NONOPERATIVE DNA COMPLEMENTARY TO RIBOSOMAL RNA*

By F. M. Ritossa

INTERNATIONAL LABORATORY OF GENETICS AND BIOPHYSICS, NAPLES, ITALY

Communicated by Sol Spiegelman, February 19, 1968

The combination of slow development and small bristles in *Drosophila* is associated with mutations or deficiencies at a large number of loci. A hypothesis has been advanced that many (perhaps all) of these loci are concerned with parts of the genetic translation system.[1] In one such case, *bobbed* (*bb*), the phenotype was indeed found to be correlated with partial deficiency of the genes that specify ribosomal RNA.[2] The *bobbed* locus is included in a heterochromatic region of the X chromosome previously shown to contain all the DNA complementary to ribosomal RNA (rDNA) inherited through the female gamete.[3]

The cytogenetic location of the *bb* locus within the heterochromatic region of the X chromosome,[4] together with other evidence on the role of the nucleolus in ribosome formation,[5–9] led us to suggest that the *bobbed* locus constituted the larger part of, or was identical with, the nucleolus organizer.[2]

The discovery of the molecular nature of the *bb* mutation pointed to a rather narrow clustering of the many copies of the genes for ribosomal RNA. Recent data are in agreement with this interpretation.[10] Furthermore there is evidence that in other organisms these genes are adjacent.[11]

One might expect, in principle, that mechanisms coordinating gene expression could evolve in a compound locus like the *bobbed* one. Alterations of such mechanisms are then conceivable which could render the genes for rRNA inactive, although still present as revealed by rRNA/DNA hybridization. The present paper describes mutants in which this appears to be the case. Data are also presented which establish a correlation between *bobbed* mutations and rDNA content in the Y chromosome of *D. melanogaster*.

Materials and Methods.—Drosophila stocks: The Y^{bbN2} was obtained from a mutated single male and the Y^{bbN3} is the chromosome of stock 389 of the Pasadena collection (*shr bw*^{2b} *abb sp*/SM5, *al*^{2} *Cy lt*^{9} *sp*^{2}). Y^{bb}*Su*-var5 comes from stock f21 of the Bowling Green Collection. From the same collection, the following stocks were also obtained: Y^{bb}/v (the Y of this stock is no longer *bb* and is here called $Y^{bb\ rev.}$); Y^{-bb}/w *sn bb* with *y v f.* = (Y^{-bb1}); Y^{-bb}/w^{m4w}(Y^{-bb2}); Y^{-bb}/y^{2} *eq* (Y^{-bb3}). *In* (1) *sc*$^{4L,\ 8R}$, *y sc*$^{4+8}$ *cv v f*/*y f.* = was obtained from the Oak Ridge National Laboratory. Tests for sensitivity to *bb*^{l} were made by crossing males carrying the Y chromosome under test with w^{a} *bb*^{l}/w^{a} *bb*^{l}/$Y^{B^{s}}$ females and tests for deficiency sensitivity were made crossing these males with *In* (1) *sc*$^{4L,\ 8R}$/*In* (1) *sc* $^{4L,\ 8R}$/$Y^{B^{s}}$ females, and scoring for round-eyed males.

DNA/rRNA hybridization: DNA was extracted from adults as previously described.[3] RNA was also labeled as previously described and the purified ribosomal fraction was obtained after MAK column chromatography. RNA was labeled with either P^{32} or H^{3}-uridine (Nuclear-Chicago). The rRNA specific activities ranged from 60,000 to 180,000 cpm/μg. Annealing was obtained according to the technique of Gillespie and Spiegelman.[24] Incubations were made in 3 ml 2 × SSC at 60°C for 12 hr with increasing amounts of rRNA. Unmatched rRNA was eliminated with RNAse digestion (20 μg/ml for 1 hr at 30°C).

Results.—On the basis of the analysis of several independently obtained bobbed mutants of the X chromosome of *D. melanogaster*, it was concluded that

this mutated phenotype was due to a partial deficiency of rDNA.[2] In order to strengthen the validity of our findings, it seemed desirable to extend the analysis to the Y chromosome, which, like the X chromosome, carries a bb^+ locus and several of its mutations.

