REGULATION OF RIBOSOMAL RNA AND 5s RNA SYNTHESIS IN *DROSOPHILA MELANOGASTER:* I. BOBBED MUTANTS[1,2]

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ABSTRACT

Analysis of the rates and amounts of rRNA and 5s RNA synthesized in *Drosophila melanogaster* bobbed mutants was done by using acrylamide-gel electrophoresis. The results show that the amounts of rRNA synthesized are constant, although the rates of rRNA synthesis in *bb*'s are reduced to 30% of the wild-type level. The rates of synthesis of 5s RNA were constant. The rate of synthesis of the two kinds of molecules that enter in equimolar amounts into the mature ribosome is non-coordinated.——The rates of rRNA synthesis were shown to be proportional to the length of the scutellar bristles, supporting the notion that in trichogen cells there is no developmental delay, but the size of the bristle depends directly on the rate of rRNA synthesis.

IN *Drosophila melanogaster*, the genes for ribosomal RNA (rRNA) are clustered, redundant and localized in or very close to the nucleolar organizer region of the *X* chromosome (RITOSSA and SPIEGELMAN 1965; PARDUE *et al.* 1970). They are transcribed as one larger unit (ribosomal transcription unit or RTU, PERRY *et al.* 1970), which is later cleaved into the smaller 28s and 18s components, which participate in the ribosomal structure and function.

The genes for 5s RNA of *Drosophila melanogaster* have been localized in the region 56EF of the second chromosome by *in situ* RNA-DNA hybridization followed by autoradiography (WIMBER and STEFFENSEN 1970). These genes were also found to be redundant and clustered (TARTOF and PERRY 1970). The number of gene copies found for 5s RNA was roughly equivalent to the number number of copies of the rRNA genes (TARTOF and PERRY 1970).

Since rRNA and 5s RNA are present in the finished ribosome in equimolar amounts, and since they are in separate loci, it was interesting to study if the synthesis and accumulation of the two gene products were correlated and how. Previous studies used the differential sensitivity of the rRNA genes to low doses actinomycin D and showed that when rRNA synthesis was inhibited, 5s RNA synthesis continued unabated (PERRY and KELLEY 1968, 1970).

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Drosophila provided us with a system where rRNA synthesis is reduced without the use of inhibitors. The group of bobbed mutations behave as partial deficiencies of ribosomal DNA (RITOSSA, ATWOOD and SPIEGELMAN 1966). Reduced rates of rRNA synthesis were found in the ovaries of flies carrying bobbed mutations (MOHAN and RITOSSA 1970). These were analyzed with regard to 5s RNA synthesis. Wide variations in the rates of rRNA synthesis did not affect significantly the rates of 5s RNA synthesis, suggesting that they are independently controlled. Bristle lengths of highly inbred lines were found to correspond closely to the rates of rRNA synthesis. This suggests that two kinds of developmental processes occur: one, where the necessary amount of RNA and protein has to accumulate in the slower growing bobbed mutants, causing developmental delay, and others, where there is just a reduced expression as in the trichogen cells that produce the bristle.

MATERIALS AND METHODS

Biological materials: The stocks used, their origin and derivation are shown in Table 1. The genetic background of the wild-type and bobbed stocks used in this study was made homogeneous by the procedure detailed in Figure 1. Additional markers were eliminated in the first steps. Bristle lengths and developmental delay were measured on females of the constitution $bb^{x}\ sc^{4}sc^{8}$. Absence of additional *Y* chromosomes was monitored throughout by the use of YB^{s}. Non-disjunction of the $y^{+}Y$ used in the last step (Figure 1) was 3%, as monitored in a separate experiment.

Scutellar bristles were measured using a graduated occular in the stereo microscope. All duplication and deficiency stocks were crossed repeatedly to attached-*X* females with homogeneous Canton S background. The rDNA deficiency $sc^{4L}sc^{8R}(y\ sc^{4}sc^{8})$ was used (RITOSSA and SPIEGELMAN 1965). All flies were kept at 25°C and grown on cornmeal-yeast-agar medium.

