Linkage in Cultured Chinese Hamster Cells of Two Genes, emtB and leuS, Involved in Protein Synthesis and Isolation of Cell Lines with Mutations in Three Linked Genes

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ABSTRACT We have determined via segregation analyses from appropriate hybrids that two genes involved in protein synthesis, one encoding for a ribosomal protein (emtB) and one encoding for leucyl-tRNA synthetase (leuS), cosegregate at a very high frequency and are linked in both Chinese hamster ovary and lung cells. In contrast, the emtA locus, defined by a second complementation group of emetine-resistant mutants which also have alterations affecting protein synthesis and probably the ribosome, is not linked to leuS. In addition, we have determined that a third gene, one that can be altered to give rise to chromate resistance, is syntenic with emtB and leuS. We have selected cell lines with mutations in each of these three linked genes and have shown that the three loci cosegregate at a high frequency. Because the mutations in these three linked genes provide easily distinguishable phenotypes, these cell lines should provide a powerful tool for examining several important questions concerning mitotic recombination in somatic cells.

The genetic and biochemical dissection of protein synthesis in mammalian cells has benefited greatly in recent years from the use of mutant methodology. A large number of cultured somatic cell mutants have been isolated with alterations in different components of the protein synthetic machinery (9, 10, 12, 16, 18, 19). One goal of this laboratory is to expand the number of such mutants in cultured Chinese hamster cells and to characterize them at the molecular level, especially mutants with alterations affecting various components of the ribosome. A second goal is to use such mutants to begin constructing a genetic map of this group of functionally related genes.

We have isolated and partially characterized three distinct types of Chinese hamster cell mutants that were selected as being resistant to the protein synthesis inhibitor, emetine (19; J Hill, L. Vock, and J. Wasmuth, manuscript in preparation). Although the phenotypes of all three types of mutants are very similar, and all have alterations affecting protein synthesis directly, they clearly define three distinct complementation groups. For all three classes, the emetine-resistant phenotypes are recessive. We have designated the loci defined by these different complementation groups as emtA, emtB, and emtC. Two laboratories (4, 8) have obtained evidence that Chinese hamster ovary (CHO), but not Chinese hamster lung (CHL), cells are functionally hemizygous for a locus that can be altered to give rise to the emetine-resistant phenotype. As shown in this report, it is almost certain that this locus corresponds to what we have designated as emtB (19). The available evidence suggests that all CHO emetine-resistant mutants reported thus far belong to this same complementation group. In addition, two laboratories have identified a 40S ribosomal protein as being electrophoretically altered in independently isolated emetine-resistant CHO mutants (2, 14).

Preliminary evidence obtained in this laboratory indicates the emtA mutants we have identified also have mutations affecting the ribosome (19). In view of the likelihood that the emtA and emtB loci both encode components of the ribosome, we were interested in determining whether these two genes might be linked. We were also interested to determine if we could establish any linkage relationships between either of these genes and other loci for which we have well-defined mutants. Experiments of this type would represent an important first step in the construction of a genetic map of the genes encoding protein synthetic components.

Toward this end, we have determined that the emtB, but not the emtA, locus is closely linked to another gene involved in protein synthesis, that encoding leucyl-tRNA synthetase (leuS). While this work was in progress, Campbell and Worton (6) reported that the emt^r locus in CHO cells, which corresponds

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to what we have designated emtB, is linked to a gene, chr, defined by mutants resistant to chromate. We therefore selected chromate-resistant mutants in a cell line that already had mutations in the emtB and leuS genes and showed that indeed all three loci are linked. Based upon our results and the data obtained by Campbell and Worton (5, 6), we can tentatively assign the leuS gene to the long arm of chromosome 2, a region that has been shown to be physically haploid in CHO cells as a result of a deletion (5).

In addition, the isolation of cell lines with mutations in three linked genes, each of which provide easily distinguishable phenotypes, represents an important technical advance in the ability to examine segregation and mitotic recombination in somatic mammalian cells.

