MOLECULAR AND CELLULAR BIOLOGY, May 1983, p. 892–902
0270-7306/83/050892-11$02.00/0

Vol. 3, No. 5

Efficient Procedure for Transferring Specific Human Genes into Chinese Hamster Cell Mutants: Interspecific Transfer of the Human Genes Encoding Leucyl- and Asparaginyl-tRNA Synthetases

RONALD E. CIRULLO, SHARON DANA, AND JOHN J. WASMUTH*

Department of Biological Chemistry, California College of Medicine, University of California, Irvine, Irvine, California 92717

Received 19 November 1982/Accepted 22 February 1983

We have developed a simple and efficient procedure for transferring specific human genes into mutant Chinese hamster ovary cell recipients that does not rely on using calcium phosphate-precipitated high-molecular-weight DNA. Interspecific cell hybrids between human leukocytes and temperature-sensitive Chinese hamster cell mutants with either a thermolabile leucyl-tRNA synthetase or a thermolabile asparaginyl-tRNA synthetase were used as the starting material in these experiments. These hybrids contain only one or a few human chromosomes and require expression of the appropriate human aminoacyl-tRNA synthetase gene to grow at 39°C. Hybrids were exposed to very high doses of γ-irradiation to extensively fragment the chromosomes and re-fused immediately to the original temperature-sensitive Chinese hamster mutant, and secondary hybrids were isolated at 39°C. Secondary hybrids, which had retained small fragments of the human genome containing the selected gene, were subjected to another round of irradiation, refusion, and selection at 39°C to reduce the amount of human DNA even further. Using this procedure, we have constructed Chinese hamster cell lines that express the human genes encoding either asparaginyl- or leucyl-tRNA synthetase, yet less than 0.1% of their DNA is derived from the human genome, as quantitated by a sensitive dot-blot nucleic acid hybridization procedure. Analysis of these cell lines with Southern blots confirmed the presence of a small number of restriction endonuclease fragments containing human DNA specifically. These cell lines represent a convenient and simple means to clone the human genomic sequences of interest.

The genes encoding various components of the protein synthetic machinery in mammalian cells represent a very large family of functionally related genes. To begin understanding how the expression of this family of genes is coordinated, we are using a combined biochemical and genetic approach to analyze the complex process of protein synthesis. The large number of different Chinese hamster cell mutants that have been isolated with alterations in various protein synthesis components, including ribosomal proteins and aminoacyl-tRNA synthetases, makes this large family of genes especially suitable for genetic analysis and genetic manipulation (1, 3, 5, 13, 28, 33, 34). In Chinese hamster ovary (CHO) cells, two of these genes, *leuS* and *asnS*, which encode leucyl-tRNA synthetase and asparaginyl-tRNA synthetase, respectively, are particularly amenable to detailed genetic studies. Conditionally lethal, temperature-sensitive mutants with alterations in either of these genes can be isolated at very high frequencies (1, 29, 31). In addition, there is a simple counterselective system, growth at an elevated temperature, to isolate revertants in which the temperature-sensitive phenotype of the mutants has been suppressed by second-site intergenic or intragenic mutations. Because of the availability of large numbers of mutants and revertants, the *leuS* and *asnS* genes are most amenable to detailed studies on the effects of mutations on gene structure and expression at the molecular level. To exploit these genetic systems fully, we are interested in cloning genomic DNA sequences encoding leucyl- and asparaginyl-tRNA synthetase. These molecular probes would provide a means to examine the effects of mutations on gene structure and expression at the DNA level and will also be useful in identifying DNA sequences that might be important in regulating the expression of the large family of genes encoding protein synthesis components.

The technique of DNA-mediated transfer of genes into mammalian cells has proven to be an effective means to enrich for and clone specific genes (16, 18, 19, 35, 36). Variations of the basic gene transfer procedure, including plasmid rescue, have increased the general usefulness of this technique considerably (18, 19). For gene transfer to be useful in enriching for specific genes, the transferred gene must provide recipient cells with some selectable or distinguishable phenotype. Mouse L-cells have proven to be by far the most efficient recipients for DNA-mediated gene transfer. Unfortunately, however, the general utility of these cells in such experiments is limited by the very small number of mutants available that provide selectable phenotypes to monitor expression of transferred genes. On the other hand, a very large number of different mutants which provide selectable phenotypes have been isolated from cell lines derived from Chinese hamsters. However, these cell lines have proven to be much less competent recipients in DNA-transfer experiments.

To circumvent this problem with Chinese hamster cells and to utilize gene transfer to enrich for the genes we wished to clone (*leuS* and *asnS*), we have devised an alternative, efficient procedure for effecting DNA transfer into mutant CHO cells. Using this procedure, we focused on transferring the human genes encoding leucyl- and asparaginyl-tRNA synthetase into the appropriate CHO cell mutants because the presence of human DNA can be monitored against the background of rodent DNA by cytological as well as nucleic acid hybridization procedures (10, 14, 15, 22). We have previously isolated interspecific somatic cell hybrids between human leukocytes and CHO cell mutants with temperature-sensitive leucyl- or asparaginyl-tRNA synthetases, which contain and express the corresponding human aminoacyl tRNA-synthetase genes (6, 8, 9). The expression of the human aminoacyl-tRNA synthetase genes in the hybrids results in their ability to grow vigorously at 39°C, a temperature which is nonpermissive for the mutant CHO parents. In both types of hybrids, the product of the human aminoacyl-tRNA synthetase gene can easily be distinguished from the product of the corresponding mutant CHO gene, based upon large differences in the thermolability of the different enzymes in vitro (6, 8). Since these hybrids contained very few human chromosomes to begin with, they represented a significant enrichment of the human genes of interest. Hybrid cell lines were subjected to high doses of γ-irradiation to extensively fragment the chromosomes and were refused immediately to the original temperature-sensitive CHO mutant, and "secondary" hybrids were isolated at 39°C to require retention and expression of the appropriate human tRNA synthetase gene. This resulted in the generation of CHO cell lines in which small fragments of the human genome containing the selected gene had integrated into the hamster genome. Successive rounds of irradiation and refusion have resulted in CHO cell lines that express the human genes of interest, yet less than 0.1% of their DNA is derived from the human genome. The amount of human DNA in the various "transformants" was monitored by using a dot-blot procedure that enables us to accurately measure as little as one part of human DNA in the presence of a 10,000-fold excess of rodent DNA. Complete λ genomic DNA libraries prepared from the cell lines can be screened to identify recombinants containing human DNA by the methods of Gusella et al. (14, 15), which will provide at least a 1,000-fold purification of the genes of interest without the use of specific probes. The procedures we describe can be applied to enrich for any gene for which a selective system is available.

