Proc. Natl. Acad. Sci. USA
Vol. 73, No. 12, pp. 4589–4593, December 1976
Cell Biology

Analysis of HeLa cell hypoxanthine phosphoribosyltransferase mutants and revertants by two-dimensional polyacrylamide gel electrophoresis: Evidence for silent gene activation

(immunoprecipitation/Lesch-Nyhan syndrome/somatic cell genetics/isoelectric focusing)

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Communicated by Howard K. Schachman, September 29, 1976

ABSTRACT **The spot corresponding to hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) has been identified in two-dimensional polyacrylamide gels of HeLa cell extracts. This spot is absent in gels of 24 HPRT deficient mutants. A missense mutant displays a new HPRT spot at the same molecular weight but different isoelectric focusing position. Five independently isolated revertants of the missense mutant display spots corresponding to both the wild-type and mutant proteins indicating that they synthesize HPRT from two separate genes. If the missense protein is synthesized from a mutated form of the initially active HPRT gene, then wild-type HPRT protein in the revertants must be synthesized from a newly activated but previously silent wild-type gene. The newly activated gene in the revertants of the missense mutation appears unstable producing a high frequency of spontaneous HPRT mutants.**

Hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) is a valuable tool for somatic cell geneticists. Cells lacking HPRT activity can be selected by their resistance to purine base analogs, and cells expressing HPRT can be selected by their resistance to chemicals which block *de novo* purine synthesis (ref. 1, see ref. 2 for review). In some cases, HPRT deficiency has been correlated with an alteration in the enzyme protein—most likely caused by a mutation in the enzyme's structural gene. Somatic cell mutants have been shown to have HPRT enzymes with altered temperature sensitivity, altered K_m for a substrate, or immunogenicity without enzyme activity (3–6). In the present work, we demonstrate that HPRT mutants and revertants can be analyzed by two-dimensional polyacrylamide gel electrophoresis of crude cell extracts. In this procedure, proteins are separated in one dimension by isoelectric focusing, and then in a second dimension by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (7).

We have identified the spot corresponding to HPRT on two dimensional gels of HeLa cell extracts even though the enzyme represents only 0.02% of the soluble protein. We have analyzed 24 HPRT deficient mutants, and for every one, the spot corresponding to the wild-type enzyme disappears. A missense mutant labeled H23 displays a new spot at the same molecular-weight location, but at a different isoelectric focusing position. Unexpectedly, five independently isolated revertants of H23 display spots corresponding to both the wild-type and mutant proteins. These results indicate that in revertant cell lines a previously silent HPRT gene is activated and produces wild-type HPRT, while simultaneously the mutant HPRT gene continues producing the mutant protein.

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; DME medium, Dulbecco's modified Eagle's medium; TG medium, DME medium containing 6-thioguanine; MTH medium, DME medium containing methotrexate, thymidine, and hypoxanthine; TH medium, DME medium containing thymidine and hypoxanthine.

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MATERIALS AND METHODS

Cell Growth Conditions. HeLa cells are grown at 37° in an atmosphere of 5% CO_2–95% air on plastic petri plates (Falcon, 100 mm style dishes) in DME medium consisting of Dulbecco's modified Eagle's medium (Gibco, 13.5 g of powdered media per liter) containing 10% calf serum (Irvine Scientific), 22 mM sodium bicarbonate, and 15 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic buffer) adjusted to pH 7.2. All cell lines discussed in this paper have a generation time of 15–20 hr and a plating efficiency of 50–70%.

Mutagen Treatment. One million wild-type HeLa cells growing exponentially are placed in plates containing 10 ml of fresh DME medium. The medium is removed after 12–24 hr and replaced with DME medium containing mutagen. The mutagens used are ethyl methanesulfonate at 400, 500, and 600 μg/ml and *N*-methyl, *N'*-nitro, *N*-nitrosoguanidine at 4, 5, and 6 μg/ml. After 24 hr, the medium containing mutagen is removed, the cells are rinsed with 5 ml of phosphate-buffered saline, and 10 ml of fresh DME medium is added to the plates. The cells are grown in DME medium for 5–10 days prior to the addition of selective medium to allow time for the residual HPRT concentration in mutants to decrease. During this time, the cells are transferred so that they do not exhaust the medium. The three levels of ethyl methanesulfonate and nitrosoguanidine kill approximately 60%, 90%, and 98% of the cells, respectively.

