*UNSTABLE REDUNDANCY OF GENES FOR RIBOSOMAL RNA**

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The present work was begun with the aim of understanding the mechanism of reversion of the *bobbed* phenotype in *Drosophila melanogaster*. We have previously shown that this phenotype is due to a partial deficiency of DNA complementary to ribosomal RNA (rRNA).[1] We know that the wild-type bb^+ locus, which probably is cytologically identifiable with the nucleolus organizer, carries at least 130 genes for ribosomal RNA.[2] Much evidence[2, 3] points to the possibility that these genes are clustered and possibly adjacent. If these multiple copies are tandemly arranged, unequal crossing-over can be expected to occur. This was indeed the mechanism we invoked to explain the frequent appearance of *bb* mutations and the reversion of the *bb* phenotype which is paralleled by gain of genes for rRNA.[1, 4] The multiplicity of the rRNA genes was explained on the basis of the comparatively high demand for ribosomal RNA.[2] The possibility that this might not be enough to satisfy all requirements was explored by comparing a variety of tissues which differed widely in their rates of ribosome synthesis. The corresponding DNA preparations showed, however, the same redundancies within experimental error.[4] A striking exception was discovered by Brown[5] in the amphibian oöcyte which exhibited a marked extrachromosomal redundancy. We have here then an example of a new type of gene regulation analogous to the one proposed some years ago[6] that involved the induced production of extrachromosomal copies of specific genes (plasmagenes).

It was of some interest to see whether evidence for a similar mechanism could be detected in *Drosophila*. As an initial attempt a search was made for abrupt changes in rDNA content in stocks carrying the *bobbed*-type deletion. Schalet[7] has shown that crossing-over in the *bb* locus can be of the unequal type and that the frequency of crossing-over within the *bb* region is of the order of 0.6 per cent. One should then expect *bb* mutations to appear in a bb^+ population with a frequency not higher than this value. Similarly, a population of *bb* individuals should also show bb^+ revertants with a frequency not higher than 0.6 per cent. The possible operation of an extrachromosomal amplification mechanism would be signaled by a restoration of the rDNA deficiency in a *bobbed* stock at a rate much faster than could be accounted for by the observed crossing-over frequency.

It is the purpose of this paper to present data showing that reversion of the *bb* phenotype can, in certain cases, occur suddenly by the accumulation of genes for ribosomal RNA which, although normally inherited through it, may not be perfectly integrated into the chromosome.

Materials and Methods.—Drosophila stocks: $g^2ty/y\ f:=$ flies were from the Pasadena collection; $In(1)sc^{4L,8R}$ came originally from the Oak Ridge collection; $w^a sn\ bb/Y^{-bb}$ and $y\ v\ f:=$ and $In(1)sc^8/y\ f:=\ Y^{B^8}$ stocks are from the Bowling Green collection; $X\ Y^L.Y^S$ (108–9), $y^2su\text{-}w^aw^aY^L.Y^S/y\ v\ bb/O$ is from the University of Rome collection.

Hybridization procedure: P^{32}- or H^3-labeled ribosomal RNA from wild-type larvae of *Drosophila melanogaster* was extracted and purified as previously described.[1, 2] DNA was

extracted from adult flies only, according to our standing procedure,[1, 2] and rRNA/DNA hybrids were made on nitrocellulose membrane filters.[8] RNase digestion (20 μg/ml at 30°C for 1 hr in 2 × SSC) was always made after hybridization.

Results.—I found that males of the g^2ty/Y and $y\,f.=$ /Y stock from Pasadena carry a strong *bb* isoallele in the X, though the mutation is not recorded in the stock list. When this X, which can hereafter be written $g^2ty\ bb$, is combined with an sc^4sc^8 chromosome which carries no rDNA, or with an X carrying a bb^l mutation, the females obtained (females Gl of Fig. 1) exhibit an extreme *bb* phenotype. One of the relevant characteristics of these females is that they are almost completely sterile and the few eggs they lay are dechorionated; they produce no progeny.