TABLE 1. List of cell lines

Cell line	Description	Relevant human genes present	Reference
Diploid CHO cells			
UCW 56	Temperature-sensitive leucyl-tRNA synthetase mutant	—	(32)
Asn5	Temperature-sensitive asparaginyl-tRNA synthetase mutant	—	(2)
Interspecific hybrids			
HHW 105	Human leukocyte × UCW 56 hybrid	*leuS*	(8)
HHW 119	Human leukocyte × Asn-5 hybrid	*asnS*	(6)

MATERIALS AND METHODS

Cell lines and cell cultures. All cell lines were grown as monolayer cultures in a humidified atmosphere of 5% CO_2–95% air at 33 or 39°C, as described previously (33, 34). The various cell lines used in these experiments are listed in Table 1 along with information concerning their phenotypes and lineage. CHO cell line Asn-5 has a mutation in the *asnS* gene, which renders asparaginyl-tRNA synthetase thermolabile and the cells nonviable at 39°C (2). UCW 56 is derived from the CHO cell line tsH-1 and has a mutation in the *leuS* gene which results in leucyl-tRNA synthetase being thermolabile (27, 32). This cell line is also nonviable at 39°C. The isolation and characterization of interspecific cell hybrids between normal human

leukocytes and either Asn-5 or UCW 56 has been described previously (6, 8). HHW 105 is a hybrid between human leukocytes and UCW 56 and was selected at 39°C to require the human gene encoding leucyl-tRNA synthetase to be retained and expressed (8). This cell line contains two forms of leucyl-tRNA synthetase, one form being very thermolabile in vitro, as is the altered enzyme from the UCW 56 parent, and one form being very resistant to thermal inactivation, as is the normal human enzyme (8). HHW 105 has retained a single human chromosome, number 5, to which the human *leuS* gene has been localized (8, 9). HHW 119 is a hybrid between human leukocytes and Asn-5, which was also selected at 39°C, to require retention and expression of the human gene encoding asparaginyl-tRNA synthetase. This cell line has retained three human chromosomes, numbers 12, 18, and 19 (6). Based upon heat inactivation experiments in vitro, HHW 119 contains both the mutant CHO asparaginyl-tRNA synthetase, which is thermolabile in vitro, and the human asparaginyl-tRNA synthetase, which is very stable in vitro (6).

γ-Irradiation of interspecific hybrids and isolation of secondary and tertiary hybrids. Replicate cultures of HHW 105 or HHW 119 containing 1.5×10^6 cells were established in 25-cm^2 flasks and incubated at 39°C. The following day, the flasks were filled with medium and the cells were exposed to 2, 4, 6, 8, or 10 krads of γ-irradiation from a ^{137}Cs source at a rate of 1.2 krads/min. Immediately after irradiation the medium was removed from flasks, and 2×10^6 cells of the Asn-5 mutant or 2×10^6 cells of the UCW 56 mutant, in 5 ml of culture medium, was added to irradiated cultures of HHW 119 or HHW 105, respectively. The cells were then transferred to 33°C. After 6 h, the mixed cultures were treated for 60 s with 45% polyethylene glycol-1,000 (wt/vol) in serum-free medium to induce cell fusion and then returned to 33°C in normal medium. After 3 days, the fused populations of cells were dispersed with trypsin-EDTA, seeded into 100-mm culture dishes at densities of 10^6 cells per dish, and transferred to 39°C. At the same time, cells of Asn-5 and UCW 56 that had not been fused to irradiated hybrids were plated at 39°C to determine the spontaneous reversion frequencies of temperature-sensitive markers in these cell lines. Temperature-resistant colonies that arose from the various fused cultures were isolated with cloning cylinders and maintained at 39°C to maintain selective pressure for expression of the appropriate human aminoacyl-tRNA synthetase gene. Secondary, temperature-resistant hybrids isolated from these experiments were subjected to a further round of irradiation and refusion in an analogous fashion, as described below.

In separate experiments, the effect of various, lower doses of γ-irradiation on viability of the hybrid cell lines was determined. Cultures containing 10^3, 10^4, 10^5, or 10^6 cells were exposed to 0, 0.25, 0.50, or 1.0 krad of γ-irradiation as described above and then placed at 33°C for 2 weeks. The flasks were then stained with 0.5% methylene blue in 50% methanol, and the number of colonies present in the various cultures was determined. Above 0.25 krad of irradiation, the plating efficiency decreased by ~3 orders of magnitude for every 0.5 krad of irradiation. Thus, at the lowest dose of irradiation used in the experiments described above to isolate secondary hybrids, the survival of the irradiated hybrids, in the absence of refusion, was decreased by a factor of $\sim 10^9$.

Dot-blot hybridizations. High-molecular-weight DNA was extracted from Chinese hamster, human, and hybrid cell lines by the methods of Pellicer et al. (21). The DNA samples were sheared by sonicating for 5 s with a Branson 185 Cell Disruptor at a power setting of 3. The sheared DNA samples were adjusted to 0.3 N NaOH and incubated at room temperature for 10 min. After neutralization, the samples were heated at 100°C for 10 min, and then rapidly cooled on ice. The samples were then serially diluted to yield solutions containing 25, 5, and 0.5 μg of DNA per ml. A 2-μl amount of the various samples was spotted onto nitrocellulose sheets that had been soaked in 5× SSC, pH 7.0 (1× SSC = 0.15 M NaCl–0.015 M sodium citrate) and dried. The filters containing DNA were baked in a vacuum oven at 80°C for 4 h. The blots were prehybridized for 8 h as described by Wahl et al. (30) and then hybridized for 16 h at 42°C with 3×10^7 cpm of ^{32}P-labeled total human DNA (10^8 to 2×10^8 cpm/μg of DNA) which had been prepared via nick translation (20). After hybridization, the blots were washed four times for 15 min at room temperature with 2× SSC–0.1% sodium dodecyl sulfate (SDS), once in 1× SSC–0.1% SDS for 30 min at 68°C, twice in 0.3× SSC–0.1% SDS for 30 min at 68°C, and once in 0.2× SSC for 30 min at 68°C. The blots were dried under a heat lamp and then exposed to X-ray film at −80°C with an intensifying screen. The resulting autoradiograms were then scanned with a densitometer (E.C. Apparatus Corp., St. Petersburg, Fla.).