Mutant Selections. Ten milliliters of selective "TG medium" containing 0.1 mM 6-thioguanine in DME-medium is added to plates containing approximately 4 to 8 × 10^6 mutagen-treated cells. After 3–7 days, the medium and cell debris are removed and fresh TG medium is added. Colonies are observed 14–21 days after addition of TG medium. Colonies are removed with a pasteur pipet and grown in TG medium. Each mutant cell line is recloned by plating the cells at a low density and isolating individual colonies. To avoid duplication of mutants, only one mutant colony is retained from each initial plate of mutagenized cells. Approximately one-half of the mutagen-treated plates of cells yield mutant colonies. More than 10^8 untreated cells were examined, and none gave spontaneous mutant colonies. Twenty-four independent mutants labeled H1 to H24 were analyzed by two-dimensional gel electrophoresis. This paper deals mainly with an apparent missense mutant H23, and revertants from this mutant.

Revertant Selection. HeLa cells lacking HPRT activity are

treated with mutagen and grown for 24 hr in DME medium. The medium is then replaced with MTH medium consisting of DME medium containing 1.0 μM methotrexate, 20 μM thymidine, and 100 μM hypoxanthine. After 3, 5, and 9 days, the medium and cell debris are removed and replaced with fresh MTH medium. Colonies appear in 10–12 days. They are removed with a pasteur pipet and grown in MTH medium. Cells to be transferred to DME medium must first recover from the effects of methotrexate by growing for 1–2 days in TH medium consisting of MTH medium lacking methotrexate. Each revertant cell line is recloned by plating the cells at low cell density and isolating individual colonies. To avoid duplication of revertants, we retained only one revertant colony from each initial plate of cells lacking HPRT activity. Approximately one-half of the mutagen-treated plates of cells yield revertant colonies. Only one revertant colony was observed from 5×10^7 untreated cells. Five revertants (labeled H23R1, H23R2, H23R3, H23R4, and H23R5) were obtained from the mutant H23.

Radioisotope Incorporation into HeLa Protein. Approximately 8×10^6 exponentially growing cells are placed in plates in 20 ml of DME medium. The medium is removed after 16–20 hr and replaced with 2.5 ml of warm DME medium lacking methionine but containing 0.2–3 mCi of [^{35}S]methionine (2.85 Ci/mmol, Schwartz/Mann). The plates are gently agitated every 20–30 min for 3 or 6 hr. The uptake of [^{35}S]methionine is generally 30–70%.

Preparation of Cell Extracts. Plates of cells are removed to a 4° room and all further procedures are performed at 0–4°. The labeling medium is removed, the cells are gently rinsed three times with 5 ml phosphate-buffered saline, and 0.75 ml of extraction buffer [10 mM $MgCl_2$, 30 mM KCl, 0.1 mM dithiothreitol, 0.5% Triton X-100 (vol/vol), and 10 mM Tris·HCl at pH 7.4] is added. The plates are gently agitated for 5 min and then tilted for 5 min to allow extract to drain to one side. The extract is centrifuged for 20 min at 19,000 × *g* in a Sorvall SE-12 rotor to remove debris, and stored in a liquid nitrogen freezer. The extracts contain 100% of the HPRT activity observed if cells were lysed by homogenization or by freezing.

Preparation of Sepharose-Conjugated HPRT Antibody. The preparation of goat antiserum has been previously described (8). The HPRT antibody is purified by precipitation with 40% saturated ammonium sulfate and attached to Sepharose 4B as described by Cuatrecasas (9). The Sepharose-conjugated antibody is capable of binding at least 35 μg of HPRT per ml. Sepharose-conjugated preimmune antibody is prepared from preimmune serum obtained from the same goat prior to immunization with HPRT.

Two-Dimensional Polyacrylamide Gel Electrophoresis. The first dimension, isoelectric focusing, is performed as described by O'Farrell with the following modifications. Biolytes (Biorad) are used in place of Ampholines (LKB). Triton X-100 (Chemical Compounding Co.) is used in place of Nonidet P-40. Acrylamide is purchased from Bio-Rad and sodium dodecyl sulfate from BDH Chemicals Limited. Concentrations of TEMED (*N,N,N′,N′*-tetramethylethylenediamine) and ammonium persulfate are increased 150%.

