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Hybrid mammalian cells assemble hybrid ribosomes

(ribosomal protein/ribosomal RNA/emetine resistance/RNA–protein interactions)

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ABSTRACT **Hybrid cell lines formed by fusion of mouse 3T3 cells and Chinese hamster ovary (CHO) cells resistant to emetine, which have an altered 40S ribosomal protein, are generally sensitive to emetine. From most hybrid lines it was possible to select sublines resistant to emetine. The ribosomal components of three lines were studied: A34, an emetine-sensitive hybrid; A34/R3, an emetine-resistant derivative of A34; and A72, an emetine-sensitive hybrid that did not give rise to emetine-resistant sublines. Genetic and biochemical evidence suggests that in A34 both the mouse emetine sensitivity gene and the hamster emetine resistance gene are active, whereas in A34/R3 only the hamster emetine resistance gene is active and in A72 only the mouse emetine sensitivity gene is active. The ribosomes of all three sublines contained both mouse and hamster RNA, predominantly mouse. However, the 60S subunits had roughly equal amounts of the three mouse and hamster proteins that could be distinguished by two-dimensional electrophoresis, suggesting the association of mouse RNA with hamster ribosomal proteins. The emetine-resistant and emetine-sensitive 40S subunits could be separated by sedimentation in 0.5 M KCl. Resistant subunits contained predominantly mouse RNA, presumably associated with the hamster protein conferring emetine resistance. We conclude that hybrid cells can form hybrid ribosomes and that the amounts of ribosomal RNA and ribosomal protein of each species are not closely coupled.**

The biosynthesis of eukaryotic ribosomes is a complex process requiring the coordinated production of several RNA species and 70 different proteins as well as their assembly in the nucleolus (1). For mammalian cells, the mechanism by which such coordinated synthesis of protein and RNA is accomplished is little understood. Analysis of this system is hampered by the fact that ribosomes are essential for growth, making mutants difficult to obtain.

Somatic cell hybrids provide an alternate route to examine the control of ribosome biosynthesis in mammalian cells. With respect to the synthesis of ribosomal RNA, hybrid cells that lose preferentially the chromosomes of one parent generally express only one type of 28S rRNA (2–4), whereas hybrids of two rodent species express both (5, 6). Recently it has been suggested that, in hybrids between Syrian hamster and mouse, individual cells contain active nucleolar organizer regions on chromosomes of both parents (7). However, in mouse–human hybrids only one parental nucleolar organizer is active, despite the presence of rDNA of both parents (4). Less attention has been paid to ribosomal proteins in hybrids. However, mouse and rat, and mouse and hamster, ribosomal proteins have been identified in hybrids between those species (8, 9). These studies did not distinguish between totipotent cells and subpopulations each of which produce the protein or RNA of a single species.

As a practical matter, hybrid cells derived from the cross of two rodent species, which retain the chromosomes of both parents and maintain the ability to synthesize ribosomal components of both species, are most amenable to study. This "coexistence" allows examination of ribosomal synthesis in cells in which failure to maintain the coordinated ribosome biosynthesis of one parent is not lethal because a second functional set of genes for ribosomal components is also present. For such investigations to be fruitful the ribosomal components of each parent should be distinguishable, and ideally they should be selectable *in vivo*. Furthermore, the extent to which these components interact to form functional "hybrid" ribosomes must be determined because hybrid cells with separate and noninteracting systems for the biosynthesis of ribosomes should behave very differently from hybrids in which ribosomal components mix freely.

In the study presented here, one parent is a Chinese hamster ovary (CHO) line requiring proline and resistant to the antibiotic emetine (10). Mutants resistant to emetine have been shown to affect the 40S subunit (11), and one such mutant has an electrophoretically altered 40S protein (12). This protein has been tentatively identified as S14 (13), in the nomenclature of McConkey *et al.* (14). The complementary parent is mouse, whose chromosomes are readily distinguishable from those of CHO (15, 16), and whose genetics have been extensively studied (17, 18).

