks were obtained from the Oak Ridge National Laboratory: *car bb*; $\gamma w bb^{ds}$; $w^a bb^l/\gamma f/B^sY$; $In(1)sc^{4L,8R}, \gamma sc^{4+8} cv v f/RA, \gamma f/Y$; $In(1)sc^{81L,4R} sc^{81} cv v B/RA, \gamma f/B^sY$. A stock called uco 3 was obtained by selection from Urbana stock, as described in RESULTS (paragraph 3). The *car bb* and $\gamma w bb^{ds}$ stocks did not show strong *bb* phenotype when received. These stocks were selected for *bb* for several generations. A B^s labeled Y chromosome from the $w^a bb^l$ stock was introduced into the $\gamma w bb^{ds}$ stock to monitor the presence of extra Y chromosomes. Analysis of the lethality of the bb^l allele was done as follows: $In(1)sc^4 sc^8/In(1)sc^4 sc^8/B^sY$ were crossed to $w^a bb^l/B^sY$. Only females with B^s eyes were obtained from this cross, and in subsequent generations, indicating that the bb^l mutation is lethal when homozygous.

RESULTS

A number of *bb* alleles of independent origin are required to test the central prediction of the hypothesis. The origins of the four stocks used was described in METHODS (last paragraph). Before being used in the experiments *car bb* and $\gamma w bb^{ds}$ stocks were rendered somewhat more homogeneous and less wild type by means of selection. We now describe the results obtained when DNA preparations from flies carrying *bb* alleles were assayed for DNA complementary to ribosomal RNA.

Carnation-bobbed DNA: Derived stocks of *car bb* used in the experiments had a large but variable proportion of females classifiable as *bb*. Saturation plateaus were obtained with DNA taken from males and females separately. No attempt was made to separate phenotypically wild-type females from the flies used for the preparation of DNA. The results are shown in Figure 1. and each experiment represents a different batch of flies from a derived *car bb* stock. Since the DNA was prepared whenever enough flies (about 10g) had been accumulated, the

FIGURE 1.—Saturation of *car bb*-DNA by H^3 -r-RNA.

populations used in the different experiments may have differed with respect to phenotype and elapsed time to eclosion. The r-DNA proportion in females ranged from 30 to 55% of the normal wild-type value of 0.27%. The DNA of males (which were all phenotypically normal because of the Y chromosome) contained higher proportions of r-DNA, 65 to 80% of normal. Males and females were not taken from exactly coincident populations, hence we have no reason to prefer any particular pair of experiments for the purpose of comparison. The observed values for males, however, fall within the range to be expected on the assumption that their X chromosomes were representative of those present in the females, and that the Y chromosome contains as much r-DNA as the wild-type X (RITOSSA and SPIEGELMAN 1965).

DNA from bobbed selected from wild type: Selection of *bb* from wild type was facilitated by crossing wild-type females to *sc⁴ sc⁸* deletion males; *bb* phenotypes were regularly found among the last deletion heterozygotes to eclose. Since the experiment was not designed to distinguish between *de novo* origination and previously accumulated alleles, the frequency with which *bb* was recovered from this cross (23% of the X chromosomes tested) is not a direct indication of the lability of the locus. A homozygous *bb* stock, designated uco 3, was established from five of the most severe *bb* deletion heterozygotes. This stock may contain alleles of more than one origin but, in any case, the origin is different from that of any other stock used. In Figure 2, saturation plateaus with DNA from uco 3 are shown for two batches of females and one of males. The results are similar to those obtained with *car bb*. It is possible to attribute the difference between the two experiments on females to causes similar to those implied in the case of *car bb*; that is, to real heterogeneity among the samples from which the DNA was obtained.

DNA from bobbed "deficiency sensitive": Stocks derived by selection *bb^{ds}* were

FIGURE 2.—Saturation of uco-3-DNA by H^3 -r-RNA. This bobbed stock was obtained as described in METHODS.