Southern blot analysis. High-molecular-weight DNA was extracted as described above and digested to completion with either *Hin*dIII or *Eco*RI. Digested DNAs were electrophoresed in 1% agarose gels, then transferred onto nitrocellulose sheets (25). The resultant blots were prehybridized at 68°C for 8 h. The prehybridization mix contained 10× Denhardt solution, 5× SSC, 0.25% SDS, 100 μg of denatured salmon sperm DNA per ml, 0.133 M glycine, and 50 mM NaH_2PO_4 (pH 6.5). The prehybridization mix was removed, hybridization mix was added, and hybridization was carried out at 68°C for 48 h. The hybridization mix contained 5× Denhardt solution, 5× SSC, 0.25% SDS, 100 μg of denatured salmon sperm DNA per ml, 10% dextran sulfate, 67 mM glycine, 25 mM NaH_2PO_4 (pH 6.5), and 3×10^7 cpm of total human DNA labeled with ^{32}P via nick translation (20). The blots were washed as described above for dot blots and then exposed to X-ray film at −80°C with an intensifying screen.

Karyological analysis. Differential staining of human and Chinese hamster chromosomes in metaphase preparations from hybrid cell lines was performed by the alkaline-Giemsa procedure described by Friend et al. (10), with the modification described by Dana and Wasmuth (9).

RESULTS

Irradiation and refusion of primary hybrids: isolation of secondary hybrids expressing the human *leuS* and *asnS* genes. The isolation and characterization of the two Chinese hamster-human cell hybrids used in these experiments

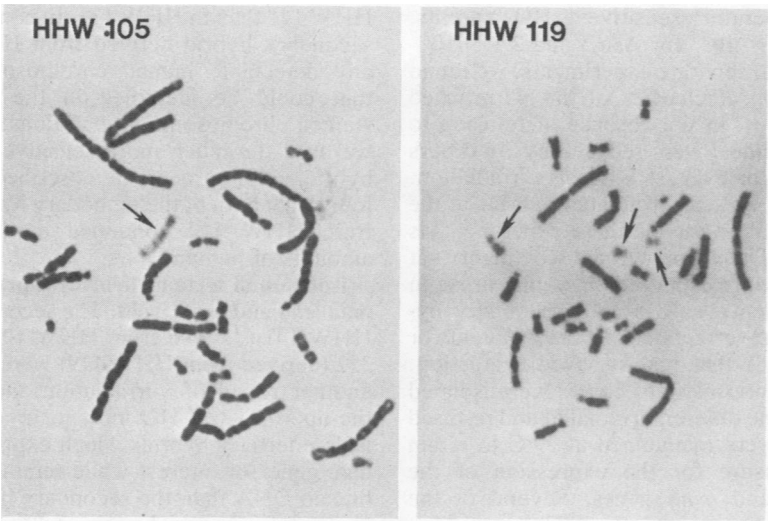

FIG. 1. Differentially stained metaphase chromosome preparations from HHW 105 and HHW 119. The alkaline-Giemsa staining procedure used to distinguish human from rodent chromosomes is described in the text. Human chromosomes, which are indicated by the arrows, stain light blue and appear lighter in these photographs than the Chinese hamster chromosomes, which stain purple.

was discussed above. HHW 105 expresses the human *leuS* gene, which encodes leucyl-tRNA synthetase, whereas HHW 119 expresses the human *asnS* gene, which encodes asparaginyl-tRNA synthetase. Photographs of metaphase chromosome preparations from these cell lines, which have been stained by using the alkaline-Giemsa procedure to distinguish human from rodent chromosomes, are shown in Fig. 1. HHW 105 contains only human chromosome 5, whereas HHW 119 contains human chromosomes 12, 18, and 19. The *asnS* gene has been localized to chromosome 18 (6). By measuring the length of the human chromosomes in these photographs and comparing that to the length of all the chromosomes combined (human and hamster), we can estimate that the human DNA represents approximately 3% of the total DNA in the hybrids.

Replicate cultures of each of these cell lines, containing 1.5×10^6 cells, were exposed to 2, 4, 8, or 10 krads of γ-irradiation from a ^{137}Cs source to fragment the chromosomes, as described above. Immediately after irradiation, 2×10^6 unirradiated cells of the temperature-sensitive CHO leucyl-tRNA synthetase mutant UCW 56 were added to the irradiated cultures of HHW 105, and the same number of cells of the temperature-sensitive CHO asparaginyl-tRNA synthetase mutant Asn-5 was added to the irradiated cultures of HHW 119. After incubation at 33°C for 5 h, the cells in the various cultures were treated with polyethylene glycol-1,000 to induce cell fusion and then returned to 33°C in normal medium. After 3 days, the fused cells were dispersed, diluted into fresh medium and transferred into 100-mm dishes and then transferred to 39°C to select secondary hybrids in which human DNA fragments containing the *leuS* or *asnS* had become integrated into the recipient genomes and were expressed. The results of these experiments are summarized in Table 2. At every dose of irradiation used for both hybrids, the number of temperature-resistant colonies that arose after refusion was at least fivefold above the reversion frequencies

TABLE 2. Recovery of temperature-resistant secondary hybrids after γ-irradiation and refusion of primary hybrids[a]

Cell line irradiated	Radiation dose (krads)	No. of temperature-resistant colonies recovered
HHW 105	2	19
	4	9
	6	3
	8	2
	10	5
HHW 119	2	25
	4	9
	6	4
	8	5
	10	1

[a] Experiments were performed as described in the text.

for the temperature-sensitive CHO parents, which are 2.4×10^{-7} for Asn-5 and 2.1×10^{-7} for UCW 56. In separate experiments, we found that the plating efficiencies of the γ-irradiated hybrid cell lines, in the absence of refusion to the CHO cell lines, were reduced by ~6 orders of magnitude for every 1 krad of γ-irradiation. Thus, survival of the γ-irradiated hybrids at the doses of irradiation used in these experiments is insignificant. Therefore, we felt confident that the temperature-resistant colonies that arose in these experiments were indeed secondary hybrids and not revertants of the CHO mutants or primary hybrids that had survived irradiation. Two temperature-resistant clones were isolated from each of the different irradiated and re-fused cultures and were maintained at 39°C to retain selective pressure for the expression of the human *leuS* and *asnS* genes. Several of the temperature-resistant colonies did not survive cloning, possibly because their temperature resistance phenotypes were unstable. From each of the primary hybrids, the two secondary hybrids that were recovered from cultures exposed to the highest doses of irradiation and that were still stable at 39°C were examined further.

