Genetic and Biochemical Distinction Among Chinese Hamster Cell *emtA*, *emtB*, and *emtC* Mutants

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Genetic and biochemical experiments have enabled us to more clearly distinguish three genetic loci, *emtA*, *emtB*, and *emtC*, all of which can be altered to give rise to resistance to the protein synthesis inhibitor, emetine, in cultured Chinese hamster cells. Genetic experiments have demonstrated that, unlike the *emtB* locus, neither the *emtA* locus nor the *emtC* locus is linked to chromosome 2 in Chinese hamster cells, clearly distinguishing the latter two genes from *emtB*. *emtA* mutants can also be distinguished, biochemically, from *emtB* and *emtC* mutants based upon different degrees of cross-resistance to another inhibitor of protein synthesis, cryptopleurine. Two-dimensional gel electrophoretic analysis of ribosomal proteins failed to detect any electrophoretic alterations in ribosomal proteins from *emtA* or *emtC* mutants that could be correlated with emetine resistance. However, a distinct electrophoretic alteration in ribosomal protein S14 was observed in an *emtB* mutant. In addition, the parental Chinese hamster peritoneal cell line of an *emtC* mutant, and the *emtC* mutant itself, are apparently heterozygous for an electrophoretic alteration in ribosomal protein L9.

Genetic analysis of protein synthesis in mammalian cells has progressed rapidly in recent years through the use of mutant methodology. Cultured Chinese hamster cell mutants have been isolated with alterations affecting aminoacyl-tRNA synthetases (1, 21, 23), elongation factors (12, 19), and ribosomes (2, 8, 10, 11, 13). Several laboratories have isolated and characterized Chinese hamster cell mutants selected as resistant to antibiotic inhibitors of protein synthesis that affect ribosomes, such as trichodermin and emetine (8, 10, 25, 26). Mutants resistant to emetine have been examined in the greatest detail (2, 11, 16, 25, 26). In previous reports from this laboratory, we described the isolation and characterization of three distinct classes, or complementation groups, of emetine-resistant (*Emt*^r) mutants (25, 26). The genetic loci defined by these three complementation groups have been designated *emtA*, *emtB*, and *emtC* (25, 26). Interestingly, we have been able to isolate *emtA* mutants only from V-79 Chinese hamster lung (CHL) cells, *emtB* mutants only from Chinese hamster ovary (CHO) cells, and *emtC* mutants only from Chinese hamster peritoneal (CHP) cells (25, 26). Our CHO-specific *emtB* mutants belong to the same complementation group as the CHO emetine-resistant mutants examined by Madjar et al. (16), in which ribosomal protein S14 has been shown to be electrophoretically altered. As shown below, at

least one of our *emtB* mutants has an alteration in this same protein. However, the relationship of the *emtA* and *emtC* loci to the *emtB* locus is not well understood. Mutations in either the *emtA* or the *emtC* locus result in phenotypes almost identical to the phenotype of *emtB* mutants. In cell-free extracts from all three types of mutants, protein synthesis is much more resistant to emetine inhibition than it is in extracts from emetine-sensitive cells (25, 26). In addition, the phenotype of emetine resistance in all three complementation groups is recessive. All of the available data suggest that *emtA* mutants and *emtC* mutants, like *emtB* mutants, have alterations that affect the ribosome directly. However, the fact that the different types of mutants complement one another indicates that the alterations in the *emtA* and *emtC* mutants must be in ribosomal components other than the protein S14, which is altered in *emtB* mutants.

To better understand the system of emetine resistance and examine possible linkage relationships among the *emtA*, *emtB*, and *emtC* loci, we performed genetic and biochemical experiments that have enabled us to more clearly distinguish the three loci. The results of these experiments demonstrate that, unlike the *emtB* locus, neither the *emtA* nor the *emtC* locus is linked to chromosome 2 in Chinese hamster cells, clearly distinguishing these latter two genetic loci from *emtB*. In addition, *emtA* mutants

show little or no cross-resistance to a related inhibitor of protein synthesis, cryptopleurine, whereas *emtB* and *emtC* mutants are considerably more resistant to this drug than their wild-type parents. Two-dimensional gel electrophoretic analysis of ribosomal proteins extracted from the different mutants revealed a distinctly altered ribosomal protein S14 in an *emtB* mutant. The result is in agreement with the results of Madjar et al. (16), who found the same protein to be altered in an independently derived *emtB* mutant. Although the available evidence suggests that *emtA* and *emtC* mutants also have alterations that affect the ribosome, no detectable electrophoretic alterations in ribosomal proteins were observed in either of these classes of *Emt*^r mutants.