We have established methods to detect differences in the ribosomal RNA and protein components of the two parents. In hybrids formed between CHO and mouse we have determined that the ribosomal components do interact to form hybrid ribosomes. Although roughly equal amounts of ribosomal proteins of both species are found, the ribosomal RNA is predominantly mouse.

METHODS

Cell Lines and Culture Techniques. A hamster CHO line requiring proline and resistant to emetine ($emt^{R1}41$), obtained from L. Siminovitch (10), and a mouse 3T3 thymidine kinase-negative line (C2F), obtained from C. Basilico (5), were routinely grown at 37°C as monolayers in 75-cm^2 flasks (Corning) and fed with Dulbecco's modified Eagle's medium (DME) supplemented with proline at 40 mg/liter and 10% fetal calf serum (North American Biologicals, Miami, FL).

Separation of Ribosomal Subunits. Cells ($1–4 \times 10^7$) were harvested, washed two times with Earle's salt solution (19), suspended in 10 ml of 50 mM Tris acetate, pH 7/50 mM NH_4Cl/12 mM $MgCl_2$/1 mM dithiothreitol (TMN) for 15 min. Nonidet P-40 was added to 0.5% and the cells were homogenized with a Dounce homogenizer to ensure lysis. The nuclei were removed by centrifugation. The supernatant was layered over 10 ml of 10% sucrose in TMN and centrifuged 2 hr at 40,000 rpm in a Spinco Ti 60 rotor at 4°C. The pellet was

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Abbreviations: DME, Dulbecco's modified Eagle's medium; TMN, Tris acetate/NH_4Cl/$MgCl_2$/dithiothreitol; KMT, KCl/$MgCl_2$/Tris·HCl; $NaDodSO_4$, sodium dodecyl sulfate; HAT, hypoxanthine/aminopterin/thymidine.

washed quickly with 0.5 M KCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.4 (KMT) buffer and resuspended in that buffer. Puromycin was added to 0.2 mM, and the suspension was incubated at 37°C for 25 min and centrifuged 10,000 × *g* for 10 min. The supernatant was layered over a 10–25% sucrose gradient in KMT buffer and centrifuged at 23,000 rpm for 23 hr in a Spinco SW 27 rotor at 4°C. The gradient was collected from the bottom and absorbance was monitored at 260 nm.

Ribosomal Proteins. Proteins were extracted from suspended ribosomes by addition of $MgCl_2$ to 0.1 M, dithiothreitol to 0.01 M, followed by 2 vol of glacial acetic acid (20). Samples were analyzed on a two-dimensional polyacrylamide gel modified (20) from that described by Mets and Bogorad (21).

Ribosomal RNA. Ribosomal RNA was extracted with phenol from cytoplasmic fractions treated wth 1% sodium dodecyl sulfate ($NaDodSO_4$) and resolved on sucrose gradients containing $NaDodSO_4$ (22). To distinguish mouse from hamster RNA, 10 μg of 1 S or 28S RNA prepared from cells incubated 24 hr with [^{32}P]phosphate in supplemented DME was mixed with 100 μg of unlabeled RNA, precipitated with ethanol, air dried, and resuspended in 20 μl of 10 mM Tris·HCl, pH 7.4/1 mM EDTA. Fifteen units of ribonuclease T1 (Calbiochem) was added, the mixture was incubated 20 min at 37°C, and the fragments were separated by two-dimensional polyacrylamide gel electrophoresis (23). The slab gel was wrapped in plastic wrap and exposed to x-ray film (Kodak NS-5) at −20°C for 1–3 days. To quantitate individual spots, the gel was laid on the developed film and the chosen spots were excised with a cork borer. A second film was exposed and developed to check the accuracy of the excision. The gel spots were digested in 0.5 ml of 30% H_2O_2 at 60°C, mixed with 10 ml of Aquasol (New England Nuclear), and assayed for radioactivity.