Metaphase chromosome preparations from these four secondary hybrids were stained by the alkaline-Giemsa procedure to determine whether any human chromosomal material could be detected. The two secondary hybrids, HHW 133 and HHW 141, which are derived from HHW 105, each contained a fragment of human chromosome 5 translocated onto a hamster chromosome (Fig. 2). The size of the human chromosome fragment is considerably smaller in HHW 141 than in HHW 133. In contrast, neither secondary hybrid derived from HHW 119 had any detectable human chromosomal material that could be identified in the differentially stained chromosome preparations. However, if we used the much more sensitive nucleic acid hybridization procedure described below, we found that both of the secondary hybrids derived from HHW 119 contained easily detectable amounts of human DNA.

Isolation of tertiary hybrids expressing the human *leuS* and *asnS* genes. The secondary hybrids HHW 141 (derived from HHW 105) and HHW 227 (derived from HHW 119) were subjected to another round of γ-irradiation and refusion to the appropriate CHO mutant, in an attempt to isolate tertiary hybrids which expressed the human genes of interest while retaining even less human DNA than the secondary hybrids. Once again, colonies which arose at 39°C after irradiation and refusion were isolated and maintained at 39°C. Four tertiary hybrids derived from HHW 141 were examined by using the differential chromosome staining procedure. None had any cytologically detectable human DNA. Tertiary hybrids derived from HHW 227 were not examined, because no human DNA was cytologically detectable HHW 227.

Quantitation of the amount of human DNA in primary, secondary, and tertiary heat-resistant hybrids. To verify that the tertiary hybrids described above contained some human DNA, we needed a more sensitive test than the relatively crude differential staining of metaphase chromosomes. Nucleic acid hybridization techniques provided a rapid and simple means to make

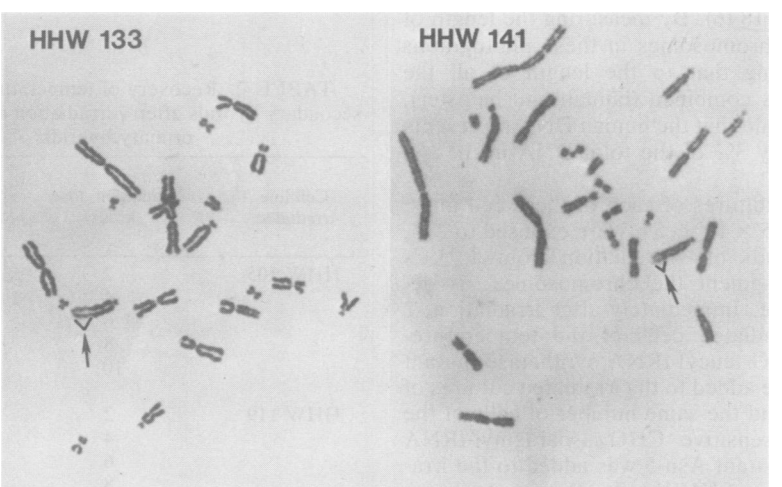

FIG. 2. Differentially stained metaphase chromosome preparations from secondary hybrids HHW 133 and HHW 141. The single fragment of human chromosome 5 translocated onto a Chinese hamster chromosome in each cell line is indicated by an arrow.

these determinations and enabled us to accurately quantitate the amount of human DNA in the whole series of hybrids from primary through tertiary. To perform these analyses, we used a slight modification of the dot-blot procedure described by Thomas (26). For these experiments, we needed a probe that would be specific for detecting small amounts of human DNA in a background of a large excess of Chinese hamster DNA. It had previously been demonstrated that human middle repetitive DNA sequences, which are distributed throughout the human genome every 2 to 3 kilobases, do not cross-hybridize to any significant extent with the analogous sequences from the Chinese hamster genome (14, 15, 23). Therefore, human middle repetitive sequences appeared to be the distinguishing probe we needed.

Partially sheared human and Chinese hamster DNAs were mixed in different proportions, and various quantities of these mixtures were spotted and fixed onto nitrocellulose filters as described above. These dot blots were hybridized to total genomic human DNA that had been labeled with ^{32}P by nick translation and then washed and exposed to X-ray film as also described above. In preparations of total nick-translated human DNA, the only sequences which are present in a high enough copy number to give a detectable hybridization signal are the highly repeated sequences (15, 16). An autoradiograph of one such blot is shown in Fig. 3. Visual inspection of the autoradiograph demonstrates that the amount of radioactive probe hybridized to the various spots is directly related to the amount of human DNA present in the sample. It can be seen that under the conditions used in these experiments, there is no detectable hybridization signal in the spots containing only Chinese hamster DNA. It should be noted that in the row of samples containing only Chinese hamster DNA (0% human DNA), three times as much DNA was spotted (150, 30, and 3 ng) as in the rows containing mixtures of human and Chinese hamster DNAs. To quantitate these experiments, three replicate blotting experiments of the type just described were performed, and autoradiograms of the lanes of DNA containing 50 ng of the various mixtures were scanned with a densitometer. The areas under the peaks on the densitometer tracings corresponding to each spot were determined and plotted against the percent human DNA in the sample. Such a plot is shown in Fig. 4. As can be seen, there is a direct linear relationship between the percent human DNA in the spots and the area of the peaks on the tracings in the range from 0 to 5% human DNA. At the higher percentages of human DNA, the linearity decreases because of complete exposure of the film to bound probe. However, simply by varying the quantities of DNA spotted and the exposure time of the film to the blots, one can easily achieve linearity over the entire range of mixtures used.