If the g^2 *ty bb* chromosome is combined with a Y^{-bb} chromosome, which is functionally equivalent to a *bb* deficiency,[9] or if X/O males are produced which carry this X, the males which appear show only traces of bristles and the abdominal integument is extremely etched. Some of these males show no traces of sexual terminalia, while the rest have very low fertility. If these males which are so strongly *bb* are crossed to $\widehat{XX}/Y^{-bb}$ females, males are obtained which have the same genetic constitution as their male parents as far as the sex chromosomes are concerned (Fig. 1). These backcross males, however, are no longer extreme *bb*.

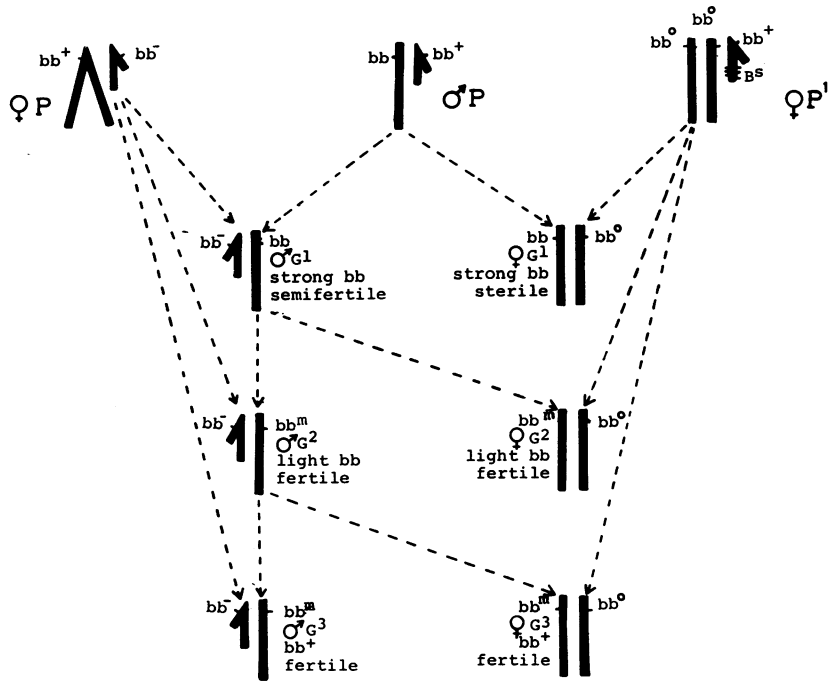


FIG. 1.—Scheme of experiments showing reversion of *bb* phenotype, while relevant sex chromosomes are the same. Only sex chromosomes are shown. The arrows show the origin of the chromosomes. Of the two arrows pointing to a certain genetic composition, one comes from a female and one from a male. Nonrelevant markers are omitted.

All the progeny have more or less the same phenotype. In parallel, males g^2 *ty* bb/Y^{-bb} which are extreme *bb* (males Gl in Fig. 1) were crosses to $sc^4sc^8/sc^4sc^8/Y^{Bs}$ females (called P females in Fig. 1). Females g^2ty bb/sc^4sc^8 from this cross are very weak *bb* in phenotype and are now fertile. In this case also, the sex chromosome composition is identical to that of the corresponding Gl females, but a partial reversion of the *bb* phenotype toward the wild type has occurred. A much better reversion of the *bb* phenotype can be obtained for both the males and the females of the above-mentioned genotypes as follows: males g^2ty bb/Y^{-bb} (G2) are further crossed with females $\widehat{XX}/Y^{-bb}$ (females P of Fig. 1) of the original stock. Males g^2 *ty* bb/Y^{-bb} (G3 of Fig. 1) are no longer *bb*. If *ty*, which itself reduces the length of the bristles, and g^2 are removed from the original X chromosome, the same results are obtained.

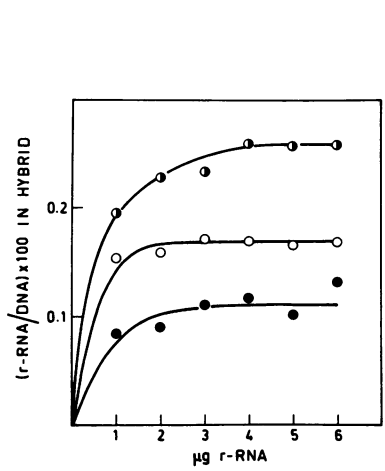
When males g^2 *ty* bb/Y^{-bb} (G2) were crossed with sc^4 $sc^8/sc^4sc^8/Y^{Bs}$ females, the great majority of progeny females of composition g^2 *ty* bb/sc^4sc^8 were also the wild phenotype.