A sample consisting of 20–30 μl of cell extract containing 100–150 μg of protein and 2 to 3×10^6 cpm of [^{35}S]methionine-labeled protein is lyophilized, dissolved in 30 μl of lysis buffer A [9.5 M urea, 2% Triton X-100 (vol/vol), 1.6% Biolytes at pH 5–7, 0.4% Biolytes at pH 3–10, and 5% 2-mercaptoethanol], and applied to prefocused gels. The gels are subjected to 400 V for 15–16 hr and 800 V for 1 hr. The focused gels display an approximately linear pH gradient from pH 4–7. The gels are placed in 5 ml of sodium dodecyl sulfate sample buffer [10% glycerol (vol/vol), 5% 2-mercaptoethanol, 2.3% sodium dodecyl sulfate, and 0.0625 M Tris·HCl at pH 6.8] for 90 min, frozen, and stored at −85°.

The second dimension, sodium dodecyl sulfate/polyacrylamide gel electrophoresis, is performed in a vertical slab gel (0.8 mm thick) consisting of a 9 cm separating gel of 12% polyacrylamide and a 2.3 cm stacking gel of 4.75% polyacrylamide, as described by Ames (10). A space of 2 mm is left between the top of the stacking gel and the top of the glass plates. A frozen isoelectric focusing gel is thawed at room temperature, and placed on the slot between the glass plates. Hot (about 80°) agarose (1% agarose in sodium dodecyl sulfate sample buffer) is applied so that it flows between the top of the stacking gel and the isoelectric focusing gel and over the isoelectric focusing gel. The agarose is allowed to solidify, and the gels are subjected to electrophoresis at 15 mA per gel for approximately 5 hr until the trailing edge of a bromphenol blue tracking dye is 3–4 mm from the bottom edge of the gel. The gel is removed, stained with Coomassie brilliant blue, and dried on filter paper under vacuum at 100°. The dried gels are exposed for 2–14 days to Kodak No-Screen medical x-ray film, and developed by standard procedures. Autoradiographs are presented with isoelectric focusing at pH 4 on the left and pH 7 on the right.

Removal of HPRT from Cell Extracts by Sepharose-Conjugated HPRT Antibody. Samples (80 μl) of [^{35}S]methionine labeled (approximately 1.5×10^7 cpm) wild-type or H23 mutant cell extract are applied both to 0.15 ml of Sepharose-conjugated HPRT antibody and to 0.15 ml of Sepharose-conjugated preimmune antibody in columns composed of a 0.5-ml syringe (Glasspak, Becton Dickinson) with a glass fiber plug. The four columns are eluted with water, and the first 500 μl eluants containing 90% of the radioactivity are collected. The eluants are lyophilized, resuspended in 60 μl of lysis buffer A, and 20 μl of each eluant containing 100–120 μg of total protein (5 to 6×10^6 cpm) is subjected to two-dimensional electrophoresis (Fig. 1). Identical experiments with unlabeled wild-type extract show that 90% of the applied HPRT activity is recovered in the eluant from the Sepharose-conjugated preimmune antibody column, but no detectable HPRT activity (less than 1%) is found in the eluant from the Sepharose-conjugated HPRT antibody column.

Immunoprecipitation of HPRT Protein. Immunoprecipitation of HPRT protein from cell extracts is a modification of the double antibody procedure previously described (8). All steps are performed at 0–4°. A 250 μl sample of [^{35}S]methionine labeled (5×10^7 cpm) cell extract is centrifuged for 2 hr at 95,000 × *g* in 1.7-ml microtest tubes in a Spinco Type-50 rotor. The supernatant is mixed with 0.6 μl of HPRT antibody and allowed to stand for 2 hr. An equivalent amount of rabbit antigoat gamma-globulin serum (6 μl) is added, and the mixture is allowed to stand for an additional 2 hr. The immunoprecipitate is sedimented by centrifugation for 10 min at 6000 × *g*, washed once with 300 μl of enzyme buffer (20 mM Tris·HCl at pH 7.8, 20 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol), centrifuged a second time, and lyophilized. The lyophilized immunoprecipitate containing approximately 10^4 cpm is dissolved in 30 μl lysis buffer A and subjected to two-dimensional gel electrophoresis.

