# *STRUCTURE AND FUNCTION OF E. COLI RIBOSOMES, VIII. COLD-SENSITIVE MUTANTS DEFECTIVE IN RIBOSOME ASSEMBLY**

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*Communicated by R. Alexander Brink, March 14, 1969*

*Abstract.*—*E. coli* mutants that fail to grow at 20°C were isolated, and three such mutants were studied in detail. Two of these are defective in 50*S* ribosome biosynthesis at low temperatures and accumulate 43*S* and 32*S* particles, respectively. The third mutant produces reduced amounts of 50*S* ribosomes and accumulates 21*S* particles at low temperatures. The 43*S* and 32*S* particles contain 23*S* RNA and appear to be precursors of 50*S* ribosomes. The 21*S* particles contain 16*S* RNA. The mutation that causes accumulation of 32*S* particles is linked to the *spc* locus. Isolation and characterization of cold-sensitive mutants should provide a fruitful approach to the study of *in vivo* ribosomal assembly.

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The biosynthesis of ribosomes has been studied by many workers, chiefly with *in vivo* systems (see ref. 1 for a review). Yet the detailed mechanism by which the component molecules are assembled into the final specific and organized structure has remained unclear. One approach to the study of ribosome assembly is to isolate mutants defective in this process and to identify the defects and the genes responsible for them. Although some mutants that show the accumulation of some 50*S* "precursor" particles (sedimenting at about 43*S*) have been isolated previously,[2, 3] the block in the biosynthetic pathway to the 50*S* particles is not complete in these mutants, and the conditions for precursor accumulation have not been defined. Moreover, no systematic method for isolating mutants defective in ribosome assembly has been available.

We have studied the mechanism of ribosome assembly, using the *in vitro* reconstitution system developed recently in our laboratory.[4] The system involves self-assembly of 30*S* ribosomal particles from 16*S* RNA and a mixture of ribosomal proteins, and the mechanism of this *in vitro* assembly reaction has been studied in detail.[5] The rate of assembly is strongly temperature-dependent, with an Arrhenius activation energy of 38 kcal/mole. The rate-determining reaction step is a structural rearrangement of an intermediate particle (21*S* "RI" particles) that is deficient in some ribosomal proteins. This step is almost infinitely slow at 10°C and below, at which temperatures the intermediate particles accumulate. Thus the assembly reaction is inherently easier at higher temperatures in this *in vitro* system. If such a principle also operates in *in vivo* assembly, many mutational defects, either in ribosomal structural components or in extraribosomal components affecting the assembly process, should be intensified at lower temperatures. Such assembly-defective mutants should be inviable at lower temperatures but viable at higher temperatures; that is, they

could be isolated as cold-sensitive mutants. This reasoning proved to be correct. We have found that a significant fraction of cold-sensitive mutants of *E. coli* isolated in a complex medium is defective in ribosome assembly. Independently, Ingraham and his co-workers[6] also found abnormal ribosomal biosynthesis at lower temperatures in some of their cold-sensitive mutants of *Salmonella typhimurium*.

*Materials and Methods.*—All mutants used were derived from *E. coli* AB1133. Cells were mutagenized with nitrosoguanidine[7] and counterselected with penicillin (500–1000 units/ml) for 24 hr at 23°C. Survivors were plated out on a medium containing *tryptone* (1.3%) and meat extract (0.5%), incubated at 42°C, and replica-plated. Colonies that failed to grow after 48 hr at room temperature were isolated and repurified.

Cell extracts were prepared for analysis as follows: Cells were harvested by centrifugation, washed in TMA buffer (0.01 *M* Tris-HCl, pH 7.8, 0.03 *M* $NH_4Cl$, $3 \times 10^{-4}$ *M* $MgCl_2$, $6 \times 10^{-3}$ *M* mercaptoethanol), and broken by alumina grinding. DNase (final concentration 10 $\mu$g/ml) and TMA were added, and the extract was centrifuged at $20{,}000 \times g$ for 20 min. Appropriate portions of the supernatant were layered immediately on 10–30% sucrose gradients (in TMA) and centrifuged for 16 hr at 25,000 rpm in a Spinco SW25.3 rotor. Six-drop fractions were collected in glass vials, to which 10 ml of scintillation fluid (0.1 gm 1,4-bis[2-4-(methyl-5-phenyloxazolyl)]benzene; 4.0 gm 2,5-diphenyl oxazole per liter of toluene) containing 10% Bio-solv (Beckman) were then added.