To test whether the *bb* reversion was attributable to an increase in content of DNA complementary to ribosomal RNA, X/O males carrying the g^2 *ty bb* chromosome from a different origin were obtained. For one of these stocks, males g^2 *ty* bb/Y^+ (P males of Fig. 1) were crossed with $\widehat{XX}$/O females, and g^2 *ty* *bb*/O were obtained which were extreme *bb* in phenotype.

In parallel, males g^2 *ty* bb/Y^{-bb} (Gl), which are extreme *bb* in phenotype, were crossed with the same $\widehat{XX}$/O females used for the previous cross, and g^2 *ty* *bb*/O males were obtained which were very weakly *bb* in phenotype. Finally, males g^2 *ty* bb/Y^{-bb} (G3) were again crossed with $\widehat{XX}$/O females, and g^2 *ty* *bb*/O males were obtained which were completely reverted to wild type from *bb*. The saturation levels in rRNA/DNA hybridization experiments, using DNA extracted from these three types of males, are reported in Figure 2. It is apparent that reversion of the *bobbed* phenotype is paralleled by an increase in the content of DNA complementary to ribosomal RNA. These experiments show that the reversion of *bb* which occurs in passing from males Gl to males G2 and to males G3 (Fig. 1) is paralleled by an increase in rDNA, and this occurs in 100 per cent of the cases. I will call the reverted *bb* locus "magnified bobbed" (bb^m).

At this point it was important to know whether the rDNA produced in the process of reversion was firmly bound to the *bb* locus or not. If g^2 *ty* bb^m/Y^{-bb} males which are reverted (G3) are maintained with females which are $\widehat{XX}/Y^{-bb}$, they never show reappearance of the *bb* phenotype. The amount of rDNA associated with the X chromosome stabilizes around 0.27 per cent. Figure 3 shows the saturation level in a rRNA/DNA hybridization experiment of the DNA from g^2 *ty* bb^m/O males which were obtained by crossing $\widehat{XX}$/O females with males g^2 *ty* bb^m/Y^{-bb}, which were kept for about ten generations with $\widehat{XX}/Y^{-bb}$ females.

I found, however, that if the reverted g^2 *ty* bb^m chromosome is put together with a partner Y chromosome carrying a bb^+ locus (in a male), then the bb^m locus, at least in part, returns to its original *bb* condition. Several experimental designs



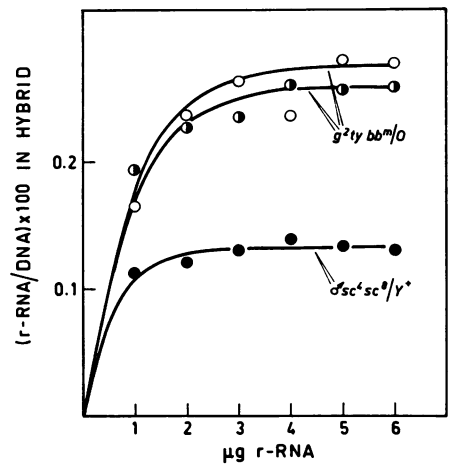


FIG. 2 (*left*).—Saturation levels in rRNA/DNA hybridization experiments with wild-type rRNA, H^3-labeled, and DNA obtained from X/O males, obtained from the following crosses:

(●) ♀ $\hat{X}\hat{X}$/O × g^2ty bb/Y^+ (♂P of Fig. 1).

(○) ♀ $\hat{X}\hat{X}$/O × g^2ty bb/Y^{-bb} (♂G1 of Fig. 1).

(◑) ♀ $\hat{X}\hat{X}$/O × g^2ty bb^m/Y^{-bb} (♂G2 of Fig. 1).