Isotope incorporation: For RNA analysis, 25 female flies, kept for seven days with *X*/0 males

TABLE 1

Phenotypic characterization of the bb *stocks used*

Name of stock	Origin of stock	Developmental delay for hemizygous females in relation to normal males from the same bottle	Bristle length in hemizygous females (microns)	Percentage of wild type
Canton	Our laboratory	None detected	452 ± 5 (16)	100
bb^{3}	$svr^{poi\ dish}\ bb^{G3}$ Caltech.	None detected	458 ± 4 (16)	101.3
bb^{2}	*lh B car bb* Caltech.	None detected	446 ± 5 (19)	98.6
bb^{5}	*Df(1)y v car* bb^{-} Caltech.	None detected	432 ± 5 (16)	95.5
bb^{11}	*gt* bb^{11} Bowling Green	12 hr	358 ± 7 (29)	79.2
bb^{V}	Dr. RUBY VALENCIA Madison, Wisconsin	32–48 hr	286 ± 9 (12)	63.2
bb^{6}	*car bb*	32–48 hr	243 ± 8 (15)	53.7
bb^{N}	*wi* f^{3} bb^{N} Caltech.	32–48 hr	223 ± 5 (20)	49.3

Standard error follows the bristle length determinations, with the number of observations in parenthesis.

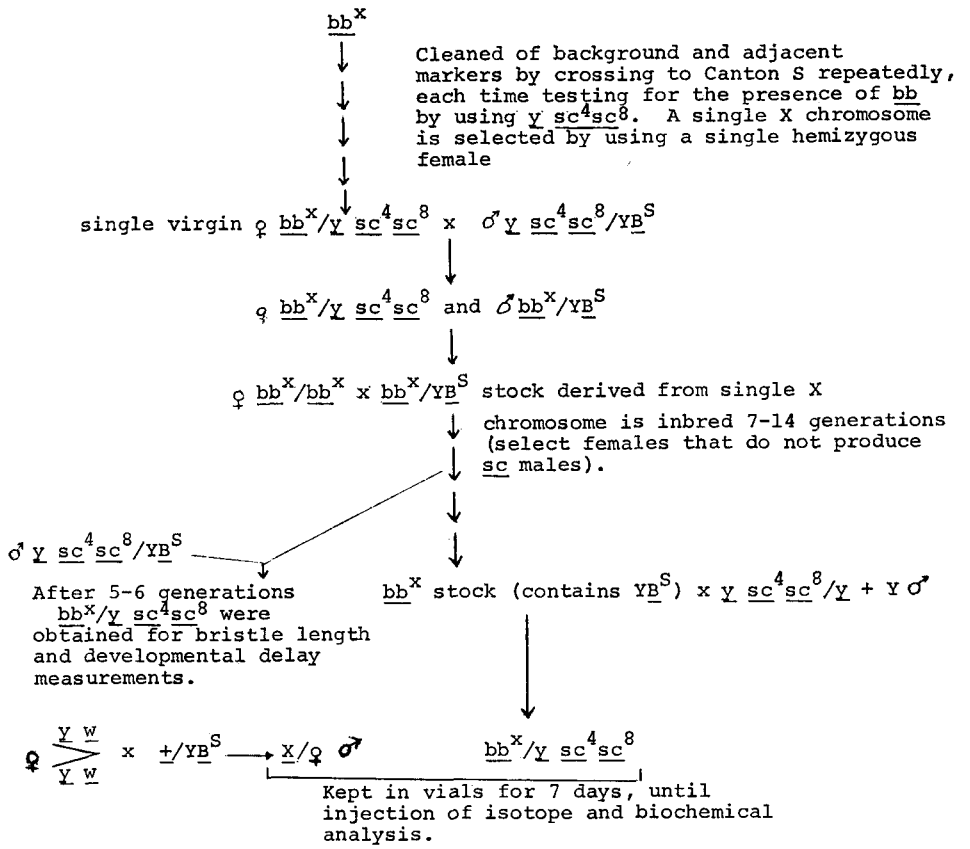

FIGURE 1.—Diagram showing the procedure used for producing the *bb* stocks analyzed. *bb*: bobbed, *y*: yellow body color, *sc*: scuttellar bristles missing, *w*: white eye, B^S: Bar of Stone, Bar eye.

were injected with 0.2 μl of ^{3}H-uridine, (24.4 CmM, New England Nuclear, 500 μc/ml) using a Hamilton microsyringe fitted with a glass needle. The flies were allowed to recover for 30 min before freezing at —20°C.

