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Mendelian and Uniparental Alterations in Erythromycin Binding by Plastid Ribosomes

Abstract. *Erythromycin binds specifically to the 52S subunit of the chloroplast ribosome of* Chlamydomonas reinhardi. *A number of erythromycin-resistant mutants whose ribosomes have lost their affinity for the antibiotic have been isolated, but the sedimentation properties of their ribosomes are indistinguishable from those of the wild-type strain. These mutants represent at least three genetic loci. Two of them show Mendelian inheritance, and one of them is inherited in a uniparental manner.*

Erythromycin resistance is a valuable genetic marker for structural components of bacterial ribosomes. In *Escherichia coli*, the alteration leading to resistance has been traced, for a number of mutants, to a single protein of the 50*S* ribosomal subunit (*1*). In a resistant mutant of *Staphylococcus aureus*, where no altered proteins could be detected, changes in the 23*S* RNA of the 50*S* subunit were found (*2*).

Sensitivity to erythromycin and loss of sensitivity by mutation seem to be common characteristics of microbial eukaryotic organisms as well. *Saccharomyces* (*3*, *4*), *Paramecium* (*5*), and *Chlamydomonas* (*6*) are among the genera in which genetic studies of these characteristics have been carried out. Linnane *et al.* (*3*) have obtained indirect evidence that some of the resistance mutations in yeast lead to functionally altered mitochondrial ribosomes. We have made a search of erythromycin-resistant mutants in *Chlamydomonas reinhardi* in order to identify and map genes coding for structural features of the sensitive ribosomes.

Wild-type, mating-type plus cells (from strain 137c obtained from R. P. Levine) were treated with ethyl methanesulfonate (*7*), distributed in portions to a large number of tubes, and kept in liquid minimal medium (*8*) for 24 hours. The cells from each tube were then transferred to individual plates of minimal medium containing $5 \times 10^{-4} M$ erythromycin. After the plates were incubated for 1 week, approximately 75 isolates were picked, one from each plate, and an attempt was made to take a representative sample of various colony sizes and morphologies. All the stable resistant isolates were resistant when grown either in the light on minimal medium or in the dark on medium supplemented with acetate. Nine of these strains, again a representative sample, were selected for further study and have been maintained in the complete absence of erythromycin for 12 months.

Ribosomes were isolated from cells in the late log phase of growth and were broken by forcing them under pressure through a phase transition at $-20°C$ in a modified Hughes press (*9*). The frozen cell lysate was thawed in three volumes of buffer (25 m*M* KCl, 25 m*M* $MgCl_2$, 25 m*M* tris-Cl, *p*H 7.5), and the cell debris and membrane material were removed by centrifugation at 27,000*g* for 20 minutes. The supernatant was layered over buffer made 1*M* in sucrose, and centrifuged at 350,000*g* for 3 hours. The clear pellet, taken up in buffer, was the standard crude preparation of ribosomes.

Table 1. Genetic characterization of the six erythromycin-resistant mutants known to have altered chloroplast ribosomes.

Mutant	Segregation	Linked to 15S4?
2L1	4 : 0	
12S3	2 : 2	No
15S4	2 : 2	(Yes)
1L5	2 : 2	Yes
11S7	2 : 2	Yes
13L1	2 : 2	Yes

In order to distinguish mutants with altered ribosomes from possible mutants which gain resistance by either reduced permeability to erythromycin or the ability to detoxify the drug, the binding of [^{14}C]erythromycin to mutant and wild-type ribosomes was studied. Erythromycin binding was measured by the retention of erythromycin-ribosome complexes by Millipore filters. The procedure was essentially that of Teraoka (*10*), with appropriate modifications of the buffer. The closed circles in Fig. 1a show the binding of erythromycin to wild-type ribosomes as a function of erythromycin concentration. This binding requires K^+ at 25 m*M* or higher concentrations. In contrast to the requirements for binding to bacterial ribosomes (*10*), NH_4^+ will not fully substitute for K^+, and at 30 m*M* or higher, NH_4^+ inhibits the binding; Na^+ does not substitute at all. The binding requires Mg^{2+} at concentrations of 10 m*M* or higher and is insensitive to change in *p*H from 6 to 9. The binding is abolished if the ribosomes are treated with Triton X-100 at any point during their preparation [a treatment which is part of the isolation procedure of Hoober and Blobel (*11*)].