The real saturation levels obtained in these experiments have been multiplied by 0.9 to take into account the lower DNA content of these individuals (which lack the Y chromosome). I expected the saturation level of g^2ty bb/O (●) males to be somewhat lower than the value observed, based on the intensity of the bb phenotype in these males. The explanation for this result can be a trivial one or involve one of the following possibilities: (*a*) certain tissues of these males have high levels of rDNA; (*b*) some genes of this particular locus are mutated.

FIG. 3 (*right*).—Saturation levels in rRNA/DNA hybridization experiments with wild-type rRNA, tritium-labeled, and DNA obtained from X/O males, obtained as follows:

(◑) $\hat{X}\hat{X}$/O × g^2ty bb^m/Y^{-bb} (this is a reproduction of the upper curve of Fig. 2).

(○) $\hat{X}\hat{X}$/O × g^2ty bb^m/Y^{-bb} (these males are obtained by keeping males G3 of Fig. 1 with females $\hat{X}\hat{X}$/Y^{-bb} for about ten generations).

(●) Males sc^4sc^8/Y^+ used as control.

were followed to test this behavior, and one is presented in Figure 4. The results illustrated in Figure 5 show that at least a fraction of the bb loci that underwent "magnification" in only one generation can return to its original condition again in only one generation. In this case, however, the reversal is not always an all-or-none phenomenon. The classification, although somewhat arbitrary, is still feasible: the main criteria is abdominal etching. Since the intensity of the bb phenotype is an expression of the partial deficiency of rDNA,[1, 9] it is apparent that the rDNA which characterized the reversion of bb can be easily lost.

These results suggested that reversion of bb in passing from G1 males to G2 males and from these to G3 males (Fig. 1) might occur by accumulation of rDNA which is free from the chromosome. Were this true, the free rDNA would then be distributed at random between the gametes. Two experiments have been carried out to test this point: (1) g^2 ty bb^m/Y^{-bb} (G3) males were crossed with sc^8bb^+/g^2 ty bb females. If "magnification" occurs by accumulation of free copies of rDNA and these are distributed at random between X and Y, one

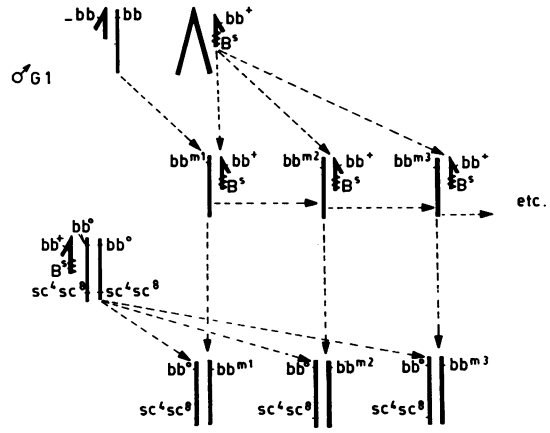


FIG. 4.—Scheme of the crosses used to keep the X chromosome carrying a bb^m locus with a Y chromosome carrying a bb^+ locus. The bb^m locus is tested, after several generations with a bb^+, by combining the chromosome which carries it with a sc^4sc^8X in a female (crosses indicated at the bottom of the figure). The sc^4sc^8 chromosome is also labeled bb^0 (no rDNA).

Substituting males P of Fig. 1 (g^2ty bb/Y^+) for males Gl (g^2ty bb/Y^{-bb}) in this scheme, one gets a control of the status of the "nonmagnified" bb after these crosses. The results of such controls are shown in Fig. 5.

should then find, from the above cross, males g^2 ty bb/Y^{-bb} which are no longer bb together with males of the same genetic composition which are extreme bb. The results of such a cross are shown in Table 1. A fraction of g^2 ty males that are no longer bb are found, while the majority are extreme bb. The bb^+ males, however, are all sterile and hence are X/O in chromosomal composition. They are the consequence of nondisjunction of the female X chromosomes. (2) sc^4sc^8/g^2ty bb^m (G3) females, phenotypically bb^+, were crossed with sc^4sc^8/Y^{Bs} males. Were some rDNA carried along by the egg containing the sc^4sc^8 chromosome, one might expect to obtain females which are homozygous for the sc^4sc^8 chromosome (which carries no rDNA by itself)[2] and possibly having nucleoli free from the chromosomes, or small free nucleoli. No females of such type were obtained (Table 2).

