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Stepwise Isolation and Properties of Unstable Chinese Hamster Cell Variants That Overproduce Adenylate Deaminase

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Addition of coformycin (0.5 μg/ml) to a culture medium containing adenine causes in Chinese hamster fibroblasts a lethal depletion of IMP. Resistant variants have been recovered, some of which exhibit increased adenylate deaminase activity. (Debatisse et al., J. Cell. Physiol., **106**:1–11, 1981). The selective medium was made more specific for the isolation of this class of variants by supplementation with azaserine. The hyperactive variants remained sensitive to coformycin concentrations above that used for their selection and were unstable. Their frequency was not increased by ethyl methane sulfonate mutagenesis. The resistant phenotype and the increased activity of adenylate deaminase behaved as semidominant traits in hybrids. No change was detected in the K_m for AMP, the cofactor requirement, or the chromatographic properties of adenylate deaminase in the variants. Through stepwise selection in media supplemented with increasing coformycin concentrations, unstable clones with adenylate deaminase activity up to 150-fold the wild-type level were isolated; from an unstable clone, a stable subclone with reduced resistance and enzyme activity was recovered. Evidence that increased adenylate deaminase activity is the manifestation of overaccumulation of the enzyme protein was supplied by the correlation of enzyme activity with the intensity of a protein band comigrating with purified adenylate deaminase during sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell extracts. Several unidentified additional bands showed comparable quantitative changes. The striking similarity between the adenylate deaminase-overproducing lines and unstable dihydrofolate reductase-overproducing lines generated by gene amplification strongly suggests that the coformycin-resistant variants also resulted from amplification of an adenylate deaminase gene.

The adenosine analog coformycin, currently used as an inhibitor of adenosine deaminase (17), does not alter the proliferation of Chinese hamster fibroblasts growing in conventional culture medium, even when supplied at concentrations above 20 μg/ml. In contrast, coformycin is toxic at concentrations above 0.05 μg/ml in medium containing adenine (7). Previous work (5) has shown that cell death is the manifestation of IMP starvation arising from a double metabolic block: adenylic nucleotides derived from adenine feedback inhibit the de novo IMP biosynthesis pathway, while coformycin shuts off both the adenosine deaminase and adenylate deaminase (AMPD) interconversion pathways. The dual mechanism by which the purine mixture exerts its toxicity was elucidated by the isolation and characterization of two classes of variants which preserved the ability to grow in the presence of coformycin (0.5 μg/ml) and adenine (2×10^{-5} M). In class 1 variants, the IMP biosynthetic pathway was desensitized to the feedback inhibition imposed by adenylic nucleotides; class 2 variants in turn had a 7- to 10-fold increase in AMPD activity. This alteration accounted for their resistance to coformycin, which has been shown to be a strong and presumably tight-binding inhibitor of AMPD (1). AMPD activity was indeed found to be markedly decreased in extracts of both wild-type and variant cells grown in medium containing 0.5 μg of coformycin per ml, but the residual activity of the enzyme in the variant under study remained comparable to the activity found in wild-type cells grown in regular medium. The class 2 variants examined were unstable, suggesting

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that they are not generated by a classical gene mutation. To study these variants and gain information on their origin under more controlled conditions, we isolated a new set of such clones and examined their genetic and biochemical properties. During this investigation, we observed that stepwise selection of resistant clones in adenine-containing medium supplemented with increasing concentrations of coformycin yielded sublines which exhibited higher and higher AMPD activity. We describe here the results of this study, show that the hyperactivity of AMPD in the resistant lines is the manifestation of enzyme overaccumulation, and discuss the striking similarity between the properties of these unstable variants and those of unstable methotrexate-resistant mutants generated by gene amplification.

MATERIALS AND METHODS

Cells and growth conditions. The GMA32 cell line is a deoxycytidine kinase (dCK)-deficient derivative (15) of the CCL39 line of Chinese hamster lung fibroblasts. Cells were grown attached to plastic petri dishes (Nunc) in ERH medium made of Eagle minimal essential medium with twice the amount of amino acids (Eurobio), 10% horse serum (Bio-Merieux), antibiotics, and the indicated supplements in humidified incubators with an atmosphere containing 10% CO_2, as previously described (6).