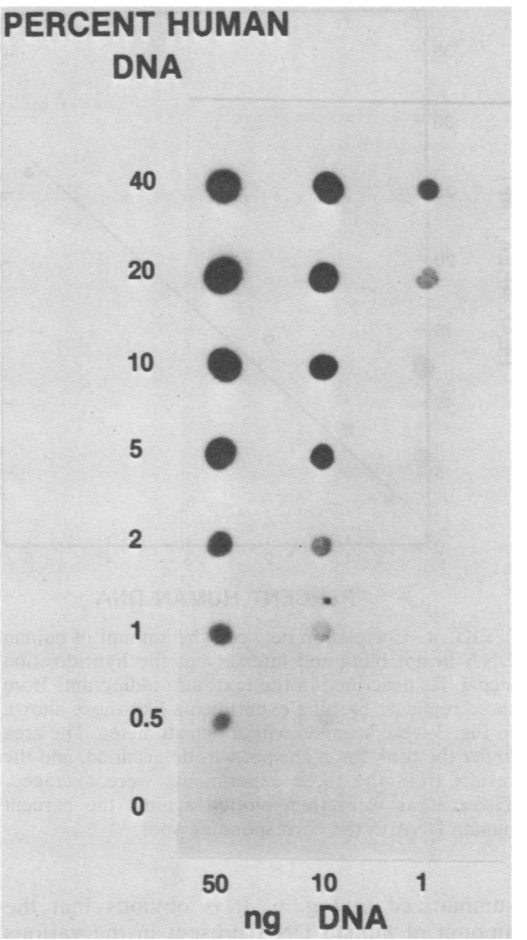

FIG. 3. Autoradiogram of dot-blot hybridization of ^{32}P-labeled total human DNA to mixtures of human and Chinese hamster DNA. Experimental details are described in the text. Human and hamster DNAs were mixed in the indicated proportions and then various amounts of DNA were attached to nitrocellulose and hybridized to labeled human DNA. The amounts of Chinese hamster DNA spotted in the row containing no human DNA were actually three times the amounts indicated for all the other samples containing various amounts of human DNA.

These same blotting experiments were carried out on DNAs from two series of primary to tertiary hybrids: one series from HHW 105 and one series from HHW 119. An autoradiograph from one such experiment is shown in Fig. 5, and the lineage and derivation of the cell lines is

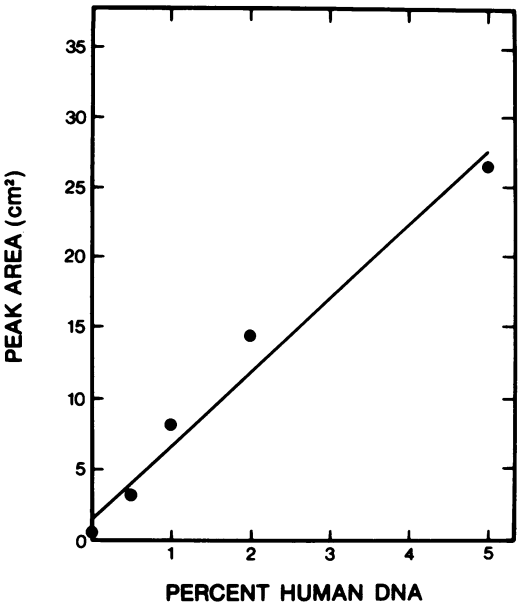

FIG. 4. Correlation between the amount of human DNA in dot blots and intensity of the hybridization signal. As described in the text, autoradiograms from three replicate blotting experiments like those shown in Fig. 3 were scanned with a densitometer. The area under the peak for each spot was determined, and the results from the three experiments were averaged. These areas were then plotted against the percent human DNA in the corresponding spot.

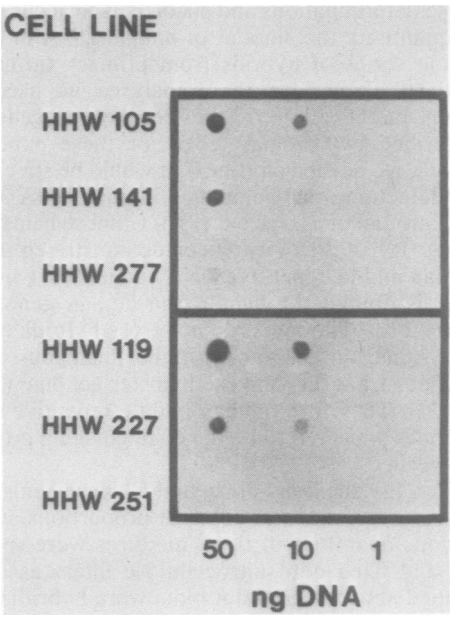

FIG. 5. Autoradiogram of dot-blot hybridization of ^{32}P-labeled total human DNA to DNA extracted from primary, secondary, and tertiary hybrids. The experiments were performed as described in the text.

summarized in Fig. 6. It is obvious that the amount of human DNA present in the various cell lines decreases dramatically in going from primary to tertiary hybrids. Autoradiographs of three replicate blots like those shown in Fig. 5 were scanned with a densitometer, along with three replicate control blots containing mixtures of human and hamster DNAs. The peak areas of the spots on the tracings from the hybrid cell line autoradiograms were then compared to the peak areas of the spots of control blots with different amounts of the mixtures of human and hamster DNAs, which enabled us to determine the percentage of human DNA in the hybrid cells. The results of these analyses are shown in Table 3. In the series of hybrids expressing the human leucyl-tRNA synthetase, the amount of DNA that is human decreases from 1.12% in HHW 105 to 0.07% in the tertiary hybrid HHW 277. In the series of hybrids expressing the human asparaginyl-tRNA synthetase, the amount of human DNA decreases from 1.61% in HHW 119 to ~0.02% in the tertiary hybrid HHW 251. In the photograph of the autoradiogram shown in Fig. 5, the hybridization signals over the spots of HHW 251 DNA are not even visible. However, when these blots were exposed to film longer, the spot of HHW 251 DNA with 50 ng of DNA gave a signal that was detectable, whereas a spot containing three times as much Chinese hamster DNA still gave no signal. The peak area of the spot with 50 ng of HHW 251 DNA was equivalent to the peak area of a spot containing 1 ng of DNA that was 1% human and 99% Chinese hamster. Thus, we estimate that in HHW 251, ~1/50 of 1%, or 0.02%, of the DNA is human. The amount of human DNA in the other cell lines was calculated in an analogous fashion.

