Mutations Affecting the Structure of Hypoxanthine: Guanine Phosphoribosyltransferase in Cultured Chinese Hamster Cells

(8-azaguanine/crossreacting material/antiserum/clones/affinity chromatography)

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ABSTRACT 14 Clones resistant to 8-azaguanine, isolated from mutagenically treated cultured cells from Chinese hamsters, were tested for loss of enzymic and immunological activities of hypoxanthine:guanine phosphoribosyltransferase. Three of the clones had no enzymic activity but reacted strongly with antiserum prepared against the enzyme (CRM⁺), indicating the presence of a defective enzyme protein, probably caused by a mutation in its structural gene. The other 11 mutants had little or no enzymic or immunologic activity. Revertant clones with positive enzymic and crossreacting immunologic activities were derived from both classes of mutants. The antiserum has been used for rapid purification of the phosphoribosyltransferase from Chinese hamster cells by immunoadsorbant affinity chromatography.

Biochemical characterization of induced phenotypic alterations of animal cells is necessary to clarify their genetic basis. We have investigated the hypoxanthine:guanine phosphoribosyltransferase activity (HGPRTase; EC 2.4.2.8.) of 35 clones of Chinese hamster lung cells (CHL) resistant to 8-azaguanine. Resistance was induced by the mutagens *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and ethylmethanesulfonate (1). Sixteen of the 35 resistant clones showed little or no HGPRTase activity (HGPRTase⁻), as determined directly *in vitro* and indirectly by the incorporation of radioactive hypoxanthine into nucleic acid of whole cells. The remaining 19 mutants had measurable HGPRTase activity. Revertant HGPRTase⁺ clones were isolated from HGPRTase⁻ clones by selection in a medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) (2). In this report, we describe the results of studies with anti-HGPRTase serum to test for immunologically crossreactive material (CRM) in 8-azaguanine-resistant clones and in their revertants.

MATERIALS AND METHODS

Cell Growth and Extract Preparation. Growth and selection of 8-azaguanine-resistant clones and extract preparation were as described (1). Resistant clones, designated as N or E, were isolated from the wild-type clone (A-3) after nitrosoguanidine or ethylmethanesulfonate mutagenesis, respectively. Revertants are designated by the parental nomenclature (e.g., N5), followed by the letters N, E, or S (spontaneous) to indicate the treatment used for reversion

(e.g., N5N4). Extracts used in precipitation-inhibition studies were dialyzed for 24 hr against 0.01 M potassium phosphate buffer (pH 7.2). Human erythrocyte lysates (3) were dialyzed in an identical manner. The type and source of other cultured cells were: Hela S3 (Dr. Saul Kit); L929 (American Type Culture Collection); NIH Balb 3T3 (Dr. Janet Butel); and human fibroblasts from male foreskin (primary isolate).

Preparation of Antibody. The livers (17.7 g) of 20 Chinese hamsters were homogenized with a Potter-Elvehjem homogenizer in 100 ml of buffer containing: 0.03 M Tris·HCl, pH 7.0–7 mM 2-mercaptoethanol. The extract was centrifuged at 30,000 × *g* for 30 min at 4°, and the supernatant was centrifuged at 150,000 × *g* for 120 min at 4°. HGPRTase was partially purified from the high-speed supernatant fraction by (a) precipitation with ammonium sulfate (35–70% saturation, 26 g/105 ml = 35%); (b) passage through a carboxymethyl-(CM)-Sephadex column (2.5 × 25 cm) equilibrated with 0.01 M potassium phosphate (pH 7.0)–7 mM 2-mercaptoethanol; (c) elution from a DEAE-cellulose column (2.5 × 25 cm) equilibrated with 0.01 M potassium phosphate (pH 7.0) by a 800-ml linear gradient from 0.01 M to 0.2 M potassium phosphate (pH 7.0) (activity was eluted at 0.09 M); and (d) treatment at 80° for 5 min, followed by centrifugation at 30,000 × *g* for 20 min and recovery of the supernatant. The HGPRTase specific activity was increased 17.5-fold, with a 17% recovery by these steps.