Mutagenesis and selection procedure. The conditions for ethyl methane sulfonate (EMS) mutagenesis and general procedures for the isolation of resistant clones have been previously reported (6). Specific aspects of the selection procedures are described below.

Cell hybridization. The technique of polyethylene glycol-induced cell fusion is that of Robert de Saint-Vincent and Buttin (16).

Enzyme assays. For the preparation of cell-free extracts, cells from cultures in petri dishes were pelleted by low-speed centrifugation and stored at −20°C until used. The pellets were then suspended in an equal volume of the appropriate buffer and disrupted by sonication in four 30-s bursts (MSE sonicator), and the extracts were cleared from the debris by centrifugation for 15 min at 40,000 × *g*. The assay conditions for the various enzymes have been previously described (5). One unit of AMPD activity is defined as that amount of enzyme required to deaminate 1 nmol of AMP per h under the experimental conditions.

AMPD purification. AMPD purification exploited the peculiar affinity of this enzyme for phosphocellulose discovered by Smiley et al. (19). Cell extract in sodium phosphate buffer (pH 6.5; 50 mM), supplemented with β-mercaptoethanol (1 mM) and EDTA (0.1 mM), was diluted in the same buffer to a protein concentration of 10 mg/ml, and ammonium sulfate was added to 35% saturation. After 1 h at 4°C, the pellet was spun down (10 min at 13,000 × *g*) and redissolved in the extraction buffer supplemented with 200 mM NaCl. The solution was adsorbed batchwise on phosphocellulose in the proportion of 1 μl of swollen resin per 200 U of enzyme. The slurry was stirred at low speed for 30 min at 4°C, poured on a Büchner funnel, and extensively washed, first with extraction buffer supplemented with 300 mM NaCl and then with sodium succinate buffer (pH 6.5; 10 mM) supplemented with β-mercaptoethanol (1 mM), EDTA (0.1 mM), and NaCl (300 mM). AMPD was eluted in the latter buffer supplemented with 0.6 mM $ZnCl_2$ and 25 mM ATP.

In earlier experiments, a partial purification of the enzyme was obtained by fractionating the sonicated cell extract on a DEAE-cellulose column. After removal of the debris by a 30-min centrifugation at 42,000 × *g*, the extract (4 mg of protein per ml) in 10 mM Tris (pH 7.5)–0.1 mM EDTA–1 mM $MgCl_2$–1 mM β-mercaptoethanol was loaded onto a DEAE-52 column; AMPD was eluted with a 0 to 0.5 M KCl gradient in the same buffer and recovered as a single sharp peak at 0.2 M KCl. The enzyme was completely free of adenylate kinase, which did not bind to the column.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Laemmli (12).

Chemicals. Bases, nucleosides, and nucleotides were purchased from P.L. Biochemicals, Milwaukee, Wis.; coformycin was obtained from Meiji Seika Kaisha Ltd., Pharmaceutical Division, Tokyo, Japan; azaserine was obtained from Serva, Heidelberg, Germany; cellulose phosphate P11 was from Whatman; NaCl (Suprapur) was from Merck, Darmstadt, Germany; ammonium sulfate (Ultrapur) was from Serlabo, Paris, France; acrylamide and bisacrylamide were from Serva. All other compounds used for electrophoresis were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

One-step selection of variants with increased AMPD activity. In a previous report (5), the regular ERH medium supplemented with 0.5 μg of coformycin per ml was designated HC medium; this designation will be used below. As described above, selection of resistant clones in HC medium containing 0.02 mM adenine and 0.05 mM uridine (routinely added to prevent possible side effects on pyrimidine synthesis of adenylic nucleotides generated from adenine) yielded both variants with altered control of IMP de novo biosynthesis (class 1) and variants with increased AMPD activity (class 2). The two types of variants were initially distinguished by their different proliferation capacity in selective medium supplemented with azaserine, a potent inhibitor of de novo IMP biosynthesis. Only class 2 variants grew in this medium; class 1 variants, which rely for their proliferation on the activity of the endogenous IMP biosynthesis pathway, were killed under these conditions. We exploited this property to define a selective medium for the more specific isolation of variants with increased AMPD activity. This medium was obtained by incorporating to regular ERH medium 0.5 μg of coformycin (HC) per ml, 0.01 mM azaserine, 0.05 mM adenine, and 0.05