*Results.—Isolation of ribosomal assembly-defective mutants:* Each of the independently isolated cold-sensitive mutants was grown at 25–30°C, which is slightly above the minimum temperature for growth. Cell extracts were examined in an analytical ultracentrifuge. Between a third and a half of the extracts showed sedimentation patterns different from that of the parental strain. Since the extract from the parental strain contained small amounts of particles (43*S* and 32*S*) other than standard ribosomal subunits (50*S* and 30*S*), differences between mutant patterns and the parental pattern were sometimes only quantitative, and their significance was not clear. Nonetheless, three new types of sedimentation patterns were definitely recognized among several mutants: (*a*) decrease in 50*S* peak and large increase in 32*S* peak (cf. upper part of Fig. 1*b*), (*b*) decrease in 50*S* peak and large increases in 43*S* peak (upper part of Fig. 1*a*), and (*c*) presence of a new peak at about 21*S*. Since these mutants show definite blocks in some steps of ribosomal subunit assembly at 20°C (see below), we shall call them *sad* (subunit assembly-defective) mutants. Mutant *sad*-19, a typical mutant of class (*a*), *sad*-68, a typical one of class (*b*), and *sad*-38, representative of the class (*c*) type, were studied in greater detail. However, because of the genetic instability of *sad*-38, it has not been analyzed as extensively as the other two.

*Accumulation of particles by sad mutants at 20°C: Sad* mutants and the parental strain were grown in complex medium at 42°C, and the exponentially growing cultures were then transferred to 20°C. *Sad*-38 does not grow at 20°C. Both *sad*-19 and *sad*-68 grew very slowly (doubling time 8 and 12 hr, respectively), whereas the parental strain continued (after a short lag) to grow much faster than the mutants (doubling time about 3 hr). Ribonucleoprotein particles that accumulate in these strains at 20°C were labeled by adding $H^3$-uracil to the cultures after the temperature shift down. After incubation for three hours at 20°C, the cultures were divided into two parts. Extracts were prepared

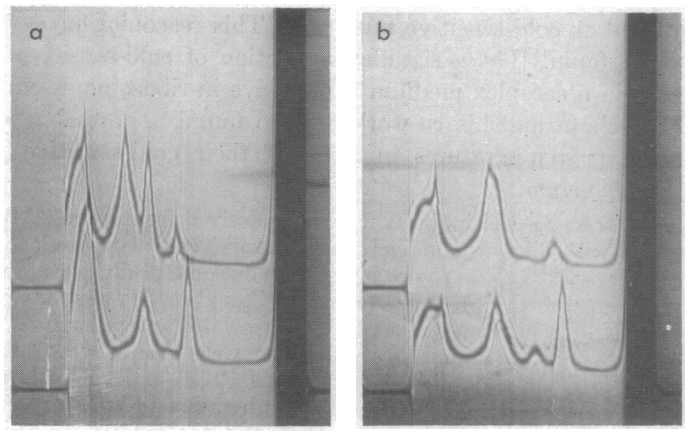


FIG. 1.—Analytical ultracentrifuge patterns using Schlieren optics. Pictures were taken 25 min after reaching maximum speed of 40,000 rpm. (*a*) Upper: *sad*-68; lower: AB1133. (*b*) Upper: a *sad*-19 *aroE*$^+$ transductant; lower: a *sad*-19$^+$ *aroE*$^+$ transductant (see Table 1). Cells used in (*a*) were grown at 37°C for two generations and then shifted to 25°C for an additional 3 hr. Cells used in (*b*) were grown at 25°C to a cell density of 1–2 × $10^8$/ml. All extracts were prepared as described in *Materials and Methods*, with omission of the DNase treatment. The extracts from the original *sad*-19 showed a pattern identical to the upper pattern of (*b*). Sedimentation is from left to right. In all pictures, the fastest-moving peak is 50*S*.