We had already shown that the wild Y chromosome of *D. melanogaster* contributes an amount of rDNA similar to that contributed by the X.[3] In accordance with expectations, we further found that in *D. simulans*, where the *bobbed* mutations are sex-linked but not sex-limited, the males had a lower DNA content than the females.[12] Several existing Y^{bb} chromosomes of *D. melanogaster* were available and two were selected in laboratory populations for our purpose.

Labeled rRNA was annealed to denaturated DNA obtained from the following stocks: Y^{bbN2}, Y^{bbN3}: Y^{bbN2} was obtained from a mutated single male and Y^{bbN3} was found in a standard stock (see *Materials and Methods*). Both behave as weak bobbed with several X^{bb} testers; there is viable male progeny with X^{bb^1} and both mutations are not deficiency-sensitive; both *bb* mutations have additive effect with themselves, namely, $X^{def.\ bb}/Y^{bb}/Y^{bb}$ males are phenotypically wild. They gave saturation levels of 0.097 and 0.096 per cent, respectively, corresponding to about 100 genes for rRNA (Fig. 1 and Table 1).

Y^{bb}Su-var5: This chromosome carries a rather strong *bb* mutation, but it gives viable males when combined with an X carrying a bb^1 locus and is not deficiency-sensitive.

This chromosome contributes rDNA for a mean of 0.047 per cent of the diploid genome or about 50 genes for rRNA (Table 1 and Fig. 2).

$Y^{bb\ rev.}$: Although this chromosome is still listed as carrying a *bb* mutation, it does not behave as bobbed in any genetic test. Its level of saturation with rRNA (0.138%) shows that the amount of rDNA it contains is also normal (Table 1).

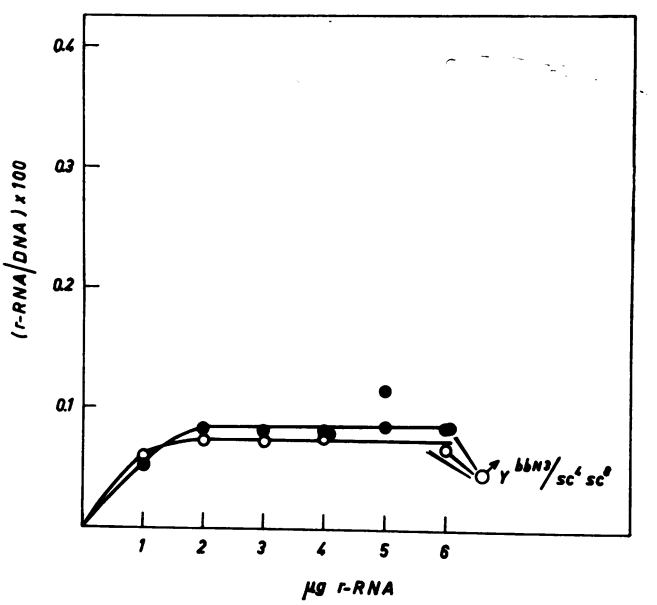

FIG. 1.—Saturation of DNA from males carrying Y^{bbN3} and an X completely deficient for the *bb* locus (*In*1)) sc^{4L}, ^{8R}) by H^3-labeled ribosomal RNA. The two curves were obtained using two series of filters, one with about 30 µg DNA per filter, the other with about 40 µg per filter.

