The Diversity of Cyanobacterial Genomes with Respect to Ribosomal RNA Cistrons

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The ribosomal RNAs were isolated from cyanobacteria (*Anacystis nidulans*, *Anabaena cylindrica*, *Anabaena* CA and *Nostoc* MAC) and labelled *in vitro* with [γ - 32 P]ATP and polynucleotide kinase. DNA from the corresponding species was isolated, digested with *Eco*RI or *Hind*III, and resolved on agarose gels. *In situ* hybridization was used to measure the number and location of the 16S and 23S ribosomal RNA genes. The comparative results are discussed in relation to the known genome size of these species and to their RNA content per cell.

INTRODUCTION

The cyanobacteria are the largest, most diverse and most widely distributed group of photosynthetic prokaryotes (Stanier & Cohen-Bazire, 1977). They display many morphological forms, ranging from unicellular species reproducing by binary fission in only one plane, through unicellular species dividing by multiple fission, filamentous species without and with heterocysts dividing in a single phase, to branching filamentous forms. Herdman *et al.* (1979) have suggested that there is a correlation between genome size, measured by renaturation kinetics, and morphological complexity: strains which exhibit a high degree of morphological differentiation almost invariably possess genomes which are larger than those strains with a simple morphological form. The distribution of genome size was discontinuous, dividing into four discrete groups, the mean values for each group corresponding closely to those which would be obtained by a process of genome evolution involving a series of fusions of a smaller ancestral genome.

The diversity in size of the genomes of cyanobacteria may be reflected in the number of rRNA genes encoded by the DNA. In *Escherichia coli* the rRNA genes are organized in seven or eight clusters, each of which contains 16S, 23S and 5S rRNA and a number of tRNA genes (Kiss *et al.*, 1977; Kenerley *et al.*, 1977). The organization is basically similar in *Bacillus subtilis* (Zingales & Colli, 1977), there probably being up to 10 copies of the rRNA operon (Potter *et al.*, 1977). In *E. coli* it has been known for some time that the rRNAs are transcribed polycistronically in the order 5'-16S, 23S, 5S-3' (Hamkalo & Miller, 1973; Pace, 1973). Such an arrangement is probably also present in *B. subtilis* (Zingales & Colli, 1977) and *B. thuringiensis* (Klier *et al.*, 1979). By contrast, in *Beneckeia harveyi*, a marine bacterium, the rRNA genes are linked, but in an order different to that in *E. coli* (Lamfrom *et al.*, 1978). The rRNAs of the cyanobacteria, like those of other prokaryotes, have molecular weights of approximately 0.04×10^6 , 0.55×10^6 and 1.05 – 1.11×10^6 (Pace, 1973; Loening, 1968; Dobson *et al.*, 1974). Except for the determination of multiple (two) rRNA cistrons, linked in the order 16S–23S–5S, in *Anacystis nidulans* (Doolittle *et al.*, 1979), nothing is known of the organization and number of rRNA genes in other species of cyanobacteria. This communication presents evidence for the diversity of cyanobacteria with respect to the multiplicity of rRNA cistrons determined, in four species, by Southern hybridization (Southern, 1975) of *in vitro* labelled RNA on to restricted DNA.

METHODS

Organisms and culture conditions. *Anabaena* CA (ATCC 33047) was grown at 39 °C and 230 $\mu\text{E m}^{-2} \text{s}^{-1}$ on BG-11 medium (Rippka *et al.*, 1979), modified by adding 75 mM-NaCl and 0.3 g HEPES l^{-1} , and by increasing the concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to 20 mM and that of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to 3.3 mM. *Anabaena cylindrica* (Culture Centre of Algae and Protozoa, Cambridge, U.K., no. 1403/2a) was grown at 29 °C and 45 $\mu\text{E m}^{-2} \text{s}^{-1}$ on the medium of Allen & Arnon (1955) modified by the addition of 4 mM- NH_4Cl . *Anacystis nidulans* (Culture Collection of Algae at the University of Texas, Austin, Texas UTEX 625) was grown at 30 °C and 90 $\mu\text{E m}^{-2} \text{s}^{-1}$ on Medium C (Kratz & Myers, 1955) with the addition of 1 g $\text{NaHCO}_3 \text{l}^{-1}$. *Nostoc* MAC (known also as *Nostoc* sp., supplied by Dr A. J. Smith, Department of Biochemistry, University of Wales, Aberystwyth and now deposited with the Culture Collection of the Institut Pasteur, PCC 8009) was grown at 37 °C and 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ on Medium C. All cultures were gassed with air/ CO_2 (95:5, v/v).