from one part of the cultures, and the radioactive ribonucleoprotein particles were examined by sucrose gradient sedimentation (Fig. 2*a* to *d*). Another part of the cultures was centrifuged, washed, and then incubated at 42°C for an additional two hours in the presence of nonradioactive uracil (Fig. 3*a* and *b*). As shown in Figure 2*b*, *sad-19* does not produce any 50*S* ribosomes at 20°C. It accumulates a large amount of particles sedimenting at about 32*S*, in addition to normal 30*S* ribosomes and a small amount of particles sedimenting at about 21*S*. After the subsequent incubation at 42°C, the radioactive 32*S* particles disappeared, and a large amount of radioactive 50*S* ribosomes was found (Fig. 3*b*). A partially purified 32*S* particle preparation was obtained by combining appropriate fractions from a gradient similar to that shown in Figure 2*b*. Sedimentation analysis of this preparation showed a clear separation of 32*S* particles from 30*S* ribosomes (Fig. 4*a*). RNA prepared from this 32*S* preparation by phenol treatment contains 23*S* RNA (Fig. 4*b*). The presence of 16*S* RNA may be explained by contamination of the preparation by 30*S* ribosomes and/or degradation of 23*S* RNA during the preparation.

In similar experiments *sad*-68 was shown to be defective in formation of 50*S* ribosomes and to accumulate 43*S* particles in large amounts at 20°C (Fig. 2*d*). The 43*S* particles were isolated and shown to contain 23*S* RNA (Fig. 5*a* and *b*). These 43*S* particles disappeared during the temperature shift up, and radioactive 50*S* ribosomes were produced (Fig. 3*a*). Since the total amount of acid-precipitable radioactive RNA was the same before and after the 42°C incubation, it would appear that both 32*S* and 43*S* particles are precursors of 50*S* ribosomes. In similar labeling experiments at 20°C for three hours, *sad*-38