RNA extraction: Homogenization and RNA extraction was done at 0–4°C with the method of HASTINGS and KIRBY (1966). Twenty-five flies were homogenized in 1 ml of cold 0.5% disodiumnaphtalene-1,5-disulphonate, 30 mM trishydroxymenthylaminomethane, 0.15 M NaCl, and 3 ml of water-saturated phenol-cresol-hydroxyquinoline (500 g of redistilled phenol, 70 ml of redistilled cresol, 0.5 g of hydroxyquinoline). A teflon-glass pestle was used to homogenize the mixture in centrifuge tubes. After spinning for 10 min at 3,000 × g, the phenol layer was re-extracted with 1 ml of 1% triisopropylnaphthalene-sulphonate, containing 5% 4-aminosalycilate. After a second centrifugation, the aqueous layers were pooled and washed with ether. Excess ether was evaporated with air, and 2 volumes of cold ethanol-Na acetate (9:1 ethanol 95%, 1 M Na acetate pH 5.5) were used to precipitate the nucleic acids overnight. The white precipitate was dissolved in 0.15 M NaCl, 0.015 M Na-citrate, 5% sucrose.

Electrophoresis: Electrophoresis was carried out in gels with an upper layer of 2.5% acrylamide and a lower layer of 10% acrylamide. Gels were cross-linked with ethylene-diacrylate and prepared as described by LOENING (1967) and BISHOP, CLAYBROOK and SPIEGELMAN (1967). Gels were cast in tubes 9 cm long with an internal diameter of 0.6 cm. Dialysis membrane was