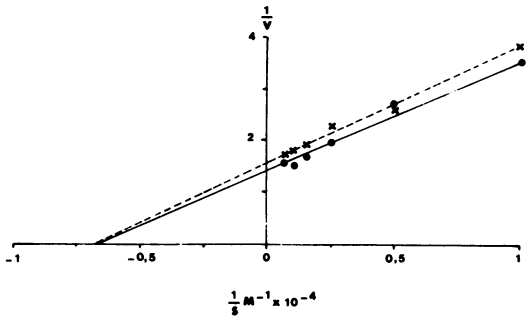

FIG. 1. Determination of the K_m for AMP of AMPD from the GMA32 (×) and HC6 (●) lines. The Lineweaver-Burke plot was constructed from the values obtained during incubation of appropriate dilutions of AMPD preparations (free of adenylate kinase), with 10^{-3} M ATP as cofactor and the indicated AMP concentrations. Ordinates are arbitrary values.

mM uridine. (The mixture of azaserine, adenine, and uridine at these concentrations will be designated below as AAU.) Cells of the parental line GMA32 were mutagenized with EMS and plated at 37°C in HC + AAU medium. Among the resistant clones recovered from this selection, three, HC4, HC5, and HC6, were further analyzed.

As expected, all three had increased AMPD activity, reaching 4,000 U/mg of protein as compared with 400 U/mg of protein in the parental line. No change was detected (not shown) in the activity of 7-purine salvage and interconversion enzymes (adenine phosphoribosyl transferase, hypoxanthine-guanine phosphoribosyl transferase, adenosine kinase, inosine phosphorylase, IMP dehydrogenase, GMP synthetase) as already observed for different clones exhibiting the same resistance phenotype (5).

To compare the properties of the enzyme in a hyperactive variant and in the wild-type line, we determined its cofactor requirement and its K_m for AMP in the GMA32 and HC6 lines. The enzyme was cleared from nucleotides and from the activity of myokinase through chromatography of cell extracts on DEAE-cellulose (see above). The enzyme from the two lines exhibited the same absolute requirement for ATP as an activator; measured in the presence of 1 mM ATP, the K_m of AMP was identical (0.15 mM) (Fig. 1). Enzyme from both sources eluted from the resin column at the same salt concentration and in association with a 5'-nucleotidase which used IMP as a substrate (M. Debatisse, unpublished data). These results suggest that the same enzyme species is expressed in the hyperactive variant and in the original line.

TABLE 1. Plating efficiency of wild-type and resistant lines in AAU medium supplemented with various concentrations of coformycin

Coformycin (μg/ml)	Plating efficiency (%)[a]			
	GMA32	HC4	HC5	HC6
0	100	100	100	100
0.05	100	100	100	100
0.1	30	100	100	100
0.2	0	100	100	100
0.5	0	88	100	100
1.0	0	27	32	40
2.0	0	0	0	0

[a] Results are expressed as percentage of plating efficiency in regular ERH medium (200 cells plated for each determination). Plating efficiencies between 90 and 110% have been recorded as 100%.

TABLE 2. Plating efficiency in AAU medium supplemented with coformycin at various concentrations of the HC6 line pregrown in selective or in regular medium

Medium (no. of transfers)[a]	Plating efficiency (%)[b] in medium with indicated amt of coformycin (μg/ml):				
	0	0.05	0.1	0.2	0.5 (HC)
AAU + HC	100	100	100	100	100
ERH (10)	100	100	100	100	70
ERH (20)	100	100	100	87	45
ERH (30)	100	100	88	48	15
ERH (40)	100	100	60	16	0
ERH (50)	100	100	22	0	0

[a] One transfer equals approximately 30 doublings.
[b] Results are expressed as the percentage of the plating efficiency in regular ERH medium. Plating efficiencies between 90 and 110% have been recorded as 100%.