The *emtA*, *emtB*, and *emtC* genes behave as single genetic loci, as determined by all criteria, and all may encode different ribosomal proteins. This is very interesting in view of the findings of Monk et al. (20) that there appear to be multiple copies of the genes encoding individual ribosomal proteins in mammalian cells.

MATERIALS AND METHODS

Cell lines and cell culture. All cell lines were grown as monolayer cultures as described previously (25, 26). The cell lines used in the genetic experiments are listed in Table 1 along with information concerning their genotypes and lineage. UCW 1 is a cloned derivative of the wild-type CHO-S cell line from L. Siminovitch's laboratory. The *emtA* mutant, UCW 288, is a ouabain-resistant (*Oua*^r) derivative of the V-79 CHL cell line UCW 163, which has been described previously (25). This cell line also has a mutation in the *leuS* gene, which renders leucyl-tRNA synthetase thermolabile and the cells nonviable at 39°C. The *emtC* mutant, UCW 482, is a derivative of the Chinese hamster peritoneal (CHP) *emtC* mutant, UCW 282, which has also been characterized before (26). The wild-type CHP parent of UCW 282 was obtained from the American Type Culture Collection (CCL14.1) and is deficient in thymidine kinase (*Tk*⁻). UCW 482 was derived from UCW 282 as follows. After mutagenesis of UCW 282 with ethyl methane sulfonate as described previously (25), a mutant was selected as resistant to 30 μ M sodium chromate (*Chr*^r). From this cell line, a spontaneous ouabain-resistant mutant, UCW 482, was isolated.

Isolation of hybrids. Hybrids between UCW 288 (*EmtA*, *LeuS*(Ts), *Hprt*⁻, *Oua*^r) and the wild-type CHO cell line, UCW 1, were selected in medium containing hypoxanthine, aminopterin, thymidine, and 1 mM ouabain, after fusion of the parental cell lines with polyethylene glycol-1000 as described previously (25). Hybrids between UCW 482 (*EmtC*, *Chr*^r, *Tk*⁻, *Oua*^r) and UCW 1 were isolated, using the same selective medium and fusion procedure. Two independent UCW 288 $\times$ UCW 1 hybrids, UCW 975 and UCW 976, and two independent UCW 482 $\times$ UCW 1 hybrids, UCW 583 and UCW 584, were used in the experiments described below.

TABLE 1. List of cell lines

Cell line	Derived from	Relevant genetic markers	Reference
UCW 1	CHO	Wild-type CHO	
UCW 288	V-79 CHL	<i>LeuS</i> (Ts) <i>EmtA</i> <i>Hprt</i> ⁻ <i>Oua</i> ^r	25
UCW 482	CHP	<i>Chr</i> ^r <i>EmtC</i> <i>Tk</i> ⁻ <i>Oua</i> ^r	26
Hybrid		Parents	
UCW 583		UCW 1 $\times$ UCW 482	
UCW 584		UCW 1 $\times$ UCW 482	
UCW 975		UCW 1 $\times$ UCW 288	
UCW 976		UCW 1 $\times$ UCW 288	

Selection of *Emt*^r and *Chr*^r segregants. To be certain that every segregant examined represented a separate segregation event, each was isolated from an independent subculture. Five independent subcultures were established at 33°C from the two UCW 288 $\times$ UCW 1 hybrids, UCW 975 and UCW 976, from inocula of 100 cells each. When each culture contained $\sim 3 \times 10^6$ cells, the cultures were diluted, and 5×10^5 cells were distributed into 100-mm culture dishes in medium containing 0.3 μ M emetine and incubated at 33°C. No more than one *Emt*^r segregant was isolated from each subculture and grown into mass culture at 33°C for subsequent analysis of the *leuS* marker, as described below.

Five independent subcultures were established from the two UCW 482 $\times$ UCW 1 hybrids, UCW 583 and UCW 584, in a similar manner, except that the cells were grown at 37°C. When each culture contained $\sim 3 \times 10^6$ cells, the cells were diluted and distributed into 100-mm culture dishes at densities of 10^5 cells per dish in medium containing 20 μ M sodium chromate or at densities of 5×10^5 cells per dish in medium containing 0.3 μ M emetine. The cultures were incubated at 37°C. One *Emt*^r and one *Chr*^r segregant were isolated from each subculture and analyzed as described below.