From experiments (1) and (2), it is apparent that both the Y^{-bb} and sc^4sc^8 chromosomes are never associated with any free rDNA, even if they were partners of the g^2 ty bb^m chromosome.

Discussion.—This paper reports that, in terms of common experience, individuals of the same relevant chromosomal composition can have different phenotypes, depending upon the previous history of these elements. The X and

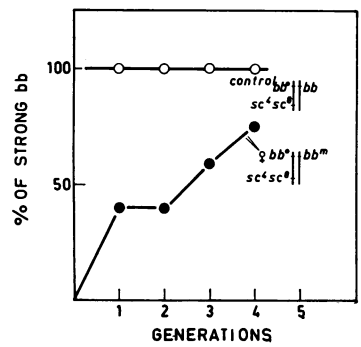


FIG. 5.—The figure shows, in successive generations, the percentage of strong bb females out of the total number of females of the following genetic constitution (sc^4sc^8/bb^{m1}; sc^4sc^8/bb^{m2}; sc^4sc^8/bb^{m3}, etc.), obtained as indicated in Fig. 4. Controls are obtained as indicated in Fig. 4. The sc^4sc^8 chromosome is also labeled bb^0 (no rDNA).

TABLE 1. *Progeny test of the following cross:* $y^{31d}sc^{8}bb^{+}car\ cv/g^{2}ty\ bb \times g^{2}ty\ bb^{m}/Y^{-bb}$.

Female Progeny		Male Progeny	
Phenotype	*N*	Phenotype	*N*
Wild	223	*y car cv*	174
$g^{2}ty$	188	$g^{2}ty$*	16
		$g^{2}ty$ strong *bb*	32

* Sterile X/O males, product of nondisjunction of the females' X's or four-strand double crossing-over.

Y chromosomes of males G1, G2, and G3 of Figure 1 are the same, as are the X's of females G1, G2, and G3 of the same figure. Phenotypic differences between males and females G1, G2, and G3 cannot be accounted for by maternal effect, simple autosomal heterozygosis, nor by unequal crossing-over. To explain the difference in rDNA content between males G1 and males G2 in terms of unequal crossing-over, one would have to postulate an extraordinary frequency of this event between X and Y at the somatic level, and the favored element should always be the X chromosome. On the contrary, the frequency of crossing-over between X and Y in males was estimated to range from 2×10^{-4} to 8×10^{-4}.[10]

I previously showed that the Y^{-bb} chromosome[11] carries about 100 genes for ribosomal RNA which are not operative. The possibility of direct involvement of these or other genes of the Y in the phenotypic differences between males G1, G2, and G3 was discounted by producing a series of X/O males through crossing $\widehat{XX}$/O females with males P, G1, and G2, respectively, of Figure 1. The males obtained from these crosses are, respectively, strong *bb*, light *bb*, and wild type. The observed phenotypic differences between females G1, G2, and G3 lead also to the same conclusion.

After these considerations I looked for template variation and found that males G1 have a lower content of genes for rRNA than males G2, and these have a lower content than males G3 (Fig. 2). The number of genes does not augment any further if males G3 are permanently kept with females P (Fig. 3). The conclusion then is that reversion of *bb* in males G2 and G3 occurs by "magnification" of genes specific for rRNA. "Magnification," furthermore, cannot occur at the level of all somatic cells. If the combination X^{bb}/Y^{-bb} were the only condition to acquire "magnification," then all body cells of males G1 should have a normal, not a *bb* phenotype: the phenomenon would be undetectable in the approach used in the present investigation. Somatic cells instead appear no longer *bb*

TABLE 2. *Progeny test of the following cross:* $y\ sc^{4}sc^{8}car\ cv/g^{2}ty\ bb^{m}$(G3) $\times$ $y\ sc^{4}sc^{8}car\ cv/Y^{B^{s}}$.