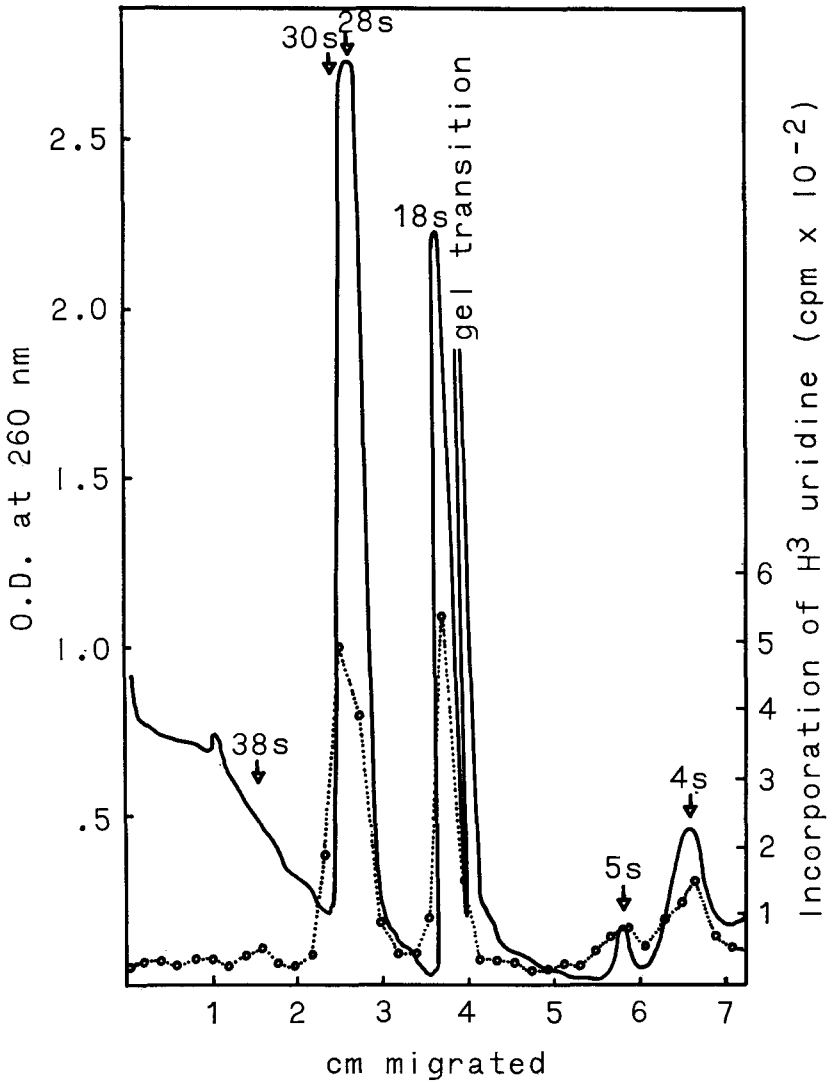

FIGURE 2.—Gel scan (—) and radioactivity profile (·····) of Canton/y sc^4 sc^s females injected with ^3H uridine and incubated for 30 min at 25°C. To the left of the gel transition, acrylamide concentration is 2.5%, to the right 10%. Gels were cross-linked with ethylene-diacrylate, and electrophoresis was for 200 min at 5mA gel. The positions of the 38s, 30s, 28s, 18s, 5s and 4s molecules are indicated. Using the published values for 28s and 18s as molecular weight standards, in this particular case we obtain for the 38s molecule a value of 2.9×10^6 daltons and for the 30s, a value of 1.58×10^6 daltons in good agreement with published values (PERRY *et al.* 1970).

stretched across the bottom before pouring. Once polymerized, the gels were prerun for 45 min in the electrophoresis buffer (30 mM Tris, pH 7.8, 30 mM NaH_2PO_4, 1 mM EDTA and 0.2% Na dodecyl sulphate). All runs and preruns were done at 5 mA/tube with a voltage gradient of 6–7 volt/cm, using a Buchler Polyanalyst electrophoresis apparatus and a Buchler current-

regulated power supply at room temperature (20–22°C). Samples were applied in a maximum volume of 50 μl and run for 200 min.

After the run, gels were pushed out of the tubes into a 0.5 × 1.5 × 10 cm quartz cuvette and scanned at 260 nm in a Gilford 240 spectrophotometer with an attached recorder, with a 0.2 mm fixed slit. The amount of each RNA specie was calculated from the scan by cutting out and weighing paper tracings of the areas corresponding to each class of RNA.

Radioactivity measurements: For radioactivity measurements the method of WIENBERG and PENMAN (1968) was used. After scanning, the gels were frozen and sliced with a manifold of razor blades spaced 1.5 mm apart. Each slide was digested in a scintillation vial with 0.5 ml of concentrated NH_4OH at 50°C for 2–4 hrs. To this, a water miscible scintillant was added that contained 6 parts of toluene based scintillator (4.75 g/l 2,5-diphenyloxazole, 0.095 g/l 1, 4-Bis-2(4 methyl-5-pheny(oxazolyl) benzene) and 4 parts of ethoxyethanol. Samples were counted in a Packard 3320 or 3380 liquid scintillation counter.

RESULTS

The success of the analysis of RNA levels and synthesis was centered on an appropriate method of separation and quantitation of all classes of RNA. Acrylamide gel electrophoresis was the method chosen because it provided adequate resolution and sensitivity for measuring RNA levels and synthesis. A compound gel, made of 2.5% and 10% acrylamide layers provided separation of 38s, 30s, 28s, 18s, 5s and 4s RNAs (Figure 2). The amounts and rates of synthesis of all classes of RNA were calculated by using data similar to those in Figure 2. The optical density scan in this Figure indicates the positions of the 28s, 18s, 5s, and 4s RNAs, while the radioactivity incorporation lines shows the positions of 38s, 30s, 18s, 5s, and 4s RNAs. For considering the incorporation into 28s RNA, the radioactivity in the 30s–28s region was all considered as 28s RNA. Radioactivity in the 38s region is very small, contrary to what occurs when tissue is incubated *in vitro*. Processing of rRNA is not the same *in vivo* as *in vitro*, since *in vitro* processing of rRNA is interrupted (MOHAN and RITOSSA 1970; PETRI *et al.* 1971; WEINMANN 1971). It was not an artifact of extraction since ovaries incubated *in vitro* and extracted by the same procedure showed that most of the counts were incorporated into 38s RNA. Adequate resolution of the 5s and 4s RNAs is also observed. For the 5s RNA specific activity determinations, the radioactivity found in the area slightly to the left of the 5s RNA optical density peak was assumed to be 5s RNA precursor, as found in *Escherichia coli* (GALIBERT *et al.* 1971). Radioactivity between the 4s and 5s RNA peaks was distributed evenly between the two species of RNA. The polyacrylamide gel electrophoresis system was checked, first measuring the molecular weights of 28s and 18s Drosophila RNAs using *E. coli* RNA as standard. The values obtained are in good agreement with published values (LOENING 1968). The molecular weight values of 28s and 18s RNA were used as standards to determine the molecular weight values of 30s and 38s RNA. The values obtained, compared to the published values, are shown in the legend of Figure 2. The comparison of the molecular weight values obtained with the results obtained by others using acrylamide gel electrophoresis supports the idea that we are analyzing rRNA. *E. coli* 5s and tRNA migrated coincidentally with Drosophila's 5s and tRNA.