Effect of emetine or cryptopleurine on rate of protein synthesis in vivo. The procedures used to determine the sensitivity of protein synthesis to inhibitors in vivo have been described previously in detail (25, 26). For each cell line examined, the rate of incorporation of [³H]lysine (L-4,5-[³H]lysine, 72.13 Ci/mmol; Amersham Corp.) into protein was determined in the presence of various concentrations of emetine or cryptopleurine. For each cell line, we determined the concentration of emetine required to reduce the rate of protein synthesis to 50% of the rate observed in the absence of the drug. For cryptopleurine, we found that the greatest distinction between the sensitivities of various cell lines was at higher concentrations of the drug. For this reason, we expressed sensitivity to cryptopleurine as the concentration of the drug required to reduce the rate of protein synthesis to 20% of the rate observed in the absence of the drug.

Determination of sodium chromate resistance or sensitivity. The procedure used to determine whether cell lines are sensitive or resistant to sodium chromate has been described previously (4). *Chr*^r cells lyse and detach from culture dishes at sodium chromate con-

centrations above 5 μ M, whereas *Chr^r* cell lines grow vigorously at sodium chromate concentrations up to 50 μ M, making the distinction between the two types of cells very clear-cut.

Effect of temperature on rate of protein synthesis *in vivo*. For each cell line examined, duplicate cultures containing 10^5 cells were established in the 16-mm wells of two 24-well culture trays and placed at 33°C. Two hours later, one of the duplicate trays was transferred to 39°C. Twenty-four hours later, the medium was removed from all wells and replaced with similar medium containing 2×10^{-5} M lysine and 2 μ Ci of [3 H]lysine per ml, and the trays were returned to 33 or 39°C. One and two hours, the cells in replicate wells were killed and fixed to dishes by adding 200 μ l of 20% trichloroacetic acid to the wells. The amounts of [3 H]lysine incorporated into protein linearly with time at 33 and 39°C were determined as described previously (4, 25).

Extraction and two-dimensional gel electrophoretic analysis of ribosomal proteins. Cells were harvested from roller bottles and washed twice with phosphate-buffered saline. Cells were lysed in buffer A (30 mM Tris, pH 7.4, 5 mM magnesium acetate, 125 mM KCl, 0.1% β -mercaptoethanol) containing 0.5% Nonidet P-40. Nuclei were removed by centrifugation at 1,500 rpm for 5 min. The postnuclear supernatant was then centrifuged at $30,000 \times g$ for 15 min. The post-mitochondrial supernatant was layered over a pad of 1 M sucrose in buffer A and centrifuged at 45,000 rpm for 4 h in a Beckman 50 Ti rotor. The ribosomal pellet was resuspended in buffer A, and ribosomal proteins were extracted with magnesium acetate and acetic acid (14, 22). Precipitated RNA was removed by centrifugation at $12,000 \times g$ for 10 min. The RNA pellet was then reextracted with magnesium acetate and acetic acid. The solubilized ribosomal proteins were dialyzed against 1 M acetic acid for 24 h and then lyophilized. The proteins were reduced and alkylated as described by Madjar and Traut (17), dialyzed for 48 h against 1 M acetic acid, and lyophilized again. Ribosomal proteins were then analyzed, using the two-dimensional gel system II described by Madjar et al. (14, 15). Their procedure was followed precisely, including the use of a water-cooled gel apparatus, which is critical to obtain reproducible gels from experiment to experiment. The nomenclature for the mammalian ribosomal proteins is that proposed by McConkey et al. (18), as amended by Madjar et al. (14).

RESULTS

Rationale. To better understand the relationships among the *emtA*, *emtB*, and *emtC* loci in Chinese hamster cells and to determine if any of these genes are linked, we took advantage of the finding that the *emtB* locus is linked to two other genetic markers, *leuS* and *chr*, on the long arm of chromosome 2 (24, 27). We have previously described the isolation of a cell line, UCW 56, with mutations in the linked *emtB*, *leuS*, and *chr* genes (24). The mutation in the *leuS* gene results in the production of a leucyl-tRNA synthetase that is thermolabile [LeuS(Ts)], making the cell