In view of the very small amount of human DNA present in HHW 251, which is at the lower

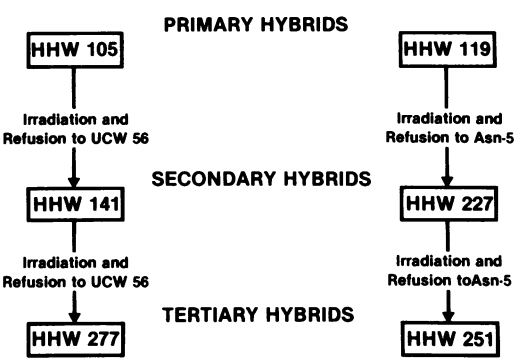

FIG. 6. Lineage of primary, secondary, and tertiary hybrids.

TABLE 3. Relative amounts of human DNA in primary, secondary, and tertiary hybrids[a]

Cell line	% human DNA
Hybrids derived from UCW 56	
HHW 105 (primary)	1.12
HHW 141 (secondary)	0.58
HHW 277 (tertiary)	0.07
Hybrids derived from Asn-5	
HHW 119 (primary)	1.61
HHW 227 (secondary)	0.27
HHW 251 (tertiary)	0.02

[a] The percentage of DNA in the various cell lines that is derived from the human genome was calculated as described in the text.

limit of detectability in the dot-blot hybridization procedure, we screened for the presence of human DNA after restriction endonuclease digestion and agarose gel electrophoresis of DNA extracted from this cell line. High-molecular-weight DNA extracted from HHW 251 and Chinese hamster cells was digested to completion with *Hin*dIII or *Eco*RI, electrophoresed, transferred to nitrocellulose, and blotted, using nick-translated total human DNA as a probe. Autoradiograms of these blots are shown in Fig. 7. Several bands that hybridized to the human DNA probe were apparent in *Eco*RI- or *Hin*dIII-digested Chinese hamster DNA. These bands correspond to repeated, apparently homologous *Eco*RI or *Hin*dIII fragments, which were apparent as unique bands on ethidium bromide-stained gels and which most likely contain hamster middle repetitive sequences. Thus, even though there is little cross-hybridization between hamster and human middle repetitive sequences, the high concentration of the hamster sequences in these specific bands results in detectable hybridization signals. However, in the *Hin*dIII-digested HHW 251 DNA, there were at least four unique bands that hybridized to the human probe and that were not present in the digested Chinese hamster DNA. The largest of these unique bands in HHW 251 (~40 kilobases) gave an especially strong hybridization signal relative to the other bands. That this band in HHW 251 was not due to incompletely digested DNA that remained near the top of the gel during electrophoresis is obvious from the blots of *Eco*RI-digested DNA. Digestion of HHW 251 DNA with *Eco*RI resulted in the production of two small fragments, both under 1 kilobase, that gave intense hybridization signals, as well as at least two other fragments which gave less intense signals and which are not present in Chinese hamster DNA. Whether the intense hybridization signals over certain of the human DNA-containing fragments in HHW 251 are due to their containing many copies per fragment of a middle repetitive sequence, or to the fact that these fragments have for some reason been amplified many-fold, remains to be determined. In any case, it is obvious from these experiments that HHW 251 does, in fact, contain human DNA sequences and that the total amount of human DNA present is probably under 100 kilobases. Similar experiments with HHW 277 also confirmed the presence of human specific DNA fragments (data not shown). As expected

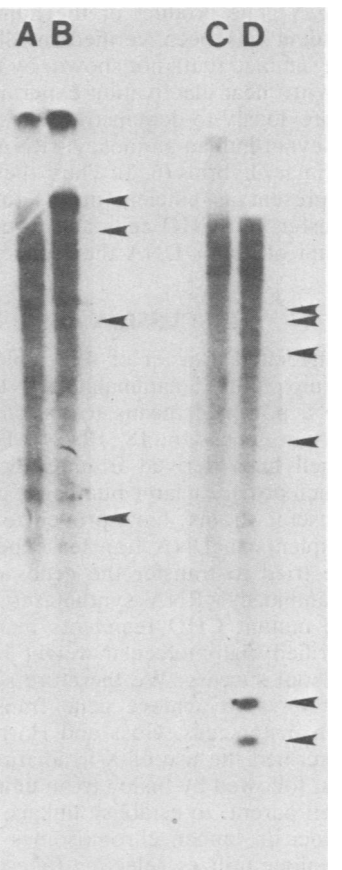

FIG. 7. Southern blot analysis of restriction endonuclease-digested Chinese hamster and HHW 251 DNA by using ^{32}P-labeled total human DNA probe. The experiments were performed as described in the text. Lanes: (A) Chinese hamster DNA digested with *Hin*dIII; (B) HHW 251 digested with *Hin*dIII, (C) Chinese hamster DNA digested with *Eco*RI; (D) HHW 251 DNA digested with *Eco*RI. After digestion, electrophoresis, and transfer to nitrocellulose, the filters were hybridized to ^{32}P-labeled total human DNA. Restriction fragments hybridizing to the human probe that are unique to HHW 251 are indicated by arrows.

from the dot-blot experiments, there are many (15 to 20) human specific fragments in *Hin*dIII- and *Eco*RI-digested DNA from this cell line.

Other secondary and tertiary hybrids derived from HHW 105 and HHW 119 have been examined in a similar manner, and all contained detectable amounts of human DNA. HHW 277 and HHW 251 represent the cell lines with the least amount of human DNA that still express the human *leuS* and *asnS* genes, respectively. In view of the very low reversion frequencies of the mutant CHO Asn-5 and UCW 56 cell lines, the possibility that some of these tertiary hybrids are revertants that have by chance also integrated nonrelevant human DNA sequences is extremely remote. In addition, the presence of the human *leuS* gene product or the human *asnS* gene product has been verified in all tertiary hybrids examined (data not shown) by using the same in vitro heat inactivation experiments described previously to demonstrate the presence of the relevant human aminoacyl-tRNA synthetase in primary hybrids (6, 8). Thus, these procedures represent an efficient means to achieve gene transfer into CHO cells and to quantitate the amount of donor DNA the recipients have retained.

DISCUSSION

DNA-mediated transfer of selectable genetic markers into cultured mammalian cells has proven to be a powerful means to enrich for and clone certain genes (16, 18, 19, 24). However, mutant cell lines derived from Chinese hamsters, which provide a large number of potentially very useful strains, have proven to be very poor recipients in DNA transfer experiments. We have tried to transfer the genes encoding various aminoacyl-tRNA synthetases into appropriate mutant CHO recipients many times using purified high-molecular-weight DNA, always without success. We therefore sought alternative ways to achieve gene transfer into Chinese hamster cells. Goss and Harris previously described the use of X-irradiation of human cells, followed by fusion to an unirradiated rodent cell parent, to establish linkage maps of specific loci on human chromosomes (11, 12). This technique utilizes selecting for expression of a specific gene on a chromosome of the irradiated human cell line in the fused cells, followed by analysis for other, unselected linked markers on the same chromosome which had been fragmented by the irradiation process. Thus, it seemed reasonable that a similar approach could be used to transfer very small chromosome fragments with specific genes into CHO cells by using very high levels of irradiation and multiple rounds or irradiation, refusion, and selection for the gene(s) of interest.