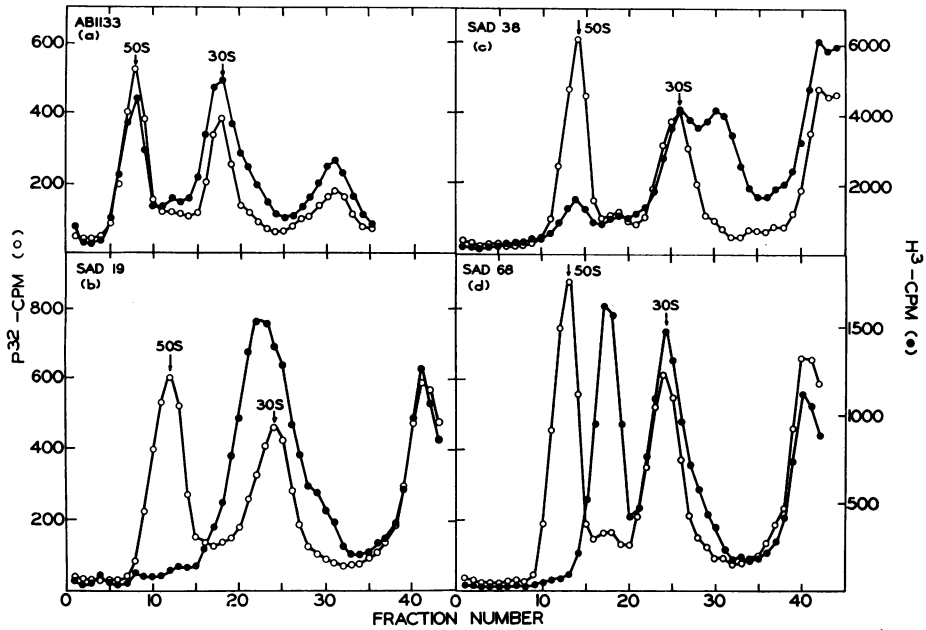


FIG. 2.—Sucrose gradient sedimentation analysis of extracts from cells incubated at 20°C in the presence of $H^3$-uracil. Overnight cultures were diluted 25-fold into 10 ml tryptone broth (1.3% tryptone, 0.7% NaCl) and grown at 42°C to a cell density of about $1 \times 10^8$/ml. The cultures were diluted 1:1 with fresh broth, and 10 ml was transferred to 20°C. After 5 min, $H^3$-uracil (0.1 mc, specific activity 5.6 c/mmole) was added, and incubation was continued for 3 hr. Nonradioactive uracil was added to 50 μg/ml, and the cells were centrifuged and resuspended in 3 ml 0.85% sterile saline containing 40 μg/ml uracil; 1 ml was then mixed with carrier cells. The remaining 2 ml was mixed with 18 ml warm broth containing 20 μg/ml nonradioactive uracil and shaken at 42°C for 2 hr (see legend for Fig. 3). Extracts were prepared and analyzed as described in *Materials and Methods*. The reference is an extract prepared from AB1133 cells grown at 20°C in the presence of $P^{32}$. (*a*) AB1133, (*b*) *sad*-19, (*c*) *sad*-38, (*d*) *sad*-68; all extracts from cells after labeling for 3 hr at 20°C.

accumulated large amounts of 21*S* particles and produced a significantly reduced amount of 50*S* ribosomes (Fig. 2*c*). In addition, particles sedimenting at about 30*S* were found, but these may actually be a mixture of 30*S* and 32*S* particles, since RNA extracted from this fraction contained both 16*S* and 23*S* RNA. The 21*S* particles were isolated (Fig. 6*a*) and shown to contain only 16*S* RNA (Fig. 6*b*). Under identical conditions (3-hr incubation at 20°C), the parental strain synthesizes both 30*S* and 50*S* ribosomes without significant accumulation of 32*S* or 43*S* particles, although small amounts of 21*S* particles are observed (Fig. 2*a*).

*Mapping of sad loci and genetic relationship between cold sensitivity and ribosome assembly defects:* The genetic determinant of the cold-sensitive character of *sad*-19 was found to map near the *spc-str* region. Transduction experiments described in Table 1 show that the cold-sensitive character can be cotransduced with $aroE^+$ from *sad*-19 to a recipient strain AB2834 ($aroE^-$ $sad^+$). Several cold-sensitive transductants ($aroE^+$ $sad^-$) were tested for ability to assemble ribosomes at lower temperatures. All showed the same defect in 50*S* assembly

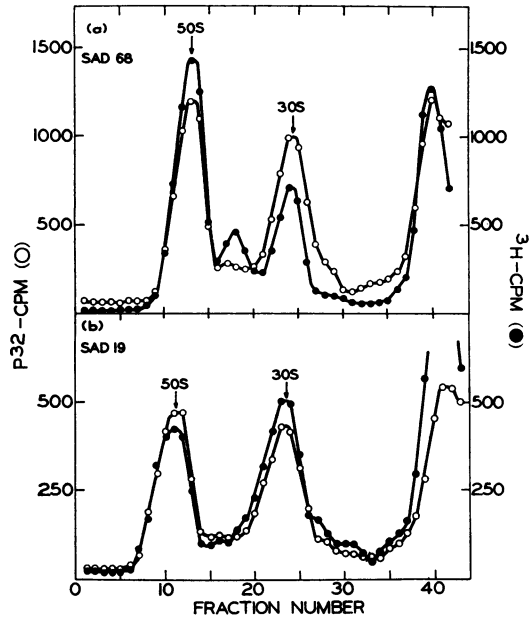


FIG. 3.—Conversion of $H^3$-43*S* particles and $H^3$-32*S* particles to 50*S* particles after temperature shift up. For experimental details, see legend for Fig. 2. (*a*) *sad*-68 extract after 2-hr chase at 42°C; (*b*) *sad*-19 extract after 2-hr chase at 42°C.

at lower temperatures as the donor strain *sad*-19 and accumulated 32*S* particles (upper part of Fig. 1*b*). On the other hand, *aroE*$^+$ transductants that were not cold-sensitive (*aroE*$^+$ *sad*$^+$) did not show such defects in ribosome assembly (lower part of Fig. 1*b*). Thus it appears that both cold sensitivity and the defect in 50*S* ribosome assembly are due to a single mutation.