Preparation of DNA. Organisms were harvested by centrifugation during exponential growth, washed in 0.15 M-NaCl, 0.1 M-EDTA, pH 8, and resuspended to 1 g wet wt cells per 10 ml in the above buffer. The suspension was incubated with 1 mg lysozyme ml^{-1} for 2 h at 37 °C. DNA was subsequently extracted by the method of Marmur (1961) and finally resuspended in 10 mM-Tris/HCl pH 8, containing 1 mM-EDTA and stored at 4 °C, over chloroform.

Isolation and radioactive labelling of RNA. All organisms were harvested during exponential growth by centrifugation at 4000 g for 20 min. RNA was isolated from *Anabaena cylindrica* after washing and resuspension of the cells in 20 mM-Tris/HCl, pH 7.8 containing 5 mM- MgCl_2 . The cells were broken at 84 MPa (12000 lbf in^{-2}) in a French pressure cell and collected into 20% (w/v) SDS to a final concentration of 1% SDS. The RNA was isolated by three extractions with an equal volume of a mixture of water-saturated phenol/cresol/hydroxyquinoline (50 g:7 ml:0.05 g) at 4 °C. To the final supernatant was added 0.1 vol. 2 M-sodium acetate, pH 6.0 and 2 vol. absolute ethanol. The precipitated RNA was collected by centrifugation and washed in 0.15 M-sodium acetate, pH 6.0, containing 0.5% SDS; the RNA was reprecipitated with ethanol, then finally resuspended in 0.15 M-sodium acetate pH 6.0 and stored at -70 °C. 16S and 23S rRNAs were prepared by precipitation with 1.5 M-NaCl for 10 h at 4 °C and each rRNA purified by electrophoresis on 2.8% polyacrylamide gels (Loening, 1968). The bands were located by UV shadowing (Hassur & Whitlock, 1974); the RNA was extracted from the gels as described by Maxam & Gilbert (1977) and further purified by passing through columns of Whatman CF-11 cellulose (Franklin, 1966).

Ribosomal RNAs were purified from *Anabaena* CA, *Anacystis nidulans* and *Nostoc* MAC by the isolation of ribosomes and subsequent dissociation into subunits. Organisms were harvested and washed with 10 mM-Tris/HCl, pH 7.6, containing 100 mM-KCl and 10 mM-magnesium acetate (buffer A). After resuspension in a small volume of Buffer A and addition of RNAase-free DNAase (Millipore) to 2 $\mu\text{g ml}^{-1}$, the cells were broken in a French Pressure cell at 84 MPa. Triton X-100 was added to a final concentration of 5% (v/v) and the suspension incubated for 15 min at 4 °C. Two centrifugations at 15000 g for 15 min removed cell debris and the supernatant was layered over equal volumes of 1 M-sucrose in buffer A and centrifuged at 165000 g for 2.5 h. The resulting ribosomal pellets were resuspended in 10 mM-Tris/HCl, pH 7.6, containing 100 mM-KCl and 0.1 mM-magnesium acetate (buffer B) and the dissociated ribosomal subunits fractionated on 36 ml 7.5–20% sucrose gradients in buffer B for 16 h at 5 °C and 70000 g. The 30S and 50S subunits were separately recovered by adjusting peak fractions to 10 mM-magnesium acetate and adding polyethylene glycol to a final concentration of 10% (w/v) (Expert-Bezancon *et al.*, 1974). After standing on ice for 1 h, the subunits were recovered by centrifugation at 15000 g for 20 min and resuspended in 50 mM-Tris/HCl, pH 8.5, containing 500 mM-KCl, 10 mM-EDTA and 0.1 mM-magnesium acetate (buffer C) and again fractionated on 7.5–20% sucrose gradients in buffer C minus the EDTA. The 50S and 30S subunits were precipitated by the addition of magnesium acetate (final concentration, 10 mM) and 1.5 vol. ethanol; the suspension was allowed to stand for 2 h at 4 °C, and subunits were recovered by centrifugation at 18000 g for 20 min.