Resistance level. In a first experiment, we determined the sensitivity of the HC4, HC5, and HC6 sublines to the AAU medium supplemented with various coformycin concentrations soon after their isolation. The plating efficiency of both the resistant lines and the sensitive parental line GMA32 was reduced from normal to zero within a narrow range of coformycin concentrations (Table 1). The three lines HC4, HC5, and HC6 were only resistant to the drug concentration which was used in their selection.

Instability of the resistant phenotype. Like the variants with increased AMPD activity previously isolated in HC + A medium, the three lines HC4, HC5, and HC6 were unstable. The kinetics of reversion of HC6 to the wild-type phenotype was studied in more detail. The reversion of coformycin resistance was an all or nothing phenomenon (Table 2). During prolonged growth in regular medium, the cell population became heterogeneous, and cells with a variety of resistance levels intermediate between the HC6 and GMA32 line were generated.

Lack of influence of EMS mutagenesis on the frequency of resistant variants. As a first attempt at determining the influence of mutagenic treat-

TABLE 3. Influence of EMS mutagenesis on frequency of AzaG^r and (HC + AAU)^r clones

Cells	EMS concn[a] ($\times 10^{-5}$ M)	Survival (%)	No. of clones recovered[b] in medium:	
			AzaG[c]	HC + AAU
Expt 1				
GMA32	0	100	0	890
	8	92	30	616
	20	53	141	713
GMA32 subclone 1	0	100	2	2
	8	60	49	9
	20	32	186	3
GMA32 subclone 2	0	100	0	778
	8	69	37	800
	20	35	192	686
Expt 2				
GMA32	0	100	1	3
	8	75	23	6
GMA32 subclone 1	0	100	0	3
	8	68	56	2
GMA32 subclone 2	0	100	0	121
	8	63	42	126

[a] Dilution factor of the commercial product.
[b] From 2×10^5 plated cells.
[c] ERH medium supplemented with 50 μg of 6-azaguanine per ml.

ments on the appearance of variants resistant to HC + AAU medium, we examined the frequency of resistant colonies growing in this medium after mutagenesis of a sensitive line with various EMS concentrations. The frequency of azaguanine-resistant (AzaG^r) mutants was measured as a control. The GMA32 line and two freshly isolated subclones were cultured and mutagenized in parallel.

As expected, the frequency of AzaG^r mutants was markedly dependent on the mutagenic treatment imposed on the cells and not significantly different in the various cultures treated with the same EMS concentration (Table 3). A quite different situation was observed when the frequency of variants resistant to HC + AAU was analyzed. The frequency of spontaneous variants was quite different from culture to culture, ranging from 1×10^{-5} to 5×10^{-3}. This did not seem to be a clonal property of sublines examined, however, as judged from the fluctuations observed in different experiments with the same subclone. A high frequency of spontaneous variants also did not reflect enrichment in cells with a growth advantage in regular medium: spontaneous variants were less numerous in experiment 2 than in experiment 1, carried out at an earlier date. More important was the observation that, even when cell populations yielding spontaneous HC + AAU-resistant and AzaG^r variants at comparable frequencies are considered, EMS mutagenesis did not stimulate the appearance of the HC + AAU-resistant clones.

Dominance of the resistant phenotype. The parental line GMA32 and its variants are deficient in the activity of dCK; this marker can be exploited to isolate hybrids after fusion of these cells with thymidine kinase-deficient (TK$^-$) cells (8). The dCK$^+$ TK$^+$ hybrid clones were selected in medium (M1 medium) supplemented with aminopterin, 1 mM thymidine, deoxycytidine, and adenine as a purine source. This selection was independent of the coformycin resistance of the dCK$^-$ cells. Also, HC + AAU-resistant dCK$^+$ TK$^+$ hybrids could be specifically selected for when the M1 medium was further supplemented with the other components of the HC + AAU medium: coformycin (0.5 μg/ml) and uridine (M2 medium). (In this medium, aminopterin replaces azaserine as an inhibitor of endogenous IMP synthesis). Thus, a direct comparison of the frequency of hybrids recovered by plating portions of a fusion mixture of M1 and M2 media allowed the determination of the dominant or recessive character of the resistance phenotype, since only hybrid clones exhibiting dominant resistance will grow on M2 medium.