New Zealand white rabbits were immunized initially and at 5 weeks by subcutaneous injection of the partially purified HGPRTase (0.48 mg in 1 ml) mixed with 1 ml of complete Freund's adjuvant. An immunoglobulin that precipitated HGPRTase activity was detected 4 weeks after the initial immunization. This immunological response was variable, since anti-HGPRTase activity was not detected in the serum of several other rabbits injected with similar preparations of HGPRTase from liver and cultured cells. All sera used in these studies were dialyzed 24 hr against 1,000 volumes of buffer containing 0.01 M potassium phosphate (pH 7.2)–75 mM NaCl.

Double-diffusion analysis (IDF Cell II, Cordis Lab.) of the serum drawn 8 weeks after HGPRTase injection revealed at least three precipitin bands with Chinese hamster liver and cultured cell extracts.

HGPRTase Immunoprecipitation and Precipitation-Inhibition. HGPRTase immunoprecipitation and precipitation-

Abbreviations: HGPRTase, hypoxanthine:guanine phosphoribosyltransferase; CRM, crossreacting material (immunologically).

inhibition reactions were performed in 1-ml conical tubes. The 95- μ l reaction mixtures contained a source of dialyzed HGPRTase, dialyzed rabbit serum, and a final concentration of 0.01 M potassium phosphate (pH 7.2)–11 or 4 mM NaCl for the precipitation or precipitation-inhibition reactions, respectively. These buffer conditions were rigidly maintained, since interference with precipitation occurred in reactions containing 0.15 M NaCl, more than 5 mM 2-mercaptoethanol, or 20% sucrose. All reactions were incubated for 30 min at 37°, then for 1.5 or 15 hr, as indicated, at 4°. 5 μ l Of anti-rabbit gammaglobulin prepared in goats, (Antibodies, Inc.) was then added, followed by 2 hr of incubation at 4°. The HGPRTase-antibody complex was sedimented by centrifugation at 12,000 $\times g$ for 20 min at 4°. All HGPRTase and adenine phosphoribosyltransferase (APRTase, EC 2.4.2.7) activities in the supernatant were determined at 37° for 20 min with 0.02- or 0.01-ml aliquots, respectively (1). The antibody precipitation does not inactivate HGPRTase, since enzyme activity could be recovered quantitatively from the precipitates.

Immunoprecipitation of HGPRTase from cultured cells by rabbit antiserum was enhanced by the presence of anti-rabbit gammaglobulin serum prepared in goats (Table 1). Precipitation of HGPRTase required the anti-HGPRTase serum from rabbit. Anti-rabbit gammaglobulin from goat improved reproducibility, shortened the precipitation time, facilitated precipitation of small amounts of HGPRTase, and conserved rabbit anti-HGPRTase serum. Goat anti-gammaglobulin was used, therefore, in all precipitation reactions. All sera were assayed and found to be free of HGPRTase activity. The apparent stimulation of HGPRTase by small amounts of rabbit antiserum and control serum was observed repeatedly, and we feel that the effect represents HGPRTase stabilization similar to that reported with albumin (4).

Precipitation-inhibition studies used wild-type HGPRTase, from cultured cells, that was partially purified by 35–70% ammonium sulfate fractionation, followed by dialysis against 0.01 M potassium phosphate (pH 7.2)–1 mM 2-mercaptoethanol. Each 95- μ l reaction contained 46 μ g of HGPRTase and 5 μ l of dialyzed rabbit antiserum. Under these conditions, 95% of the HGPRTase activity was precipitated. Different amounts of individual extracts were added to these reactions, and the reaction mixtures were incubated and centrifuged as indicated above. The immunological activity (CRM) of an extract was quantitated by the amount of wild-type HGPRTase activity that was displaced from the antibody complex and appeared in the supernatant fraction. The CRM activity corresponds to nmol of IMP formed per min per mg of extract protein added. Extracts from all HGPRTase⁺ clones used in precipitation-inhibition assays had no HGPRTase activity, and did not stimulate or inhibit wild-type HGPRTase (data not shown).