Female Progeny		Male Progeny	
Phenotype	*N*	Phenotype	*N*
B^{s}*‡	81	*y car cv* B^{s}†‡	171
y car cv B^{s}*‡	58	$g^{2}ty$ B^{s}‡	126
Wild†	184	$g^{2}ty$*	161
Light *bb*†	109	Wild*	13
Strong *bb*†	5		

* Nondisjunctional products.
† Some might come from nondisjunction in both males and females.
‡ Few, nonrelevant, double crossovers have been included in the main group.

when the X^{bb} chromosome comes from males G1. Since a *bb* phenotype is the expression of the number of genes for rRNA, it seems quite reasonable to conclude that the "magnification" of rDNA occurred in the germ line cells of males G1. From the observation that all cells of males G2 show a reversion of the *bb* character, and remembering that *bb* is a cell-autonomous character,[12] we can also conclude that the rDNA produced in the "magnification" process is maintained in all progeny cells; it is hence self-duplicating. A possible explanation of the phenomenon would assume a strong selection at the level of the original X^{bb}/Y^{-bb} spermatogonia; only those in which unequal crossing-over has led to an increased number of genes for rRNA would generate active sperm. On this assumption, however, the bb^m locus should, afterward, behave in a completely normal fashion. This kind of rDNA cannot, however, be considered perfectly integrated into the chromosome. Were it so, one would expect the bb^m locus to be as stable as any *bb* locus and hence to produce partial deletions with a frequency lower than 0.6 per cent. On the contrary, if a bb^+ locus is a partner of bb^m, bb^m shows a high frequency of reversion to the original *bb* condition. The frequencies of revertants shown in Figure 5 are possibly underestimated, since a fraction of strong *bb* individuals die before hatching. A control to this phenomenon is the daily experience of the stability of the bb^+ locus. However, a direct measure of it is illustrated in Figure 4. Were the bb^+ locus of the Y^{B^8} chromosome unstable as the bb^m locus, one should find *bb* mutations associated to this chromosome as frequently as observed in its partner. This was never observed to be the case in this and similar controls involving X^{bb^+}/X^{bb^m} females. From the behavior of bb^m in the presence of a bb^+ partner, one could visualize the bb^m locus as constituted of two parts: one stably integrated within the chromosome (the original *bb* locus), and the other relatively free (diluted 30–50% per generation). On these bases one could expect to find some of these hypothetical free copies to be inherited along with the partner of the bb^m locus after segregation. This was found to be not so when the partners were the sc^4sc^8 and the Y^{-bb} chromosomes (Tables 1 and 2).

One could imagine that the amount of rDNA measured in the adults of *Drosophila* is itself a product of a "tissue-directed magnification" of a few chromosomally integrated genes for rRNA; *bb* mutations could hence be conceived as alteration of the normal "magnification" mechanism. The data presented here, in this case, could involve alteration of this kind of a mechanism. Data pointing to chromosomal integration of many copies of genes for rRNA are, however, rapidly accumulating.[3, 4, 7]

Summary.—The data presented here show that sudden reversion of *bb* (*bb* to bb^m) can occur in certain circumstances and that its molecular explanation is due to an accumulation of rDNA; bb^m, however, can return with high frequency to its original *bb* condition if matched with a normal bb^+ locus. A direct test to see whether the return to the original *bb* condition is paralleled by loss of rDNA has not yet been made. Considerable data[1, 9] on the parallelism between *bb* phenotype and rDNA content suggest, however, that this is probably the case. Given these observations, two working hypotheses can be considered to explain the observed phenomenon. (1) Certain chromosomes can undergo selective increase

and selective loss of rDNA. While selective increase could be easily accounted for by spermatogonial selection of bb^+ cells originated after unequal crossing-over or intrachromosomal exchange within the *bb* loci, it is difficult at present to conceive how directed losses of rDNA in a particular one of the chromosomes of a spermatogonia could have selective advantage. (2) Mechanisms exist which allow independent duplication of specific chromosomal sections. The genome fractions, thus duplicated, are capable of only loose integration within the chromosome. Examples of differential duplication of specific chromosomal sections are known,[13-16] as are examples of instability of certain genetic situations (see, for example, Brink *et al.*[17]). The molecular events leading to "magnification" of rDNA in our case are obscure. The phenomenon, however, is not restricted to the particular case presented here but always occurs, even if the efficiency can variate, in males of strong *bb* phenotype (to be published elsewhere).

The possibility that this phenomenon is not restricted to the genes for rRNA must be entertained.

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