The experiment was carried out by fusing HC6 cells to cells of the GMF11 line, which is a TK$^-$ derivative of the same wild-type line (CCL39) from which GMA32 was also isolated. The selection was carried out at both 37 and 39°C; as shown previously (5), decay of the AMPD activity in coformycin-containing media is less drastic at 39 than at 37°C (Table 4). At 39°C, the number of colonies recovered on the two media was not significantly different. In contrast, when the selection was made at 37°C, only 75% of the colonies growing in M1 medium developed on M2 medium, and they were of smaller size (not shown), indicating restricted growth of the cells.

To verify the hybrid character of the cells surviving these selections, two colonies from the fusion HC6 × GMF11 were randomly picked from those growing at 39°C on M1 medium and two from those growing on M2 medium; the karyotype of the cells was determined. The HC6 and GMF11 lines have a modal chromosome number of 21; in the four clones examined, the modal chromosome number was 40 to 42. AMPD activity was measured in these clones, and the specific activity of the enzyme was close to 2,500 U/mg of protein, as compared with 400 U for GMF11 and 4,000 U for HC6. Thus, both the resistance of the HC6 clone to the HC + AAU medium and the increased level of AMPD activity behaved as semidominant traits.

TABLE 4. Clones recovered from fusion of the GMF11 and HC6 cell lines and from control fusions after plating in selective media[a]

Cross	Dilution of fusion mixture ($\times 10^{-3}$)	No. of clones recovered in plating medium:	
		M1[b]	M2[c]
Expt 1			
GMF11[d] × GMF11	4	0	0
	2	0	0
	1	0	0
HC6[e] × HC6	4	3	1
	2	0	1
	1	0	0
GMF11 × HC6	4	80	84
	2	39	46
	1	19	26
Expt 2			
GMF11 × GMF11	4	0	0
	2	0	0
	1	0	0
HC6 × HC6	4	5	3
	2	1	2
	1	0	0
GMF11 × HC6	4	78	81
	2	40	46
	1	23	23

[a] For fusion, GMF11 was plated at 1.5×10^6 cells per dish, and HC6 was plated at 1.5×10^5 cells per dish. Cells were plated in M1 and M2 media at 39°C.

[b] Contained aminopterin, thymidine (1 mM), deoxycytidine, and adenine (selective for hybrids sensitive to HC + AAU medium).

[c] Contained same supplements as M1 plus coformycin (0.5 μg/ml) and uridine (selective for hybrids resistant to HC + AAU medium).

[d] Selective markers were TK^- dCK^+.

[e] Selective markers were TK^+ dCK^-.

Stepwise isolation of variants with progressively increasing coformycin resistance and AMPD activity. We showed above (Table 1) that the HC4, HC5, and HC6 lines are killed in AAU medium supplemented with concentrations of coformycin higher than that used for their selection. We therefore attempted to isolate variants able to grow in medium containing a ten times higher (5 μg/ml) concentration of the drug (designated below as HC10). Cultures of HC4 and HC6 lines were plated in HC10 + AAU medium. No mutagenic treatment was applied to the cells before selection. Two resistant clones, HC1042 and HC1047, derived from HC4, and two resistant clones, HC1061 and HC1063, derived from HC6, were isolated and propagated for further study. Like their parents, all four clones remained sensitive to coformycin concentrations above that chosen for their isolation (not shown). AMPD activity was measured in the four lines soon after their isolation. The values ranged from 15,600 to 16,800 U/mg of protein, i.e., about 40-fold the activity in wild-type cells. For the HC1061 subline, which was more extensively analyzed, the secondary variants were unstable: prolonged growth in regular medium yielded cells with a variety of reduced resistance levels (Table 5). Reversion appeared to take place faster than in the parental line HC6. AMPD activity decreased during reversion, reaching nearly the wild-type level in cell populations approaching reversion to complete drug sensitivity.

