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Amplification and Expression of Heterologous Ornithine Decarboxylase in Chinese Hamster Cells

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We have developed an amplifiable mammalian expression vector based on the enzyme ornithine decarboxylase (ODC). We show greater than 700-fold amplification of this vector in ODC-deficient Chinese hamster ovary cells. A passive coamplified marker, dihydrofolate reductase (*dhfr*), was amplified and overexpressed 1,000-fold. This ODC vector was a dominant marker in a variety of cell types and displayed at least 300-fold amplification in wild-type Chinese hamster ovary cells.

Selectable genetic markers have allowed the introduction of cloned DNA into animal cells, allowing study of eucaryotic gene regulation and function. Amplification vectors based on dihydrofolate reductase (*dhfr*) can be used to obtain high levels of expression of a variety of heterologous genes, since genes linked to it can be coamplified and thus overexpressed (14, 15, 18, 34). However, amplification of *dhfr*-based vectors is generally limited to DHFR-deficient Chinese hamster ovary (CHO) cells, and dominant vectors based on *dhfr* have reduced ability to amplify (20, 27). An amplifiable vector based on adenosine deaminase (ADA) (13) is more easily used in wild-type cells. However, the selections required for the ADA markers are complicated, and some cell types do not grow well under selective conditions (13).

To independently amplify more than one polypeptide in the same cell requires the use of more than one amplifiable marker. There are currently a limited number of amplifiable markers. We have developed a simple dominant amplifiable vector system, based on ornithine decarboxylase (ODC; EC 4.1.1.17), the initial enzyme in the synthesis of polyamines by animal cells and an essential enzyme for cellular growth (32).

MATERIALS AND METHODS

Derivation of the ODC expression vector. pdhOD1 (Fig. 1) contains the *Hin*dIII-*Pvu*I fragment of pSVMdhfr (16) that includes the *dhfr* expression unit and the 626-base-pair (bp) *Eco*RI-*Pvu*I fragment of pBR322. The *Hin*dIII-*Xho*I fragment containing the simian virus 40 (SV40) early promoter and the *Xho*I-*Pvu*I fragment containing the SV40 late polyadenylation sequences and flanking pBR322 sequences were obtained from pCD-X (21). The *Taq*I-*Pvu*II fragment of pOD20.7 (11, 17), containing the ODC cDNA adapted with *Xho*I linkers is between the SV40 early promoter and SV40 polyadenylation signals of the pCD-X-derived sequences so that ODC is expressed from the SV40 early promoter.

Cell culture. ODC-deficient (ODC^-) CHO cells (clone C55.7) were grown as described (31). Other cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Cells were transfected with calcium phosphate-precipitated DNA as described (9, 10) with the following modifications. CHO cells in 100-mm dishes were exposed to 20 μg of precipitated DNA for 3 h and then shocked for 3 min in 15% glycerol. African green monkey kidney (American Type Culture Collection) CV1 cells were treated with the precipitated DNA for 16 h and were not treated with glycerol. Mouse fibroblast NIH 3T3 cells were exposed to the precipitated DNA for 16 h and then shocked for 3 min in 15% glycerol. C127 mouse mammary cells (7) were transfected by the method of Chen and Okayama (4).

Selection for DFMO-resistant cells. Cells were selected for resistance to serially increasing levels of difluoromethylornithine (DFMO), a suicide-substrate inhibitor of ODC, as follows: 10^5 cells were plated per 100-mm dish into medium containing DFMO, and the plates were refed with fresh medium every 5 days until a resistant population emerged.

RNA analysis. Total and polyadenylated [poly(A)$^+$] RNAs were prepared as described previously (5). For Northern (RNA blot) analysis, RNA was electrophoresed in 1.5% agarose gels containing formaldehyde (23) and transferred to nitrocellulose filters (28). R-dot hybridization analysis was performed as described previously (33). The ODC-specific probe used was nick-translated pOD20.7 (11, 17), a plasmid containing mouse ODC cDNA in pBR322. RNA filters were prehybridized in hybridization buffer (50% formamide, 5× SSC [0.45 M NaCl plus sodium citrate], 5× Denhardt [6] solution, 200 μg of yeast RNA per ml, 1% Sarkosyl) for 2 h and hybridized in the same buffer with the ODC probe at 45°C overnight. Filters were washed four times in 2× SSC–0.1% sodium dodecyl sulfate (SDS) for 10 min at room temperature and two times in 0.1× SSC–0.1% SDS for 30 min at 50°C. The vector-specific mouse *dhfr* probe was an SP6 RNA polymerase-synthesized (19) probe homologous to the 714-bp *Bgl*II-*Hpa*I fragment of pdhOD1 containing the small t splice sequences. The synthesis of RNA probe, prehybridization, hybridization, and washing of RNA filters were carried out as described by Zinn et al. (35) except that 170 μCi of [α-^{32}P]UTP (800 Ci/mmol, 20 mCi/ml) was used in 20 μl of the transcription reaction, 7% dextran sulfate was in the hybridization buffer, and the second washing of filters was done in 0.1× SSC–0.1% SDS.