Cell Hybridization and Selection. Techniques utilized to fuse rodent cells and select hybrids were as described by Pontecorvo (24). Briefly, 7×10^5 of each cell type were plated together on a 60-mm diameter dish (Falcon), grown overnight, washed repeatedly with polyethylene glycol (BDH 6000) in DME, and then incubated 24 hr in DME supplemented with proline and 10% fetal calf serum. The cells were then replated in 100-mm diameter dishes at concentrations of 1×10^4 or 5×10^5 cells per dish in DME with 100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine (HAT) and 10% dialyzed fetal calf serum but without proline. Appropriate controls for reversion of both parental markers were treated similarly. Colonies that arose from the hybrid cross were picked with cloning rings and cultured in the same medium. Clones were prepared by seeding cells on glass fragments and picking fragments with only one cell. Metaphase chromosomes were prepared as described by Kozak *et al.* (16), enabling us to differentiate the small telocentric mouse chromosomes from hamster chromosomes and thus to verify the hybrid nature and homogeneity of the clone. To determine emetine resistance, 10^3–10^5 cells were seeded in 100-mm dishes containing DME with HAT and dialyzed fetal calf serum with or without emetine at 0.1 μM and examined after 5–14 days. At this concentration of emetine the plating efficiency of emetine-resistant CHO cells is 50%, while that of 3T3 cells is less than 10^{-5}. We designated as sensitive the hybrids with a plating efficiency of less than 1%. To select emetine-resistant clones from sensitive hybrids, 5×10^6 cells were plated at 5×10^5 cells per 100-mm dish in the presence of 0.1 μM emetine. After 2 weeks colonies that arose were picked and cultured several days in the presence of 0.1 μM emetine. Emetine-resistant cells were then routinely grown in the same media as other hybrids. During prolonged growth, emetine resistance was occasionally verified.

RESULTS

Ribosomal RNA and Proteins of Mouse and Hamster Cells. Ribosomal proteins from CHO and 3T3 were separated by two-dimensional electrophoresis at pH 5 in the first dimension, with $NaDodSO_4$ in the second. As shown by using another gel system (9), the patterns (Fig. 1 *A* and *B*) are similar but not identical. At least three proteins of the mouse, all from the 60S subunit (Fig. 1*A*), are resolved from their hamster counterparts (Fig. 1*B*) under these conditions. These differences are seen most clearly in the pattern of proteins from the hybrid line A34 (Fig. 1*C*), which contains both parental types. The proteins in the upper pair of spots, which are probably L6 (13, 14), differ slightly in molecular weight. Those in the middle pair appear to differ in size by several thousand daltons, on the basis of molecular weight standards in the second-dimensional gel. The proteins in the lower pair, which stain rather faintly but are clear on the original gel, differ in charge but not in size. The basic identity of the proteins in each pair has been verified by proteolytic digestion of the spots followed by a third dimension of electrophoresis, as in the method of Cleveland *et al.* (25). S14, the protein thought to be responsible for emetine resistance (12),