nonviable at 39°C, whereas mutations in the *chr* locus render cells resistant to normally cytotoxic concentrations of sodium chromate (24). In hybrids between UCW 56 and wild-type CHO cells, the *leuS*, *emtB*, and *chr* genes segregate coordinately at a very high frequency (24). Therefore, if either the *emtA* locus or the *emtC* locus was linked to *emtB* on the long arm of chromosome 2, these genes should segregate from hybrids along with other markers on this chromosome. If the *emtA* and *emtC* loci do not segregate coordinately with markers on chromosome 2, we could be certain they were distinct from, and unlinked to, the *emtB* locus. To examine these questions, we isolated an *emtA* mutant from a cell line with a mutation in the *leuS* gene, and we isolated, from an *emtC* mutant, a cell line with a mutation in the *chr* locus. Each of these multiply marked cell lines was fused to a wild-type CHO cell line, and hybrids were isolated. We then determined whether the wild-type *emtA* locus or the wild-type *emtC* locus from the CHO parent segregated coordinately from these hybrids with either the wild-type *leuS* or *chr* gene of the CHO parent. There are two important points to keep in mind concerning these experiments. First, the *leuS* and *chr* genes in the CHO cell line are located in a region on the long arm of chromosome 2 that has been rendered haploid as the result of an interstitial deletion that has removed about one-half the long arm of one chromosome 2 homolog (5, 24, 27, 28). Thus, only a single segregation event, involving the normal, undeleted chromosome 2, is required to render any of these three loci (*emtB*, *leuS*, *chr*) homozygous (or hemizygous) from heterozygous hybrid cells in which the wild-type genes are donated by a CHO parent. Second, in all of the segregation experiments described below, we are selecting for loss of wild-type gene functions (emetine sensitivity or chromate sensitivity) from hybrids in which these genes are donated by the wild-type CHO parent. In this cell line, we know for certain that the *emtB*, *leuS*, and *chr* genes are linked to the long arm of chromosome 2. If the wild-type *emtA* or *emtC* locus in such hybrids fails to segregate coordinately with either the *leuS* or *chr* marker on chromosome 2, we could be certain that *emtA* or *emtC* are genetically distinct from *emtB*, since the former genes would have to be located at completely different regions of the genome.

Noncoordinate segregation of *emtC* and *chr*: nonlinkage of *emtB* and *emtC*. A *Chr^r* mutant was isolated from the previously described *emtC* mutant, UCW 282, as described above. This mutant, UCW 482, failed to complement the *Chr^r* CHO cell line UCW 56. Thus, the *Chr^r* phenotype of UCW 482 is due to a mutation in

the same gene, *chr*, which is altered in CHO Chr^r mutants and which is linked to *emtB* on the long arm of chromosome 2. Hybrids between UCW 482 and the wild-type CHO cell line, UCW 1, were isolated as described above. All of the hybrids examined were phenotypically Emt^s and Chr^s, as expected, since the mutations in both the *emtC* and *chr* loci in UCW 482 behave as recessive markers. To determine whether or not the wild-type *emtC* and *chr* genes of the CHO parent segregated coordinately, we isolated Chr^r and Emt^r segregants from independent subcultures of two different UCW 482 × UCW 1 hybrids, UCW 583 and UCW 584, as described above. The number of Emt^r and Chr^r segregants in each subculture was determined, and no more than one Emt^r and one Chr^r segregant were isolated from each independent culture. The average frequency of Chr^r segregants was very high (2.7×10^{-3}), as expected, since the CHO parent is haploid for the wild-type *chr* locus. In contrast, the frequency of Emt^r segregants was more than 300-fold lower (7.8×10^{-6}) than the frequency of Chr^r segregants. This result indicated that the *emtC* locus, unlike the *chr* locus, is dizygous in the CHO parent and therefore is not linked to the haploid region on the long arm of chromosome 2 where *chr* and *emtB* are localized. To verify this, each of the cloned Chr^r segregants was examined to determine its phenotype with respect to *emtC*. In all 10 of the Chr^r segregants, protein synthesis in vivo was as sensitive to emetine inhibition as in the Emt^s parent hybrids (Table 2). Thus, the wild-type *emtC* locus from the CHO parent did not segregate coordinately with the wild-type *chr* locus of the CHO parent, indicating that the two markers are unlinked. In addition, all seven of the independent Emt^r segregants (three subcultures contained no Emt^r segregants) were as sensitive to sodium chromate as the Chr^s parent hybrids, determined as described above. These results clearly demonstrate that the wild-type *chr* and *emtC* loci of the CHO parent segregate independently from these hybrids, in contrast to the high frequency with which the *chr* and *emtB* genes cosegregate (24, 27). In addition, the fact that all of the Emt^r segregants from these hybrids are Chr^s indicates that the *emtC* locus is not located anywhere on chromosome 2. Thus, *emtB* and *emtC* are clearly distinct genes and are located on different chromosomes.