Bobbed mutants were ordered according to the intensity of the phenotype.

TABLE 2

Molar ratios of RNAs synthesized with rRNA genes of different size

Genotype of females	Bristle length (μ)	Number of observations	Number of 5s RNA molecules/28s molecule	Percentage of wild type	Number of tRNA molecules/28s molecule	Percentage of wild type
Canton/$y\ sc^4sc^8$	452	10	1.53 ± 0.18	100	17.8 ± 1.5	100
$bb^3/y\ sc^4sc^8$	458	3	1.39 ± 0.18	90.8	16.4 ± 2.0	92.1
$bb^2/y\ sc^4sc^8$	446	1	1.42	92.8	14.8	83.1
$bb^5/y\ sc^4sc^8$	432	3	2.50 ± 0.08	163.3	24.6 ± 3.4	138.2
$bb^{11}/y\ sc^4sc^8$	358	3	1.64 ± 0.07	107.1	17.1 ± 1.2	96.0
$bb^V/y\ sc^4sc^8$	286	9	1.47 ± 0.04	96.0	15.5 ± 1.2	87.0
$bb^6/y\ sc^4sc^8$	243	3	.71 ± 0.18	46.4	12.0 ± 0.6	67.4
$bb^N/y\ sc^4sc^8$	223	3	2.31 ± 1.08*	150.8	24.02 ± 9.22*	134.5

The number of 5s RNA and transfer RNA molecules relative to the number of 28s molecules in bobbed mutants is shown. Data calculated from areas of 260 nm scan. Standard error is shown after values of 3 or more observations. Bristle lengths are shown to evaluate the intensity of the *bb* phenotypes.

* Standard error of this set of data is unusually large because of low viability of the stock (it is a very extreme *bb*, semilethal). The flies of this genotype for each experiment had to be collected over several days.

Bobbed is a pleiotropic, sex-linked recessive that shows abdominal etching, bristles decreased in length and thickness and developmental delay. These last two characteristics can be measured quantitatively. Bristle length was chosen however because it was easier to measure. As is shown in Table 1, using flies with a homogeneous genetic background, sex, and age, bristle-measurement determinations have a very small standard error. Bristle length shows little variability under the conditions used in these experiments. Approximate developmental delays are also shown in Table 1. It can be seen that bb^2, bb^3, and bb^5 show no significant differences in bristle length from wild type, and thus can be classified as revertants. The existence of additional *Y*s has been precluded in these experiments by the use of YB^s in bristle measurements.

Table 2 shows the results obtained from the measurements of the amount of RNA present. These are expressed as molar ratios. MOHAN and RITOSSA (1970) showed that the RNA/DNA ratios were independent of the number of rRNA genes, i.e. did not depend on the presence of *bb* mutations or their intensity. Table 2 shows that the relative molar amounts of 4s RNA per ribosome are constant. Because the bulk of the RNA is rRNA and the RNA/DNA ratios are constant (MOHAN and RITOSSA 1970), the constancy of the 4s/28s ratio indicates that there is no preferential accumulation of tRNA or rRNA. This is the same as saying that the rRNA/DNA and tRNA/DNA ratios are independent of the number of rRNA genes.