The subunits were resuspended in 20 mM-Tris/HCl, pH 7.8, containing 5 mM- MgCl_2 , and the RNA isolated as described for *Anabaena cylindrica*. The RNA preparations were checked for purity by electrophoresis on 2.8% polyacrylamide gels.

High specific activity *in vitro*-labelled 16S and 23S rRNAs were prepared by partial hydrolysis in 50 mM-Tris/HCl, pH 9.5, containing 0.1 mM-spermidine (95 °C for 15 min) and 5'-end labelling by the method of Silberklang *et al.* (1979) with polynucleotide kinase (Boehringer-Mannheim) and 1.85 MBq [γ - ^{32}P]ATP (74 TBq mmol^{-1} ; Amersham).

Hybridization of rRNAs to DNA. DNA (9–12 μg) was digested in 100 μl reaction volumes at 37 °C for 16 h, with either *Eco*RI (Boehringer-Mannheim) or *Hind*III (Bethesda Research Laboratories), each at 30–40 units μg^{-1} . The incubation buffer for *Eco*RI contained 100 mM-Tris/HCl, pH 7.5, 50 mM-NaCl and 10 mM- MgCl_2 , and that for *Hind*III, 20 mM-Tris/HCl, pH 7.4, 60 mM-NaCl and 7 mM- MgCl_2 . In each case the reaction was terminated by the addition of 20 μl of a mixture containing 0.2% (w/v) agarose, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol FF. Phage λ DNA, digested with *Eco*RI or *Hind*III (4 units μg^{-1}) was used as a molecular weight marker.

The digested cyanobacterial DNA and the λ DNA marker were resolved on 0.7% agarose gels dissolved in either 50 mM-Tris/HCl, pH 8.0, containing 2 mM-sodium acetate and 18 mM-NaCl, or 160 mM-Tris/acetate, pH 8.0, containing 8 mM-EDTA, 80 mM-sodium acetate and 80 mM-NaCl. Gels were run for 16–24 h at 100 mA, at room temperature. The DNA fragments in the gels were denatured, neutralized and transferred to cellulose nitrate filters as described by Southern (1975). Filter strips, 1 cm wide, containing approximately 2 μ g DNA, were hybridized with up to 1 μ g of *in vitro*-labelled rRNAs (usually greater than 10^6 c.p.m. μ g⁻¹) in 300 μ l 4 $\times$ SSC (0.6 M-NaCl, 0.06 M-sodium citrate), 50% (v/v) formamide and *E. coli* tRNA (60 μ g ml⁻¹). The filters were sealed with Cling-film and hybridization carried out at 42 °C for 16 h. The strips were then washed twice for 1 h in 4 $\times$ SSC/50% (v/v) formamide, at 42 °C, rinsed in 2 $\times$ SSC, digested with pancreatic ribonuclease (20 μ g ml⁻¹) in 2 $\times$ SSC at 20 °C for 30 min, then finally washed three times for 10 min in 2 $\times$ SSC. After air-drying, the filter-strips were mounted on a glass plate and the radioactive bands detected by autoradiography using Ilford Red Seal 100 FW X-ray film.