FIGURE 3.—Saturation of $\gamma w bb^{ds}$ -DNA by H^3 -r-RNA. The Y chromosome of the original stock was replaced with a Y^{Bs} in order to monitor possible accumulation of extra Y.

persistently heterogeneous in phenotype and never contained a high frequency of deficiency-sensitive alleles. Although we have retained the symbol bb^{ds} to designate these stocks, they differ from the original bb^{ds} in producing viable $bb^{ds}/sc^4 sc^8$ heterozygotes. Figure 3 shows saturation plateaus for a derived population in which the female parents, but not the flies used for DNA preparation, were selected for *bobbed* phenotype. The r-DNA content of females is subnormal, 0.18%. In the males the r-DNA contribution of the Y chromosome alone is expected to be 0.135% of the total DNA. An X chromosome representative of those in the females would have contributed about 0.09%; that is, half the female saturation value. Thus the observed 0.25% in males exceeds somewhat the expected 0.225% in this instance.

DNA from the bb^1 homozygous lethal allele: The bb^1 allele proved to be lethal when homozygous and, as expected, lethal with the $sc^4 sc^8$ deletion which contains no r-DNA. It was of interest, therefore, to see whether bb^1 could be distinguished from the full deletion by its r-DNA content. Males that were bb^1/Y were crossed to bb^{ds} homozygotes of approximately uniform *bb* phenotype. At the same time $sc^4 sc^8/Y$ males were crossed to similarly selected females bb^{ds} from the same population. Thus, saturation plateaus for bb^{ds}/bb^1 and $bb^{ds}/Df sc^4 sc^8$ afford an estimate of the r-DNA content of bb^1 on the assumption that bb^{ds} is approximately equivalent in the progeny of the two crosses. In the first experiment, shown in Figure 4, the female flies for DNA preparation were collected in bulk without exclusion of wild-type exceptions. The experiment was repeated in the same way except that the DNA was prepared from flies selected to exclude those containing extra Y chromosomes or any other phenotypic revertants. The proportions of r-DNA were lower, as shown in Figure 5. Evidently the bb^1 allele is associated with a partial rather than complete deletion of r-DNA. A similar

FIGURE 4.—Comparison of saturation r-RNA plateaus of DNA carrying bb^1 with that carrying $sc^4 sc^8$ when each is combined with bb^{ds} . Females ($\gamma w bb^{ds}$) were separately crossed with males $Y^{B8}/w^a bb^1$ and males $Y/sc^4 sc^8$. The DNA from the daughters were hybridized to H^3 -r-RNA. For comparison, a hybridization was also performed with DNA from males carrying three NO doses ($Y/sc^{S1L} sc^4 R$).

FIGURE 5.—Comparison of r-RNA saturation plateaus of DNA carrying bb^l with DNA carrying $sc^4 sc^8$ when each is combined with bb^{ds} . This is the same cross as the experiment of Figure 4. However, here the females were carefully screened for the bobbed phenotype.

FIGURE 6.—Comparison of r-RNA saturation plateaus of DNA from females bb^{ds}/bb^l and DNA from males (bb^{ds}/Y^{Bs}). Females $\gamma w bb^{ds}$ were crossed with $w^a bb^l/Y^{Bs}$ males. The male and female progeny were separated and the DNA prepared from each.

inference can be made from an experiment in which bb^{ds} homozygotes were crossed to bb^l/Y^{Bs} males. The marked Y chromosome facilitated the exclusion of XXY females. Assuming that the Y^{Bs} chromosome has a normal r-DNA content, and that bb^{ds} is equivalent in the sibs, comparison of the saturation plateaus for bb^{ds}/bb^l and bb^{ds}/Y^{Bs} (Figure 6), indicates that the r-DNA contribution of bb^l is about 0.04% of the total DNA. This is in fair agreement with the second of the preceding experiments on bb^l . If, as we suspect, the first experiment gave a spuriously high value for bb^l , it may be tentatively concluded that deletion of about 70% of the region passes the threshold of lethality when homozygous.

Summary comparison of DNA from bobbed and wild type: In Table 1 the available data on bobbed are compared with those of similar experiments on flies in which all the NO regions are wild type with respect to bb . For easier comparison, the same data are given in Figure 7 as a rank order plot of r-RNA/DNA obtained with wild type and bobbed DNA. Where the regions are alike, the calculated r-DNA per NO region is the ratio of the saturation level to the number of NO regions. In $bb/+$ the content of the bb mutant region is calculated on the assumption that any normal region present contributes 0.135%. In bb^{ds}/bb^l the r-DNA content of bb^l is estimated with reference to that of bb^{ds} determined in a parallel experiment.