TABLE 1. *Estimate of rDNA content of various Y chromosomes.*

Genetic constitution of DNA source	(rRNA/DNA) × 100 at saturation	X contribution (%)	Y contribution (%)	Genetic constitution of DNA source	(rRNA/DNA) × 100 at saturation	X contribution (%)	Y contribution (%)
Y^{bbN2}/X^{bb}	0.198	0.101	0.097	$Y^{bb\ rev}/v$	0.272	0.127	0.145
Y^{bbN2}/X^{bb}	0.220	0.120	0.100	$Y^{bb\ rev}/v$	0.250	0.119	0.131
Y^{bbN2}/X^{bb}	0.180	0.101	0.079				*0.138*
Y^{bbN2}/X^{bb}	0.222	0.110	0.112	Y^{+} Urbana stock (12)			0.131
			0.097	Y^{-bb1}/X^{bb}	0.207	0.101	0.106
Y^{bbN3}/X (*shr, abb*)	0.257	0.164	0.093	Y^{-bb1}/X^{bb}	0.205	0.101	0.104
Y^{bbN3}/X (*shr, abb*)	0.269	0.185	0.084	Y^{-bb1}/X^{bb}	0.182	0.101	0.081
Y^{bbN3}/X^{bb}	0.215	0.101	0.114	Y^{-bb}/X^{bb1}	0.200	0.090	0.110
Y^{bbN3}/X^{bb}	0.222	0.101	0.121	Y^{-bb1}/X^{bb}	0.185	0.090	0.095
Y^{bbN3}/X^{bb}	0.218	0.101	0.117	Y^{-bb1}/X^{+}	0.228	0.135	0.093
$Y^{bbN3}/sc^{4}sc^{8}$	0.070	—	0.070				*0.098*
$Y^{bbN3}/sc^{4}sc^{8}$	0.076	—	0.076	Y^{-bb2}/w^{m4w}	0.276	0.191	0.085
			0.096	Y^{-bb2}/w^{m4w}	0.236	0.176	0.060
$Y^{bb}Su$-var5$/w^{m4}$	0.156	0.118	0.038	Y^{-bb2}/w^{m4w}	0.268	0.203	0.065
$Y^{bb}Su$-var5$/w^{m4}$	0.176	0.119	0.057	Y^{-bb2}/w^{m4w}	0.234	0.177	0.057
			0.047				*0.066*
				$Y^{-bb2}/y^{2}eq$	0.428	0.307	0.121
				$Y^{-bb2}/y^{2}eq$	0.396	0.267	0.129
							0.125

The above Y chromosomes behaved exactly as expected: those giving saturation levels below 0.135 per cent of the diploid genome showed the bobbed phenotype when in single dose, while those contributing 0.135 per cent or more of this DNA allowed the wild phenotype to appear even if combined with an X chromosome completely deficient for the *bb* locus. On the other hand, rather unexpected results were obtained with another set of Y chromosomes, all classified as carrying complete deficiencies of the *bb* locus.

Y^{-bb}: This mutated Y chromosome was obtained by Schultz.[13] Cytological analysis showed that its short arm was reduced to about one third of its normal length.[13] The deficiency thus includes chromatin different from the *bb* locus. Three different stocks carrying the same chromosome have been analyzed.

Y^{-bb1}/w sn bb: The Y chromosome of this stock behaves genetically as a bobbed

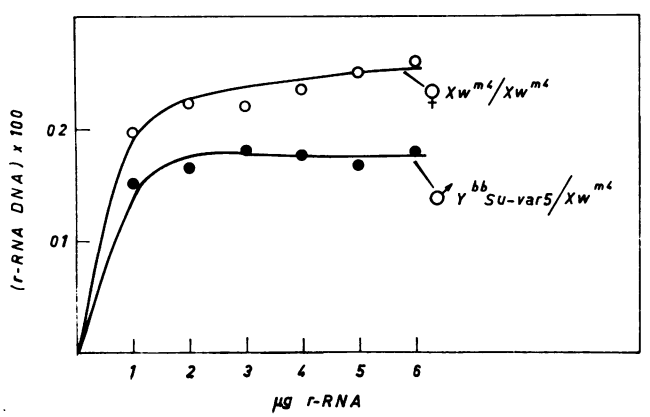

FIG. 2.—Comparison of the saturation levels in hybridization experiments with ribosomal RNA of the DNA's obtained from females having homozygous the X w^{m4} chromosome and from males carrying one of these X's and the Y^{bb}-*Su*-var5 chromosome. The X w^{m4} chromosome of this stock carries itself a weak *bb* locus.