MATERIALS AND METHODS

Cell Lines and Culture

Cell lines were grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 5% bovine calf serum. Routine cell culture techniques have been described previously (19). All cell lines used in these studies are listed in Table I. UCW 10 is an emetine-resistant CHO mutant belonging to the emtB complementation group, it is auxotrophic for proline (pro^-) and has a temperature-sensitive leuS. UCW 10 was selected from the tsH1 cell line (15) and has been characterized in some detail previously (19). UCW 15 is a subclone of the N3 cell line isolated by Waye and Stanners (20). This cell line is also pro^-, is deficient in asparagine synthetase (asn^-) and has a defect in folate metabolism that renders it auxotrophic for glycine, adenosine, and thymidine (gat^-). All of the CHL cell lines used in these studies are derived from the wild-type V-79 strain as described previously (19). The CHL cell line UCW 113 is deficient in hypoxanthine-guanine phosphoribosyl transferase ($hprt^-$) and does not grow in medium containing hypoxanthine, aminopterin, and thymidine (HAT). UCW 288 is an emetine-resistant CHL mutant belonging to the emtA complementation group. This cell line is also $hprt^-$, has a temperature-sensitive leuS, a temperature-sensitive asparagyl-tRNA synthetase, and is resistant to 1 mM ouabain. The ouabain-sensitive parent of this cell line UCW 183 has been characterized in some detail previously (19).

Cell Fusion and Isolation of Hybrids

The procedures used to fuse various cell lines with polyethylene glycol 1,000 have been described in detail (19). Hybrids between UCW 10 and UCW 113 were selected at 39°C in medium containing HAT. Only hybrids having a functional hprt and leuS will grow under these conditions. Presumptive hybrid colonies that arose were cloned and subsequently cultured at 33°C unless otherwise stated. Hybrids between the two CHO cell lines, UCW 10 and UCW 15, were selected at 39°C in medium containing proline and 10% dialyzed fetal calf serum but lacking asparagine, adenosine, and thymidine. Again, the elevated temperature selects against the UCW 10 parent and the lack of asparagine, adenosine, and thymidine selects against the UCW 15 parent. Hybrids between UCW 288 and UCW 100 or UCW 15 were selected at 33°C in medium containing HAT and 1 mM ouabain.

Karyotypic analyses were done on all presumptive hybrid clones to be used for further experiments, as described previously (17). All the hybrids used in the experiments described in this report were near tetraploid, with modal chromosome numbers of 38-44.

Selection of Emetine-resistant Segregants

Cells from cultures of the various hybrids were distributed into 100-mm culture dishes at densities of 2–5 × 10^5 cells/dish in medium containing 3 × 10^{-7} M emetine, and incubated at the temperature indicated in specific experiments. Any manipulation of the culture conditions is described in the Results. The spontaneous mutation frequency to emetine resistance is so low (9), that we can be certain that virtually all the emetine-resistant colonies arising in these cultures are segregants and do not represent new mutations. As described in the Results, some of the segregants were grown into mass culture and examined for leuS activity. For all these segregants, their karyotypes were determined to ensure that they did not represent a diploid parent that was somehow recovered from the hybrid population. For all three such segregants, the chromosome number was near tetraploid and not noticeably distinguishable from the emetine-sensitive tetraploid parent hybrid.

Aminoacyl-tRNA Synthetase Assays

The procedures used to prepare extracts are described in detail elsewhere (18). Reaction mixtures contained, in a final volume of 150 μl; 20 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 20 mK KCl, 0.35 mM cytidine 5'-triphosphate, 5 mM ATP, 140 μg of yeast tRNA, 50–200 μg of extract protein and either 3 × 10^{-5} M [^{14}C]leucine (342 Ci/mmol) or 2 × 10^{-5} M [^{14}C]threonine (236 Ci/mmol). The assays were performed at 33.5°C exactly as described before (18). Each extract was assayed using at least two different protein concentrations, and the amount of ^{14}C-aminoacyl-tRNA formed linearly with time was determined. Background values were determined in complete reaction mixtures that were stopped at zero time with trichloroacetic acid, or in which the extract had been inactivated by heating at 60°C. These values were subtracted from experimental values. Specific activities are expressed as picomoles of ^{14}C-amino acid attached to tRNA per minute per milligram protein.