RESULTS

Anacystis nidulans

The hybridization of *Anacystis nidulans* rRNAs to *A. nidulans* DNA digested with *Eco*RI is shown in Fig. 1(a) (lanes 2 and 4). 16S rRNA gave hybridization signals with fragments of approximately 15 MDal and 12 MDal and with a double band of 5.2/5.1 MDal, whereas 23S rRNA only hybridized with the 15 and 12 MDal fragments. The 23S rRNA and 16S rRNA genes must, therefore, be closely linked since, except for the 5.2/5.1 MDal fragments, they hybridized to identical fragments of DNA. The hybridization of only 16S rRNA to the smaller 5.2/5.1 MDal fragments showed that there must be an *Eco*RI site within the 16S cistron. A possible explanation for this hybridization pattern is given in Fig. 1(b). The approximately equal intensity of the 12 MDal and 15 MDal bands (as measured by a Zeiss scanning densitometer) suggested an equal number of cistrons on each fragment; the simplest explanation for this would be that there are two rRNA cistrons in *A. nidulans*, as also suggested by Doolittle *et al.* (1979). If there were more than two rRNA cistrons, flanking sequences at some distance (as much as 18 kb = 12 MDal) must have been conserved. The 12 and 15 MDal fragments were created because of the heterogeneity in the flanking sequence of the 23S rRNA cistron, the *Eco*RI site therefore being at a different position. The 5.2/5.1 MDal double band could be explained in the same way. The rRNA cistrons must be at least 25 kb apart (equivalent to 16.7 MDal of double-stranded DNA). The size of the spacer DNA between the 16S and 23S rRNA cistrons cannot be determined from these results. The analogous spacer in *E. coli* is quite small, being approximately 0.2 MDal (Lund *et al.*, 1976). The exact position of the *Eco*RI site within the 16S rRNA cistron cannot be determined, but it is assumed to be near the centre, since each of the smaller 5.2/5.1 MDal fragments were of approximately equal intensity to each of the two larger fragments.

When *A. nidulans* DNA was digested with *Hind*III, the 16S rRNA hybridized to a 2.8 MDal fragment, while 23S rRNA hybridized to five fragments of 5.7, 2.3, 0.9, 0.7 and 0.5 MDal (Fig. 1a, lanes 1 and 3). Since the 16S and 23S rRNA hybridized to no common fragment, a *Hind*III site must be present in the spacer region between the 16S and 23S cistrons. A tentative map of the approximate *Hind*III sites in relation to the 16S and 23S RNA cistrons is given in Fig. 1(b), again assuming that there are two rRNA cistrons. The 5.7 and 2.3 MDal fragments hybridizing to 23S rRNA were of equal intensity, but no comparison can be made with the three small fragments, since the bands were very diffuse. Therefore, it is not possible to determine the exact *Hind*III sites within the 23S rRNA cistron.

Anabaena CA

Both 16S rRNA and 23S rRNA from *Anabaena* CA gave hybridization signals with DNA fragments of 7.8, 5.3, 4.2, 3.0 and 2.4 MDal after digestion of *Anabaena* CA DNA with *Eco*RI (Fig. 2a). There was also evidence of two faint hybridization bands of low molecular weight for the 23S rRNA, but possibly not for the 16S rRNA. These results show that the 16S and 23S rRNAs are closely linked and that there are a minimum of five rRNA cistrons. Since the 3.0 and 2.4 MDal fragments are smaller than the length of DNA needed to code for the 16S rRNA plus

Fig. 1. (a) Hybridization of ^{32}P -labelled *Anacystis nidulans* rRNAs to *A. nidulans* DNA. The DNA was digested with *Hind*III (lanes 1 and 3) or *Eco*RI (lanes 2 and 4), resolved on a 0.7% agarose gel and following transfer to nitrocellulose filter strips was hybridized with 16S rRNA (10^6 c.p.m., 200 ng; lanes 1 and 2) or 23S rRNA (10^6 c.p.m., 100 ng; lanes 3 and 4). The sizes of the DNA fragments are given in MDal.

(b) Diagram showing the arrangement of the *A. nidulans* rRNA cistrons with respect to the tentative *Eco*RI and *Hind*III restriction sites. The numbers indicate the sizes of the DNA fragments (MDal).

23S rRNA (minimum of 3.32 MDal) it follows that there must be one (or two) *Eco*RI sites within two rRNA cistrons. This points to minor differences in the rRNA cistrons. The two faint hybridization signals with fragments of low molecular weight for the 23S rRNA could account for the small portions of the 23S rRNA cistron, produced by one *Eco*RI site within two 23S rRNA cistrons.