The data are unselected and hence provide an estimate of the variability of wild-type r-DNA content, including experimental error. The results summarized in Table 1 and Figure 7 leave no doubt that the r-DNA content of the mutant alleles of bb is significantly lower than that of wild type. The relatively homogeneous results with wild type permit the conclusion that the greater variability of bb (e.g., Figures 1, 2) is not caused by experimental error, but by differences in r-DNA content among batches of flies from the same stock. The origin of such

TABLE 1

Summary of RNA/DNA ratios obtained with DNA from bobbed and wild-type flies of various genetic constitution

Source of DNA	Nucleolus organizer regions, + and <i>bb</i>	(r-RNA/DNA) $\times$ 100 at saturation	(r-DNA/DNA) $\times$ 100 per NO region [‡]
♀ (4)	4+	.472	.118
♂ (3)	3+	.393	.131
		.402	.134
		.399	.133
		.420	.140
		.368	.123
♀ (2)	2+	.284	.142
		.317	.159
♂ (2)	2+	.283	.142
		.244	.122
♀ & ♂ (2)	2+	.251	.126
		.249	.125
		.253	.127
♂ (1)	1+	.140	.140
		.188	.188
Average = 0.137 $\pm$.018			
<i>car bb</i> ♀	2 <i>bb</i>	.122	.061
		.090	.045
		.136	.068
<i>car bb/Y</i> ♂	1+, 1 <i>bb</i>	.199	.064 <i>bb</i> only
		.211	.076 <i>bb</i> only
uco3 ♀	2 <i>bb</i>	.098	.049
		.149	.075
uco3 ♂	1+, 1 <i>bb</i>	.210	.075 (<i>bb</i> only)
<i>bb^{ds}</i> ♀	2 <i>bb</i>	.172	.086
		.184	.092
<i>bb^{ds}/Y</i> ♂	1+, 1 <i>bb</i>	.250	.115
<i>bb^{ds}/Y^{Bs}</i> ♂	1+, 1 <i>bb^{ds}</i>	.220	.085 (<i>bb^{ds}</i>)*†
<i>bb^{ds}/bb^l</i> ♀	1 <i>bb^{ds}</i> , 1 <i>bb^l</i>	.125	.040(<i>bb^l</i>)*†
<i>bb^{ds}/sc⁴ sc⁸</i> ♂	1 <i>bb^{ds}</i>	.102	.102(<i>bb^{ds}</i>)*
<i>bb^{ds}/bb^l</i> ♀	1 <i>bb^{ds}</i> , 1 <i>bb^l</i>	.176	.074(<i>bb^l</i>)*
<i>bb^{ds}/sc⁴ sc⁸</i> ♂	1 <i>bb^{ds}</i>	.065	.065(<i>bb^{ds}</i>)*†
<i>bb^{ds}/bb^l</i> ♀	1 <i>bb^{ds}</i> , 1 <i>bb^l</i>	.098	.033(<i>bb^l</i>)*†
Average = 0.071 $\pm$.022			

* Parallel experiments.

† Nondisjunctional Y chromosomes excluded.

‡ In calculating r-DNA/DNA ratios for *bb*, the value for + was rounded to 0.135.

FIGURE 7.—Rank order plot of RNA/DNA per NO obtained with bobbed and wild type of various genetic constitutions. The data are taken from the last column of Table 1.

differences was not investigated, but we surmise that differences in the proportion of nondisjunctional Y chromosomes and in the rank order of eclosion may have been influential.

DISCUSSION

Corroborative evidence relating r-DNA to the nucleolus organizer: The association of r-DNA with the NO is suggested not only by the dosage effect of the *sc^ssc^s* region—which includes both NO and *bb*—in r-RNA annealing experiments, but also by several independent lines of evidence that lend considerable generality to the concept. Since it has not been rigorously demonstrated that the r-DNA occupies precisely the site of the NO, the likelihood that this is the case rests upon these several lines of evidence taken together.

Radioactive label incorporated into r-RNA precursors can be detected autoradiographically in the nucleolus before it appears elsewhere in the cell (PERRY 1962). Partial inhibition of RNA synthesis with actinomycin-D can lead to a relatively greater impairment of r-RNA than of total RNA synthesis, and under these conditions the appearance of label in the nucleolus is preferentially inhibited (PERRY 1962).