Spontaneous Mutants of H23 Revertants. HeLa wild-type and revertants H23R1, H23R2, H23R3, and H23R4 are grown in MTH medium to eliminate HPRT mutants. On day 0, each cell line is transferred into twelve 100 mm plates, three each at 10^6, 10^5, 10^4, and 10^3 cells per plate. All plates contain 10 ml nonselective TH medium. On days 1, 2, and 4, one plate of each

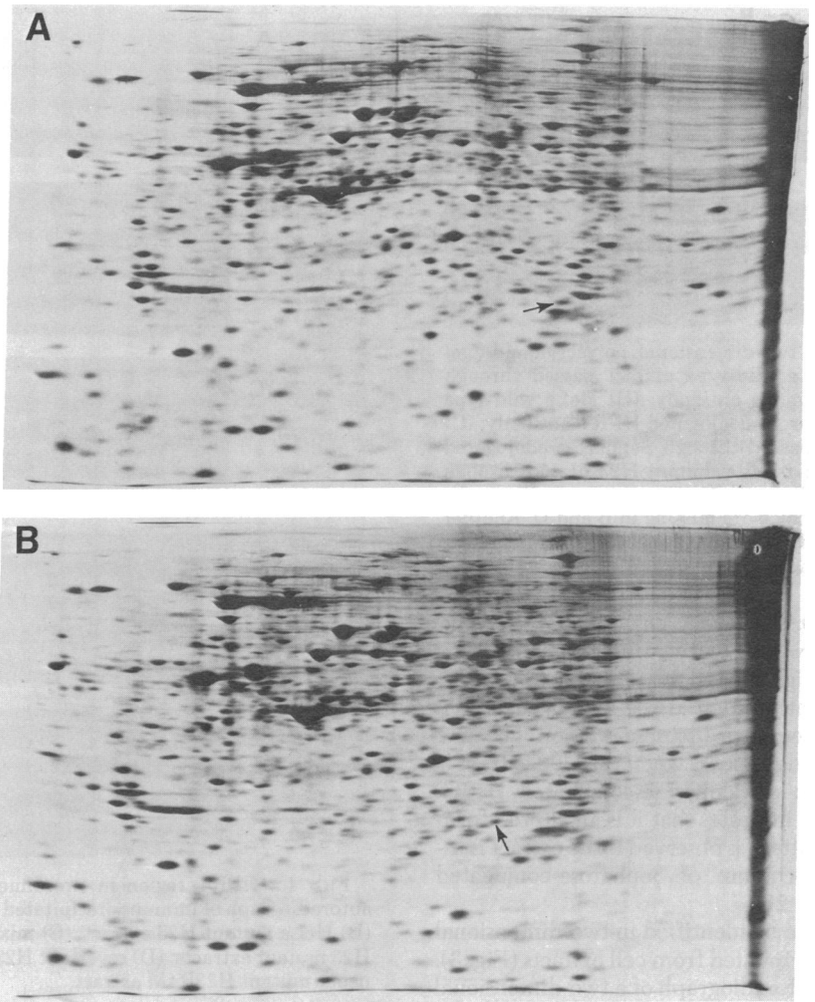

FIG. 1. Two-dimensional polyacrylamide gel autoradiograph of (A) HeLa wild-type extract and (B) HeLa mutant H23 extract. The position of HPRT protein is indicated by the arrows.

cell line at each density (20 plates) is switched into 10 ml mutant selection TG medium. On day 10, the medium is replaced with 10 ml of fresh TG medium. On day 14, colonies are counted. The average number and mean deviation of colonies in each plate is presented in Table 1. Spontaneous mutants of H23R1, H23R3, and H23R4 were cloned and their extracts were examined by two-dimensional polyacrylamide gel electrophoresis.