deficient chromosome. It is neither viable with an X chromosome fully deficient for *bb* (*sc*^{4}*sc*^{8}) nor with an X carrying a lethal mutation of *bb*. Furthermore, when combined with a series of X^{bb} tester chromosomes, it shows no sign of additivity in the expression of the *bb* character. Attempts have also been made to produce $X^{bb\ def.}/Y^{-bb1}/Y^{-bb1}$ males but with no success. For this purpose, $X^{bb\ def.}/Y^{B^s}/Y^{-bb1}$ males were crossed with $\widehat{xx}/Y^{-bb1}$ females. Unexpectedly, however, the Y^{-bb} chromosome contains an amount of DNA complementary to ribosomal RNA, which is 0.098 per cent of the diploid genome. This amount of rDNA is of the same order of magnitude as that carried by the Y^{bbN2} and Y^{bbN3} chromosomes, which is sufficient to ensure survival. The above estimate is the mean of a series of experiments in which different X's have been used as carriers for this mutated chromosome (Figs. 3 and 4 and Table 1).

Since no $X^{bb\ del.}/Y^{-bb1}/Y^{-bb1}$ males have been obtained and we know that additive effect of the bobbed loci of different Y chromosomes occurs, we can conclude that even an amount of rDNA of the order of 0.196 per cent of the diploid genome is lethal if carried by this mutated Y. Such an amount of rDNA is more than enough to guarantee wild-type phenotype (Table 1).

Y^{-bb2}/w^{m4w}: This Y chromosome behaves as a *bb* deficiency on genetic analy-

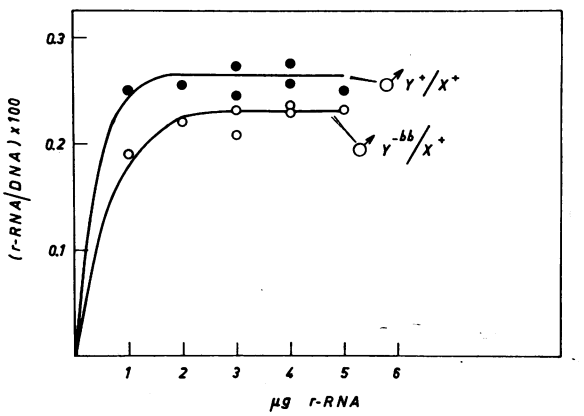

FIG. 3.—Sib females of the Urbana wild type were independently crossed to males carrying a Y^{+} chromosome (Urbana stock) and to males carrying the Y^{-bb1} chromosome. The males were independently collected and their DNA hybridized by H^{3}-labeled rRNA.

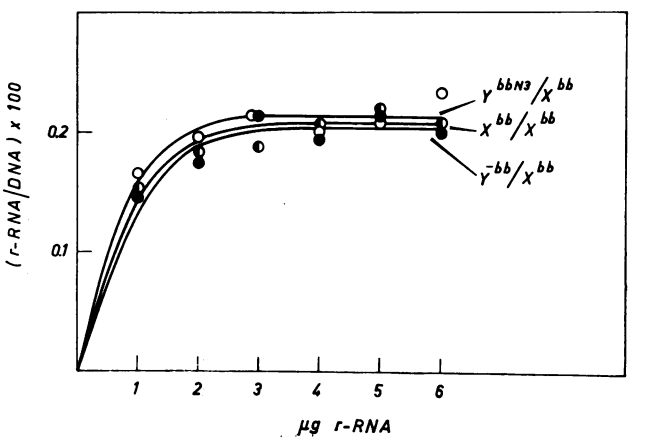

FIG. 4.—Comparison of the saturation levels using DNA from X^{bb}/X^{bb} females and from males obtained by combining one of these X^{bb}'s with Y^{bbN3} and Y^{-bb1}. Males carrying the Y^{-bb1} chromosome have a much stronger *bb* phenotype than males carrying the Y^{bbN3} chromosome.

sis. In this case also the combination $X^{bb\ def.}/Y^{-bb2}/Y^{-bb2}$ is not viable. As shown in Table 1 and Figure 5, the amount of rDNA carried by this chromosome is 0.066 per cent of the diploid genome.