Immunoabsorbent Affinity Chromatography. 2 ml Of rabbit anti-HGPRTase serum was precipitated with ammonium sulfate (33%, 0.47 g). The precipitate was dissolved in 1.5 ml of 0.1 M NaHCO₃ (pH 8.0) and dialyzed against this solution (1000 volumes) for 16 hr. Immunglobulin was linked to agarose with cyanogen bromide (5, 6). Cyanogen bromide (500 mg) was added to 5 ml of Sepharose 4B in a total volume of 11 ml of 0.1 M NaHCO₃ (pH 8.0). After 8 min of incubation at 20°, during which the pH was maintained at 11 $\pm$ 0.5 with 2 M NaOH, the Sepharose was washed with 450 ml of cold 0.1 M NaHCO₃ (pH 8.0). Sepharose (2 ml) was mixed

FIG. 1. Anti-HGPRTase enzyme specificity. Precipitation reactions contained 124 μ g of unfractionated cultured cell extract and control serum (○) or anti-HGPRTase serum (●) as indicated; they were incubated 15 hr at 4° before addition of anti-rabbit gammaglobulin. The procedures for HGPRTase precipitation and quantitation of HGPRTase and APRTase in the supernatant are described in *Methods*. The values for reaction mixtures incubated without enzyme added (0.016 and 0.031 nmol) were subtracted from HGPRTase (*top*) and adeninephosphoribosyltransferase, (*bottom*) values, respectively. Data are given as nmol per 20 min per 20 μ l of supernatant (*top*) or per 10 μ l of supernatant (*bottom*).

immediately with the partially purified globulin at 4°, resulting in 80% linkage of the immunoglobulin to the Sepharose.

Immunoglobulin affinity columns containing 1 ml of Sepharose-globulin were equilibrated with 0.01 M potassium phosphate (pH 7.2). Extracts equilibrated in the same buffer were applied to the columns and allowed to adsorb for 10 min. Columns were washed with 0.75 ml of 0.15 M NaCl in the above buffer, and the HGPRTase was eluted with 0.08 M NH₄OH in 20% sucrose. 0.75-ml Fractions were collected in tubes containing 0.15 ml of 1 M potassium phosphate (pH 7.2 or 6.5) for the NaCl and NH₄OH washes, respectively. These buffers adjust the pH of all fractions to 7.2. The HGPRTase activity of an aliquot of each fraction was further stabilized by addition of bovine-serum albumin and 2-mercaptoethanol to final concentrations of 0.5 mg/ml and 7 mM, respectively. All column operations were conducted at 4°. Protein determinations were performed by the method of Lowry (7).

Materials. Sources of material were: [8-¹⁴C]hypoxanthine (53.7 Ci/mol) and [8-¹⁴C]adenine (41.3 Ci/mol), Schwarz/Mann; Sepharose 4B, Pharmacia; cyanogen bromide, Eastman; *p*-chloromercuribenzoate, K and K Laboratories; Freund's adjuvant, Difco; all others as described (1).

RESULTS

The enzymic specificity of rabbit anti-HGPRTase serum is demonstrated in Fig. 1. Antiserum from a rabbit immunized with Chinese hamster liver HGPRTase precipitated HGPRTase from cultured cells (shown) and liver (not shown)

FIG. 2. Specificity of anti-HGPRTase serum for HGPRTase from various organisms. Each precipitation reaction was incubated 15 hr at 4° before addition of anti-rabbit gammaglobulin from goat, and contained—where indicated—124 μ g of Chinese hamster lung cell extract (○), 77 μ g of L929 extract (□), or 472 μ g of human erythrocyte lysate (Δ). Reactions containing control and anti-HGPRTase sera are indicated by closed and open symbols, respectively. The 100% values were: Chinese hamster lung cell extract, 0.967 nmol; L929 extract, 1.137 nmol; and human erythrocyte lysate, 1.165 nmol.

equally well. This anti-HGPRTase serum had no effect on adenine phosphoribosyltransferase (APRTase, EC 2.4.2.7), a related purine “salvage” enzyme, from cultured cells (shown) and liver (not shown). Control serum had little effect on immunoprecipitation of either enzyme.