Selection of Chromate-resistant Mutants

UCW 10 was mutagenized with ethylmethane sulfonate as described previously (7), then grown at 33°C in nonselective medium for 4 d. Cells were then distributed into 100-mm culture dishes at a density of 4 × 10^5 cells/dish in nonselective medium. The next day, medium containing 25 μM sodium chromate was added and the plates were incubated at 33°C until colonies appeared. Several colonies were isolated, grown in nonselective medium for ~12 generations, then tested to determine their degree of chromate resistance. The two most resistant cell lines, UCW 56 and UCW 57, were chosen for further study. Both of these cell lines grow at near normal rates in medium containing 40 μM sodium chromate, whereas the parental cell line, UCW 10, is killed at chromate concentrations above 5 μM. Hybrids between the two chromate-resistant cell lines and UCW 113 were formed and selected as described above for hybrids between UCW 10 and UCW 113. The chromate-resistant phenotypes of UCW 56 and UCW 57 are recessive in such hybrids.

RESULTS

Linkage of emtB and leuS in CHO and CHL cells

As discussed in the Introduction, we were interested in examining possible linkage relationships between the emtA and emtB loci, and other genes as a first step in constructing a genetic map of genes encoding for protein synthetic components. We have previously isolated several different emtA and emtB mutants from cell lines with a number of other recessive genetic markers (19), some of which are listed in Table I. Because both the $emtA^r$ and $emtB^r$ phenotypes are recessive when either type of mutant is fused to wild-type cells, we were able to examine whether the loss, or segregation, of the wild-

TABLE I

List of Cell Lines

Strain number	Phenotype*	Parent
Derived from CHO		
UCW 10	pro^-; $leuS^{ts}$; $emtB^r$	tsH1
UCW 15	pro^-; gat^-; asn^-	GAT^-
UCW 56	pro^-; $leuS^{ts}$; $emtB^r$; chr^r	UCW 10
UCW 57	pro^-; $leuS^{ts}$; $emtB^r$; chr^r	UCW 10
Derived from CHL		
UCW 100	Wild type	—
UCW 113	$hprt^-$	UCW 100
UCW 288	$hprt^-$, $leuS^{ts}$, $asnS^{ts}$, $emtA^r$, ouB^r	UCW 183

More detailed descriptions of the cell lines and references concerning their derivations and characterizations are provided in Materials and Methods.

* The abbreviations are: *pro*$^-$, auxotrophy for proline; *asnS*^{ts}, temperature-sensitive asparagyl tRNA synthetase; *leuS*^{ts}, temperature-sensitive leucyl-tRNA synthetase; *emtA*^{r}, resistance to emetine-complementation group A; *emtB*^{r}, resistance to emetine-complementation group B; *gat*$^-$, auxotrophy for glycine, adenosine and thymidine; *asn*$^-$, auxotrophy for asparagine; *chr*^{r}, resistance to sodium chromate; *hprt*$^-$, deficiency in hypoxanthine-guanine phosphoribosyl transferase; *ouB*^{r}, resistance to 1 mM ouabain.