The binding constant for the reaction

$$\text{Ribosome} + \text{erythromycin} \rightleftharpoons \text{erythromycin-ribosome complex}$$

and the number of binding sites in the reaction mixture can be directly determined from the double reciprocal plot shown in Fig. 1b. The equilibrium constant, as determined from the average of several experiments, is about 8×10^4 mole^{-1}, and the typical preparation of crude ribosomes contains one binding site for every 8×10^6 to 9×10^6 daltons of nucleic acid. For ribosomes from wild-type cells, this type of plot always yields a straight line, indicating the homogeneity of the binding sites in their affinity for the antibiotic.

For the resistant mutants listed in

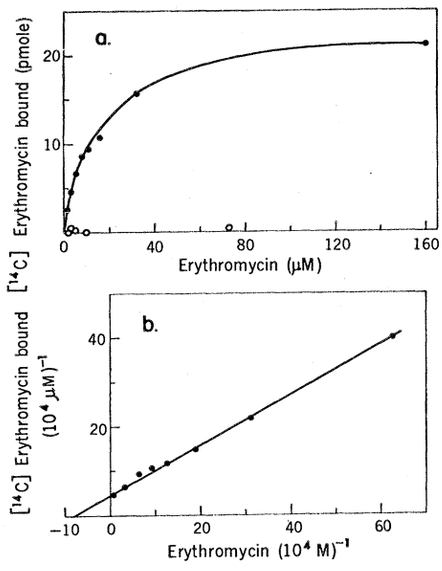

Fig. 1. (a) Binding of [^{14}C]erythromycin to crude ribosomes; (●) binding to wild-type ribosomes; (○) binding to ribosomes from mutant 11S7. Reaction mixtures of 0.1 ml containing buffer, [^{14}C]erythromycin, and 0.27 mg of nucleic acid (*18*) in a crude ribosome preparation were incubated for 30 minutes at 25°C, diluted with 3 ml of buffer, and poured over Millipore HA filters. The filters were washed six times with 3-ml portions of buffer, dried, and counted in toluene-based scintillation fluor. The [^{14}C]erythromycin, 24 count min^{-1} pmole^{-1}, was the generous gift of Dr. R. H. Williams (Lilly Research Laboratories). (b) Double reciprocal plot of the data for wild-type ribosomes in Fig. 1a. The binding constant is the negative of the intercept on the abscissa.

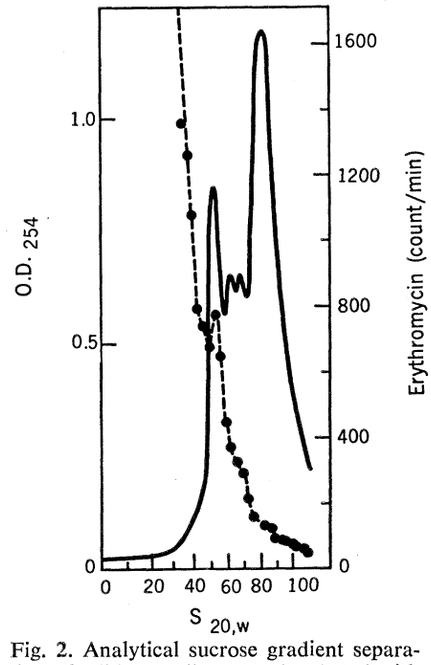

Fig. 2. Analytical sucrose gradient separation of wild-type ribosomes incubated with 120 μM [^{14}C]erythromycin. Fractions (0.25 from the gradient were counted in Aquafluor. The s_{20w} axis was calibrated by measuring the s_{20w} of the 52*S*, 60*S*, and 81*S* ribosomes in a model E analytical ultracentrifuge; O.D., optical density.

Table 1, no binding of erythromycin to ribosomes in crude preparations could be detected. Data for binding to ribosomes from mutant 11S7 are included in Fig. 1a as an example for comparison with wild-type binding. The resistance mutation in each case has apparently led to either alterations in the erythromycin binding sites or the loss of the ribosomes carrying them from the crude ribosome preparation.