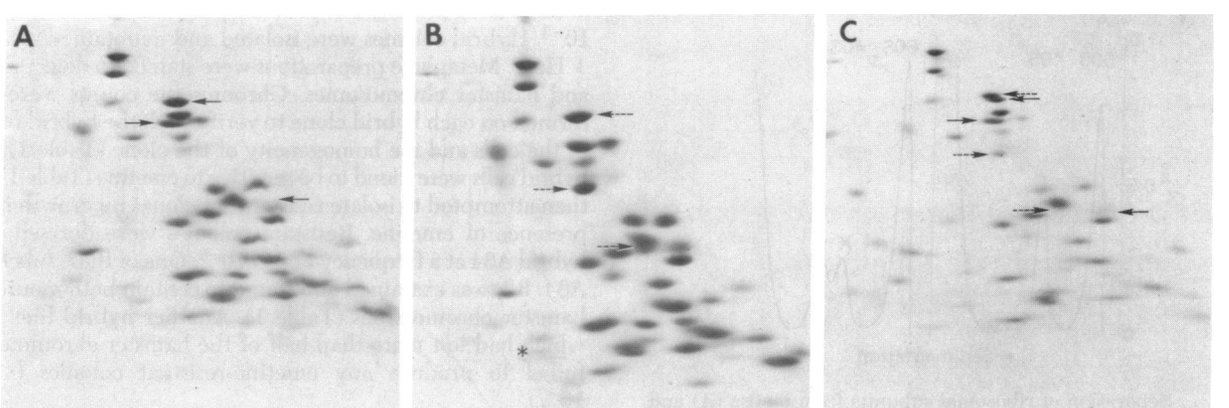

FIG. 1. Ribosomal proteins of mouse, hamster, and hybrid. The 80S ribosomes were prepared from cultures containing 1×10^7 cells, and proteins were extracted by 67% acetic acid as described (4). Samples containing approximately 100 μg of protein were lyophilized and separated by two-dimensional polyacrylamide gel electrophoresis (20). Separation from left to right was toward the cathode at pH 5, and separation from top to bottom was in the presence of $NaDodSO_4$. (*A*) Mouse; (*B*) hamster. Unique mouse proteins are marked by solid arrows, their hamster counterparts by dashed arrows. The protein shown to be altered in other emetine-resistant CHO lines is marked with an asterisk (12). (*C*) Proteins extracted from the emetine-sensitive hybrid A34. Mouse proteins are indicated by solid arrows, hamster proteins by dashed arrows.