A culture of HC1061 cells in regular medium was cloned after 10 transfers, and 16 colonies were randomly picked. These clones were propagated in regular medium, and the clonal cell populations were quickly tested for their ability to grow in AAU medium supplemented with coformycin at various concentrations. Two had fully reverted both resistance and AMPD activity; 14 remained resistant to various levels of the drug, unambiguously demonstrating the clonal segregation of cells with intermediate resistance levels. Further analysis showed that 13 of the subclones remained unstable. The last one, designated HC1061-S1, preserved during prolonged growth in regular medium a resistance level close to that of the HC4, HC5, and HC6 lines and comparable AMPD activity (Table 5). Thus, stable subclones can be occasionally segregated from the unstable population.

The plating of a culture of HC1061 in AAU medium supplemented with 12.5 μg of coformycin per ml yielded a clone, HC2561, with an AMPD activity of 30,000 U/mg of protein. From a culture of this clone, it was again possible to isolate a subclone, HC5061, able to grow in AAU medium supplemented with 25 μg of coformycin per ml. AMPD activity in these cells reached 60,000 to 70,000 U/mg of protein, i.e., about 150-fold the activity of the enzyme in the sensitive GMA32 cells.

Overaccumulation of AMPD protein in the resistant clones. AMPD was purified from the HC2561 line and used as a marker to compare the amount of this protein present in extracts of sensitive and resistant clones. AMPD purification exploited the ability of the enzyme to remain bound to phosphocellulose under conditions (see above) at which apparently no other proteins are bound. The purification from HC2561 yielded with 50% recovery an enzyme purified 130-fold. After electrophoresis on SDS-polyacrylamide gels of fresh preparations of the purified fraction, the enzyme was revealed as one protein band of apparent molecular weight

TABLE 5. Resistance level of an unstable and a stable subclone in AAU medium supplemented with various concentrations of coformycin

Cell line	No. of transfers in regular medium before plating	Plating efficiency (%)[a] in AAU medium plus coformycin (μg/ml):				AMPD specific activity (U)
		0	0.5	1	5	
GMA32		100	0	0	0	405
HC1061	0	100	100	100	100	15,690
	7	100	100	100	60	—[b]
	29	100	25	6	0	620
HC1061-S1	5	100	87	0	0	4,950
	36	100	100	0	0	5,170
	44	100	88	0	0	5,156

[a] Results expressed as percentage of the plating efficiency in coformycin-free medium (values between 90 and 110% are recorded as 100%).
[b] —, Not determined.

120,000; two faint bands with very close migration coefficients (apparent molecular weight, 95,000) were just discernible in these preparations. After storage at 4°C for 2 to 3 weeks, the major band spontaneously became converted into the two minor bands, and after 2 months of storage, all protein was present as the lowest-molecular-weight component. This pattern of spontaneous alteration of the purified AMPD protein is strikingly similar to that observed by Ranieri-Raggi and Raggi for AMPD purified from rabbit muscle (14). These observations indicate that the AMPD preparation was essentially free of contaminating proteins, implying that AMPD represents nearly 0.3% of the total protein content of the HC2561 cells.

Purified AMPD was used as a marker to analyze the electrophoretic pattern of GMA32, HC6, HC1061, HC2561, and HC5061 cell extracts (Fig. 2). Under these experimental conditions, which reveal only major proteins as discrete bands, no band could be seen in the GMA32 extract at the AMPD marker level. In contrast, bands of increasing intensity were clearly discernible in lanes containing extracts from lines with increasing AMPD activity, showing that increased activity is the manifestation of an overaccumulation of enzyme molecules. Bands of markedly increased intensity were also observed at the levels designated X and Y on the electrophoretic pattern of the HC5061 cell extract and less clearly seen on the pattern of the extracts with intermediate activity. Not only the AMPD band but also the bands at levels X and Y disappeared in extracts from cells of the HC1061 line which regained the sensitive phenotype during growth for 36 transfers in regular medium, or from clones isolated from this revertant population, indicating that the accumulation of these protein species is related to that of AMPD (Fig. 3).