Figure 2 shows a typical optical density profile of a crude preparation of ribosomes centrifuged on an analytical sucrose gradient. The major absorbance peak at 52*S* is attributable to the large subunit of the 68*S* chloroplast ribosomes (*11, 12*). The peak at 81*S* represents the cytoplasmic ribosomes, and the small peak at 60*S* is the large subunit of the cytoplasmic ribosome. The optical density profile of ribosomes prepared from each of the mutants is indistinguishable from that of wild type.

The ribosomal profile differs from that obtained by Hoober and Blobel (*11*) in that most of the 68*S* ribosomes are dissociated to subunits. This is apparently due to oxidation during resuspension of the crude pellet, since inclusion of 0.01M β-mercaptoethanol in the buffers preserves the 68*S* ribosome. The small subunits produced in this dissociation apparently form aggregates, since the 16*S* RNA which they contain can be recovered from a pellet in the bottom of the gradient tube. A similar dissociation on exposure to molecular oxygen has been found in *E. coli* ribosomes (*13*).

Also shown in Fig. 2 is the profile of radioactivity recovered from the gradient after centrifugation of wild-type cell ribosomes first incubated in 120 μM [^{14}C]erythromycin. The peak of radioactivity associated with the 52*S* subunits indicates that they carry erythromycin binding sites. The high radioactivity trailing down into the gradient toward the position of the 52*S* subunits probably arises from dissociation of the complex during centrifugation. A very slight deflection in the curve in the 68*S* region may represent binding to the few remaining intact chloroplast ribosomes.

In order to further establish which type of ribosome binds erythromycin, we fractionated crude ribosomes on preparative gradients, and the binding properties of the various fractions were studied. All of the binding sites present in the mixture can be recovered from the gradient. About 70 to 80 percent of the sites are associated with the region of the gradient containing the 52*S* subunits, with one binding site per 1.2×10^6 daltons of nucleic acid. The apparent molecular weight of the RNA found in the 52*S* subunits is 1.08×10^6, as estimated by the sodium dodecyl sulfate–acrylamide electrophoresis procedures of Loening (*14*). Hence, there is about one binding site per 52*S* subunit. The rest of the binding sites are found in the 68*S* region of the gradient. The binding constant in each of these two regions was indistinguishable from that found for the crude mixture. There is essentially no binding to ribosomes in the 81*S* region. Our results imply that the mutations to resistance involve alterations of the 52*S* subunits.

The binding of erythromycin to the large subunit of the chloroplast ribosome of *C. reinhardi* parallels the binding specificity for the 50*S* subunit of bacterial ribosomes (*15, 16*). However, the binding constant for these chloroplast ribosomes is five to ten times lower than for bacterial ribosomes, and the salt requirements for binding are qualitatively different.

Since *Chlamydomonas* contains nuclear, chloroplastic, and possibly other cytoplasmic genomes, a localization of these genes within the cell is of major interest. Tetrad analysis of various crosses (*17*), has yielded the following results. (i) Each of the mutants, except mutant 2L1, behaves as a single normal Mendelian gene, giving 2 : 2 segregation in meiosis. (ii) Mutant 2L1 behaves like a normal uniparental gene. In a mating-type plus strain, it is transmitted to all four progeny, and in a mating-type minus strain, it is transmitted to none. This mutant locus is probably associated with the uniparental linkage group described by Sager and Ramanis (*6*), and is possibly allelic with the erythromycin-resistant marker which they studied. (iii) No recombination has yet been detected among any of the markers listed as linked to mutant 15S4 in Table 1. The number of tetrads analyzed has not, however, been large enough to distinguish between closely linked nonallelic markers and alleles of the same gene. (iv) Crosses of mutant 12S3 with any of the other Mendelian markers yields three types of tetrads: (a) four resistant products; (b) two re-

sistant, one sensitive, and one lethal product; and (c) two sensitive and two lethal products. Type a is scored as parental ditype. Types b and c are scored as tetratype and nonparental ditype, respectively, with the lethal products taken as recombinants carrying both resistance markers. No evidence for linkage between this mutant and the other Mendelian markers has been found.