Table 1. Spontaneous mutation of H23 revertants

Cells	Initial number per plate	Days in nonselective media*		
		1	2	4
Wild-type	10^6	0	0	0
	10^5	0	0	0
	10^4	0	0	0
	10^3	0	0	0
H23 revertants	10^6	4 ± 2	89 ± 49	344 ± 64
	10^5	0	11 ± 5	55 ± 15
	10^4	0	1 ± 1	6 ± 4
	10^3	0	0	2 ± 1

The number of spontaneous mutant colonies from H23 revertants or HeLa wild-type cells was determined as a function of exposure time to nonselective media as described in *Materials and Methods.*
* Results are number of colonies in TG medium ± mean deviation.

RESULTS

The location of HPRT in two-dimensional polyacrylamide gels of crude HeLa cell extracts is indicated by the arrows in the autoradiographs in Figs. 1A and 2A. The HPRT spot is also clearly visible in Coomassie brilliant blue stained gels which are not shown. The following evidence demonstrates that the spot is HeLa HPRT: the HPRT spot appears at the same molecular weight (26,000) and isoelectric focusing position (pH 6) as human red blood cell HPRT. Moreover, when purified HPRT from human red blood cells is added to HeLa cell extracts, the major HPRT isozyme from red blood cell, corresponding to the most basic isozyme focusing at pH 6 (11), coincides with the HeLa HPRT spot. We have examined two-dimensional autoradiographs of 24 mutants lacking HPRT activity, and in every one, the HPRT spot is the only spot in this region which is missing. Finally, when extracts of wild-type HeLa cells are passed through a column containing Sepharose-conjugated HPRT antibody, the two-dimensional pattern is identical except that the HPRT spot disappears (Fig. 2B). No change in pattern is seen if the extract is instead passed through a column of Sepharose-conjugated preimmune antibody (Fig. 2A).

We have isolated a potential missense mutant H23 which produces a normal amount of antigenic HPRT protein, but has

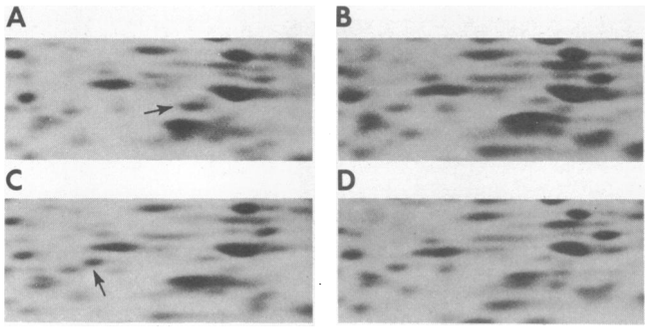

FIG. 2. HPRT region in two-dimensional polyacrylamide gel autoradiographs of (A) HeLa wild-type extract passed through Sepharose-conjugated preimmune antibody; (B) HeLa wild-type extract passed through Sepharose-conjugated HPRT antibody; (C) HeLa mutant H23 extract passed through Sepharose-conjugated preimmune antibody; and (D) HeLa mutant H23 passed through Sepharose-conjugated HPRT antibody. The arrows indicate the HPRT proteins in A and C which are missing in B and D. Another protein appears very close to HPRT, but at a slightly higher molecular weight and isoelectric focusing position.

no detectable (less than 0.1%) HPRT enzyme activity. The mutant H23 displays a new spot on two-dimensional gels indicated by the arrows in Figs. 1B and 2C. The spot appears at the same molecular-weight position as the wild-type HPRT spot, but at a more acidic isoelectric focusing position. The new spot is the only one which disappears when the H23 extract is passed through a column of Sepharose-conjugated HPRT antibody (Fig. 2D), which indicates that it is an altered form of HPRT. No change in pattern is observed if the extract is instead passed through a column of Sepharose-conjugated preimmune antibody (Fig. 2C).

The HPRT spots can also be identified in two-dimensional gels of HPRT immunoprecipitated from cell extracts (Fig. 3). The HPRT region in an autoradiograph of a two-dimensional gel of immunoprecipitated wild-type HeLa extract is shown in Fig. 4A. The major spot in the pattern corresponds in position to the location of the HPRT spot in two-dimensional gels of crude HeLa extracts (see Fig. 1). Similarly, the major spot in the pattern from immunoprecipitated H23 extract (Fig. 4B) occurs at the location of the H23 HPRT spot in two-dimensional gels of crude H23 extracts. A two-dimensional gel of a mixture of immunoprecipitated wild-type HeLa and H23 extracts is shown in Fig. 4C.