type emtA or emtB genes from such hybrids was accompanied by loss of the wild-type allele for the other genetic markers in the emetine-resistant parents. UCW 10, a CHO emtB mutant (emtB^r) which also has a temperature-sensitive leuS (leuS^{ts}) (19), was fused to a CHL cell line, UCW 113, that is wild type with respect to both loci, and hybrids were selected as described in Materials and Methods. Because both markers in the CHO parent are recessive the hybrid is phenotypically emetine resistant and grows at 39°C (leuS$^+$). The hybrids were maintained, after cloning, at 33°C. Four emetine-resistant segregants were isolated at 33°C from each of three independent cultures of a UCW 10 × UCW 113 hybrid, which were initiated from inocula of ~100 cells each. The 12 emetine-resistant segregants were grown into mass culture at 33°C. If the emtB$^+$ and leuS$^+$ genes of the wild-type parent were linked and cosegregated, many of the emtB^r segregants should have simultaneously become temperature sensitive (leuS^{ts}). If the genes were not linked, the emetine-resistant segregants should be phenotypically leuS$^+$ and able to grow at 39°C. When cultures of the emtB^r segregants were transferred to 39°C, the cells from all 12 cultures lysed and detached from the culture dishes within 48 h, indicating that all were, in fact, temperature sensitive. The parental emetine sensitive hybrid grew perfectly well under the same conditions. This result suggested the emtB$^+$ and leuS$^+$ genes had cosegregated from the hybrid, indicating the genes are linked to at least the same chromosome.

To be certain that the temperature-sensitive segregants had in fact lost the wild-type leuS gene, extracts were prepared from three independent segregants, as well as from the parent hybrid and the two diploid parents of the hybrid, and leuS activity was determined, as described in Materials and Methods. The results of these assays are shown in Table II. As previously reported for its emetine-sensitive parent, tsH1 (11), the leuS activity in UCW 10 is barely detectable, <5% that of the wild-type CHL cell line, UCW 113. Thus, even though this enzyme functions well enough at 33°C in vivo to support normal growth of tsH1 or UCW 10, its activity in vitro is extremely poor. The activity of leuS in extracts from the parental UCW 10 × UCW 113 hybrid is ~50% that of UCW 113. This is in agreement with the notion that part of the leuS in this hybrid is wild-type and part is of the labile, less active UCW 10 type. However, in all three emetine-resistant segregants leuS activity is virtually undetectable, <10% of the activity in the parent hybrid. Thus, the temperature sensitivity of these emetine-resistant segregants is owing to their having defective leuS activity. As also shown in Table II, the specific activity of another aminoacyl-tRNA synthetase, threonyl-, is the same in all six extracts. Thus, the lowered leuS activity in UCW 10 and the three segregants is not caused by a technical problem in preparation of the extracts. It seems clear, therefore, that in these cell lines the wild-type leuS gene has cosegregated with the wild-type emtB gene, rendering the cell lines temperature sensitive.

TABLE II

Specific Activity of Leucyl and Threonyl-tRNA Synthetases in UCW 10, UCW 113, UCW 10 × UCW 113, and Emetine-resistant Segregants

Cell line	Specific activity	
	Leucyl-tRNA synthetase	Threonyl-tRNA synthetase
UCW 10	6.0	125.5
UCW 113	155.5	122.4
UCW 10 × UCW 113 Hybrid 1	74.5	136.0
UCW 10 × UCW 113 em^{r}1	<5.0	114.5
UCW 10 × UCW 113 em^{r}11	<5.0	124.3
UCW 10 × UCW 113 em^{r}12	6.4	111.2

Preparation of the extracts and enzyme assays are described in Materials and Methods. Specific activities are expressed as picomoles of ^{14}C-amino acid attached to tRNA per minute per milligram protein. UCW 10 × UCW 113 em^{r}1, em^{r}11, and em^{r}12 are three emetine-resistant segregants isolated from UCW 10 × UCW 113 hybrid 1, as described in the text.

As mentioned in Materials and Methods, karyotypic analyses were done on both the parent UCW 10 × UCW 113 hybrid and the same three emetine-resistant segregants in which leuS had been assayed. Like the parent hybrid, all three emetine-resistant segregants were near tetraploid, thus discounting the possibility that we were simply recovering diploid CHO cells as segregants. However, cytogenetic analyses have not yet been detailed enough for us to detect specific chromosomal alterations in the emetine-resistant segregants.