The case of $bb^5/y\ sc^4sc^8$ (line 4) shows an increase in the relative amount of the tRNA and also of 5s RNA. The reason for this is unknown. Also, $bb^N/y\ sc^4sc^8$ shows a huge standard error, probably due to the semilethality of this genotype. Collection of this kind of female was very difficult because of inviability. In general, however, the relative number of 5s molecules seems to be remarkably

TABLE 3

Rates of RNA synthesis with rRNA genes of different size

Genotypes of females	Bristle length (μ)	Number of observations	cpm/mg-area of 28s rRNA per cpm/mg-area of tRNA	Percentage of wild type	cpm/mg-area of 5s RNA per cpm/mg-area of tRNA	Percentage of wild type
Canton/$y\ sc^4sc^8$	452	7	1.47 ± .39	100	3.83 ± .31	100
$bb^5/y\ sc^4sc^8$	432	2	1.44	97.9	3.81	99.4
$bb^{11}/y\ sc^4sc^8$	358	2	1.11	75.5	3.30	86.1
$bb^V/y\ sc^4sc^8$	286	5	.71 ± .06	48.2	3.28 ± .40	85.6
$bb^6/y\ sc^4sc^8$	243	3	.54 ± .10	36.7	4.01 ± .88	104.6
$bb^N/y\ sc^4sc^8$	223	1	.41	27.8	2.49	65.1

The number of cpm/mg-area of ribosomal or 5s RNA relative to cpm/mg-area tRNA incorporated in 30 min in different *bb* genotypes is indicated. Standard errors follow the values when 3 or more observations were made. Bristle lengths were used to evaluate the intensity of the *bb* phenotype.

constant. On the other hand, in the case of $bb^6/y\ sc^4sc^8$ we find a decreased number of 5s molecules.

The rates of rRNA and 5s RNA synthesis were analyzed as described in MATERIALS AND METHODS. Specific activities were calculated for 28–30s rRNA, 5s RNA, and 4s RNA, and the values relative to 4s RNA were used for comparison. This provides an internal standard of isotope incorporation, so that without measurement of the pools of precursor, from which all classes of RNA are presumed to draw at the same rate, a relative rate of RNA synthesis is obtained. A comparison of the second and fourth columns of Table 3 shows the excellent correlation between bristle length and the relative rates of rRNA synthesis (Figure 3).

The analysis of the last column of Table 3 shows that the rates of 5s RNA synthesis relative to tRNA are constant. Since the rates of 5s RNA synthesis remain constant even with a wide range of variation in the rates of rRNA synthesis, it seems that the rates of synthesis of the two classes of RNA are independently controlled.

DISCUSSION

MOHAN and RITOSSA (1970) propose that the action of bobbed mutations (reduced RTU redon size) is of two kinds. In some tissues, a delay in development occurs until enough ribosomes are accumulated for development to proceed. In other tissues development can continue but at necessarily lower rates of protein synthesis because of the scarcity of ribosomes, which, in turn, results in a phenotypic effect. The bristle is generated by a single trichogen cell (LEES and WADDINGTON 1942) and requires a burst of protein synthesis. The slower rate of rRNA synthesis in bobbed mutants (reduced size of the RTU redon) very likely does not provide enough ribosomes for this amount of protein synthesis to occur, and a smaller bristle results. The proportionality between bristle lengths and rates of rRNA synthesis in ovaries supports this idea. Qualitative analysis of bristle length was first reported by STERN (1929). Our results on bristle length