Digestion of *Anabaena* CA with *Hind*III also gave multiple hybridization signals. Four fragments of molecular size 2.8, 2.3, 1.9 and 1.6 MDal were common to both 23S and 16S rRNA (Fig. 2b). 23S rRNA also hybridized to a fragment of 8 MDal, but 16S rRNA only gave a very faint signal with this fragment. Since all the fragments, except for that at 8 MDal, are below the minimum cistron size for 16S and 23S rRNA, one would expect to see smaller, but non-identical

Fig. 2. Hybridization of ^{32}P -labelled rRNAs to *Anabaena* CA DNA resolved on 0.7% agarose gels. The DNA was digested with (a) *Eco*RI or (b, c) *Hind*III, and following transfer to nitrocellulose filter strips was hybridized with: (a) *Anabaena* CA 16S rRNA (1.6×10^5 c.p.m., 210 ng) or *Anabaena* CA 23S rRNA (1.6×10^5 c.p.m., 70 ng); (b) *Anabaena* CA 16S rRNA (3.9×10^5 c.p.m., 540 ng) or *Anabaena* CA 23S rRNA (3.7×10^5 c.p.m., 270 ng); (c) *Anabaena cylindrica* 16S rRNA (5×10^5 c.p.m., 30 ng) or *Anabaena cylindrica* 23S rRNA (5×10^5 c.p.m., 30 ng). The sizes of the DNA fragments are given in MDal.

fragments that hybridize with the 16S and 23S rRNAs. These were not evident but it is possible that they had not been retained on the cellulose nitrate filter during the stringent washing procedure. However, these results confirm those obtained with *Anabaena* CA DNA digested with *Eco*RI, in that they show a minimum of five rRNA cistrons.

Hybridization of rRNAs from *Anabaena cylindrica* to *Hind*III-digested *Anabaena* CA DNA gave the same signals as for the rRNA from *Anabaena* CA (Fig. 2c). This demonstrates the high degree of conservation between the rRNA of these two species of cyanobacteria.

Anabaena cylindrica and *Nostoc* MAC

The hybridization of rRNAs from *Anabaena cylindrica* to DNA digested with *Eco*RI again showed that 16S and 23S rRNA cistrons are closely linked, since, excepting two that were faintly labelled, they hybridized to identical DNA fragments (Fig. 3a). The predominant bands are of molecular size 1.75 and 1.63 MDal, pointing to minor heterogeneity within the rRNA cistrons. No good explanation can be found for the less intensely labelled fragments of both higher and lower molecular weights. The 2.85 MDal fragment only hybridized to 16S rRNA, as did the (probable) double fragment of approximately 0.55 MDal. Degradation of the DNA could explain the presence of smaller fragments, although it is unlikely that one would be able to detect well-defined labelled fragments resulting from this process. Incomplete digestion may explain the larger fragments; however, the experiment was repeated a number of times, with the same results.

The hybridization pattern obtained when *A. cylindrica* DNA was digested with *Hind*III is shown in Fig. 3(b). Both 16S and 23S rRNAs hybridized to six identical fragments of 4.95, 3.95, 2.50, 2.27, 1.80 and 1.28 MDal. This result suggested a minimum of six rRNA cistrons for *A. cylindrica*. Ribosomal RNAs from *Nostoc* MAC hybridized to DNA digested with *Eco*RI gave identical hybridization signals, although these signals were not of the same relative intensity

Fig. 3

Fig. 4

Fig. 3. Hybridization of ^{32}P -labelled *Anabaena cylindrica* rRNAs to *A. cylindrica* DNA. The DNA was digested with (a) *Eco*RI or (b) *Hind*III, resolved on 0.7% agarose gels, and following transfer to nitrocellulose strips was hybridized with: (a) 23S rRNA (10^6 c.p.m., 800 ng) or 16S rRNA (10^6 c.p.m., 685 ng); (b) 16S rRNA (1.5×10^6 c.p.m., 100 ng) or 23S rRNA (1.5×10^6 c.p.m., 94 ng). The sizes of the DNA fragments are given in MDal.

Fig. 4. Hybridization of ^{32}P -labelled *Nostoc* MAC rRNAs to *Nostoc* MAC DNA. The DNA was digested with *Eco*RI, resolved on a 0.7% agarose gel, and following transfer to nitrocellulose filter strips was hybridized with 16S rRNA (10^6 c.p.m., 310 ng) or 23S rRNA (10^6 c.p.m., 218 ng). The molecular sizes of the DNA fragments are given in MDal.