Such experiments identify the nucleolus as the initial repository of newly synthesized r-RNA precursors if not the actual site of synthesis. Since the r-DNA is necessarily the site of synthesis of r-RNA, the presence of r-DNA in or attached to the nucleolus would indicate that nucleolar r-RNA is produced locally rather than recruited from more or less remote sources. The DNA from isolated nucleoli of HeLa cells was found by McCONKEY and HOPKINS (1964) to be significantly

enriched in r-DNA. A prior experiment of the same sort in *Pisum* (CHIPCHASE and BIRNSTIEL 1963) failed to reveal such enrichment. In view of the technical difficulties involved in the isolation of nucleolar DNA, this is an instance in which a positive result is significant whereas a negative result is inconclusive.

Both the r-RNA synthetic role of the nucleolus and the spatial proximity of r-DNA to NO have been demonstrated by means of experiments in which genetic manipulation of the region replaced the more difficult procedures of physical isolation. Anucleolate lethal homozygotes of *Xenopus* were shown by BROWN and GURDON (1964) to synthesize no r-RNA although synthesis of amino acid transfer RNA (t-RNA) proceeded normally. Following the demonstration by RITOSSA and SPIEGELMAN (1965) that in *Drosophila* the proportion of r-DNA per genome was directly related to the dosage of the *sc^ssc^s* segment containing the "NO" region, WALLACE and BIRNSTIEL (1966) compared the r-DNA content of wild-type *Xenopus* with anucleolate heterozygotes and homozygotes. The heterozygotes had about half the normal amount of r-DNA, and the homozygotes had a negligible quantity that may well be attributed to background noise. If the anucleolate mutant of *Xenopus* is a deletion of NO, the extent of the deletion beyond the boundaries of NO is not known and hence it cannot be stated with absolute certainty that the r-DNA is in NO rather than closely adjacent to NO. Even so, the obviously close association of r-DNA and NO in such phylogenetically remote organisms as *Xenopus* and *Drosophila* suggests that the association is necessary rather than fortuitous.

The redundancy of r-DNA provides a noteworthy point of correspondence between r-DNA and NO, since it has been shown on several occasions that NO is itself redundant. Chromosome rearrangements have been reported in which one of the breaks transects NO, resulting in widely separated but functional organizers derived from a single organizer. (McCLINTOCK 1934; BEERMANN 1960; KRIVSHENKO 1959). In particular, BEERMANN (1960) was able to test the functional sufficiency of NO fragments of unequal length obtained in *Chironomus* by radiation-induced breakage and rearrangement. These fragments were associated with nucleoli and were individually capable of supporting development, whereas the anucleolate zygotes were, of course, lethal. This clearly demonstrates the redundancy of NO as a cytological and functional entity. Since an r-RNA synthetic function must be attributed to each isolated nucleolar fragment, bizarre assumptions concerning the location of r-DNA would be required to avoid the conclusion that fragmentation of NO is equivalent to fragmentation of a redundant segment of r-DNA. The assumption that r-DNA—known to be in the vicinity of NO and to be redundant—is the material basis of NO, provides a simple explanation for the redundancy of NO.

Finally, some observers may prefer to define NO as a singularity of chromosome morphology, such as the secondary constriction that is considered to mark the locus in a condensed nucleolar chromosome. It may be noted that the volume occupied by the r-DNA in *Drosophila*, per NO, is less than 10^{-3} cubic microns. Many different arrangements of this material in relation to neighboring structures would be compatible with the microscopic appearance of the region.

Implicit in the foregoing discussion is the concept that all the r-DNA is intercalated in the chromosome. Alternatively, it might be proposed that the r-DNA is single in the ultimate chromonemata and the redundancy represents DNA copies separated from these sites. This model does not account for the divisibility of NO and the graded hypomorphism of *bb* mutants. Moreover, in the chicken the redundancy of r-DNA is similar in DNA from various tissues and from spermatozoa, as expected if it is all chromosomal (RITOSSA, BLEYMAN and SPIEGELMAN 1966). These considerations are not meant to deny the possible existence in some instances, e.g., the annular DNA structures in the nucleoli of certain amphibian oocytes (MILLER 1964) of a superimposed mechanism for amplifying the capacity for r-RNA synthesis by further increasing the number of available templates.