The results presented in this report demonstrate that the procedures we used represent a simple and efficient means of effecting transfer of specific human genes into mutant Chinese hamster cells with the cotransfer of minimal amounts of extraneous human DNA sequences. Selection for the transfer and expression of human genes in these experiments, coupled with nucleic acid hybridization techniques, affords a simple and convenient way to monitor the quantity of human DNA present in the various secondary and tertiary hybrids. As discussed below, the ability to distinguish hamster from human DNA also provides a powerful means to enrich for and clone the specific human genes of interest. The first objective in experiments to clone the genes encoding leucyl- and asparaginyl-tRNA synthetase was to construct cell lines which expressed the human genes of interest, yet contained very small amounts of extraneous human DNA. For this reason, our first rounds of irradiation and refusion were performed with human-CHO hybrids which had one or a few chromosomes. In these primary hybrids, only a small portion of the human genome was present, which represented a significant initial enrichment for the human *leuS* and *asnS* genes. Exactly the same procedures could be used to transfer any human gene into mutant CHO cells, providing the transferred gene would provide the recipients with a selectable phenotype. If interspecific hybrids of the type we started with were not available for other selectable markers, human cells could be used as the irradiated parent in the first round of irradiation and refusion. The only drawback would be that more rounds of irradiation and refusion would probably be required to significantly reduce the amount of human DNA retained in the subsequent hybrids.

The next step in attempts to clone the *leuS* and *asnS* genes will be to prepare complete genomic DNA libraries from tertiary hybrids in a λ phage vector. These libraries will be screened by using the Benton and Davis plaque hybridization technique with total nick-translated human DNA as a probe (4). As described by Gusella et al., this procedure distinguishes phage recombinants containing human DNA from recombinants containing only rodent DNA, again by virtue of differential hybridization of human and rodent middle repetitive DNA sequences (14, 15). Since middle repetitive sequences are dispersed in an apparently random manner every 2 to 3 kilobases through the human genome, virtually every phage recombinant (with inserts of ~20 kilobases) that contains unique, single-copy human sequences will most likely also contain at least one middle repetitive sequence and will be identified in the screening process (23). In fact, a random sampling of a λ phage human genomic

DNA library confirmed that approximately 99% of the phage recombinants contained sequences that hybridized to a human middle repetitive DNA probe (17). A complete library of all the DNA sequences present in a mammalian cell would represent ~800,000 λ recombinants with average size inserts of ~20 kilobases (7). In the tertiary hybrid HHW 251, for example, about 0.02% of the DNA is derived from the human genome. Thus, a complete library of all the human DNA in this cell line should be contained in ~160 λ recombinants. This sublibrary of 160 λ recombinants containing all the human DNA sequences present in HHW 251, including those encoding asparaginyl-tRNA synthetase, would represent a ~5,000-fold purification of the human *asnS* gene without having used any specific probes. The enrichment of the human *leuS* gene in HHW 277 is somewhat less, because this cell line contains about 3.5 times as much human DNA as HHW 251. However, we have recently isolated derivatives of HHW 277 that were selected as being resistant to 4-azaleucine, an inhibitor of leucyl-tRNA synthetase, which have apparently amplified the human *leuS* gene (S. Dana and J. Wasmuth, unpublished data). In one such cell line, the specific activity of the human leucyl-tRNA synthetase is increased ~10-fold, and a subset of the total human DNA sequences present in HHW 277 has been amplified by approximately the same amount (Dana and Wasmuth, unpublished data). A genomic DNA library from this cell line should be significantly enriched for human *leuS* sequences relative to the other, nonrelevant human sequences. Human DNA-containing λ recombinants isolated from libraries prepared from this cell line and HHW 251 will be screened with a variety of different techniques to identify those containing human *leuS* and *asnS* specific sequences. Hopefully, these procedures will enable us to isolate the complete genomic sequences encoding leucyl- and asparaginyl-tRNA synthetase.

While this work was in progress, two laboratories reported using an approach similar to the one outlined here to clone specific human DNA sequences after transfection of rodent cells with human DNA (16, 24). Thus, although some technical problems may be encountered, we believe the cell lines we have isolated and the approaches we plan to use will enable us to isolate human sequences encoding asparaginyl- and leucyl-tRNA synthetase. Using these human sequences, which will almost certainly cross-hybridize to the corresponding sequences from Chinese hamsters, we will isolate clones containing the Chinese hamster *asnS* and *leuS* sequences from genomic DNA and cDNA libraries. As discussed previously, these probes would provide valuable tools to study, in detail, two mammalian genes that are among the most amenable to classic genetic analyses and mutational studies in cultured somatic cells.

ACKNOWLEDGMENTS

We are grateful to Linda Vock and Douglas Skarecky for expert technical assistance and Darlene Wise for excellent help in manuscript preparation. We thank Leon Carlock for help, advice, and discussions during the course of this work.

This work was supported by Public Health Service grant GM 25339 from the National Institute of General Medical Sciences and grant PCM-8103600 from the Genetic Biology Program of the National Science Foundation. S.D. and R.E.C. were supported in part by predoctoral training grant GM 07134 from the National Institute of General Medical Science.