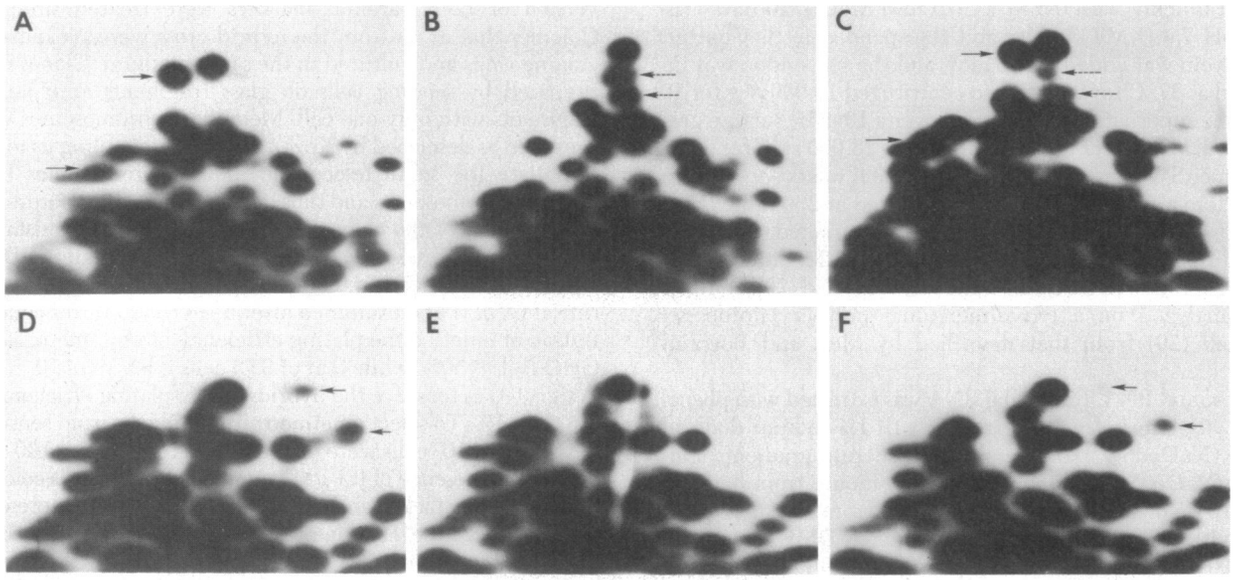

FIG. 2. Ribosomal RNA of mouse, hamster, and hybrid cells digested with ribonuclease T1. Cells of each type (2×10^6) were incubated for 2 hr with 1 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{32}P]phosphate in DME with reduced phosphate concentration and then for 12 hr in complete DME. RNA was prepared in $NaDodSO_4$/phenol from mouse (*A*, *D*) or hamster (*B*, *E*) cytoplasm or separated hybrid ribosomal subunits (*C*, *F*) (22). The 28S and 18S species were separated on sucrose gradients, precipitated from 67% EtOH, digested with 15 units of T1 ribonuclease for 20 min at 37°C, separated by two-dimensional polyacrylamide gel electrophoresis (23), and autoradiographed. Mouse RNA is shown in *A* (28S) and *D* (18S), with solid arrows indicating unique mouse spots; hamster RNA is shown in *B* (28S) and *E* (18S), with dashed arrows indicating unique spots. RNA obtained from hybrid A34/R3 60S subunits (*C*) and degraded 40S subunit (*F*) (see peak Y of Fig. 5*B*) shows spots unique to both mouse (solid arrows) and hamster (dashed arrows).

is indicated in Fig. 1*B* by the asterisk. In the mutant we are studying, however, that protein is indistinguishable from wild-type hamster or mouse protein S14.

One can distinguish RNA species of hamster and mouse cells by analysis of T1 digests of ^{32}P-labeled RNA (Fig. 2). For the 18S species mouse-specific spots are clearly seen and in the 28S pattern spots unique both to mouse and to hamster are clearly resolved. When mouse and hamster RNA are mixed and then analyzed, the radioactivity present in these species-specific spots is an accurate measure of the amount of each parental type in the input.

40S Subunits of Emetine-Resistant Cells. An unexpected instability of emetine-resistant 40S subunits has permitted us to separate 40S subunits containing a hamster protein from those containing the mouse counterpart. This instability is observed after sedimentation through a sucrose gradient containing 0.5 M KCl (Fig. 3). A single 40S peak is obtained from mouse or wild-type CHO (not shown) under these conditions (Fig. 3*A*). However, two particles containing 18S RNA are obtained from emetine-resistant cells (Fig. 3*B*). The heavier species (X) contains all but three of the normal 40S proteins (Fig. 4 *A* and *B*) and sediments about 10% slower than a normal 40S subunit. The lighter species (Y), which sediments well away from the 40S region, contains only about half the normal proteins (Fig. 4*C*). The loss of proteins is specific and nearly quantitative, suggesting their dissociation as discrete groups. The ribosomal protein associated with emetine resistance (12) is absent from both the X and Y species. The missing proteins can be detected at the top of the gradient. They are also found in intact 80S ribosomes (Fig. 1).

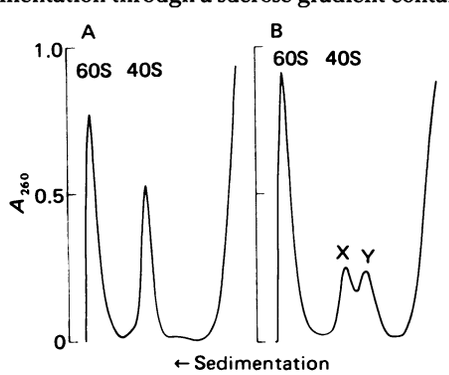

FIG. 3. Separation of ribosomal subunits from mouse (*A*) and emetine-resistant hamster (*B*) cells. Cells of each species (1×10^7) were swollen and lysed in TMN buffer. The nuclei were removed by centrifugation. Ribosomes were sedimented through 10% sucrose in that buffer. The pellet was resuspended in KMT and incubated at 37°C for 20 min with 0.2 mM puromycin. This was layered over a 10–25% sucrose gradient in KMT buffer and centrifuged at 23,000 rpm in a Spinco SW 27 rotor for 23 hr at 4°C.