The nature of contrary evidence: If the foregoing lines of evidence are taken to indicate that r-DNA has the same position on the chromosome as the nucleolus organizer, then the present experiments revealing the usual genetic basis of *bb* to be partial deletion of r-DNA indicate that the loci of *bb* and NO are also identical. On the other hand, the experiments could be interpreted to mean that *bb* and NO are closely adjacent but separable regions, each redundant, with r-DNA at the *bb* locus and constituents of uncertain nature at NO. This alternate interpretation would be necessitated by cytogenetic evidence of separation of *bb*⁺ from NO by chromosome rearrangement. We have not found unequivocal examples of such separation in the published literature, but unpublished experiments of COOPER and KRIVSHENKO can be interpreted to mean that separation has occurred. In a series of about 50 large X-chromosome deletions, five of the remaining fragments appeared genetically to carry the wild-type allele of *bb*, but cytologically did not appear to be nucleolus associated. The question remains open whether such observations can be reconciled with the coincidence of NO and *bb*⁺. Ordinarily, the genetic test for *bb*⁺ in free duplications would lead to some partial deletions being scored as wild type, since in the presence of *bb* alleles (on normal X chromosomes) the phenotype would be returned to normal by the addition of less than a full complement of r-DNA. Thus it is possible that NO fragments too small to appear associated with a nucleolus under the usual conditions of observation might still be sufficient to tip the balance to wild type phenotypically. In any event, the further investigation of such instances could in principle provide a critical test of our conjecture.

The question of functional differentiation of r-DNA: A possible explanation for the apparent redundancy of r-DNA is that it is not truly redundant, but instead comprises a large number of different sequences, permitting the formation of ribosomes with different specificities. The existence of viable partial deletions shows, however, that the redundancy of r-DNA cannot be largely attributed to functional differentiation of the region, since each indispensable part is represented in both the deleted and the remaining undeleted portion. This conclusion is consistent with the additive bristle-forming capacity found by STERN (1929) for mutant (including lethal) alleles. A functionally differentiated region would be expected to have mutants that are complementary rather than additive in their interactions. Complementation has not been reported for *bb*. The hypomorphism

of different partial deletions supports the conjecture that the concurrent transcription of equivalent templates is necessary to meet the normal requirement for r-RNA synthesis. Although we have no reason to believe that the r-DNA of *Drosophila* is differentiated so that it leads to the formation of different kinds of ribosomes, we do not mean to imply that such differentiation may not have evolved elsewhere.

The proposed origin of variation in r-DNA redundancy: A simple model to explain the spontaneous occurrence of partial deletions of the locus, and the reversion of such deletions, is a linear array in which the templates for 18S and 28S r-RNA are interspersed, perhaps in alternating fashion. The presence of tandem repeats affords the opportunity for unequal pairing, and this can lead to crossover products of unequal length (STURTEVANT 1925). Similarly, crossing over with intrachromosomal pairing can produce length changes in either direction (PETERSON and LAUGHAN 1963). The frequency of crossing over in the region need not be especially high to render it far more labile than nonredundant loci with normal mutation rates. The hypothesis that crossing over is the agency responsible for changes in redundancy is amenable to test by conventional genetic techniques.

Given a source of natural variation in redundancy, it should be possible to demonstrate that the optimal redundancy is maintained by natural selection. The rather constant r-DNA content of the wild-type *bb* locus suggests that not only deficient individuals, but also those with excessive redundancy, have a selective disadvantage. Thus, we predict that excessive redundancy produced by genetic manipulation, as in the ♀ (4) ♂ (3) stock used by RITROSSA and SPIEGELMAN (1965) will be unstable; indeed, preliminary indications of a return toward wild type are already apparent in these stocks.

Predicted sex differences in amount of r-DNA: The hypothesis predicts that the equality of r-DNA in males and females, as seen in wild-type *D. melanogaster*, will not be universal. Where the content of nucleolar chromosomes differs in males and females, the amount of r-DNA per genome and the number of *bb* loci should exhibit corresponding differences. In *D. ananassae*, for example, males are expected to have 1.5 times as much r-DNA per genome as females, provided the nucleolar regions on the fourth and Y chromosomes are equivalent. In *D. simulans*, *bb* is located on the X chromosome in the same position as in *D. melanogaster*, but the Y chromosome of *D. simulans* does not carry a wild-type allele of *bb* (STURTEVANT 1929). We predict, therefore, that females of *D. simulans* will have more r-DNA per genome than males; twice as much in the limiting case of a completely deficient Y chromosome. In *D. pseudoobscura*, STURTEVANT and TAN (1937), have reported that in race A the Y chromosome intensifies *bb* (as is usual in *D. simulans*), while in race B it has no effect on *bb*. In both of these cases a sex difference in r-DNA content is predicted in the same direction as for *D. simulans*, and perhaps differing in degree in the two races.