The results to this point have demonstrated that in UCW 113 the genes for leuS and emtB are linked. Thus, when selective pressure is applied to the UCW 10 × UCW 113 hybrid, allowing only cells which have segregated the wild-type emtB gene to grow, the wild leuS gene is lost simultaneously. We next wanted to examine two questions: (*a*) Was the result unique to the one particular UCW 10 × UCW 113 hybrid clone used? (*b*) Would the same result be obtained using a hybrid between UCW 10 and an emetine-sensitive CHO, rather than CHL, cell line? The first question is relevant because it is possible that in the single UCW 10 × UCW 113 hybrid used in the initial experiments described above, a translocation had occurred establishing an "artificial" linkage between leuS and emtB. The second question is important, since there are considerable karyotypic differences between V-79 CHL cells and the CHO cells. Thus, owing to chromosome breaks or translocations, linkage relationships that exist in one cell line may be disrupted in the other. However, if the two genes are linked in both cell lines, one could be confident that linkage of these genes is the normal situation in the species.

Five different UCW 10 × UCW 113 hybrids and two different UCW 10 × UCW 15 (an emetine-sensitive CHO cell line, see Table I and Materials and Methods) hybrids were grown into mass culture at 33°C. Cells from the different cultures were then replated at lower densities in medium containing 3×10^{-7} M emetine and were returned to 33°C. 14–21 d later, macroscopically visible emetine-resistant colonies that had appeared on the various plates were marked. The medium was aspirated, the cells were washed with a balanced salts solution, then transferred to 39°C in medium containing 2×10^{-4} M leucine and no emetine. 2 d later, each marked colony was examined under the microscope and scored as alive or dead. The scoring of these colonies is clear-cut and unequivocal, since the temperature-sensitive cells lyse at the elevated temperature and only cell fragments or cell ghosts remain where colonies had been. In most cases, when colonies comprised of temperature-sensitive cells are transferred to 39°C, the colonies seem to simply disappear. The results of this experiment, summarized in Table III, show that the vast majority of emetine-resistant segregants from all seven hybrids are temperature sensitive. Totaling the results from the five UCW 10 × UCW 113 hybrids, 320 out of 324 segregants, or 98.7%, are temperature sensitive. From the two UCW 10 × UCW 15 hybrids, 232 of 233 emetine-resistant segregants, or over 99%,

TABLE III

Cosegregation of leuS and emtB from UCW 10 × UCW 113 and UCW 10 × UCW 15 Hybrids

Hybrids	No. of cells plated in medium with emetine	No. of emetine-resistant segregants	No. temp. sensitive/No. emetine resistant
CHO emtB^r × CHL emtB$^+$			
UCW 10 × UCW 113 1	2×10^6	28	28/28
UCW 10 × UCW 113 2	3×10^6	36	36/36
UCW 10 × UCW 113 3	5×10^6	91	89/91
UCW 10 × UCW 113 5	2×10^6	55	53/55
UCW 10 × UCW 113 7	5×10^6	114	114/114
			Total 320/324
CHO emtB^r × CHO emtB$^+$			
UCW 10 × UCW 15 2	2×10^6	89	88/89
UCW 10 × UCW 15 5	2×10^6	144	144/144
			Total 232/233

The selection of emetine-resistant segregants, and subsequent determination of their temperature sensitivity or temperature resistance are described in the text.

are temperature sensitive. Thus, in both CHL and CHO cells the leuS and emtB loci cosegregate at a very high frequency and are linked.

Linkage of chr to emtB and leuS: Isolation of Cell Lines with Mutations in Three Linked Genes