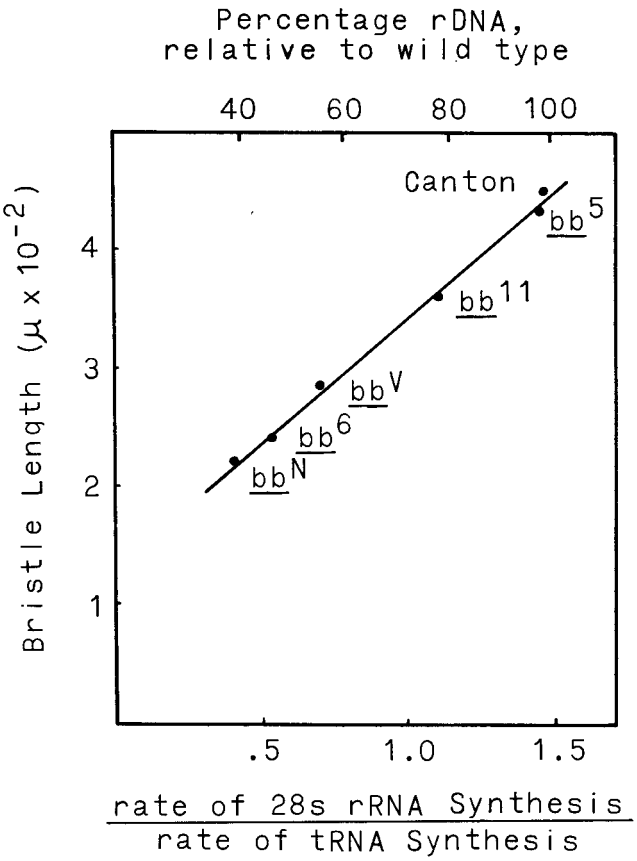

FIGURE 3.—Relation between the relative rate of rRNA synthesis and the intensity of the bobbed phenotype, as measured by bristle length. The genotype of the respective hemizygous females is indicated. Prediction of RTU size (% rDNA) would be bb^N:39%; bb^6 (data from QUAGLIARROTTI and RITOSSA 1968):45%; bb^V:55%; bb^{11}:77%; Canton and bb^5:100%.

measurement showed a very small standard error (Table 1). Conditions were used such that if alterations in the number of copies of the rRNA genes occurred, it was the same for all stocks tested. This precaution, as well as a homogeneous genetic background probably are responsible for the high repeatability of bristle length measurements. The observations on the quantification of the phenotypic effect (bristle length) give us further proof of a tight relationship with the molecular basis (slower rate of rRNA synthesis) in the cell.

Since the amounts of rDNA of *Canton S* and bb^6 (QUAGLIAROTTI and RITOSSA 1968) are known and assuming that no variations in redundancy occurred since the measurements were made, the amount of rDNA of unknown *bb* mutations can be predicted. This prediction would only be valid for rDNA deletions, not for nonoperative rDNA regions as found in Ybb^- (RITOSSA 1968). It can be predicted (Figure 3) that the size of the rDNA in bb^5 is 100% that of Canton, bb^{11} is 77%, bb^V is 55%, bb^N is 39% from the wild type. Actually, since rDNA values

for these mutants (bb^5, bb^{11}, bb^V and bb^N) have not been measured, it can be said only that they behave as if they had 100%, 77%, 55% and 39% that of the wild-type amount of functional rDNA.

There seems to be no direct correlation between the rates or the amounts of rRNA and 5s RNA synthesized in a particular bobbed mutant. In Table 2, data on the relative molar amounts of tRNA per ribosome are presented. Because RNA/DNA ratios are constant for different *bb* mutants (MOHAN and RITOSSA 1970) and the molar ratio of tRNA per ribosome also is constant, we can conclude that the rRNA/DNA ratios are independent of the amount or rDNA. However, the 5s RNA/DNA ratios are not completely independent of redon size, being reduced in the case of bb^6 and probably also bb^N, although it cannot be determined for this last mutant, because of inviability.

When the rates of RNA synthesis were analyzed (Table 3) it is observed that there is a constant rate of 5s RNA synthesis, while the rate of rRNA synthesis varied from 100% to 30% according to the intensity of the bobbed phenotype. The fact that the synthesis of 5s RNA occurs in the nucleus and it is not altered with wide variations in rates of rRNA synthesis, indicates that the synthesis of these two classes of RNA are independently controlled. Although not enough evidence is available here, it may be possible that the low levels of accumulation of 5s RNA could be related to very low rates of rRNA synthesis, maybe because it is degraded. The fact that 5s RNA cannot be integrated into a ribosome may activate some specific degradation mechanism.

When rRNA synthesis is reduced below the levels indicated here, as in bb^N a semilethal effect occurs, or below that, lethality ensues.

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