Noncoordinate segregation of *emtA* and *leuS*: nonlinkage of *emtA* and *emtB*. We have previously reported preliminary results indicating that the *emtA* locus is not linked to chromosome 2 in V-79 CHL cells (24). To confirm and extend this finding to CHO cells, we isolated hybrid cell lines between the CHL *emtA* mutant, UCW 288, and the wild-type CHO cell line UCW 1, as

described above. As described previously, the CHL cell line has, in addition to the mutation in the *emtA* locus, a mutation in the *leuS* gene, rendering leucyl-tRNA synthetase thermolabile and the cells nonviable at 39°C (25). The *leuS* mutation in UCW 288 does not complement the *leuS* mutation in the CHO mutant UCW 56, demonstrating that the mutation altering leucyl-tRNA synthetase in UCW 288 is in the *leuS* gene, which is linked to *emtB* on the long arm of chromosome 2. The mutations in both the *leuS* and *emtA* genes in UCW 288 behave as recessive markers, so hybrids between this cell line and the wild-type CHO cell line UCW 1 are phenotypically temperature resistant (LeuS⁺) and Emt^s. If the *emtA* and *leuS* genes in the CHO parent are not linked, the majority of Emt^r segregants selected from such hybrids should remain LeuS⁺. If, however, *emtA* is linked to the *leuS* gene on the long arm of chromosome 2, the majority of segregants selected as Emt^r should simultaneously become temperature sensitive.

Five independent subcultures from two different UCW 288 × UCW 1 hybrids, UCW 975 and UCW 976, were established at 33°C from inocula of ~100 cells each, as described above. One independent Emt^r segregant was isolated from eight subcultures (two cultures contained no Emt^r segregants) and grown into mass culture at 33°C. Each of these segregants was examined to determine if it was temperature sensitive or temperature resistant. As shown in Table 3, the rate of protein synthesis in each of the eight Emt^r segregants was about twofold greater at 39°C than at 33°C, like a LeuS⁺ parent hybrid, UCW 976, but in marked contrast to the

TABLE 2. ID₅₀ for emetine in UCW 482, UCW 584, and Chr^r segregants^a

Cell line	ID ₅₀ for emetine (μM)	Emt phenotype
UCW 482	1.70	r
UCW 584	0.10	s
Chr ^r segregants		
1	0.09	s
2	0.11	s
3	0.15	s
4	0.09	s
5	0.10	s
6	0.10	s
7	0.11	s
8	0.12	s
9	0.08	s
10	0.13	s

^a ID₅₀ is the concentration of emetine required to reduce the rate of protein synthesis in vivo to 50% of the rate observed in the absence of the drug.

LeuS(Ts) UCW 288 cell line, in which the rate of protein synthesis at 39°C is about 2% the rate at 33°C. In addition, all eight of the *Emt*^r segregants grew vigorously at 39°C, indicating that none has lost the wild-type *leuS* gene of the CHO parent. Thus, the wild-type *emtA* and *leuS* genes of the CHO parent segregate independently from hybrids, confirming that *emtA* is not linked to *leuS*. Therefore, *emtA* and *emtB* are unequivocally distinct genetic loci and are not linked.

Biochemical distinction among *emtA*, *emtB*, and *emtC* mutants. Gupta and Siminovitch (9) have shown that *Emt*^r CHO mutants are cross-resistant to another inhibitor of protein synthesis, cryptopleurine. Cryptopleurine, like emetine, inhibits protein synthesis by binding to a site(s) on the 40S ribosomal subunit and blocking translocation (7). We assumed that the *Emt*^r mutants examined by Gupta and Siminovitch were all *emtB* mutants, so we decided to examine the three different classes of *Emt*^r mutants to determine whether or not they were all cross-resistant to cryptopleurine. If the biochemical alterations that give rise to the *Emt*^r phenotypes in the three different classes of mutants are slightly different, these might be reflected in different responses to other, related inhibitors of protein synthesis, such as cryptopleurine. The effect of various concentrations of cryptopleurine on the rates of protein synthesis *in vivo* was determined for three independent *emtA* mutants, three *emtB* mutants, and two *emtC* mutants and compared to the effect of emetine on the same cell lines, as described above. The results of these experiments are summarized in Table 4. The three classes of *Emt*^r mutants show very similar degrees of resistance to emetine, with one exception, an *emtB* mutant that was considerably more sensitive to the drug than the other seven mutants. In contrast, the mutants

are quite different with respect to their degrees of cross-resistance to cryptopleurine. The three *emtB* mutants and the two *emtC* mutants were all quite resistant to cryptopleurine, ranging from 4.6- to 7.7-fold more resistant to the drug than their wild-type parents. However, none of the three *emtA* mutants exhibited any significant degree of cross-resistance to cryptopleurine, even though their resistance to emetine was comparable to that of the *emtB* and *emtC* mutants. Thus, based upon the criteria of cross-resistance to cryptopleurine, mutants belonging to the *emtA* complementation group appear to be quite different from mutants belonging to the *emtB* and *emtC* complementation groups. The possible significance of these differences is discussed below.