(Fig. 4). The most intensely labelled fragments were 1.6 and 0.4 MDal, with additional labelled fragments of 1.0, 1.2, 2.1 and 3.9 MDal. Again, the results indicate that the 16S rRNA and 23S rRNA cistrons are closely linked. The generation of the fragments of molecular size 2.1 MDal and less, hybridizing to both 16S and 23S rRNAs must be the result of two *Eco*RI sites within the rRNA cistrons, and also suggests the presence of multiple cistrons. As with the results from *Anabaena cylindrica*, it is unlikely that degradation or incomplete restriction of the DNA explains the multiple bands.

DISCUSSION

In the four species of cyanobacteria studied, the cistrons for 16S rRNA and 23S rRNA are closely linked. This is in agreement with the arrangements found in other prokaryotes such as *Escherichia coli* (Kiss *et al.*, 1977; Lund *et al.*, 1976), *Bacillus subtilis* (Zingales & Colli, 1977), *Bacillus thuringiensis* (Klier *et al.*, 1979) and *Beneckea harveyi* (Lamfrom *et al.*, 1978). In addition, the results also demonstrate the diversity of the cyanobacteria with respect to the number of rRNA cistrons. In *Anacystis nidulans* there are probably only two cistrons, whereas in *Anabaena*

CA there appears to be a minimum of five. *Anabaena cylindrica* and *Nostoc* MAC both gave complicated hybridization patterns with *Eco*RI-digested DNA, suggesting the presence of multiple rRNA cistrons, although the exact number could not be determined. The hybridization of *A. cylindrica* rRNAs with *Hind*III-digested DNA indicated the presence of at least six cistrons.

Although the position of the 5S rRNA cistron in relation to the 16S and 23S rRNA cistrons was not unequivocally determined, preliminary experiments with *A. cylindrica* and *Nostoc* MAC suggested that they were closely linked, since 5S rRNA hybridized to the same DNA fragments as the 16S and 23S rRNAs. Results obtained for *Anabaena* CA and *A. cylindrica* cannot be adequately interpreted without the assumption that there is minor heterogeneity within the rRNA cistrons themselves. Indeed, this was found to be the case in *Bacillus thuringiensis* (Klier *et al.*, 1979). It has been demonstrated that the rRNA genes in *Beneckea harveyi* are linked in a different order to that in other prokaryotes (Lamfrom *et al.*, 1978), therefore the transcriptional organization of rRNA genes in cyanobacteria should be investigated, since it is of evolutionary importance (Doolittle, 1979).

Renaturation kinetics give an average mean value for genome size of 2.21×10^9 Dal for the unicellular, non-nitrogen fixing type of cyanobacteria exemplified by *A. nidulans* and a value of 3.56×10^9 Dal for heterocyst-forming, nitrogen-fixing species, such as the two *Anabaena* species used in this work (Herdman *et al.*, 1979). Assuming that the increase in genome size had been by a process of gene evolution involving a series of fusions of a smaller ancestral genome (Herdman *et al.*, 1979) and there had been no mutational alteration in the flanking sequences between the ribosomal RNA cistrons, the mapping of rRNA genes on to endonuclease-digested DNA would be identical with respect to location and number of sites in the cyanobacteria containing the small and larger genomes. However, if, as one would expect from the significant differences between the different groups of cyanobacteria, extensive mutation had occurred, giving rise to new restriction sites, then a more complex arrangement of rRNA genes would be presented. This appears to be the case, although it should be noted that the increase in ribosomal RNA cistrons from two to five does not exactly coincide with the measured increase in the genome size itself.

Cyanobacteria, like other prokaryotes, have increased amounts of RNA per cell in rapidly, relative to slowly, growing exponential cultures (Mann & Carr, 1974). However, greater differences in RNA content per cell are revealed in a comparison between species, and these no doubt reflect the considerable divergence in cell volume. Thus *A. nidulans* has an RNA content of 20×10^{-14} g per cell (Mann & Carr, 1974) as compared with 300×10^{-14} g per cell in *A. cylindrica* (Simon, 1977; J. M. Nichols & N. G. Carr, unpublished observations), each value being derived from cultures growing at about half maximum growth rate. Even taking into account the fact that the growth rate of the latter organism was approximately four times slower, it is clear that a larger number of gene sites would be necessary to synthesize the requisite amount of rRNA. It may, therefore, be reasonable to interpret the increased number of rRNA cistrons in *Anabaena* species relative to *A. nidulans* in terms of a gene dosage effect.

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