Fusion and Selection. Mouse 3T3 and hamster CHO cells were fused and hybrids were selected by growth in HAT medium lacking proline. The efficiency of hybridization was 3×10^{-3}. Hybrid colonies were isolated and maintained in DME + HAT. Metaphase preparations were stained to detect mouse and hamster chromosomes. Chromosome counts were performed on each hybrid clone to verify both the hybrid nature of the cells and the homogeneity of the clone (Table 1). The hybrid cells were found to be sensitive to emetine (Table 1). We then attempted to isolate resistant subclones by growth in the presence of emetine. Resistant colonies were derived from hybrid A34 at a frequency of 3×10^{-6}. One of these subclones. A34/R3, was examined and found to contain both mouse and hamster chromosomes (Table 1). Another hybrid line, A72, which had lost more than half of the hamster chromosomes, failed to produce any emetine-resistant colonies ($<2 \times 10^{-7}$).

Ribosomal Protein and RNA of Hybrid Cells. Ribosomal proteins extracted from the three hybrid clones—A34, A34/R3, and A72—were separated on two-dimensional polyacrylamide gels. The three patterns were essentially identical and the pattern obtained from hybrid A34 is shown in Fig. 1*C* as a representative. From the Coomassie blue-stained patterns it

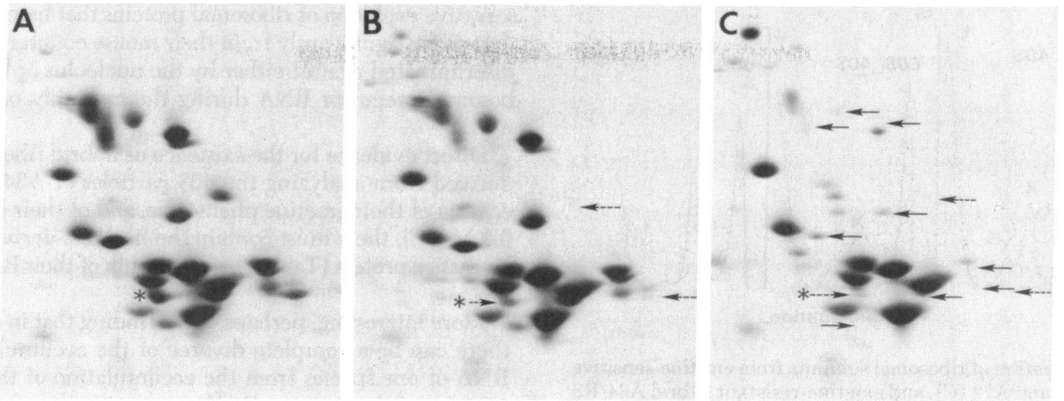

FIG. 4. Proteins from intact and degraded 40S subunits. Proteins were extracted from 40S ribosomal subunits prepared from mouse and from the X and Y peaks from emetine-resistant hamster cells and separated by two-dimensional gels. (*A*) Proteins from mouse. S14, the protein whose mutation can cause emetine resistance, is marked with an asterisk. (*B*) Proteins from the heavier (X) hamster peak. Proteins present in 40S subunits but missing here are indicated by dashed arrows. (*C*) Proteins from the lighter (Y) hamster peak. Proteins present in peak X, but missing in Y, are indicated by solid arrows.

appeared that equal amounts of both parental species were present. To quantitate this observation, ^{14}C-labeled ribosomal proteins from hybrid cells were mixed with unlabeled mouse and hamster ribosomal proteins and separated electrophoretically. The specific spots were excised, the radioactivities were determined, and the ratios are shown in Table 2. The ratio for these proteins is nearly 1:1 in all three hybrids.

^{32}P-Labeled ribosomal RNA was isolated from all three hybrid lines. It was digested with T1, fragments were separated on two-dimensional gels, and the radioactivity present in species-specific spots was determined. The ratios of the two parental RNAs were calculated (Table 2). For hybrids A34 and A34/R3 both parental types were clearly present, though mouse accounted for 80–90% of the total. For hybrid A72, mouse again predominated and hamster RNA, if present, was at the limit of resolution.