Analysis of ribosomal proteins from *emtA*, *emtB*, and *emtC* mutants. In all three of the complementation groups of *Emt*^r mutants, protein synthesis *in vitro* is resistant to emetine inhibition, indicating that in each of the three classes of mutants, a component of the protein synthesis machinery is altered (25, 26). Thus, the *emtA*, *emtB*, and *emtC* loci must all encode protein synthesis components. Furthermore, since the *Emt*^r phenotype of all three types of mutants is partially expressed *in vitro* by *Emt*^r × *Emt*^s hybrids, it seemed likely that all three types of mutants had alterations affecting the ribosome directly, probably ribosomal proteins. To examine this possibility directly, we analyzed ribosomal proteins prepared from wild-type cells and *emtA*, *emtB*, and *emtC* mutants, using the two-dimensional polyacrylamide gel electrophoresis system described above. Representative gels from the wild-type parents and one *emtA*, *emtB*, and *emtC* mutant are shown in Fig. 1. There are not detectable differences in the electrophoretic mobilities of the ribosomal proteins among the parental CHL, CHO, and CHP cell lines, with one exception, which is discussed below. Analysis of three *emtA* mutants failed to detect an alteration in any ribosomal protein, as shown in Fig. 1 for the mutant UCW 181. Similarly, analysis of ribosomal proteins extracted from two different *emtC* mutants failed to detect any alterations in a ribosomal protein, as shown in Fig. 1 for the *emtC* mutant UCW 282. However, we noticed in the course of these experiments that UCW 104, the wild-type CHP parent of UCW 282, and UCW 282 as well, are apparently heterozygous for an electrophoretic alteration in ribosomal protein L9, as evidenced by a double spot on gels from these cell lines at the position corresponding to L9 (Fig. 1). This alteration cannot be responsible for the *Emt*^r phenotype of UCW 282, since the *Emt*^s UCW 104 parent has the same alteration. Whether this heterozygosity of CHP cells for

TABLE 3. Effect of temperature on the rates of protein synthesis in UCW 288, UCW 976, and *EmtA*^r segregants

Cell line	Rate of protein synthesis at 39°C/rate of protein synthesis at 33°C	LeuS phenotype
UCW 288	0.02	Ts
UCW 976	1.61	+
<i>Emt</i> ^r segregants		
1	1.72	+
2	1.96	+
3	2.14	+
4	2.21	+
5	1.60	+
6	2.03	+
7	1.85	+
8	1.34	+

TABLE 4. Resistance of *emtA*, *emtB*, and *emtC* mutants to emetine and cryptopleurine^a

Cell line	ID ₅₀ for emetine (μM)	Relative resistance to emetine	ID ₂₀ for cryptopleurine (pM)	Relative resistance to cryptopleurine	Relative resistance to emetine/relative resistance to cryptopleurine
Wild-type CHL	0.12	1.0	2.4	1.0	
<i>emtA</i> mutants					
1	1.9	15.8	3.2	1.3	12.2
2	1.8	15.0	3.9	1.6	9.4
3	1.6	13.3	2.5	1.0	13.3
Wild-type CHO	0.10	1.0	2.6	1.0	
<i>emtB</i> mutants					
1	0.6	6.0	16.1	6.2	0.9
2	2.0	20.0	16.2	6.2	3.2
3	1.6	16.0	20.0	7.7	2.1
Wild-type CHP	0.09	1.0	2.2	1.0	
<i>emtC</i> mutants					
1	1.8	20.0	10.1	4.6	4.4
2	1.0	11.1	12.0	5.4	2.0

^a The effect of various concentrations of emetine or cryptopleurine on the rates of protein synthesis in vivo was determined as described in the text. The results represent the average of duplicate experiments with both emetine and cryptopleurine for each cell line. The degree of resistance to emetine and cryptopleurine for each of the different mutants is expressed relative to the wild-type cell line from which it was derived (CHL for *emtA* mutants, CHO for *emtB* mutants, and CHP for *emtC* mutants). ID₅₀, See footnote a, Table 2; ID₂₀, concentration of cryptopleurine required to reduce the rate of protein synthesis to 20% of the rate observed in the absence of the drug.

ribosomal protein L9 has any significance with respect to our finding that only *emtC* mutants can be isolated from this cell line (26) remains to be determined.