Y^{-bb3}/y^2 eq: The Y chromosome from this stock is no longer *bb*-deficient on genetic analysis. In combination with $X^{bb\ def.}$ and X^{bbl}, it gives viable males which are very slightly bobbed. Males which are $X^{bb\ def.}\ Y^{-bb3}/Y^{-bbl}$ show a wild-type phenotype. The amount of rDNA carried by this Y chromosome is 0.125 per cent of the diploid genome (Table 1).

Discussion.—The conclusion that the bobbed mutation involves a partial deficiency of rDNA[2] is confirmed for the Y chromosome also. Three independently obtained mutations (Y^{bbN2}, Y^{bbN3}, Y^{bb}*Su-var*) show deficiency of rDNA when compared with the amount of this DNA carried by the wild Y chromosome. One special case of interest is the Y chromosome listed as carrying a *bb* mutant but found to be reverted to wild-type.

The ability of the *bb* locus to revert to wild-type is well known,[14] but opinions on the molecular events leading to *bb*-curing are rather vague. It is often thought that reversion occurs by accumulation of extra Y chromosomes or by successive formation of suitable modifiers. The analysis presented here (see Table 1) suggests that *bb*-curing is paralleled by an increase in rDNA content. Tests for extra Y presence were negative. The conclusion that the effective mechanism of *bb* curing does in fact involve gain of genes for rRNA in this case may be contradicted if one assumes that: either (*a*) the *bb* phenotype was formerly due to something different from an rDNA partial deficiency, or (*b*) the Y chromosome is no longer the original one. In addition to the published data, the correlation *bb* = rDNA deficiency holds in a large additional series of data and no *bb*-resembling mutant has been reported for the Y chromosome.

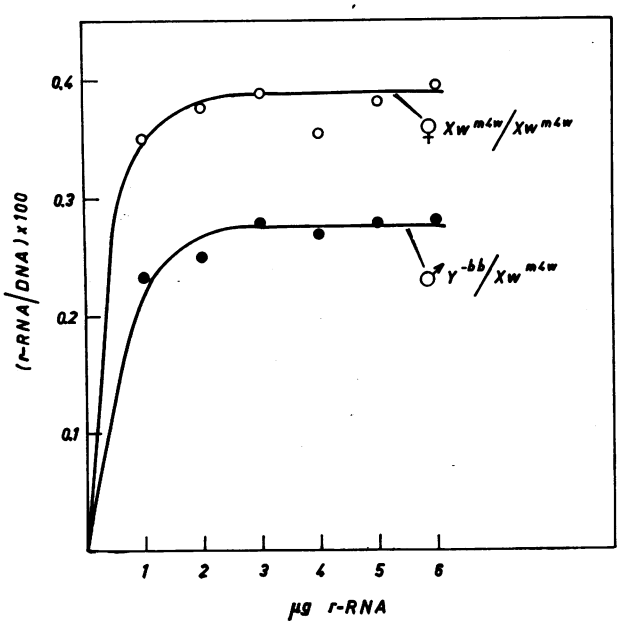

FIG. 5.—Comparison of the saturation levels of female and male partners of the stock carrying the Y^{-bb2} chromosome.

Concerning possibility (*b*), it can be stated definitely (to be published) that gain of rDNA parallels *bb*-curing also in the X chromosome and in cases in which the suitably labeled X is genetically isolated from any other X.

As is the case for *bb* mutants in X, those in Y show a correlation between the extent of the deficiency of rDNA and the intensity of phenotype, with the clear-cut exception of the Y^{-bb} mutants presented here.