We have isolated five independent revertants of mutant H23.

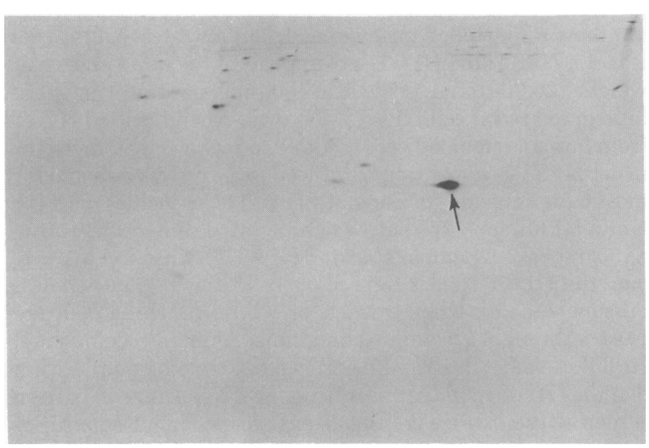

FIG. 3. Two-dimensional polyacrylamide gel autoradiograph of immunoprecipitated HeLa wild-type extract. The major spot (arrow) occurs at the position of HPRT in gels of crude cell extracts.

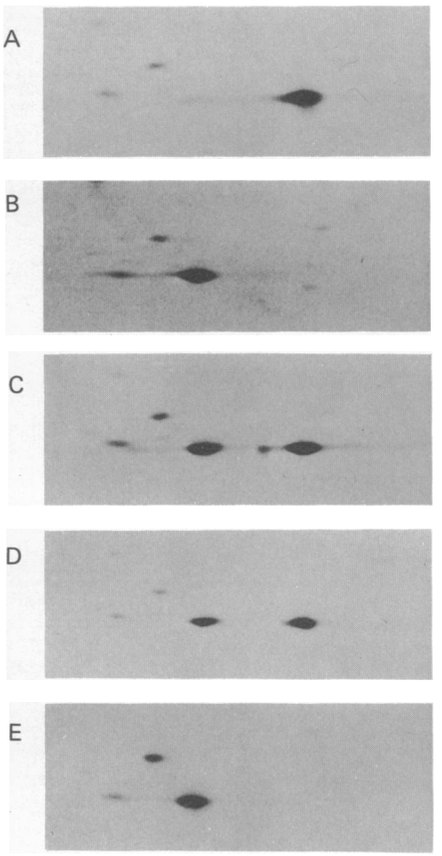

FIG. 4. HPRT region in two-dimensional polyacrylamide gel autoradiograph of immunoprecipitated (A) HeLa wild-type extract; (B) HeLa mutant H23 extract; (C) mixture of HeLa wild-type and H23 mutant extracts; (D) revertant H23R5 extract; and (E) spontaneous mutant H23R4M extract.

Two-dimensional gels of immunoprecipitated HPRT from all five revertants give the same pattern which is shown in Fig. 4D. The revertant pattern is identical to the composite pattern of the wild-type HeLa and H23 extracts. It is unlikely that the H23 revertants contain H23 mutant cells coexisting by metabolic cooperation, because the revertants have been successively cloned from single cells and grown at low density in MTH media which does not support H23 mutant growth. Thus, revertants appear to synthesize HPRT from two separate genes; one producing the wild-type protein and the other producing the mutant protein.

The H23 revertants also differ from wild-type HeLa in that they produce a high frequency of spontaneously arising 6-thioguanine resistant colonies (HPRT mutants), whereas we have observed no spontaneous HPRT mutants from wild-type HeLa. The H23 revertants are routinely grown in MTH media which does not support the growth of HPRT mutants. When H23 revertants are switched from MTH media into nonselective TH media, there is an apparent exponential increase with time in the number of mutant colonies which are 6-thioguanine resistant (Table 1). We have examined three spontaneous mutants of H23 revertants on two-dimensional gels, and they all give the identical pattern illustrated in Fig. 4E. The wild-type HPRT spot disappears and only the H23 mutant spot remains.