The possible occurrence of a dominant bobbed mutant: A dominant bobbed*

* It has been pointed out by JACK SCHULTZ (personal communication) that this may represent a Y-translocation linked to a "Minute".

(Bb^Y) located on the Y chromosome of *D. funebris* has been described by LÜERS (1937). It is possible for a deletion to behave as a dominant if the redundancy in the species concerned is insufficient to give a normal phenotype when the region is present in single dose. In *D. funebris* X0 males are not phenotypically bobbed, however, and Bb^Y is dominant to two doses of the wild-type allele; that is, $X+X+Y^{Bb}$ and $X+Y+Y^{Bb}$ are phenotypically bobbed. Therefore, Bb^Y cannot have the genetic basis that we believe to be typical of recessive bb . While it is not proved that Bb^Y is a bobbed mutant in the sense that its phenogenesis is congruent to that of bb at the level of the primary bb gene product, the assumption that Bb^Y is related to bb in this way leads to a useful conjecture: Since the bobbed phenotype is produced in individuals with more than adequate redundancy, Bb^Y may affect the regulation of r-DNA transcription. Thus the occurrence of atypical bobbed mutants having normal redundancy may afford a means of identifying elements of a regulatory system.

It may be noted that in the presence of such a system two distinct forms of regulation would be simultaneously in force; first, the regulation by natural selection of the redundancy itself, which sets an upper limit to the rate of r-RNA synthesis, and second, a control of the rate of transcription at submaximal levels.

Ubiquity of mutants analogous to bb: Since r-RNA is among the components required for genetic translation, mutations affecting it are expected in all organisms that have a complete gene action system. Those that possess redundant r-DNA, affording the possibility of partial deletion, must have mutant forms that are analogous to bb . The r-DNA has proved to be redundant in every case tested, and although the instances are few, they include representatives of the vertebrates, arthropods, higher plants and bacteria; hence it seems a reasonable extrapolation to say that redundancy of r-DNA is practically universal. The phenotypic manifestation expected to be common to r-DNA partial deletions in different organisms is slow growth and development. At best, this can only serve to define the general category of mutants within which those analogous to bb may be found.

In the particular case of *Drosophila* the bristle size acts as an indicator phenotype, probably because it represents the maximum protein-synthesizing capacity of trichogen cells during a limited interval in development. Special features of the developmental patterns in other organisms may lead to different indicator phenotypes associated with a limited capacity for protein synthesis. These phenotypes will be mimicked by mutations that affect the special aspect of development from which they arise, but do not produce a general retardation of development.

Finally, at least some of the mutations affecting such components as transfer RNA, amino acid activating enzymes, polymerases, and ribosomal proteins may be expected to have phenotypic effects rather similar to those that result from r-RNA insufficiency. Thus it seems possible that other mutants of *Drosophila*, e.g., Minutes, bearing a phenotypic resemblance to bobbed, may eventually be identified with other components of the gene action system.

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SUMMARY

The proportion of DNA complementary to ribosomal RNA was estimated by means of annealing experiments in four bobbed (*bb*) mutants of independent origin in *D. melanogaster*. All were found to be partially deficient in this DNA. These experiments, together with other evidence concerning the localization of this DNA and the characteristics of *bb* mutants, have led us to offer the conjecture that the *bb* locus is the nucleolus organizer and that the typical genetic basis of *bb* is partial deletion of the nucleolus organizer.