DNA analysis. High-molecular-weight DNA was isolated as described (1). For Southern analysis, digested DNA was electrophoresed in 0.8 or 0.2% (8) agarose gels and transferred to nitrocellulose filters (29). For D-dot hybridization analysis, DNA was denatured with 1/10 volume of 3 M NaOH for 10 min at room temperature, neutralized with an equal volume of 2 M ammonium acetate, and applied to nitrocellulose filters (12). The *Hin*dIII-*Xho*I fragment of pdhOD1 containing the SV40 origin (*ori*) sequences was nick

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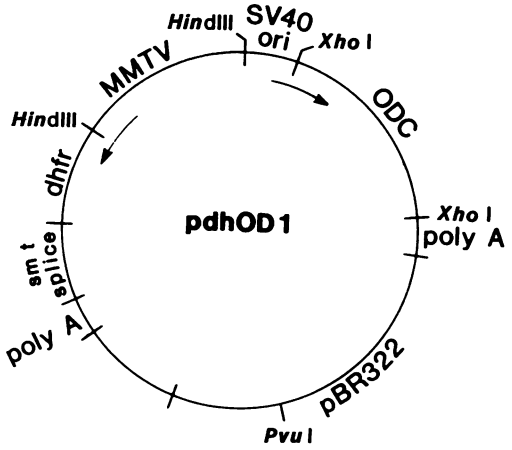

FIG. 1. Structure of plasmid pdhOD1. sm t, SV40 small t antigen gene from SV40; poly A, polyadenylation site; MMTV, mouse mammary tumor virus long terminal repeat.

translated. When DNA filters were probed with this nick-translated probe, the prehybridization, hybridization, and washing steps were carried out as described for RNA analysis with the nick-translated probe except that hybridizations were carried out at 42°C in the presence of 5% dextran sulfate. When DNA filters were probed with the *dhfr*-specific RNA probe, prehybridization and hybridization steps were performed as described for RNA blots probed with this probe except that they were done at 50°C.

The amount of pdhOD1-specific DNA per cell was calculated from D-dot hybridization analysis of cellular DNA, assuming that the average cell contains 6 pg of DNA (28).

RESULTS

Expression of heterologous ODC in ODC-deficient hamster cells. To develop an amplifiable vector based on ODC, we constructed a vector (pdhOD1) that contains mouse ODC cDNA expressed from the SV40 early promoter and, as a marker of coamplification, the mouse *dhfr* cDNA expressed from the mouse mammary tumor virus long terminal repeat (Fig. 1).

ODC-deficient (ODC^-) mutants of CHO cells have an absolute requirement for the ODC product putrescine. ODC^- CHO cells were transfected either with control plasmid lacking the ODC cDNA or with pdhOD1, and ODC-containing (ODC^+) transfectants were obtained by selection in medium lacking putrescine. Control plasmid conferred the ODC^+ phenotype on ODC^- cells at a frequency of less than 10^{-5}. pdhOD1 transformed ODC^- cells to the ODC^+ phenotype at a frequency of 4×10^{-3}.

Northern blot analysis showed that a pooled population of ODC^+ transfectants expressed the vector-specific ODC and *dhfr* genes (Fig. 2A and B, lanes 1 to 4). The ODC probe hybridized both to the endogenous CHO ODC mRNA and to a vector-specific ODC mRNA. A vector-specific *dhfr* probe, made from the SV40 small t splice sequences contained within the *dhfr* transcription unit, hybridized only to vector-specific mRNAs. We do not know why there were multiple vector-specific *dhfr* mRNAs.