In contrast, the CHO *emtB* mutant UCW 56 has a distinct alteration in a ribosomal protein, S14 (Fig. 1 and 2). The S14 protein from UCW 56 migrates more slowly in the first-dimensional gel than S14 protein from wild-type cells, indicating it is less basic than the wild-type S14 protein. This result is in agreement with the findings of Madjar et al. (16), who showed the same protein to be electrophoretically altered in a CHO Emt^r mutant belonging to the *emtB* complementation group. This result, together with other results discussed below, provides very strong evidence that the *emtB* locus is the structural gene for ribosomal protein S14. In addition, it should be pointed out that the inability to detect alterations in ribosomal proteins in *emtA* and *emtC* mutants does not argue against the idea that the *emtA* and *emtC* loci also encode ribosomal proteins.

DISCUSSION

The results of genetic experiments described here demonstrate that neither the *emtA* locus

nor the *emtC* locus is linked to chromosome 2 in CHO cells. The *emtA* locus does not segregate coordinately from hybrid cells with the *leuS* gene, which is known to be located on the long arm of chromosome 2. Similarly, the *emtC* locus does not segregate coordinately with the *chr* locus, which is also linked to the long arm of chromosome 2. Therefore, these results clearly distinguish the *emtA* locus and the *emtC* locus from *emtB*, which is linked to *leuS* and *chr* on chromosome 2. However, at this point we have no information concerning the genetic location of either *emtA* or *emtC*, nor do we have any information as to whether these two genes are linked or unlinked. *emtA* mutants and *emtC* mutants can still be distinguished by at least two criteria, however. First, mutants from the two classes complement one another, such that hybrids between an *emtA* mutant and an *emtC* mutant are sensitive to emetine inhibition (26). Secondly, as shown in this report, *emtC* mutants are cross-resistant to cryptopleurine, whereas *emtA* mutants are not.

All of the evidence to date indicates that the *emtB* locus is the structural gene for ribosomal protein S14. Thus, as shown above and reported by others, *emtB* mutants have electrophoretic alterations in this protein (2, 16). In addition,

FIG. 1. Two-dimensional gel electropherograms of ribosomal proteins from wild-type cells and *emtA*, *emtB*, and *emtC* mutants. The ribosomal proteins were extracted and electrophoresed as described in the text. The nomenclature for mammalian ribosomal proteins is that proposed by McConkey et al. (18), as amended by Madjar et al. (14). The arrowheads on the electropherograms for the wild-type CHP cells and the *emtC* mutant UCW 282 indicate the double spot at the position corresponding to protein L9, indicating probable heterozygosity of the two cell lines for this ribosomal protein. Note that only a single L9 spot is present on the gels from all of the other cell lines. The arrowheads on the electropherograms from the wild-type CHO cell line and the *emtB* mutant UCW 56 indicate the position of the normal and altered S14 proteins, respectively. The area within the box is enlarged in Fig. 2 to more clearly show the difference in migration of the normal and mutant S14 proteins.

mRNA extracted from *emtB* mutants translates in vitro into an altered S14 protein (16). In hybrids between *emtB* mutants and wild-type cells, both the mutant and normal S14 proteins can be detected after two-dimensional gel electrophoresis (3; unpublished data). In addition, we have recently found that in interspecific hybrids between normal human cells and the *emtB* mutant UCW 56 which express the human *emtB* gene function, both the mutant hamster S14 and the normal human S14 proteins are present (4; S. Dana, S. Chang, and J. Wasmuth, manuscript in preparation). These results make it seem very unlikely that the mutation in *emtB* mutants affects post-translational modification of ribosomal protein S14. If this were the case, in hybrid cell lines between normal cells and *emtB* mutants, the wild-type *emtB* gene function

could modify the S14 protein normally, and only the wild-type protein would be detected on gels. This is clearly not the case. In view of the similarity of the phenotypes of *emtA* and *emtC* mutants to the phenotype of *emtB* mutants, we suspect that the former two types of mutants also have alterations that affect ribosomal proteins. Thus, one could imagine that the *emtA*, *emtB*, and *emtC* loci all encode proteins that are near the site on the ribosome to which emetine binds. An alteration in any of the three proteins could alter this binding site, rendering ribosomes resistant to the effect of emetine. In this regard, the differences between the three classes of mutants with respect to cross-resistance to cryptopleurine is of interest. Cryptopleurine, like emetine, specifically affects the 40S ribosomal subunit (7). If some of the structural determinants