The specificity of antisera for HGPRTase from various organisms is illustrated in Fig. 2. The HGPRTase activities of human erythrocytes, cultured human fibroblasts, and HeLa cells (last two not shown) were not precipitated by the antiserum to Chinese hamster HGPRTase. The discriminatory capacity of this antibody could be useful in studies of human-Chinese hamster cell hybrids. The slopes of the pre-

FIG. 3. Immunoprecipitation of HGPRTase from Chinese hamster lung cell mutants. Each precipitation reaction contained extracts as indicated, and was incubated 15 hr at 4° before addition of anti-rabbit gammaglobulin from goat. The quantity and source of protein for each determination was as follows: *upper panel*; 124 μ g of wild-type cell extract (○), 63 μ g of clone N9 (Δ), 237 μ g of clone E47 (□); *lower panel*; 124 μ g of wild-type cell extract (○), 232 μ g of clone E31S1 (Δ), 158 μ g of clone N20S1 (□), 119 μ g of clone N15N1 (◇). Reactions containing control and anti-HGPRTase sera are indicated by closed and open symbols, respectively. Extracts in the upper panel were prepared from 8-azaguanine-resistant cell clones, while those in the lower panel were prepared from HGPRTase⁺ revertant clones derived from HGPRTase⁻ mutants.

TABLE 1. Immunoprecipitation of Chinese hamster lung cell HGPRTase

μ l of anti-HGPRTase	Supernatant HGPRTase	μ l of anti-HGPRTase	Supernatant HGPRTase
<i>Minus anti-rabbit gammaglobulin from goat</i>		<i>Plus anti-rabbit gammaglobulin from goat</i>	
0	0.777	0	0.787
0.27	1.006	0.27	0.455
0.67	1.094	0.67	0.106
1.33	0.324	1.33	0.041

Each 95- μ l precipitation reaction contained 124 μ g of unfractionated HGPRTase, 5 μ l of anti-rabbit gammaglobulin from goat, rabbit anti-HGPRTase, as indicated, and buffer. These reactions were incubated 1.5 hr at 4° before addition of anti-rabbit gammaglobulin, followed by precipitation and HGPRTase assay of the supernatant fraction, as described in *Methods*. Supernatant HGPRTase is given as Δ nmol of [¹⁴C]IMP per 20 min per 20 μ l. The value for reaction mixtures incubated without enzyme added (0.016 nmol) was subtracted from all values.

cipitation curves of mouse L929 and NIH Balb 3T3 (not shown) HGPRTase differ significantly from that of Chinese hamster HGPRTase, indicating their lack of structural homology. The lack of complete precipitation of L929 and 3T3 HGPRTase at high concentrations of antiserum could be a further reflection of the lack of homology, or an indication of the presence of HGPRTase isozymes in the mouse cells. Isozymic forms of HGPRTase have been detected in other species (3, 4, 8).

The immunological precipitation properties of HGPRTase from several 8-azaguanine-resistant CHL clones are given in Fig. 3. The cell lines are of two types: (i) HGPRTase⁺ clones resistant to 8-azaguanine and (ii) HGPRTase⁺ clones (revertants) derived from HGPRTase⁻ clones. The HGPRTase activities in extracts from all HGPRTase⁺ cell lines thus far studied (five 8-azaguanine-resistant clones and six revertants) were precipitated by the antibody; thus, they possess the HGPRTase immunological determinants. Alterations in HGPRTase structure that affect immunological reactivity or enzyme specific activity or stability could theoretically give altered immunoprecipitation. Slight alterations in the HGPRTase precipitation curves for some clones were observed. Because these changes are small as compared with the striking difference between Chinese hamster lung cell and L929 HGPRTase (Fig. 2), they are of questionable significance and require further investigation.

The immunological properties of HGPRTase⁻ clones were compared by a modification of the immunoprecipitation method. Since three immunoprecipitin lines were detected by immunodiffusion analysis, an assay method was used that measures competition for the HGPRTase-antibody complex. This immunologic method, precipitation-inhibition, avoids the study of unrelated antibodies when HGPRTase activity in the supernatant is used as the endpoint (8–10). The inhibition of precipitation of wild-type HGPRTase⁺ by extracts from HGPRTase⁻ clones is shown in Fig. 4. The wild-type HGPRTase was displaced from the antibody by increasing amounts of extract in a linear manner after the slight antibody excess was overcome. The crossreacting activity (CRM) of each extract was calculated from the linear portion

TABLE 2. Immunologic characteristics of Chinese hamster lung cell mutant extracts

