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Amplification of the IMP Dehydrogenase Gene in Chinese Hamster Cells Resistant to Mycophenolic Acid

FRANK R. COLLART AND ELIEZER HUBERMAN*

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439-4833

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The regulation of IMP dehydrogenase (IMPDH) was analyzed in Chinese hamster V79 cell variants that exhibit different degrees of resistance to the cytotoxic effect of mycophenolic acid, a specific inhibitor of IMPDH. Western blot (immunoblot) analysis with an IMPDH antiserum revealed a 14- to 27-fold increase in the amount of enzyme in the mycophenolic acid-resistant cells. The antiserum was also used to screen for a phage containing the IMPDH cDNA sequence from a λgt11 expression library. Northern blot (RNA blot) analyses of total cellular and poly(A)$^+$ RNA showed that an IMPDH cDNA probe hybridized to a 2.2-kilobase transcript, the amount of which was associated with increased resistance. Southern blotting with the probe indicated an amplification of the IMPDH gene in the mycophenolic acid-resistant cells. Our findings suggest that the acquired mycophenolic acid resistance of the V79 cell variants is associated with increases in the amount and activity of IMPDH and the number of IMPDH gene copies.

IMP dehydrogenase (IMPDH) (EC 1.2.1.14), an enzyme that regulates guanine nucleotide biosynthesis, catalyzes the reaction of the branch point in the synthesis of adenine and guanine nucleotides. In both normal and malignant cells, the activity of this enzyme is positively correlated with cellular growth rate (26, 27). Furthermore, in tumor cells IMPDH inhibitors cause a dose-dependent reduction in growth (10, 13, 22) and in the human promyelocytic HL-60 leukemia cell line also cause a dose-dependent induction of cell maturation (12, 20). These observations suggest that IMPDH activity and the production of guanine nucleotides are involved in the regulation of growth and differentiation in mammalian cells.

To study the control of IMPDH in mammalian cells, we isolated from the Chinese hamster V79 cell line, variants with altered IMPDH activity. The variants were obtained by treating the V79 cells with 0.5 μg of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and then selecting colonies of cells in the presence of 1 μg of mycophenolic acid (MPA), a cytotoxic IMPDH inhibitor (4), per ml as described previously (6). The resistance level of one of these cell variants was further increased by a stepwise selection in the presence of increasing concentrations of MPA. After adaptation to the higher concentration of MPA, the cells were seeded in medium containing an increased concentration of MPA at 200 cells per 60-mm petri dish, and MPA-resistant colonies were isolated 8 days later. This procedure, which was repeated a number of times, resulted in four cell clones, VM1 through VM4, which were resistant to 5, 10, 25, and 50 μg of MPA per ml, respectively, whereas the parental V79 cells were resistant to only 0.1 μg of MPA per ml (Table 1). The increased resistance to MPA cytotoxicity in the variant cells was associated with an increased IMPDH activity in their cell homogenates, with VM1 cells exhibiting about a 6-fold increase in IMPDH activity over the parental cells and VM2, VM3, and VM4 cells expressing about 7-, 9-, and 11-fold increases, respectively, in IMPDH activity (Table 1).

To further investigate the basis for the increased enzyme activity in the MPA-resistant cells, we isolated and characterized the enzyme from VM2, one of the resistant variants. Two-dimensional gel electrophoresis established a molecular weight of 56,000 and verified the purity of the enzyme. The K_m values of the VM2 cell enzyme for IMP and NAD were similar to those reported previously for partially purified IMPDH from V79 cells (6) and human placenta (5), as well as those of purified IMPDH from Yoshida sarcoma ascites cells (17). Moreover, the VM2 cell enzyme retained a high sensitivity to MPA with a K_i in the nanomolar range. The similarity of the kinetic parameters of the VM2 and parental V79 cell enzymes indicates that the gene coding for the enzyme in the VM2 cells has not undergone an alteration that would change the affinity of the enzyme for the substrate or its sensitivity to MPA. Other reports with MPA-resistant cells have attributed the acquired resistance to either changes in the structure of the gene coding for the enzyme (24) or altered regulation of enzyme synthesis resulting from either gene amplification or changes in transcriptional or posttranscriptional controls (6). Our results suggest that the

TABLE 1. Activity and amount of IMPDH in cell homogenates from cells of the V79 cell line and its MPA-resistant cell variants