FIG. 2. Enlargement of the region of gel electropherograms from wild-type CHO and UCW 56 containing protein S14. Reference lines are drawn through the spots corresponding to proteins L25, S15, and L11. Note that the S14 protein from wild-type cells migrates to the right of protein L25, such that the reference line between L25 and S15 goes through the S14 spot. In contrast, the S14 protein from the *emtB* mutant UCW 56 migrates to a position directly above L25, such that it lies almost in the middle of the "V" generated by the reference lines. This electrophoretic shift was observed in every gel (six) run on proteins extracted from UCW 56, and an apparently identical shift has been observed in another independent *emtB* mutant.

that define the emetine and cryptoleurine binding sites on the ribosome are common, but each binding site also has some unique structural determinants, it is not surprising that different classes of mutants are affected by cryptoleurine to different degrees. Thus, alterations in either the *emtB* gene product (S14) or the *emtC* gene product might alter structural determinants common to both the emetine and the cryptoleurine binding sites, rendering cells resistant to both drugs. Alterations in the *emtA* gene product could, however, alter a determinant that was unique to the emetine binding site, leaving

the cryptoleurine binding site relatively unperturbed. In addition, we now have evidence to indicate that whereas the products of the *emtA* and *emtC* loci are distinct, they almost certainly interact somehow. We recently constructed *emtA*, *emtC* double mutants and found that they are resistant to approximately 500-fold higher concentrations of emetine than either of the single mutants alone (J. Wasmuth, manuscript in preparation).

Although mutations in the *emtB* locus can clearly be associated with a specific altered ribosomal protein, no such clear correlation can be made for *emtA* or *emtC* mutants. That definitive alterations in ribosomal proteins have not yet been observed in *emtA* and *emtC* mutants does not argue against the idea that these genes encode ribosomal proteins, however. Thus, if the mutations do affect ribosomal proteins, they must not only render the ribosomes resistant to inhibition by emetine, they must also leave the resultant protein functional, or the mutations would be lethal. In view of the evolutionary conservation of ribosomal proteins, it seems likely that many mutations in genes encoding ribosomal proteins might be lethal, and only a few types of alterations would result in emetine-resistant proteins that are still functional. If most of these types of mutations do not produce amino acid substitutions that result in electrophoretic changes in the protein, no alteration in the gel patterns of ribosomal proteins from the mutants would be detected. However, we are continuing to analyze ribosomal proteins from more *emtA* and *emtC* mutants in attempts to definitively determine if these loci encode ribosomal proteins.

If, in fact, the *emtA* and *emtC* genes, like the *emtB* gene, encode ribosomal proteins which are altered in the various classes of mutants, the findings of Monk et al. (20) and D'Eustachio et al. (6) are difficult to reconcile with ours. These workers have cloned cDNA copies of mRNAs encoding seven different mouse ribosomal proteins (20). Using these probes, they found that there are multiple copies (7 to 20) of genomic sequences that hybridize to each of the seven different ribosomal protein cDNAs, in both mouse and human cells. Furthermore, they determined that genomic sequences hybridizing to a specific ribosomal protein cDNA probe were usually distributed on a number of different chromosomes in the mouse (6). From this work, they concluded that for each of the ribosomal proteins they examined, there are multiple, usually unlinked, genes encoding them. It is reasonable to assume that the same situation probably exists for most, if not all, of the ribosomal proteins in mammalian cells, including Chinese hamster cells. If this indeed is true, it is hard to

understand how it is possible to isolate mutants, in a single-step selection, that have altered ribosomal proteins. In addition, the fact that *Emt^r* segregants can be isolated at reasonable frequencies from hybrids between wild-type cells and *emtA*, *emtB*, and *emtC* mutants argues strongly against there being any more than two functional copies of the wild-type *emtA*, *emtB*, or *emtC* genes in the wild-type parent of the hybrids. For the *emtB* locus in particular, there is very strong evidence that (i) it is the structural gene for ribosomal protein S14, (ii) there is only a single functional copy of this gene in CHO cells, and (iii) the single functional copy of the gene is localized specifically to the long arm of chromosome 2. Since ribosomal proteins are required in stoichiometric amounts, it seems unlikely that there would be a single locus encoding some proteins and multiple copies of the genes encoding others. The most reasonable explanation to reconcile these apparently conflicting results is that each ribosomal protein is encoded by one locus and the other genomic sequences that hybridize to ribosomal protein cDNAs are pseudogenes or evolutionary remnants which are no longer functional. We are presently attempting to clone a cDNA copy of the mRNA encoding Chinese hamster ribosomal protein S14 in attempts to answer some of these questions.

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