Two of these Y's (Y^{-bb1}, Y^{-bb2}) carry enough rDNA to ensure survival (see Table 1), but when combined with an X deficient for the *bb* region (sc^4sc^8) or carrying a *bb* lethal locus, they give no viable progeny. The rDNA they carry is functionally not operative. A number of explanations for this behavior may be suggested: (*a*) Since the Y^{-bb} chromosome originated by a deletion of a large part of the short arm of Y, one may suppose that the deleted part of the Y, other than rDNA, contains some gene needed for the transcription of the rDNA carried by the Y chromosome or for the utilization of the specific rRNA of the Y. In this case, if the gene can act when in a *trans* relation to the rDNA, one would expect Y^{-bb} to become active in the presence of a normal Y. This possibility must be discounted because, when males were produced of the composition $X^{bb\ def.}/Y^{-bb1}/Y^{bbN3}$, they showed a bobbed and not a wild-type phenotype; not even in the presence of normal Y is the rDNA of the Y^{-bb} used. (*b*) The Y^{-bb} can carry unbalanced proportions of ribosomal components. One might suppose the genes for the 18S ribosomal component to be adjacent to each other and to represent some 0.043 per cent of the diploid genome; similarly, the genes for the 28S ribosomal component might constitute a block representing about 0.086 per cent of the diploid genome. These fractions of rDNA correspond to 130 genes for the 18S molecule and 130 genes for the 28S ribosomal component. The Y^{-bb2} (0.066% of rDNA) could thus be a deletion involving all genes for 18S rRNA, thus behaving like a complete deletion where ribosomal formation is concerned. This interpretation is not tenable, however, in the case of the Y^{-bb1} chromosome. The rDNA it carries is 0.098 per cent, which guarantees the presence of at least 30 genes for 18S rRNA in the most extreme cases. If this number is the threshold of lethality, one should have viable progeny when two Y^{-bb1} are present, namely, when the presence of 60 genes for 18S is assured. Evidence is also accumulating against this kind of organization of the genes for 28 and 18S RNA (manuscript submitted for publication). (*c*) A large fraction of the rDNA carried by the Y^{-bb} chromosome is mutated so that the ribosomal RNA is incapable of forming active ribosomes, but is still able to hybridize fully with wild-type rDNA. This possibility cannot at present be completely ruled out. However, there are two observations which point against it. The first is the complete absence of additive effect to other *bb* loci. We know that as few as 20–30 genes of the bb^1 locus have additive effect.[15, 2] More than 80 per cent of the genes of the Y^{-bb} should be mutated to justify its behavior. However, a mechanism could be invoked like that proposed by Callan and Whitehouse,[16, 17] with the assumption that a "master" gene is mutated. Secondly, there is the report that the Y^{-bb} mutation was a single-step mutation. (*d*) Finally, the possibility must be mentioned that the rDNA of the Y^{-bb} chromosomes is inoperative because of the absence of an "operator,"[18] triggering off the function

of the entire locus, or because of some kind of position effect. The formation of the Y^{-bb} chromosome involved the loss of a considerable part of the short arm of the Y chromosome. If the genes for rRNA are contiguous, then one point of breakage could have occurred within the *bb* locus, because the Y^{-bb} carries a partial deficiency of rDNA. The other point of breakage should then be distal to this point but before the K2 fertility factor, since the males carrying this chromosome are fertile (Fig. 6). The possibilities are that either the deleted region contains a point triggering the activity of the entire locus or the insertion of the distal part of the Y chromosome, which then becomes adjacent to the rDNA, is causing a blockade of transcription. Against the first and most attractive possibility are the known instances in which the nucleolus organizer is split into functional portions.[19-21] It is perhaps significant, however, that no such mutation has ever been reported in the long genetic history of *D. melanogaster*.

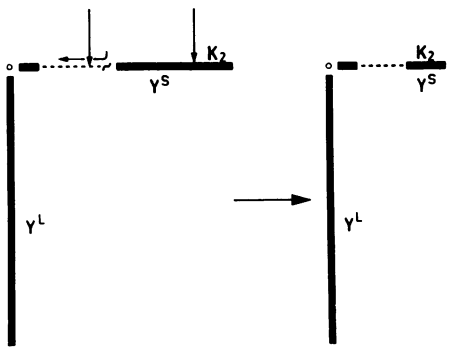

FIG. 6.—Diagram showing a possible organization of the *bb* locus in the Y^+ chromosome and of the events leading to the formation of the Y^{-bb} chromosome (see text for discussion).