Cell type	Resistance to MPA (μg/ml)	Enzyme activity[a] (mU/mg of total protein)	Enzyme amt[b] (μg/mg of total protein)
V79	0.1	0.7	0.3
VM1	5.0	4.0	4.1
VM2	10.0	4.7	4.8
VM3	25.0	6.3	5.4
VM4	50.0	7.4	8.1

[a] IMPDH activity was determined as continuous IMP-dependent NAD reduction at 37°C by monitoring the change in absorbance at 340 nm as described by Proffitte et al. (18) as well as by measuring the conversion of [^{14}C]IMP into [^{14}C]XMP (6). In the latter case, the reaction mixture was similar to that described for the spectrophotometric assay, except for the addition of 0.2 mM α,β-methylene adenosine 5′-diphosphate to inhibit 5′-nucleotidase activity and 0.04 μCi of [8-^{14}C]IMP. One unit of enzyme activity was defined as the amount forming 1 μmol of either [^{14}C]XMP or NADH per min at 37°C under the prescribed assay conditions.

[b] The amount of cellular IMPDH was quantitated by densitometric scanning of Western blots. Blot analyses (23) were carried out with the IMPDH-specific immunoglobulin G. Immune complexes were visualized by incubation with goat anti-rabbit immunoglobulin G followed by rabbit immunoglobulin G conjugated with horseradish peroxidase and 4-chloronapthol (3 mg/ml in 20% methanol with 0.01% H_2O_2) used as the substrate (9). Peak areas corresponding to the amounts of IMPDH in the cells were determined by comparison with standards of the purified protein electrophoresed through the same gel.

* Corresponding author.

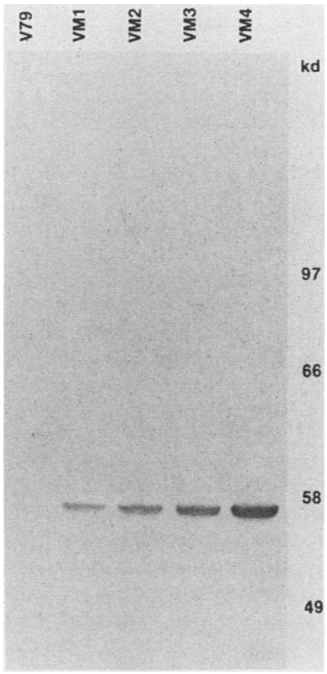

FIG. 1. Western blot of proteins from the cells of the V79 cell line and its MPA-resistant cell variants. Cell homogenates containing 25 μg of protein were electrophoresed through a 7.5% polyacrylamide gel (11) and then transferred to nitrocellulose. Western blot analyses were performed as described in footnote *b* of Table 1. kd, Kilodaltons.

basis for the increased resistance of the VM2 cells is an alteration in the regulation of IMPDH.

The purified IMPDH from the VM2 cells was used to prepare a high-titer specific antibody (25). This antiserum, in conjunction with the Western blotting (immunoblotting) technique, was used to determine the amount of IMPDH in parental V79 cells and in cells from the MPA-resistant variants (Fig. 1). This antiserum reacted, in both the V79 and variant cells, with a protein of 56 kilodaltons, corresponding to IMPDH. Furthermore, the amount of enzyme in MPA-resistant cell variants was positively correlated with the degree of resistance and was more than 1 order of magnitude higher than the amount of enzyme detected in the parental V79 cells (Table 1). A comparison between cells from the MPA-resistant cell variants and from the parental V79 cell line revealed that the amount of enzyme in the variant cells was about 14- to 27-fold higher than the amount detected in V79 cells.

The specific anti-IMPDH antiserum was also used to screen a λgt11 expression library derived from mouse bone marrow (7). A phage with a 750-base-pair cDNA insert was isolated from this library as outlined by Maniatis et al. (14), and the insert was subcloned into a pUC8 vector designated pUC8/IMPDH5. The confirmation of IMPDH coding sequences in this cDNA probe was obtained by translational arrest. These experiments used poly(A)$^+$ RNA isolated from the VM2 cells as a source for IMPDH mRNA. Analysis of the results indicated that the pUC8/IMPDH5 probe effectively blocked the translation of an immunoprecipitable IMPDH product in a dose-dependent manner (Table 2). The pUC8/IMPDH5 DNA at 0.6 and 6 μg per assay reduced the translation product by 71 and 85%, respectively, compared with a reduction of only 24 and 29%, respectively, with the same amount of pUC8 vector DNA without the insert used per assay (Table 2). These results validated the identity of the clone as a cDNA probe for IMPDH.