As discussed in the Introduction, Campbell and Worton (6) reported that the emt locus in CHO cells is linked to a gene (chr) that can be altered to give rise to chromate resistance. Because we suspected that their emt locus corresponds to what we have designated emtB, we were interested to determine if the chr locus cosegregated from hybrids along with the emtB and leuS genes. Therefore, we isolated a number of mutants from UCW 10 that were selected as being resistant to 25 μM sodium chromate. We then isolated hybrids between UCW 113 and two such mutants, UCW 56 and UCW 57. Because chromate resistance is also recessive, these hybrids are phenotypically chromate and emetine sensitive, and temperature resistant. We determined whether or not the chr locus cosegregated with emtB and leuS from such hybrids as follows. Cells of a UCW 56 × UCW 113 hybrid or a UCW 57 × UCW 113 hybrid were distributed into 100-mm dishes in medium containing 3×10^{-7} M emetine. The cultures were incubated at 33°C until colonies of emetine-resistant segregants appeared. The emetine-resistant colonies were marked and the medium was replaced with the same medium that also contained 20 μM sodium chromate. This concentration of chromate causes lysis of the chromate-sensitive UCW 56 × UCW 113 and UCW 57 × UCW 113 hybrids within 2–3 d, but allows near normal growth of both UCW 56 and UCW 57. Thus, if the wild-type chr locus cosegregates from hybrids along with the wild-type emtB locus, many of emetine-resistant segregants should also be resistant to chromate. If the two loci do not cosegregate, almost all of the emetine-resistant segregants would be killed by chromate. As shown in Table IV, the former situation is the case. Thus, 26 out of 31 emetine-resistant segregants derived

TABLE IV

Cosegregation of the chr, emtB, and leuS Genes

Hybrid	No. emt^r segregants isolated	No. chr^r segregants/No. emt^r segregants	No. ts segregants/No. emt^r chr^r, segregants
UCW 56 × UCW 113	31	26/31	26/26
UCW 57 × UCW 113	15	8/15	8/8

The selection of emetine-resistant segregants and subsequent determination of their chromate resistance or sensitivity and their temperature resistance or sensitivity is described in the text.

from the UCW 56 × UCW 113 hybrid are chromate resistant and 8 out of 15 emetine-resistant segregants derived from the UCW 57 × UCW 113 hybrid are chromate resistant. The chr and emtB loci are therefore linked and by inference, chr is also linked to leuS. To verify this latter point, the plates containing the emetine- and chromate-resistant segregants were transferred to 39°C to determine if they had lost or retained the wild-type leuS gene, as described above. As shown in Table IV, all of the emetine- and chromate-resistant segregants from both hybrids were temperature sensitive and died at 39°C. In addition, we isolated a total of 17 segregants from the same two hybrids at 33°C which were selected as being resistant to 25 μM chromate and 16 of these died at 39°C, indicating they had also lost the wild-type leuS gene. These results confirm that all three loci, emtB, leuS, and chr, are linked.

Nonlinkage of emtA and emtB

Having established that the emtB locus was linked to leuS, we were able to determine if the same was true for the emtA locus. We have previously described the isolation of an emetine-resistant mutant, which belongs to the emtA complementation group, from a CHL cell line that was already hprt$^-$, leuS^{ts}, and has a temperature-sensitive asparagyl-tRNA synthetase (asnS^{ts}) (19). The leuS^{ts} marker in this cell line does not complement the leuS^{ts} marker in UCW 10, indicating the mutations in the two cell lines are allelic (S. Chang and J. Wasmuth. Unpublished results). A ouabain-resistant derivative, UCW 288, of this cell line was fused to the wild-type CHL cell line, UCW 100, and to the CHO cell line, UCW 15, which is wild type with respect to all pertinent loci. Hybrids were selected as described in Materials and Methods and maintained at 33°C in medium containing 0.1 mM asparagine. Emetine-resistant segregants (emtA^r) were isolated at 33°C from two different UCW 288 × UCW 100 hybrids and from two different UCW 288 × UCW 15 hybrids, as described above. Again, macroscopically visible emetine-resistant colonies on the culture dishes were marked, and the cultures were transferred to 39°C in medium lacking asparagine. 2 and 4 d later, the emetine-resistant colonies were scored as alive or dead as described above for the segregants from hybrids involving UCW 10. If either the leuS$^+$ or asnS$^+$ genes of the wild-type parent cosegregated with the emtA$^+$ locus, cells would die at 39°C because they would have only a temperature-sensitive leuS or asnS gene product. However, only 2 out of over 400 emtA^r segregants from the four different cultures were temperature sensitive. Since we have previously determined that either the leuS^{ts} or asnS^{ts} marker of the UCW 288 parent will cause lysis of cells within 48 h after they transferred to 39°C, the results clearly indicate that the emtA locus does not cosegregate with, and is not linked to, either leuS or asnS. By inference, therefore, emtA is not linked to emtB. In similar experiments

we have also determined that the emtA and hprt loci do not cosegregate, indicating that emtA is not X-linked.