DISCUSSION

In a previous report (5), we showed that unstable variants with increased AMPD activity can be isolated from the GMA32 cell line growing in medium containing coformycin and adenine. This selection also yielded at a comparable frequency variants which survived because AMP generated from adenine was no longer able to inhibit endogenous IMP synthesis in these cells. In the present work, we added azaserine to the selective medium to eliminate the latter class of variants. The modified selective medium imposed two independent blocks on the operation of the endogenous IMP biosynthetic pathway, thereby limiting the recovery of resistant cells to variants altered in the salvage and interconversion purine pathways. Such a selection appears to be essentially specific for the isolation of variants with increased AMPD activity: all (14 of 14) clones recovered so far from cultures in this selective medium and examined for the enzyme level did exhibit this biochemical alteration (unpublished data).

The molecular mechanism which leads to AMPD overproduction in the resistant cells remains to be analyzed. It may, a priori, correspond to oversynthesis of the enzyme or to its protection against a degradative activity present in wild-type cells. Both possibilities are of interest, but we favor the first hypothesis because the properties of the coformycin-resistant variants appear so far to be remarkably similar to those of mutants selected for stepwise resistance to a toxic analog and subsequently shown to have an amplified number of copies of the gene coding for a drug target enzyme (2, 3, 20). Evidence suggesting that AMPD overproduction in the coformycin-resistant lines results from amplification of its structural gene can be summarized as follows. (i) Variants with gradually increasing

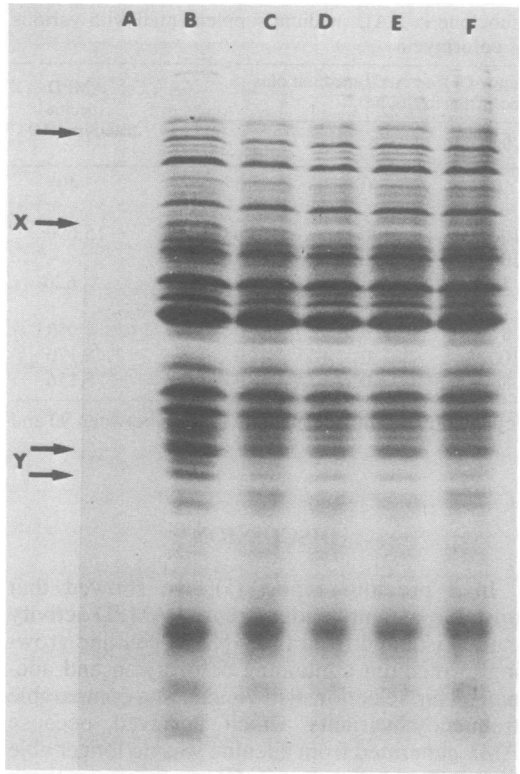

FIG. 2. SDS-polyacrylamide gel electrophoresis pattern of crude extracts from the GMA32 line and from variants with increasing resistance. Lanes: A and G, purified AMPD (see the text); B, HC5061 extract; C, HC2561 extract; D, HC1061 extract; E, HC6 extract; F, GMA32 extract. Arrows indicate levels X and Y on the gel at which unidentified protein bands are visible on the electrophoresis pattern of variant cell extracts.

AMPD levels—up to 150-fold (or more) above the wild-type level—can be isolated through stepwise selection. The enzyme level is a clonal property of the variants at the time of their isolation, but they spontaneously segregate some subclones with increasing enzyme levels when maintained in selective medium, whereas they revert stepwise to the wild-type level when grown in nonselective medium. These fluctuations in AMPD activity within populations of dividing cells may all be simply explained by the gradual accumulation or loss of extra copies of the AMPD gene, as observed for the dihydrofolate reductase (DHFR) gene in unstable methotrexate-resistant mutants (4, 9, 10). (ii) Subclones, such as HC1061-S1, which are stabilized at an intermediate enzyme level, can be recovered from clones with higher enzyme activity. This remarkable exception to the pattern of segregation of the unstable variants is similar to the situation which has been observed for the DHFR genes (10, 18). (iii) EMS does not increase the frequency at which AMPD-overproducing lines are recovered. This is unexpected if such variants are generated by conventional mutations; it is consistent instead with the lack of influence of common mutagens in two well-characterized systems of drug-resistant mutants produced by gene amplification (11, 18).