LITERATURE CITED

1. **Adair, G. M., L. H. Thompson, and S. Fong.** 1979. [^{3}H]amino acid selection of aminoacyl-tRNA synthetase mutants of CHO cells: evidence of homo- vs. hemizygosity at specific loci. Somatic Cell Genet. **5**:329–344.
2. **Andrulis, I. L., C. S. Chiang, S. M. Arfin, T. A. Miner, and G. W. Hatfield.** 1978. Biochemical characterization of a mutant asparaginyl-tRNA synthetase from Chinese hamster ovary cells. J. Biol. Chem. **253**:58–62.
3. **Ashman, C. R.** 1978. Mutations in the structural genes of CHO cell histidyl-, valyl-, and leucyl-tRNA synthetases. Somatic Cell Genet. **4**:299–312.
4. **Benton, W., and R. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180–182.
5. **Boersma, D., S. McGill, J. Mollenkamp, and D. J. Roufa.** 1979. Emetine resistance in Chinese hamster cells. Analysis of ribosomal proteins prepared from mutant cells. J. Biol. Chem. **254**:559–567.
6. **Cirullo, R. E., F. X. Arredondo-Vega, M. Smith, and J. J. Wasmuth.** 1983. Isolation and characterization of interspecific heat-resistant hybrids between a temperature-sensitive Chinese hamster cell asparaginyl-tRNA synthetase mutant and normal human leukocytes: assignment of the human *asnS* gene to chromosome 18. Somatic Cell Genet. **9**:215–233.
7. **Clarke, L., and J. Carbon.** 1976. A colony bank containing synthetic Col E1 hybrids plasmids representative of the entire *E. coli* genome. Cell **9**:91–99.
8. **Dana, S., and J. J. Wasmuth.** 1982. Linkage of the *leuS*, *emtB* and *chr* genes on chromosome 5 in humans and expression of human genes encoding protein synthetic components in human-Chinese hamster hybrids. Somatic Cell Genet. **8**:245–264.
9. **Dana, S., and J. J. Wasmuth.** 1982. Selective linkage disruption in human-Chinese hamster cell hybrids: deletion mapping of the *leuS*, *hexB*, *emtB*, and *chr* genes on human chromosome 5. Mol. Cell. Biol. **2**:1220–1228.
10. **Friend, K., S. Chen, and F. Ruddle.** 1976. Differential staining of interspecific chromosomes in somatic cell hybrids by alkaline Giemsa stain. Somatic Cell Genet. **2**:183–188.
11. **Goss, S. J., and H. Harris.** 1977. Gene transfer by means of cell fusion. I. Statistical mapping of the human X-chromosome by analysis of radiation-induced gene segregation. J. Cell Sci. **25**:17–37.
12. **Goss, S. J., and H. Harris.** 1977. Gene transfer by means of cell fusion. II. The mapping of 8 loci on human chromosome 1 by statistical analysis of gene assortment in somatic cell hybrids. J. Cell Sci. **25**:39–57.
13. **Gupta, R. S., and L. Siminovitch.** 1976. The isolation and characterization of somatic cell mutants resistant to the protein synthesis inhibitor—emetine. Cell **9**:213–219.
14. **Gusella, J. F., C. Keys, A. Varsanyi-Breiner, F.-T. Kao, C. Jones, T. Puck, and D. Housman.** 1980. Isolation and localization of DNA segments from specific human chromosomes. Proc. Natl. Acad. Sci. U.S.A. **77**:2829–2833.

15. **Gusella, J., A. Varsanyi-Breiner, F.-T. Kao, C. Jones, T. T. Puck, C. Keys, S. Orkin, and D. Housman.** 1979. Precise localization of human β-globin gene complex on chromosome 11. Proc. Natl. Acad. Sci. U.S.A. **76**:5239–5243.
16. **Jolly, D. J., A. C. Esty, H. U. Bernard, and T. Friedman.** 1982. Isolation of a genomic clone partially encoding human hypoxanthine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U.S.A. **79**:5038–5041.
17. **Kao, F.-T., J. A. Hartz, M. L. Law, and J. N. Davidson.** 1982. Isolation and chromosomal localization of unique DNA sequences from a human genomic library. Proc. Natl. Acad. Sci. U.S.A. **79**:865–869.
18. **Lowy, I., A. Pellicer, J. F. Jackson, G.-K. Sim, S. Silverstein, and R. Axel.** 1980. Isolation of transforming DNA: cloning the hamster aprt gene. Cell **22**:817–823.
19. **Lund, T., F. G. Grosveld, and R. A. Flavell.** 1982. Isolation of transforming DNA by cosmid rescue. Proc. Natl. Acad. Sci. U.S.A. **79**:520–524.
20. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **75**:1184–1188.
21. **Pellicer, A., M. Wigler, R. Axel, and S. Silverstein.** 1978. The transfer and stable integration of the HSV thymidine kinase gene into mouse cells. Cell **14**:133–141.
22. **Rodgers, A.** 1979. Detection of small amounts of human DNA in human-rodent hybrids. J. Cell Sci. **38**:391–403.
23. **Schmid, C. W., and P. L. Deininger.** 1975. Sequence organization of the human genome. Cell **6**:345–358.
24. **Shih, C., and R. A. Weinberg.** 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29**:161–169.
25. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
26. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
27. **Thompson, L. H., J. L. Harkins, and C. P. Stanners.** 1973. A mammalian cell mutant with a temperature-sensitive leucyl-transfer RNA synthetase. Proc. Natl. Acad. Sci. U.S.A. **70**:3094–3098.
28. **Thompson, L. H., D. J. Lofgren, and G. M. Adair.** 1977. CHO cell mutants for arginyl-, asparagyl-, glutaminyl-, histidyl- and methionyl-transfer RNA synthetases: identification and initial characterization. Cell **11**:156–168.
29. **Thompson, L. H., C. P. Stanners, and L. Siminovitch.** 1975. Selection by [^{3}H]amino acids of CHO-cell mutants with altered leucyl- and asparagyl-transfer RNA synthetases. Somatic Cell Genet. **1**:187–208.
30. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
31. **Wasmuth, J. J., and C. T. Caskey.** 1976. Selection of temperature-sensitive CHL asparaginyl-tRNA synthetase mutants using the toxic lysine analog, S-2-aminoethyl-L-cysteine. Cell **9**:655–662.
32. **Wasmuth, J. J., and L.-Y. Chu.** 1980. 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. J. Cell Biol. **87**:697–702.
33. **Wasmuth, J. J., J. M. Hill, and L. S. Vock.** 1980. Biochemical and genetic evidence for a new class of emetine resistant Chinese hamster cells with alterations in the protein biosynthetic machinery. Somatic Cell Genet. **6**:495–516.
34. **Wasmuth, J. J., J. M. Hill, and L. S. Vock.** 1981. Identification and characterization of a third complementation group of emetine-resistant Chinese hamster cell mutants. Mol. Cell. Biol. **1**:58–65.
35. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single copy eucaryotic genes using total cellular DNA as donor. Cell **14**:725–731.
36. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.