Hybrid Cells Assemble Hybrid Ribosomes. Sucrose gradient profiles of the ribosomal subunits of two hybrid lines, A72 and A34, both emetine-sensitive, and the resistant subline A34/R3 are shown in Fig. 5. For A72, only the normal 40S subunit was seen (Fig. 5*C*), suggesting that it contains only the mouse-derived emetine-sensitive protein. The pattern shown by hybrid A34 (Fig. 5*A*) is consistent with an equal mixture of sensitive and resistant subunits. This was demonstrated directly by acrylamide gel analysis of the proteins of the "40 + X" peak. On the other hand, the emetine-resistant subline, A34/R3 (Fig. 5*B*), showed only the resistant pattern (compare with Fig. 3*B*), suggesting the absence of the emetine-sensitive protein derived from mouse.

To demonstrate further the existence of hybrid 40S subunits, we grew cells of the emetine-resistant hybrid A34/R3 in the presence of [^{32}P]phosphate and isolated subunits by sedimentation through 0.5 M KCl. RNA was extracted from both the 60S subunit and the smaller peak (Y). This RNA was digested and the autoradiogram is shown in Fig. 2 *C* and *F*. The 28S pattern shows both mouse- and hamster-specific spots, with mouse predominating. The 18S RNA extracted from the Y peak, which arises only from 40S subunits containing the hamster emetine-resistant protein, clearly shows the mouse-specific spots. Quantitative determination of the mouse 18S RNA is hampered by the absence of hamster-specific spots in the T1 digest (Fig. 2). However, by comparing the radioactivity in mouse-specific spots with that in common spots, we estimate that more than 80% of the 18S RNA in both peak Y and peak X is mouse, in agreement with the ratio for 28S RNA from this hybrid (Table 2). Thus, mouse 18S RNA is clearly shown to be associated in a 40S subunit with hamster protein S14.

The presence of electrophoretically separable 60S proteins allows us to quantitate directly their proportions in the hybrid. From the ratios shown in Table 2 both parental types appear

Table 1. Characteristics of parental and hybrid cells

Cells	No. of chromosomes* Mode	No. of chromosomes* Mouse/CHO	Plating efficiency in 0.1 μM emetine, %	Emetine sensitivity of 40S subunits†
Parents				
Mouse	69 ± 4	—	<0.1	Sensitive
CHO	21 ± 1	—	50	Resistant
Hybrids				
A34	71 ± 5	54/17	<1	Sensitive + resistant
A34/R3	68 ± 4	51/17	50	Resistant
A72	66 ± 3	57/ 9	<1	Sensitive

* Values shown represent means of eight to ten determinations; ± is maximum variation.

† Determined by sedimentation through 0.5 M KCl. Sensitive subunits sedimented as intact 40S particles; resistant subunits as two distinct smaller species.

Table 2. Ratio of mouse to hamster ribosomal proteins and ribosomal RNA from hybrid lines

Cell line	Protein *A*	Protein *B*	Protein *C*	28S RNA
A72	0.96	0.94	1.03	29.1
A34	1.01	0.73	1.82*	6.7
A34/R3	0.88	1.38	0.81	8.0

The relative amounts of mouse and hamster species-specific ribosomal proteins and ribosomal RNA from the 60S subunit are presented. To obtain the protein data, hybrid cells were labeled for 5 hr with [^{14}C]leucine and grown for an additional 14 hr without radioactivity; total protein was extracted, mixed with mouse and hamster ribosomal proteins, and analyzed on two-dimensional acrylamide gels, and the amount of ^{14}C in the mouse spot was compared to that in the hamster spot for the three distinguishable pairs of proteins (see text and Fig. 1). Column *A*, upper pair; *B*, middle pair; *C*, lower pair. The values represent the average of three determination, except * denotes a single determination. The RNA values were obtained by measuring ^{32}P in mouse-specific (M), hamster-specific (H), and selected common (C) spots from a T1 digest of 28S RNA isolated from hybrid lines. The values represent [(M hybrid/C hybrid)/(M mouse/C mouse)] ÷ [(H hybrid/C hybrid)/(H hamster/C hamster)].