Taken together, these observations are difficult to reconcile with the operation of altered regulatory mechanisms acting at the transcriptional level or at later stages of AMPD biosynthesis. In the unstable murine lines with amplified DHFR genes, the multiple gene copies could be assigned to extrachromosomal elements designated "double-minute" chromosomes, whereas they appear to have a chromosomal location as "homogenously staining regions" in stable derivatives of these lines and in unstable lines of Chinese hamster origin (4, 13). Preliminary karyotypic studies did not dis-

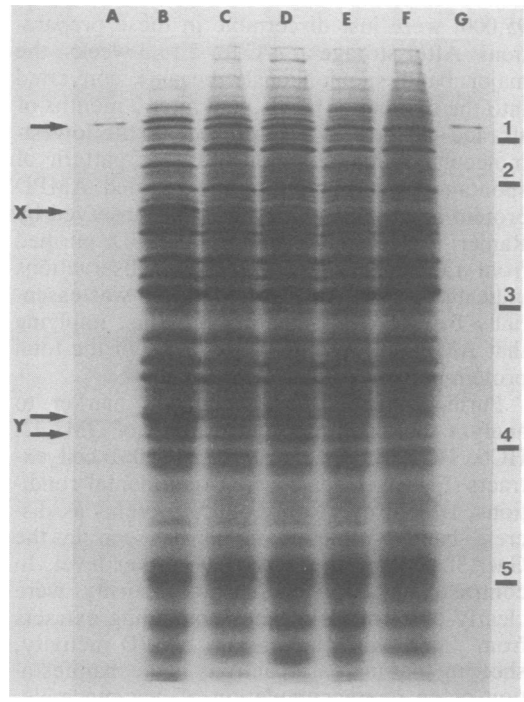

FIG. 3. SDS-polyacrylamide gel electrophoresis pattern of crude extracts from the HC1061 line and revertants. Lanes: A, purified AMPD; B, HC1061 extract; C and D, extracts made from two revertant subclones (HC1061 cells cloned after 10 transfers in ERH medium and propagated in this nonselective medium); E, HC1061 extract made from a culture propagated for 36 transfers in ERH medium; F, GMA32 (wild-type) extract. Arrows indicate levels X and Y on the gel at which unidentified protein bands are visible on the electrophoresis pattern of variant extracts. Bars indicate the position of reference molecular weight markers: 1, phosphorylase B (93,000); 2, bovine albumin (66,000); 3, pepsin (34,700); 4, pepsinogen (24,000); 5, β-lactoglobulin (18,400).

close the presence of double-minute chromosomes in clones overproducing AMPD, but examination of clone HC1061 revealed the existence of a homogenously staining region segment located at the point at which a small chromosome is translocated to one long chromosome of pair 1 (unpublished data). Although the presence of AMPD genes on this abnormal chromosome has not been established so far, this situation encourages us to believe that a common mechanism may govern overproduction of AMPD and DHFR in Chinese hamster cells. Work in progress to estimate, with the help of a DNA probe, the actual number of AMPD gene copies in the coformycin-resistant lines should clarify this point. The availability of a convenient selection medium for the isolation of AMPD-overproducing variants and of an easy purification procedure for the enzyme make this system an invaluable model for genetic and molecular investigations on the general mechanisms leading to acquired drug resistance through overproduction of a target enzyme. The examination of gel electrophoresis patterns of variant cell extracts has shown that several protein bands vary in their intensity just as the AMPD band does; this is of special interest if the mechanism leading to enzyme overproduction is gene amplification, since one attractive hypothesis would be that several linked genes with detectable products are coamplified in this system.

Other more specific developments are also suggested by the remark that AMPD is an enzyme widely distributed as a variety of isozymic species in mammalian cells of different histotypic origin. It will be of particular interest to determine whether the selection exploited above can be utilized to overproduce these different isozymes. This may be a unique source of information on their possibly specific physiological functions and on their genetic determinism.

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