Similar experiments showed that a genetic determinant of cold sensitivity in *sad*-68 also maps near the *spc-str* region. It was found, however, that these cold-sensitive transductants do not accumulate 43*S* particles but synthesize normal (parental) amounts of 50*S* ribosomes. Although the relationship

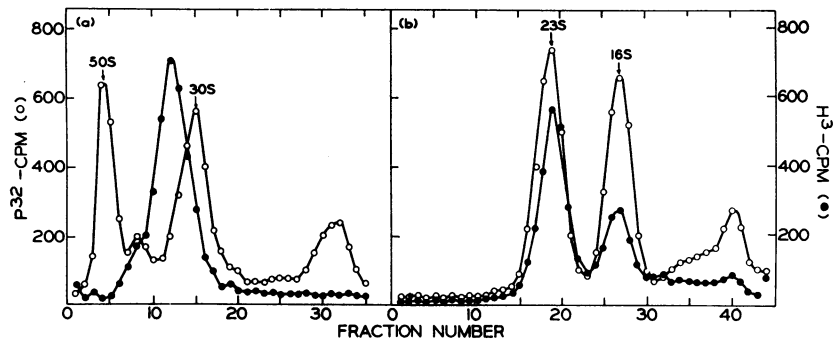


FIG. 4.—(*a*) Sucrose gradient sedimentation analysis of isolated $H^3$-labeled 32*S* particles from *sad*-19 (see text). (*b*) RNA from the $H^3$-labeled 32*S* particle preparation. The RNA was prepared by phenol treatment as described previously.[13] An aliquot was mixed with reference $P^{32}$-labeled RNA and layered on a 5–20% sucrose gradient in SSC (standard saline citrate: 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7) −0.01 *M* EDTA and centrifuged in a Spinco SW25.3 rotor for 16.5 hr at 25,000 rpm.

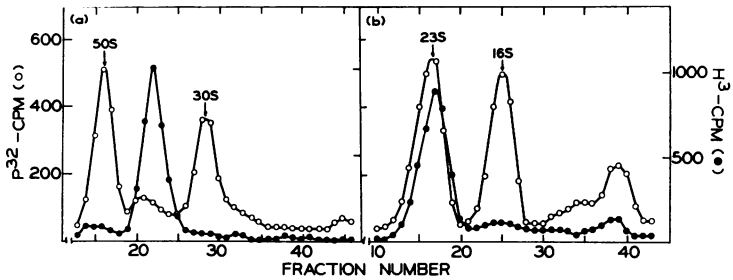


FIG. 5.—(*a*) Sucrose gradient sedimentation analysis of isolated $H^3$-labeled 43*S* particles from *sad*-68. (*b*) RNA from the isolated $H^3$-labeled 43*S* particle preparation. The RNA was prepared and analyzed as described in the legend for Fig. 4.

between cold sensitivity and the *sad*-68 phenotype is thus still unclear, low temperature appears to be required for expression of this phenotype. When grown at high temperature in synthetic medium with acetate as the carbon source, *sad*-68 did not accumulate 43*S* particles although the generation time is about six hours, and normal amounts of 50*S* ribosomes were synthesized. Thus slow growth is not sufficient to produce the *sad*-68 phenotype.

Preliminary tests with *sad*-38 also suggested linkage of the cold-sensitive phenotype with the *spc-str* region. Detailed analysis has not yet been completed.

*Incorporation activity of ribosomes from sad-19 and sad-68:* 70*S* ribosomes were isolated from these cells grown at 37°C and tested for their poly U-directed phenylalanine incorporation activity at 22°C and 37°C. No significant difference was observed from the activity of parental ribosomes at either temperature. It is concluded that the defects in *sad*-19 and *sad*-68 are in the assembly of 50*S* ribosomes at 20°C and that the ribosomes, once assembled at higher temperatures, are able to function in protein synthesis even at lower temperatures. This conclusion is consistent with the observation that *in vivo* both *sad*-19 and *sad*-68 continue making protein at least for the first hour after a temperature shift from 42°C to 20°C, at a rate similar to that of the parental strain.

*Discussion and Conclusions.*—Several ribosomal biosynthetic intermediates have been postulated from short pulse-labeling experiments.[1, 8] These intermediates include 32*S* and 43*S* precursors of 50*S* and 26*S* and 22*S* precursors of 30*S*. However, with the exception of 43*S* particles isolated from chloramphenicol-treated cells[9] or from mutant strains,[10] these various particles have not been characterized, and the nature of the rate-limiting reactions allowing their detection has thus remained unclear.