TABLE 2. Arrest of specific poly(A)$^+$ RNA translation by IMPDH cDNA

Translational arrest[a] by:	DNA amt (μg)	Relative amt of immunoprecipitated IMPDH (%)
	0	100[b]
pUC8	0.6	76
	6.0	71
pUC8/IMPDH5	0.06	60
	0.6	29
	6.0	15

[a] In vitro translation reactions were performed with poly(A)$^+$ RNA obtained from the VM2 MPA-resistant cell as recommended by the supplier (Promega Biotec Co., Madison, Wis.). Polypeptides were immunoprecipitated in 50 mM Tris hydrochloride buffer (pH 7.6) containing 150 mM NaCl, 5 mM EDTA, 1 mM methionine, 0.5% Triton X-100, and 0.5% sodium deoxycholate by the addition of 5 μg of IMPDH-specific immunoglobulin G. Immune complexes were recovered by adsorption to IgGsorb (The Enzyme Center, Malden, Mass.) and brief centrifugation. Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography of the dried gel.

[b] Relative amount of immunoprecipitated IMPDH was derived by dosimetric analysis of the fluorograph. Numbers represent the averages of two independent experiments. The translational activity of the pUC8/IMPDH5-arrested RNA samples was restored to the level of the pUC8 samples by heat dissociation of the hybrids.

Two methods were used to analyze the relationship between degree of resistance and IMPDH mRNA levels in both V79 cells and MPA-resistant cell variants. One method involved the use of an in vitro translation system as a means

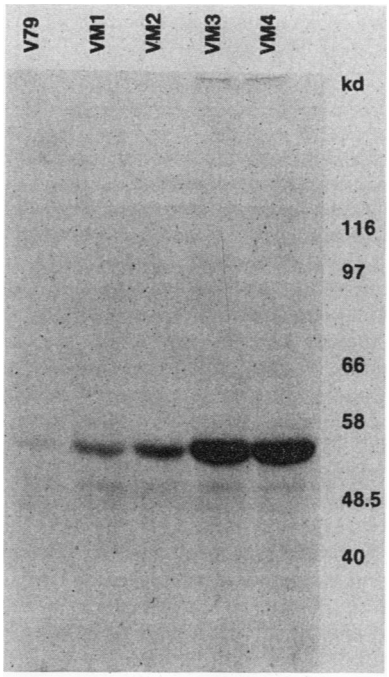

FIG. 2. RNA purified by centrifugation through a CsCl cushion as described by Chirgwin et al. (2). poly(A)$^+$ RNA was prepared by two cycles of oligodeoxythymidylate cellulose (Collaborative Research, Inc., Lexington, Mass.) chromatography (14). In vitro translation reactions were performed as described in footnote *a* of Table 2. kd, Kilodaltons.

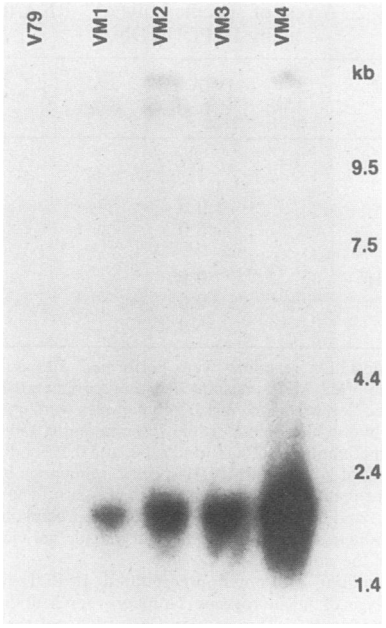

FIG. 3. Northern blot of RNA from cells of the V79 cell line and its MPA-resistant cell variant performed by electrophoresis of 12 μg of total cellular RNA through a 2.2 M formaldehyde–1% agarose gel and blotting to Nytran (Schleicher & Schuell Co., Keene, N.H.). The blot was hybridized to a labeled insert from pUC8/IMPDH5, which was isolated by an electroelution technique (28) and labeled to high specific activity as described by Feinberg and Vogelstein (3). The same RNA samples were also hybridized with a control DNA probe, which was composed of a fragment of the rat β-actin cDNA. Unlike the results obtained with the IMPDH probe, the intensity of the hybridization signals obtained with the β-actin probe was similar for RNA from V79 and the variant cells. After being washed, the blots were dried, sealed in Saran Wrap, and exposed to X-ray film (Fuji RX) with an intensifying screen (Dupont Cronex Lightning Plus) at −70°C (21). kb, Kilobases.