This genetic analysis indicates that at least three loci in *C. reinhardi* can mutate to yield erythromycin resistance expressed at the level of the chloroplast ribosome. If the suggestion of Sager and Ramanis (*6*) that uniparental genes are carried on chloroplast DNA is proved correct, our results are similar to those of studies on erythromycin resistance in yeast (*4*). Mendelian and "cytoplasmic" resistance mutations have now been found in both organisms. A major difference is that, in the *Chlamydomonas* mutants described here, it has been possible to trace both classes of mutations to expression as altered ribosomes. In *E. coli* many independently isolated resistant strains all gained resistance by alteration of one and the same ribosomal protein (*1*); in contrast, our results show that in *Chlamydomonas* several loci may be involved.

Since a combination of mutant 12S3 with any of the other Mendelian genes in the same cell is lethal, it appears that there is a strong interaction between their gene products. A reasonable interpretation is that the ribosomal component which is altered in each case, whether protein or RNA, leads to resistance without seriously affecting the function of the ribosome. However, when the gene products are combined in the same cell, the result is either no ribosome or one that does not function in protein synthesis. An implication of this analysis is that a functional chloroplast ribosome is required for the growth and division of the cell under the zygote germination conditions used. A search for conditions which might "save" these cells is necessary. A contrasting situation is found in cells containing both gene 2L1 and either of the two types of Mendelian genes; these cells grow normally. It will be valuable to identify the nature of the alterations in the ribosomes for each of these three different types of mutations.

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Mammalian Motor Units: Physiological-Histochemical Correlation in Three Types in Cat Gastrocnemius

Abstract. *The correlation among a variety of physiological properties and the histochemical characteristics of muscle fibers belonging to single motor units in a mixed mammalian muscle is directly demonstrated. The population of motor units making up the cat gastrocnemius was classified into three nonoverlapping groups on the basis of a combination of physiological parameters. The muscle fibers belonging to motor units of each physiological type exhibited a distinctive histochemical profile, such that the three basic histochemical "fiber types" exactly matched the three physiologically defined groups. Within each individual motor unit, the muscle fibers were histochemically uniform.*

Many mammalian muscles are composed of muscle fibers that differ from one another in a variety of morphological and histochemical characteristics (*1*). Most attempts to relate the histochemical characteristics of muscle fibers to their physiological properties have depended on indirect evidence based on the properties of whole muscle (*1*, *2*), a process sometimes leading to conflicting conclusions (*3*). The muscle fibers making up a given skeletal muscle are organized into functional entities, the motor units, each consisting of a group of muscle fibers and the single motoneuron innervating them (*4*). More direct evidence of a correlation between one physiological parameter, resistance to fatigue, and oxidative enzyme activity of the active muscle fibers has been obtained by stimulating individual motoneuron axons and examining both physiological responses and muscle fiber histochemistry in the same motor unit (*5*). Utilizing a similar technique, we have now obtained direct correlation of a variety of physiological parameters of single motor units with the reactivity of their muscle fibers in a number of histochemical stains. The motor units studied both physiologically and histochemically were representative of the entire popu-

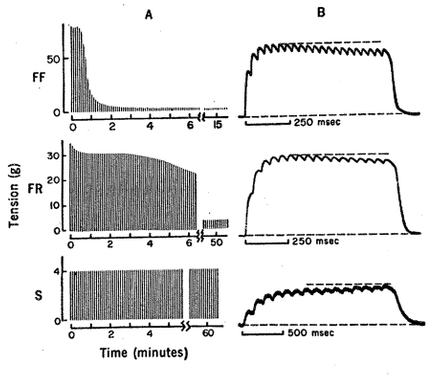

Fig. 1. (A) Graphs of the tension (ordinates) produced by three different motor units during 40 per second tetani, each lasting 330 msec and repeated every 1 second throughout the durations shown on the abscissas. Note rapid decline in tension within 2 minutes in the type FF motor unit (row 1) and greater resistance to fatigue in the FR (row 2) and S (row 3) units. (B) Records of unfused tetanus responses in the same three motor units, showing the slight decline in tension ("sag") in late portions of the tetani in FF and FR units, and the absence of "sag" in the S unit. Tension scales same in A and B.