Amplification of heterologous ODC in ODC^- CHO cells. Cells were transfected with pdhOD1, and about 400 of the ODC^+ transfectants were pooled. This pooled population was selected for resistance to serially increasing concentrations (from 0.1 to 15 mM) of the ODC inhibitor DFMO for 7 weeks. Northern blot (Fig. 2) and R-dot (data not shown) hybridization analyses showed that signal from the vector-specific ODC mRNA in the highly drug-resistant population was 400-fold greater than the signal from the endogenous hamster- and vector-specific ODC mRNAs of the initial population. Quantitation of the amount of vector-specific ODC mRNA in the initial population was not possible because a significant amount of signal was due to hybridization of the ODC probe to the hamster ODC mRNA. Therefore, the vector-specific ODC mRNA was overexpressed at least 400-fold in the highly drug-resistant population.

To determine the underlying mechanism of overproduction of ODC mRNA, total cellular DNA was analyzed by Southern blot (Fig. 3) and D-dot (data not shown) hybridization analyses with either an SV40 *ori* or a vector-specific *dhfr* (data not shown) probe. Digestion of the DNA with *Eco*RI,

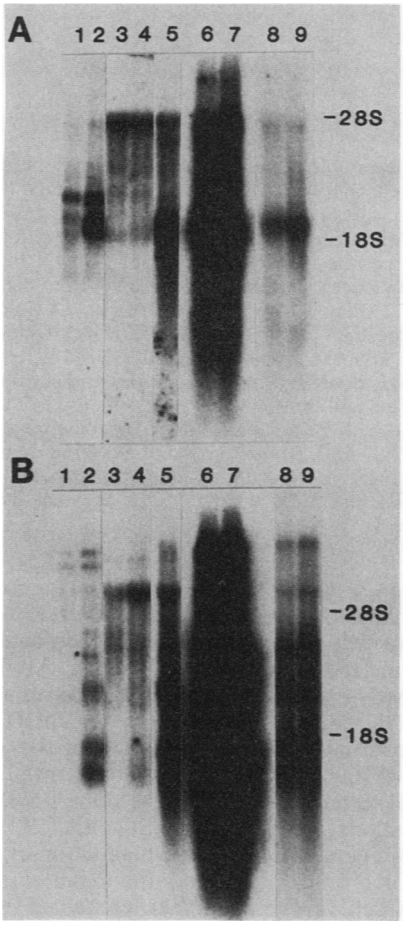

FIG. 2. Northern blot analysis of RNA from pdhOD1-transfected ODC^- cells and their DFMO-resistant variants. (A) Northern blot probed with ODC cDNA probe. Lanes 1 and 2, poly(A)$^+$ RNA; lanes 3 to 9, 10 μg of total RNA; lanes 5 to 9, 5 μg of total RNA; lane 1, parental ODC^- cells; lane 2, pooled population of ODC^- cells transfected with pdhOD1; lanes 3 and 4, same as lanes 1 and 2, respectively; lanes 5 to 7, pdhOD1-transfected cells selected in 1 mM DFMO (lane 5), 9 mM DFMO (lane 6), or 15 mM DFMO (lane 7); lanes 8 and 9, lower exposures of lanes 6 and 7, respectively. (B) Northern blot probed with a vector-specific *dhfr* probe. Lanes: Same as in panel A. The size standards are mouse rRNAs: 28S is equivalent to approximately 5.1 kb, and 18S is equivalent to approximately 1.9 kb.

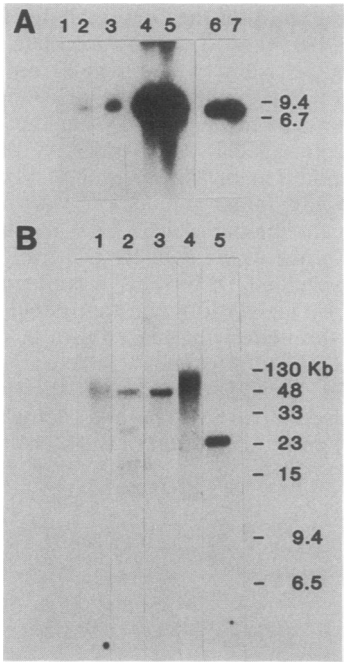

FIG. 3. Southern blot analysis of DNA from pdhOD1-transfected ODC$^-$ cells and their DFMO-resistant variants. (A) Cellular DNA (1 μg) cut with *Eco*RI was electrophoresed in a 0.8% agarose gel and transferred to nitrocellulose by blotting. The blot was probed with the nick-translated 353-bp fragment of pdhOD1 that contains the SV40 *ori* sequences. Lanes 1 to 5 are DNA from the same cells as in lanes 3, 4, 5, 6, and 7, respectively, of Fig. 2A. Lanes 6 and 7 are shorter exposures of lanes 4 and 5, respectively. The size markers are in kilobase pairs. (B) Cellular DNA (1 μg) cut with *Kpn*I, *Sal*I, *Xba*I, *Eco*RV, *Nru*I, and *Sma*I was electrophoresed in a 0.2% agarose gel and transferred to nitrocellulose by blotting. The blot was probed with a fragment of pdhOD1 containing the small t splice. Lanes 1, 2, and 3, DNA from the same cells as lanes 2, 3, and 4 of panel A; lane 4, DNA from an independent pool of pdhOD1-transformed cells; lane 5, DNA from DFMO-resistant derivatives of the cells in lane 4.