LITERATURE CITED

- BEERMANN, W., 1960 Der Nukleolus als Lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* **11**: 263-296.
- BRIDGES, C. B., and K. S. BREHME, 1944 The mutants of *Drosophila melanogaster*. Carnegie Inst. Washington Publ. **552**.
- BROWN, D. D., and J. B. GURDON, 1964 Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc. Natl. Acad. Sci. U.S.* **51**: 139-146.
- CHIPCHASE, M. I. H., and M. L. BIRNSTIEL, 1963 On the nature of nucleolar RNA. *Proc. Natl. Acad. Sci. U.S.* **50**: 1101-1107.
- COOPER, K. W., 1959 Cytogenetic analysis of major heterochromatic elements (especially Xh and Y) in *Drosophila melanogaster* and the theory of "heterochromatin." *Chromosoma* **10**: 535-588.
- GILLESPIE, D., and S. SPIEGELMAN, 1965 A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12**: 829-842.
- KAUFMANN, B. P., 1936 The chromosomes of *Drosophila ananassae*. *Science* **83**: 39.
- KIKKAWA, H., 1938 Studies on the genetics and cytology of *Drosophila ananassae*. *Genetics* **20**: 458-516.
- KRIVSHENKO, J., 1959 The divisibility of the nucleolus organizer and the structure of the nucleolus in *Drosophila busckii*. *Genetics* **44**: 520-521.
- LÜERS, H., 1937 A contribution to comparative genetics using the dominant *bobbed* on the Y chromosome of *Drosophila funebris*. *Z. Ind. Abst. Vererb.* **74**: 70-90.
- MARMUR, J., 1961 A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**: 208-218.
- MCCLINTOCK, B., 1934 The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z. Zellf. Mikr. Anat.* **21**: 294-328.
- MCCONKEY, E. H., and J. W. HOPKINS, 1964 The relationship of the nucleolus to the synthesis of ribosomal RNA in HeLa cells. *Proc. Natl. Acad. Sci. U.S.* **51**: 1197-1204.
- MEAD, C. G., 1964 A deoxyribonucleic acid-associated ribonucleic acid from *Drosophila melanogaster*. *J. Biol. Chem.* **239**: 550-554.
- MILLER, O. L., 1964 Extrachromosomal nucleolar DNA in amphibian oocytes. *J. Cell Biol.* **23**: 60A.
- MULLER, H. J., 1932 Further studies on the nature and causes of gene mutation. *Proc. 6th Intern. Congr. Genet.* **1**: 213-255.

- PERRY, R. P., 1962 The cellular sites of synthesis of ribosomal and 4S RNA. *Proc. Natl. Acad. Sci. U.S.* **48**: 2179-2186.
- PETERSON, H. M., and J. R. LAUGHAN, 1963 Intrachromosomal exchange at the Bar locus in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.* **50**: 126-133.
- RITOSSA, F. M., and S. SPIEGELMAN, 1965 Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.* **53**: 737-745.
- RITOSSA, F. M., M. BLEYMAN, and S. SPIEGELMAN, 1966 Chromosomal redundancy of DNA complementary to ribosomal RNA. (In manuscript.)
- RUDDIN, G. T., 1963 The structure and function of heterochromatin. *Proc. 11th Intern. Congr. Genet.* **2**: 359-374.
- SPENCER, W. P., 1944 Iso-alleles at the bobbed locus in *Drosophila hydei* populations. *Genetics* **29**: 520-536.
- STERN, C., 1927 Ein genetischer und zytologischer Beweis für Vererbung in Y-chromosome von *Drosophila melanogaster*. *Z. Ind. Abst. Vererb.* **44**: 187-231. — 1929 Über die additive Wirkung multipler Allele. *Biol. Zbl.* **49**: 261-290.
- STURTEVANT, A. H., 1925 The effects of unequal crossing over at the Bar locus in *Drosophila*. *Genetics* **10**: 117-147. — 1929 The genetics of *Drosophila simulans*. *Carnegie. Inst. Washington Publ.* **399**: 5-62.
- STURTEVANT, A. H., and C. C. TAN, 1937 The comparative genetics of *Drosophila pseudoobscura* and *Drosophila melanogaster*. *J. Genet.* **34**: 415-432.
- VERMEULEN, C. W., and K. C. ATWOOD, 1965 The proportion of DNA complementary to ribosomal RNA in *Drosophila melanogaster*. *Biochem. Biophys. Res. Comm.* **19**: 221-226.
- WALLACE, H., and M. L. BIRNSTIEL, 1966 Ribosomal cistrons and the nucleolar organizer. *Biochim. Biophys. Acta* **114**: 296-311.
- YANKOFSKY, S. A., and S. SPIEGELMAN, 1963 Distinct cistrons for the two ribosomal RNA components. *Proc. Natl. Acad. Sci. U.S.* **49**: 538-544.