DISCUSSION

Concerning the construction of a genetic map of genes encoding for components of the protein synthetic machinery in Chinese hamster cells, the results presented in this report have demonstrated that: (*a*) Two genes involved in protein synthesis, emtB and leuS, are linked in CHO and CHL cells. As discussed below, this linkage may be quite close. (*b*) Another locus, emtA, encoding for some protein synthetic component and probably a component of the ribosome is not linked to leuS or emtB, nor is it linked to the gene encoding for asparagyl-tRNA synthetase (asnS) or the X-chromosome. To understand how a group of functionally related genes, such as those encoding for protein synthetic and ribosomal components, is coordinately expressed, it is essential that we learn more about their structural organization within the mammalian genome. Although the experiments described in this report are certainly a step in that direction, an enormous amount of work remains to be done. In this laboratory we are continuing to carry out experiments of the type described here with a number of other protein synthetic mutants we have isolated, including a third class of emetine-resistant mutants we have recently identified.

Campbell and Worton (6) have recently demonstrated that a locus defined by an emetine-resistant mutant, emt^r, and a locus defined by mutants resistant to chromate, chr, are linked in CHO cells. Because the chr locus is also linked to the emtB locus in our CHO mutants, it appears quite probable that their emt^r locus corresponds to our emtB locus. Based upon polarized segregation patterns and karyotypic analyses, Campbell and Worton have proposed that the emt^r and chr genes are located on the long arm of chromosome 2, with the gene order being: centromere-emt-chr (6). From the experiments reported here, it appears that the leuS and emtB genes are at least as closely linked as are emtB and chr, and possibly closer. Even though the emtB and leuS genes cosegregate 99% of the time from the UCW 10 × UCW 113 hybrids, we believe there are two factors which actually make this frequency an underestimate. First, emetine-resistant segregants that have also lost the wild-type leuS gene are at a distinct growth disadvantage as compared to the rare segregants that have retained the $leuS^+$ gene. Thus, segregants with only the defective leuS gene have a generation time ~10–15% longer than segregants with the normal leuS gene, even at 33°C (J. Wasmuth. Unpublished observations). This factor would obviously tend to make the emetine- and temperature-resistant segregants appear to be more common than they actually are, since once they arise they proliferate more rapidly than emetine-resistant, temperature-sensitive segregants. Secondly, we have determined the segregation rate of the emtB locus from a hybrid between another CHO $emtB^r$ mutant, with a normal leuS, and UCW 113 and have obtained results that indicate the emtB locus is dizygous in CHL cells, as we previously suspected (19). It is therefore likely that there are two copies of the genes for both leuS and emtB in UCW 113, all of which must cosegregate from the UCW 10 × UCW 113 hybrids in order for a segregant to become emetine resistant and temperature sensitive. Thus, each emetine-resistant, temperature-sensitive segregant would represent two cosegregation events rather than one. This idea is supported by the results shown in Table III, since the frequency of emetine-resistant segregants from the CHL × CHO hybrids is lower than the frequency of segregants from the CHO × CHO hybrids. However, because these experiments were not designed to accurately determine the segregation rates or frequencies of emetine resistance from hybrids, the data cannot be interpreted in a quantitative way. It should be noted in fact, that the difference in the frequency of emetine-resistant segregants between the CHL × CHO and the CHO × CHO hybrids is not as great as one might expect if the CHO cells are hemizygous and the CHL cells are dizygous for the emtB locus. In particular, the frequency of emetine-resistant segregants from the CHO × CHO hybrids is lower than the frequencies reported by Gupta et al. (8). There are at least two possible reasons for this difference. (*a*) As discussed above, emetine-resistant segregants that have also become temperature sensitive are at a growth disadvantage as compared to nontemperature-sensitive cells, even at 33°C. This fact alone makes quantitative estimates of segregation frequencies very difficult. In addition, we cannot be certain that under nonselective conditions, emetine-sensitive hybrids and emetine-resistant segregants have similar growth rates. (*b*) The frequency of emetine-resistant segregants from CHO × CHO hybrids reported by Gupta et al. (8) was determined using a two-step emetine-resistant mutant (emt^{rII}) that is resistant to very high concentration of emetine ($<10^{-5}$ M). The segregants they obtain are also resistant to these high concentrations of emetine. The emetine-resistant mutant used in our experiments is not a second-step mutant, and its plating efficiency begins to decrease at emetine concentration above 5×10^{-7} M. Since in both cases, segregants were selected at 3×10^{-7} M emetine, it is quite possible that the recovery of emetine-resistant segregants from a population of hybrid cells is significantly higher in the experiments reported by Gupta et al. (8) simply because the segregants that arise in their experiments are resistant to much higher concentrations of emetine than the segregants that arise in our experiments.