The Y^{-bb} chromosomes analyzed in this paper carry different amounts of rDNA (Table 1). If the three Y's have the same origin, as is the case (so far as is known), one has to conclude, as for the Y^{bb} which reverted, that gain and loss of rDNA is possible also in the Y chromosome.

The mechanism(s) responsible may well be similar to those operating in the X chromosome. The *bb* region of the Y chromosome must not be viewed indeed as isolated from the homologous region of the X. The region of homology between X and Y chromosomes encompasses the *bb* region; hence exchanges between the rDNA of the X and the rDNA of the Y are possible, and even unequal distributions of parts of the *bb* locus have been reported following this event.[22, 23]

As to the Y^{-bb3} chromosome, which has functional rDNA and is still carrying the K2 fertility factor, it can be seen as the result of double crossing-over.

Summary.—A series of *bb* mutants of the Y chromosome of *D. melanogaster* was analyzed by the rRNA/DNA hybridization technique. It was confirmed that the molecular basis of the *bb* mutation is a partial deficiency of DNA complementary to ribosomal RNA. A mutant was found in which rDNA was present but not operative. Possible interpretations of this mutation are discussed.

I am indebted to Profs. S. Spiegelman and K. C. Atwood for reviewing this manuscript and acknowledge the skillful technical assistance of Mr. G. Scala.

* Work carried out under the Association Euratom-CNR-CNEN contract no. 012-61-12 BIAI.

[1] Atwood, K. C., and J. L. White, personal communication.
[2] Ritossa, F. M., K. C. Atwood, and S. Spiegelman, *Genetics*, **54**, 819 (1966).
[3] Ritossa, F. M., and S. Spiegelman, these PROCEEDINGS, **53**, 737 (1965).
[4] Cooper, K. W., *Chromosoma*, **10**, 535 (1959).
[5] Edström, J. E., W. Grampp, and N. Shor, *J. Biophys. Biochem. Cytol.*, **11**, 549 (1961).
[6] Perry, R. P., these PROCEEDINGS, **48**, 2179 (1962).
[7] Birnstiel, M. L., and M. I. H. Chipchase, *Federation Proc.*, **22**, 473 (1963).
[8] Brown, D. D., and J. B. Gurdon, these PROCEEDINGS, **51**, 139 (1964).
[9] *Natl. Cancer Inst. Monograph*, **23** (1966).
[10] Schalet, A., *Genetics*, **56**, 587 (1967).
[11] Birnstiel, M. L., H. Wallace, J. L. Sirlin, and M. Fishberg, *Nat. Cancer Inst. Monograph*, **23**, 447 (1966).
[12] Ritossa, F. M., and K. C. Atwood, these PROCEEDINGS, **56**, 496 (1966).
[13] Schultz, J., in *Carnegie Inst. Wash. publ.*, **552** (1915) p. 233.
[14] Bridges, C. B., and K. S. Breheme, in *Carnegie Inst. Wash. publ.* **552** (1915), p. 233.
[15] Stern, C., *Biol. Zbl.*, **49**, 261 (1929).
[16] Callan, H. G., *J. Cell Sc.*, **2**, 1 (1967).
[17] Whitehouse, H. L. K., *J. Cell Sci.*, **2**, 9 (1967).
[18] Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).
[19] McClintock, B., *Zeitschr. Zellf. Mikr. Anat.*, **21**, 294 (1934).
[20] Beermann, Z., *Chromosoma*, **11**, 263 (1960).
[21] Krivshenko, J., *Genetics*, **44**, 520 (1959).
[22] Neuhaus, M. E., *Biol. Zh.*, **15**, 63 (1936).
[23] Lindsley, D. L., *Genetics*, **40**, 22 (1955).
[24] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).