an enzyme that cuts once within pdhOD1, released a unit-sized fragment that was present in increased abundance in the DNA from DFMO-resistant cells (Fig. 3A). The initial and final DFMO-resistant populations contained an average of 20 and 5,000 copies, respectively, of pdhOD1 per cell (data not shown). Clearly, amplification of the vector-encoded SV40 *ori* and *dhfr* sequences had occurred.

Because these measurements were done on DNA from pooled populations, we cannot quantitate the fold amplification from these data. An accurate quantitation of amplification requires determination of both the number of copies of pdhOD1 per cell and the number contained within the amplified unit. Multiple copies of plasmid are inserted into the same genomic location during the initial transfection integration event (22, 25). During subsequent selection, a large region containing the plasmid copies and flanking cellular sequences is amplified (22, 24). We quantitated the number of copies of pdhOD1 in the amplified unit by Southern analysis of DNA which had been digested with a battery of restriction enzymes that do not cut within pdhOD1 but do cut the flanking cellular DNA sequences of the amplified unit. When this analysis was carried out on DNA from the initial unamplified population, a smear of DNA ranging in size from 15 to 130 kilobases (kb) hybridized to the vector-specific probe (Fig. 3B, lane 1). This reflects the heterogeneity in number of plasmids inserted into the genomes of the cells in the initial population. When this analysis was carried out on DNA from the amplified population, vector-specific sequences ran as one band at 50 to 60 kb (Fig. 3B, lane 3). The 60-kb fragment can contain at most five to seven copies of pdhOD1. The amplification is therefore at least 5,000/7 to 5,000/5, or 700- to 1,000-fold.

The single band suggests that the final DFMO-resistant "population" arose from one or at most two cells that came to dominate the population during selection. Five different highly amplified populations analyzed in this manner each displayed a single unique band. This strongly suggests that populations selected for many rounds of amplification tend to become dominated by one or at most two initial transfectants.

Southern blot analysis of this type showed that an intermediate population displayed a series of bands (Fig. 3B, lane 2). Four independent populations analyzed at intermediate stages of amplification all contained from two to seven bands. At final stages of amplification, all of these populations contained only one band. The 14 amplified bands observed ranged from 15 to 60 kb and therefore contained between two and seven copies of the plasmid inserted (Fig. 3B and data not shown). This range is clearly smaller than the range of number of copies inserted into the genomes in the initial populations from which the amplified populations were derived.

The expression of DHFR from the coamplified *dhfr* gene was measured by Northern blot (Fig. 2B) and R-dot hybridization analyses. These analyses indicated that *dhfr* mRNA was over-produced about 1,000-fold in the amplified population.

Amplification of the ODC vector in wild-type cells. The ODC$^-$ CHO cells transfected with pdhOD1 were resistant to higher concentrations of DFMO than wild-type CHO cells (data not shown). This suggests that pdhOD1 could confer a DFMO-resistant phenotype on wild-type cells and therefore be used as a dominant marker.

Wild-type CHO K1 cells were transfected with pdhOD1, and 200 stable transfectants resistant to 160 μM DFMO were selected (Table 1), pooled, and selected sequentially for resistance to 600 μM, 1 mM, 3 mM, 9 mM, and 15 mM DFMO.

Northern blot and R-dot hybridization analyses were performed to measure the expression of ODC and *dhfr* in wild-type transfectants relative to that in the transfectants derived from ODC$^-$ cells (Fig. 4). Wild-type CHO cells transfected with pdhOD1 expressed more ODC and *dhfr* mRNAs than did ODC$^-$ CHO cells transfected with pdhOD1. This is because the selection for pdhOD1 in wild-type cells, DFMO resistance, requires more ODC expression than the selection for pdhOD1 in ODC$^-$ cells. DFMO-resistant derivatives of pdhOD1-transfected wild-type cells expressed amounts of ODC and *dhfr* mRNAs equivalent to those expressed in amplified derivatives of pdhOD1-transfected ODC$^-$ CHO cells.