Based on our segregation data and the observation by Campbell and Worton (5) that segregation of the emt^r locus in CHO × CHO hybrids does not involve loss of any detectable chromosome fragments, it appears very likely that leuS is located on the long arm of chromosome 2. More recent preliminary experiments in this laboratory support this notion and indicate the gene order is: centromere-leuS-emtB-chr. Experiments now in progress will enable us to verify this order and to better determine the relative distances between the three genes. Another interesting feature concerning the linkage of emtB and leuS, besides a possible functional significance, is that good evidence exists for both loci being hemizygous in CHO cells (1, 4, 8). Interestingly, Worton et al. have pointed out that the region of chromosome 2 thought to contain the emt^r and chr genes is karyotypically deleted in one homolog of chromosome 2 in CHO cells (5). These findings, when taken together, make it seem very likely that the reason emt^r (emtB) and $leuS^{ts}$ mutants can be isolated from CHO cells at such high frequencies is that the two loci are both located in a region that is truly haploid in CHO as a result of the deletion of part of the long arm of one chromosome 2. It is also interesting to note that in a CHO cell line with amplified dihydrofolate reductase (DHFR) genes, the amplified genes could be localized to a unique homogeneously staining region on the short arm of chromosome 2 (13). Thus, the structural gene for DHFR may normally be located in this region, bringing the number of genes localized to chromosome 2 in Chinese hamsters to four.

The finding that the emtA and emtB genes are not linked also raises another interesting point. All of the emetine-resistant mutants we have isolated from a number of different clones of

V-79 CHL cells belong to the emtA complementation group (19). However, emetine-resistant mutants we have isolated from different clones of CHO cells all belong to a different complementation group, emtB (19). We have since isolated many more emetine-resistant mutants from both V-79 CHL and CHO cells and find absolute cell line specificity with respect to the different complementation groups. These results are in marked contrast with those obtained by two other laboratories, in that all the emetine-resistant mutants they isolated form both V-79 CHL and CHO cells were reported to belong to a single complementation group (3, 4). In view of the results presented in this report demonstrating that emtA and emtB are not linked, our previous definition of the two distinct complementation groups remains unequivocal (19). Thus, the different results in our laboratory and the others remain very difficult to explain.

In addition to the results already discussed, it should be noted that the cell lines UCW 56 and UCW 57 represent the first cultured mammalian cell lines described with mutations in three linked genes. These cell lines, and hybrids derived from them, should prove to be very valuable in examining many questions concerning segregation and mitotic recombination in cultured somatic cells, especially since the mutations in each of the three genes provide easily distinguishable phenotypes. This system may also prove to be a useful tool for testing the ability of various components suspected of inducing gross chromosomal alterations such as breaks or translocations. Thus, the ability of such suspected carcinogens or teratogens to disrupt the normally coordinate segregation of three linked genes may provide a quantitative estimate of their chromosome-damaging potential.

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