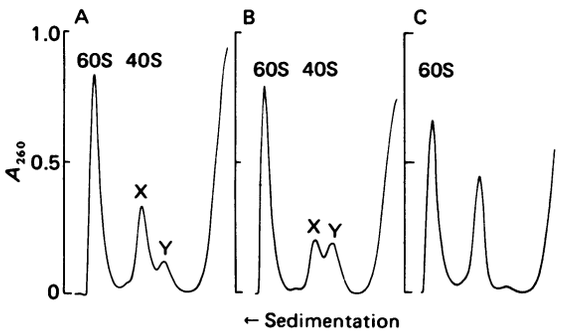

FIG. 5. Separation of ribosomal subunits from emetine-sensitive hybrids A34 (*A*) and A72 (*C*), and emetine-resistant hybrid A34/R3 (*B*), prepared and analyzed as in Fig. 3.

in approximately equal amounts. However, the 28S RNA is predominantly mouse, indicating that some hamster 60S proteins must be present on subunits containing mouse RNA. This is particularly true for hybrid A72, which has virtually no hamster RNA but does contain hamster proteins.

DISCUSSION

The emetine-resistant protein causes the 40S subunit to be unstable at high ionic strength, due to the loss of discrete groups of proteins. This occurs in 40S particles containing either hamster or mouse 18S RNA. Such instability was presaged by the finding that 40S subunits isolated from emetine-resistant cells had low activity for protein synthesis (11). The resistance to emetine, therefore, may arise from an alteration in the architecture of the 40S subunit, rather than from an alteration in binding to the mutated protein. As a practical matter, the instability and the concomitant ability to separate physically wild-type and emetine-resistant subunit is useful in the analysis of hybrid cells.

Interspecific hybrids between emetine-sensitive mouse cells and emetine-resistant hamster cells are phenotypically emetine-sensitive. Similar results were obtained for intraspecific hybrids (26), in spite of the fact that *in vitro* experiments suggest the presence of some emtine-resistant ribosomes. Fig. 5*A* demonstrates directly that hybrid A34 contains both resistant and sensitive 40S subunits. Its sensitive phenotype therefore must be due to cooperativity between resistant and sensitive subunits, presumably as they traverse the same mRNA.

It is possible, however, to select emetine-resistant hybrid lines that contain both mouse and hamster chromosomes. Because these arise at a level of about 3×10^{-5}, and because no emetine-resistant cell of mouse origin has ever been isolated, we conclude that the mouse genes responsible for the protein conferring emetine sensitivity have been either lost or efficiently repressed. These cells appear to have few, if any, sensitive 40S subunits (Fig. 5*B*). Conversely, we were unable to isolate emetine-resistant clones from some hybrid lines—e.g., A72—suggesting that these cells have lost or repressed the gene responsible for the protein conferring emetine resistance. This suggestion is supported by the finding that these cells have few if any resistant 40S subunits (Fig. 5*C*).

Ribosomes from hybrid cells contain both mouse and hamster RNA and both mouse and hamster proteins. However, because mouse RNA predominates (Fig. 2 *C* and *F*), whereas there are roughly equal amounts of those proteins we can differentiate (Fig. 1*C*), there is no strict coordination by species. On the contrary, it is clear for the 60S particle that hamster proteins must be assembled with mouse RNA. In fact, hamster 60S proteins are present in equal amounts even when hamster RNA is barely detectable, as in clone A72. It is testament to the conservative evolution of ribosomal proteins that hamster proteins that differ significantly from their mouse counterparts are not discriminated against either by the nucleolus or by mouse ribosomal precursor RNA during the assembly of a 60S subunit.

Direct evidence for the existence of hybrid ribosomes is also derived from analyzing the 40S particles of A34/R3. By the criteria of their emetine phenotype, and of their instability in 0.5 M KCl, these must contain the hamster-derived emetine-resistance protein (Table 1), yet the bulk of their RNA is mouse 18S.

More interesting, perhaps, is the finding that in a hybrid cell there can be a complete divorce of the accumulation of the RNA of one species from the accumulation of the ribosomal proteins of that species. Further experiments are necessary to determine whether this occurs at the level of synthesis of the macromolecules or in the processing and assembly steps involved in the formation of the ribosome.

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