To determine whether gene amplification was the underlying mechanism for this increased expression of ODC and *dhfr* mRNAs, genomic DNAs from the initial and DFMO-resistant populations were analyzed. D-dot hybridization analysis (Fig. 5B) indicated that there were an average of 20 and 1,150 copies of pdhOD1 per cell in the initial and final DFMO-resistant populations, respectively. Southern blot analysis (Fig. 5A) of DNA from the final population digested with a battery of enzymes showed that vector-specific se-

TABLE 1. pdhOD1 transformation of various cell types to DFMO resistance

Cell line	Plasmid	No. of colonies/10^5 cells					
		DFMO (μM)					G418 (400 μg/ml)
		50	70	75	160	240	
NIH 3T3	pdhOD1	178		122			
	Control	0		0			
C127	pdhOD1	193					
	Control	0					
	pSV2neo						96
CV1	pdhOD1		44				
	Control		0				
	pSV2neo						25.5
CHO	pdhOD1				101	39	
	Control				18	0	

quences ran as a single 35-kb band. Therefore, at most four copies of pdhOD1 were contained within the amplified unit, and the amplification was at least 1,150/4, or 290-fold.

The ability of the pdhOD1 vector to be dominant in NIH 3T3, CV1, and C127 cells was investigated. Cells were transfected with pdhOD1 or control plasmid and subjected to a DFMO concentration that was lethal for each untransfected cell line, and surviving colonies were counted. The frequency with which pdhOD1 transformed each cell line to DFMO resistance is listed in Table 1. The frequencies with which pSV2neo (30) transformed CV1 and C127 cells to G418 resistance are also noted. pdhOD1 was a dominant marker in all of these cell lines; resistant colonies were scored with an efficiency equivalent to or greater than that seen with pSV2neo.

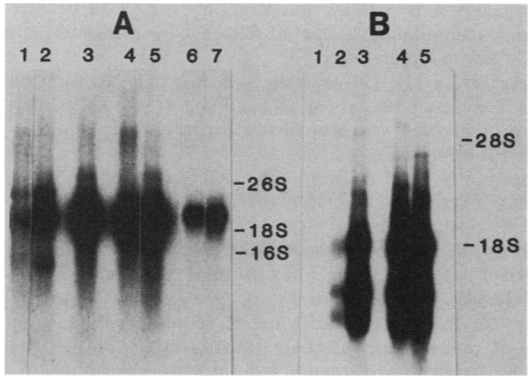

FIG. 4. Northern blot analysis of wild-type CHO cells transfected with pdhOD1 and their DFMO-resistant variants. (A) Northern blot probed with ODC cDNA probe. Lane 1, Pooled population of ODC$^-$ cells transfected with pdhOD1; lane 2, wild-type cells transfected with pdhOD1 and selected in 160 μM DFMO; lane 3, wild-type-derived population selected in 1 mM DFMO; lane 4, pdhOD1-transfected ODC$^-$ cells selected in 9 mM DFMO; lane 5, pdhOD1-transfected wild-type cells selected in 15 mM DFMO; lanes 6 and 7, shorter exposures of lanes 4 and 5, respectively. Lanes 1 to 3 contain 1 μg of poly(A)$^+$ RNA; lanes 4 to 7 contain 0.1 μg of poly(A)$^+$ RNA. (B) Northern blot probed with a vector-specific *dhfr* probe. Lane 1, wild-type CHO cells, untransfected; lanes 2, 3, 4, and 5, RNA from the same cells as lanes 1, 2, 4, and 5, respectively, of panel A. The amounts of poly(A)$^+$ RNAs loaded are the same as in panel A. The size standards were mouse rRNAs (28S and 18S) and *E. coli* rRNAs (26S and 16S). The 28, 26, 18, and 16S markers are equivalent to approximately 5.1, 3, 1.9, and 1.5 kb, respectively.