Phenotypic characteristics	Clone	CRM activity
Wild type	A-3	0.57
8-azaguanine-resistant, <i>in vitro</i>	N-1	0.02
HGPRTase ⁻ , fails to	N-5	0.33
incorporate [¹⁴ C]hypoxanthine into	N-6	0.02
whole cells	N-10	0.00
	N-15	0.09
	N-17	0.01
	N-19	0.02
	N-20	0.01
	E-26	0.02
	E-27	0.02
	E-31	0.00
	E-33	0.01
	E-36	0.00
	E-37	0.00
	E-43	0.01
8-azaguanine-resistant, <i>in vitro</i>	N-2	0.00
HGPRTase ⁻ , and incorporates	N-21	0.00
[¹⁴ C]hypoxanthine into whole cells	N-22	0.01
	E-28	0.00
	E-39	0.44

CRM specific activity was determined as given in the legend to Fig. 4 and *Methods*. CRM activity is expressed as nmol of [¹⁴C]IMP formed per min per mg of extract added, and was determined from a linear region of each graph. All values were based on total HGPRTase and extract protein added to 0.10-ml precipitation reactions.

of the curve. All mutant extracts used did not have HGPRTase activity, and did not stimulate or inhibit wild-type HGPRTase (see *Methods*). Since attempts to alter wild-type enzyme to an HGPRTase⁻, CRM⁺ state by heating, aging, and treating with *p*-chloromercuribenzoate were not successful, we used enzymically active HGPRTase from the wild type for comparison. Consequently, differences between HGPRTase⁻ extracts are comparable, but absolute differences from the wild-type extract are affected by the enzyme activity present in the latter case. It is clear, however, that there are large differences in the CRM activity of different HGPRTase⁻ clones. The precipitation-inhibition activities of extracts from CRM⁺ HGPRTase⁻ and the wild-type clones were eliminated completely when they were heated for 2 min at 100°, suggesting that inhibition is due to proteins in the extracts.

The CRM activities of several 8-azaguanine-resistant cell extracts are summarized in Table 2. With the exception of the wild-type (A-3), there was no detectable HGPRTase enzymic activity in any of the extracts studied. Furthermore, most clones do not incorporate [¹⁴C]hypoxanthine from culture medium into nucleic acid. A second group of clones listed in the lower portion of Table 2 have no detectable HGPRTase activity *in vitro*, but do incorporate [¹⁴C]hypoxanthine into nucleic acid and, thus, have *in vivo* HGPRTase activity. Three HGPRTase⁻ clones (N-5, N-15, and E-39) are significantly CRM⁺ and are, therefore, felt to represent examples of mutations in the structural gene for HGPRTase. The re-

FIG. 4. Precipitation-inhibition by HGPRTase⁻ mutants. All precipitation reactions contained 46 μg of wild-type Chinese hamster lung cell HGPRTase, partially purified by ammonium sulfate precipitation (35–70%); 1.33 μl of anti-HGPRTase; and, as indicated, different amounts of dialyzed extracts from mutants E-39 (▲), N-5 (○), N-21 (□), E-31 (△), and wild-type A-3 (●). All precipitation reactions are incubated 1.5 hr at 4° before addition of anti-rabbit gammaglobulin from goat. The supernatant HGPRTase activity in the absence and presence of anti-HGPRTase serum were 1.094 and 0.062 nmol per 20 min per 20 μl, respectively. The 0.062 nmol background was subtracted from all values.

maining clones have very low or absent CRM activity by this test. The molecular basis for the HGPRTase⁻, CRM⁻ results is discussed below. We have previously concluded that mutants that undergo reversion do not represent large deletions. Revertants (HGPRTase⁺) have been obtained from the following mutants: N-1, N-5, N-6, N-10, N-15, N-17, N-19, N-20, E-26, E-27, E-31, E-37, and E-43 (unpublished results). No revertants of E-33 and E-36 have been isolated. The six revertants selected on HAT medium studied to date are HGPRTase⁺ and CRM⁺. Four of these revertants (N20S1, E31S1, Fig. 3; N14N1, E31N4, not shown) were derived from HGPRTase⁻, CRM⁻ clones, and two revertants (N15N1, Fig. 3; N5N3, not shown) were derived from HGPRTase⁻, CRM⁺ clones.