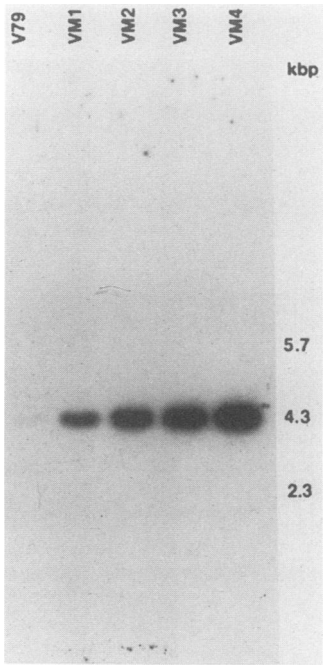

FIG. 4. Southern blot analysis of DNA from cells of the V79 cell line and its MPA-resistant cell variants of IMPDH. DNA, purified and digested with the *Hin*dIII restriction enzyme, was electrophoretically separated in a 0.8% agarose gel. The DNA was fragmented by acid depurination, transferred to a nylon membrane by the procedure of Reed and Mann (19), and hybridized with the insert from pUC8/IMPDH5. In a control experiment with a c-*fos* probe (16), hybridization signals with similar intensity were obtained for the same DNA from both V79 and the variant cells, which confirmed that equivalent amounts of DNA were analyzed. kbp, Kilobase pairs.

of detecting in total cellular poly(A)$^+$ RNA, the amounts of specific mRNA capable of directing the synthesis of immunoprecipitable IMPDH. On the basis of the levels of the immunoprecipitable proteins (Fig. 2), we concluded that the amount of translatable mRNA, which directs the synthesis of IMPDH, was increased in the resistant cells. The increase in the amount of this translatable message roughly corresponded to the increase in the amount of cellular enzyme in the parental and variant cells (Fig. 1 and 2). The molecular weight of the in vitro translated protein was also similar to that of the protein purified from the VM2 cells, suggesting that the enzyme does not result from a major proteolytic processing of an IMPDH proenzyme. The other approach involved the use of the pUC8/IMPDH5 cDNA probe to characterize the steady-state level of IMPDH mRNA synthesis (Fig. 3). Hybridization signals corresponding to a 2.2-kilobase message were detected in both total cellular RNA and poly(A)$^+$ RNA samples. This 2.2-kilobase transcript is sufficient to code for the enzyme, which has an estimated molecular weight of 56,000. The steady-state level of this message in the various cell types correlated to their degree of resistance. These mRNA levels were similar to those estimated from the amount of protein measured by the in vitro translation assay (Fig. 2 and 3).

The pUC8/IMPDH probe was also used to determine possible major changes in IMPDH gene copy number or structure. Analysis of the Southern blots of the high-molecular-weight DNA isolated from the parental V79 cells and from the MPA-resistant cell variants after digestion with the *Hin*dIII restriction enzyme revealed that the gene for IMPDH, like other genes (1, 8, 15), is amplified in the drug-resistant cells (Fig. 4). Furthermore, the degree of gene amplification in the different cell variants was positively correlated to their order of resistance to MPA and closely resembled the amounts of cellular enzyme. The level of IMPDH gene amplification in the cells from the most resistant MPA cell variant, VM4, was approximately 20-fold higher than the level of this gene in the parental V79 cells. Southern blot analysis of the high-molecular-weight DNA digested with other restriction enzymes showed a similar variation in the intensity of the hybridization signals for V79 and the variant cell DNA. Using the pUC8/IMPDH5 probe, we obtained identical fragment patterns for each restriction enzyme, which suggested that no major internal rearrangement of the IMPDH gene had occurred.

Thus, our results show that amplification of the IMPDH gene appears to be closely involved in establishing resistance in the V79 cell variants, with each selection step of increased MPA concentration resulting in a corresponding increased level of IMPDH gene amplification. These variant cells should be useful to study the effects of altered IMPDH regulation on cellular growth. Furthermore, the cDNA for IMPDH will provide a valuable tool for elucidating the role of a growth regulatory enzyme in cell growth and differentiation.

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