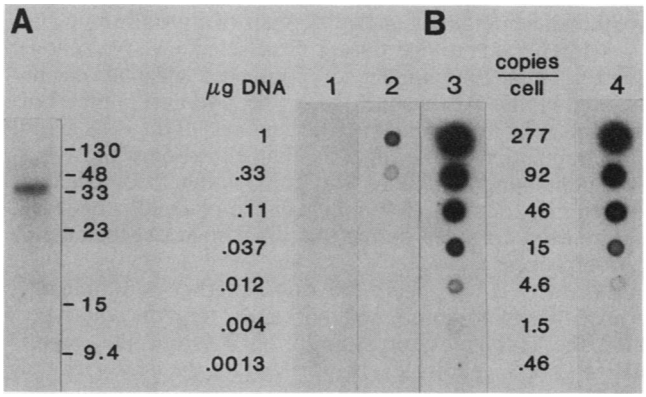

FIG. 5. Southern blot and D-dot hybridization analyses of DNA from wild-type CHO cells transfected with pdhOD1 and their DFMO-resistant variants. (A) DNA from the pooled population of wild-type cells transfected with pdhOD1 and selected for resistance to 15 mM DFMO was analyzed by the multirestriction Southern analysis described in the legend to Fig. 3B. The size markers are shown in kilobases. (B) DNAs from various cells were analyzed by D-dot hybridization analysis with a vector-specific *dhfr* probe. Lane 1, Untransfected wild-type cells; lane 2, wild-type cells transfected with pdhOD1 and selected in 160 μM DFMO; lane 3, wild-type-derived cells selected in 15 mM DFMO; lane 4, various amounts of pdhOD1 with carrier salmon sperm DNA were spotted onto nitrocellulose to provide a standard for quantitation of the copy number of pdhOD1 per cell.

DISCUSSION

We have developed an amplifiable vector based on ODC. A 1,000-fold increase in expression of a coamplified marker can be obtained in ODC$^-$ CHO cells. This ODC marker can be used as a dominant amplifiable marker in wild-type CHO cells and as a dominant marker in a variety of cell types. Final levels of expression of vector-specific ODC and coamplified *dhfr* in wild-type CHO cells were equivalent to those achieved in ODC$^-$ CHO cells.

The physiology involved in the ODC selection system is simple, direct, and conserved in animal cells. Therefore, selective conditions for amplification of the ODC marker are easy to set up in multiple cell types. The ADA system requires complicated selective conditions that make ADA essential. The selection required for successful amplification of the ADA gene is not well tolerated by some cell types, and they do not grow at maximal rates during selection (13). Variants that overexpress ODC grow well under selective conditions. The *Escherichia coli* asparagine synthetase (AS) gene is a useful dominant marker for both animal and bacterial cells (2). However, the AS gene was shown to amplify only 20-fold in wild-type cells and is more difficult to amplify to high copy number than is *dhfr* (2). The *E. coli pyrB* gene has been shown to function as an amplifiable marker in mutant cells, but greater than 4-fold amplification of this marker was not demonstrated (26).

Cells that were resistant to very high levels of DFMO (above 2 mM) showed some dependence on DFMO to grow at maximal rates. S49 mouse lymphoma cells resistant to 20 mM DFMO reverted to DFMO independence at a high frequency. These variants were stable and retained parental levels of ODC expression (McConlogue and Coffino, unpublished).

Initial transfectants that expressed higher amounts of ODC tended to have higher numbers of plasmid copies per insertion in their genomes (data not shown). Therefore,

transfectants containing multiple copies of plasmid should be at a selective advantage during drug selection. We found, however, that the number of copies of plasmid in the amplified units was smaller than the average number of copies of plasmid inserted into the genomes of the cells in the initial population. This suggests that either only those regions containing relatively low copy numbers of plasmid inserted can successfully amplify or the plasmid-containing regions are rearranged during the initial stages of the amplification process.

Like the ADA marker, the ODC marker is dominant, because this vector expressed more ODC than the wild type. Thus, the level of expression of ODC from the vector relative to endogenous levels is crucial for the dominance of the ODC marker. The efficiency of transfection of pdhOD1 in wild-type cells was similar to that of pSV2neo. Therefore, the stringencies of the selections for pdhOD1 and pSV2neo are similar. We have not yet shown, however, whether one copy of pdhOD1 per cell is sufficient for the dominant behavior, as has been demonstrated for the *neo* marker.

Because pdhOD1 expresses more than wild-type levels of ODC, it is possible to design an amplifiable ODC vector for ODC^- cells that expresses less ODC than is expressed by pdhOD1. The advantages of this type of vector would be that one could potentially amplify such a vector further than 1,000-fold or use DFMO concentrations below 2 mM or both.

The ODC marker is dominant in a variety of cell types and can be amplified 1,000-fold. Because the physiology of the selection is simple, the ODC marker may be more useful in some systems than the *dhfr* or ADA markers. The ODC marker can be used in conjunction with other amplifiable markers when multiple amplifiable markers are required, for example, to independently coamplify two or more genes in the same cell.

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