The *sad* mutants now provide a system in which conditions for the accumulation and conversion of ribosomal assembly intermediates are well defined. It should be possible to characterize these particles, identify the defects caused by the different *sad* mutations, and perform critical experiments to test the validity of the ribosome assembly pathways proposed previously.[1, 8] Although we have not yet identified the specific defects in the *sad* mutations under study, it ap-

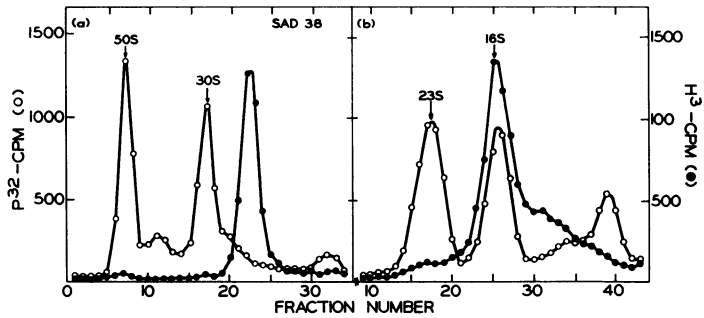


FIG. 6.—(*a*) Sucrose gradient sedimentation analysis of isolated $H^3$-labeled 21*S* particles from *sad*-38. (*b*) RNA from the isolated $H^3$-labeled 21*S* particle preparation. The RNA was prepared and analyzed as described in the legend for Fig. 4.

pears likely that structural alterations in ribosomal components (or in some hypothetical extraribosomal components participating in the assembly reaction) hamper the assembly of ribosomes at lower temperatures.

The close linkage of the *sad*-19 locus to the *spc* and *str* loci was unexpected. The *spc* and *str* loci determine each of two different 30*S* ribosomal proteins,[11, 12] and this region appears to contain a single clustered genetic unit coding for many 30*S* ribosomal proteins. Two possibilities can be considered: (*a*) a 50*S* ribosomal component or some other extraribosomal component is altered by the mutation, and the *sad* locus is linked to genes controlling 30*S* ribosomal protein; (*b*) a 30*S* ribosomal component is altered by the *sad*-19 mutation, and the assembly of the 50*S* ribosome is somehow dependent on the "correct" structure of the 30*S* ribosome or its precursor. It is interesting to note that *sad*-38 appears to have defects in both 30*S* and 50*S* assembly at low temperatures. If this phenotype is proved to be caused by a single mutation, as it now appears, the second alternative would be strongly favored.

Further speculation would be premature, but it is clear that the present genetic system, coupled with the *in vitro* ribosome reconstitution techniques now available, provides a fruitful approach to the study of the *in vivo* ribosomal assembly process.

TABLE 1. *Linkage of sad-19 locus to spc and str loci.*

| | | Numbers in Subclasses | | | |
|---|---|---|---|---|---|
| *sad* Phenotype | Total | $spc^r str^s$ | $spc^s str^s$ | $spc^s str^r$ | $spc^r str^r$ |
| $sad^-$ (cold-sensitive) | 141 | 0 | 26 | 112 | 3 |
| $sad^+$ (cold-insensitive) | 37 | 34 | 1 | 1 | 1 |

| Cotransduction Frequencies | | |
|---|---|---|
| With selected marker | With unselected marker | |
| $sad^-/aroE^+$ | $spc^s/sad^-$ | $str^r/sad^-$ |
| 80% | 98% | 82% |

P1 transduction was performed according to the method of Adler and Kaiser.[14] P1 was grown on *sad*-19 ($aroE^+spc^s str^r$), and the lysate was used to transduce strain AB2834 ($aroE^-spc^r str^s$). $AroE^+$ transductants were selected on minimal plates and spot-tested for characterization. Colonies were scored for antibiotic resistance on nutrient plates containing 200 μg/ml of spectinomycin or streptomycin. The $sad^-$ phenotype was scored by failure of colonies to grow on nutrient plates after 48-hr incubation at 20°C.

We thank Miss Susan Schmidt and Mrs. Margaret Leppik for technical assistance, Dr. J. Davies for supplying a bacterial strain and spectinomycin and for advice in the mapping experiments, and Dr. M. Susman for a critical reading of the manuscript. The work was supported by a U.S. Public Health Service research grant (GM-15422) and by a National Science Foundation grant (GB-3947).

* Paper number 1296 of the Laboratory of Genetics, University of Wisconsin. Paper VII in this series is reference 11.

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