Structural analysis of HGPRTase from CRM⁺ clones may be facilitated by an additional use of anti-HGPRTase serum. The HGPRTase of cultured cells was purified by affinity chromatography with anti-HGPRTase linked to Sepharose 4B (Fig. 5). Crude cellular extracts of HGPRTase were

FIG. 5. Immunoadsorbent affinity chromatography of HGPRTase. An unfractionated A-3 cell extract (1.55 mg in 0.75 ml) was applied to and eluted from an anti-HGPRTase-Sepharose 4-B column. 0.03-ml aliquots were assayed (1) for 20 min for HGPRTase activity (○). The total protein in each fraction (●) is indicated. The value for reaction mixtures incubated without enzyme added (0.011 nmol) was subtracted from all HGPRTase values.

applied to and recovered from the immunoadsorbent affinity column with considerable purification. Recovery of HGPRTase activity varied from 19 to 25% in the elution peak, and depended on the method of enzyme elution and stabilization. Because of limited quantities of cellular extract and the extremely small amount of protein present in the elution peak, the overall purification of the HGPRTase protein has not been determined accurately. The HGPRTase purified by this technique was analyzed by Na dodecyl sulfate-acrylamide gel electrophoresis (11); only three protein bands were detected, while innumerable bands were detected in void fractions. With further development, this method should provide a rapid and simple means of purifying HGPRTase of CRM⁺ azaguanine-resistant clones and revertants for structural analysis.

DISCUSSION

The molecular basis of the stable phenotypic variants of cultured animal cells is currently under investigation in many laboratories. Acquired drug resistance, particularly to 8-azaguanine, has been the most extensively studied phenotype. The data reported here suggest that three of our 8-azaguanine-resistant Chinese hamster clones (N-5, N-15, and E-39) were caused by a mutation in the HGPRTase structural gene. Extracts from these clones showed no detectable HGPRTase enzymic activity, although immunologic procedures detected the presence of HGPRTase protein. Thus, these clones synthesize an immunologically recognizable, but enzymically inactive, form of the HGPRTase molecule.

In humans with the genetic disease Lesch-Nyhan syndrome, cells that are 8-azaguanine resistant and HGPRTase⁻ have been detected (12). In several Lesch-Nyhan patients, the cells show a HGPRTase⁻ CRM⁺ phenotype, similar to the three Chinese hamster clones (8, 13).

For the clones that are CRM⁻ HGPRTase⁻, it is difficult to establish the mechanism causing this phenotype, since drug-resistant variants may result from stable epigenetic events—in addition to mutations. Recently, the possible epigenetic origin of tissue culture variants was emphasized by two reports that examined the relationship between the frequency of drug resistance and the chromosomal ploidy of the cell lines. Harris found that the frequency of 8-azaguanine resistance did not vary significantly between diploid, tetraploid, and octaploid clones of Chinese hamster cells (14). Mezger-Freed reported that the frequency of bromodeoxyuridine resistance for diploid frog cells did not approach the square of the frequency for haploid cells (15). These studies suggest that in certain cases the frequency of drug resistance does not correlate readily with a simple mutational model.

Nevertheless, we favor the possibility that at least some of our HGPRTase⁻ CRM⁻ clones are mutants, since these were

induced with mutagens and isolated with selective techniques identical to those used for the CRM⁺ HGPRTase⁻ mutants. It is unlikely that CRM⁺ mutants should occur exclusively. If any of the HGPRTase⁻, CRM⁻ clones are HGPRTase structural gene mutants, we might expect that some premature chain-termination (nonsense) mutations have occurred in the group. Studies with *Escherichia coli* alkaline phosphatase indicated that nonsense mutations are frequently CRM⁻ (16, 17). Similar immunological studies of β -galactosidase mutants from *E. coli* have indicated that this correlation is not absolute, since nonsense mutations closer to the carboxyl terminus may be CRM⁺ (18). Furthermore, the relative CRM reactivity varies with the type of immunological assay used. Subject to these reservations, some of our CRM⁻ HGPRTase⁻ mutants have three characteristics suggestive of nonsense mutants, namely extreme negative phenotype (1), revertibility (unpublished results), and CRM negativity. Our data indicate that the HGPRTase locus of Chinese hamster cells is a genetically useful marker for the study of mutational events in cultured mammalian cells.

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