DISCUSSION

Two-dimensional polyacrylamide gel electrophoresis can potentially detect any event which produces an HPRT protein

with an altered charge or size. These types of alterations provide strong evidence for a genetic mutation in the enzyme's structural gene. An advantage of the technique is that it is not dependent upon the enzymatic activity or immunoreactivity of the altered protein. However, locating a new spot in a crude extract pattern is difficult, and we were fortunate that the missense mutants H23 protein migrated to a location where it was visible. When the altered molecules are immunoreactive, two-dimensional gels of immunoprecipitated extracts provide clear and easily interpreted patterns; e.g., the demonstration of two types of HPRT molecules in H23 revertants.

Two-dimensional polyacrylamide gel electrophoresis is a powerful method to search for potential protein chain-termination mutations, amber- or ochre-type mutations which produce amino terminal fragments of the protein. These fragments are difficult to detect since they may have a short-half-life and most would not be expected to react with antibody to the native enzyme. However, radioisotope-labeled fragments in the molecular-weight range between 8000 and 25,000 might be identified as new spots on two-dimensional gels. Two of the mutants we have examined appear to have new spots in this region. Additional evidence such as tryptic peptide maps will be necessary to prove that the new spots are derived from HPRT.

All 24 mutants we have examined by two-dimensional gels are missing the wild-type HPRT spot. Only one is an apparent missense mutant; two may be chain-termination mutants; and the remaining 21 mutants show no additional spots. This last class of mutants may be short-lived missense or chain-termination mutants, missense or chain-termination mutants outside the isoelectric pH range of the gels, chain-termination mutants with fragment proteins less than 8000 molecular weight, mutants with altered proteins which coincide with other spots on the gels, or regulatory mutants or variants in which the HPRT gene is turned off. Examination of revertants could eliminate a regulatory explanation. Identification of a revertant which synthesizes an altered HPRT enzyme would imply that the original mutation was in the structural gene.

The human gene for HPRT is X-linked and presumably present as only a single active copy in diploid cells (12). As HeLa cells are polyploid, there is no *a priori* reason why they should not have more than one active HPRT gene. However, the apparent mutation frequency for HeLa is not greatly different than that for pseudodiploid or diploid cells which suggests that a single event in HeLa can eliminate HPRT activity. The data in Figs. 4A–C clearly demonstrate that mutant H23 produces an altered HPRT protein. Moreover, the altered protein is the only HPRT product in the mutant cells. Because it is unlikely that multiple HPRT genes would all mutate to produce the identical mutant product, the presence of a single altered HPRT protein in the mutant H23 strongly suggests that this mutant contains only a single active HPRT gene.

The simultaneous synthesis of wild-type and mutant H23 protein in H23 revertants is best explained if the revertants have two active HPRT genes. Alternative explanations such as a tRNA missense suppressor or partial enzyme modification (e.g., phosphorylation, processing) seem unlikely as five independently isolated revertants all display the same phenotype. If the revertants synthesize mutant H23 protein from the original HPRT gene which has been mutated, then wild-type HPRT protein must be synthesized from a newly activated and previously silent wild-type gene.

Compared to the wild-type HPRT gene in parental HeLa cells, the newly activated gene in H23 revertants appears unstable producing a high frequency of spontaneous mutants (Table 1). It is unlikely that the mutants arise by a mutation in the activated wild-type gene as the frequency of appearance is too high. Moreover, two-dimensional gels demonstrate that the spontaneous mutants all synthesize only the mutant H23 gene product, whereas one might expect other types of altered proteins from structural gene mutations. Probably, the spontaneous mutants arise from an event which reverses or negates the initial process which activated the gene. Such an event could be the loss of an inducer required for gene or chromosome expression, the activation of a repressor turning off gene or chromosome expression, or the loss of the chromosome (13, 14) containing the activated wild-type gene. Further study of HPRT gene activation may help elucidate general mechanisms of gene regulation.

Other investigators have suggested that a silent HPRT gene may be activated under some conditions (15–18). Although Kahan and DeMars (18) suggest activation of HPRT on an inactive X-chromosome, it is usually assumed that the silent gene is synonymous with the normal wild-type gene which has been turned off. Our data indicate that an HPRT gene which is normally off can be activated. This observation should serve as a caveat in the interpretation of apparent HPRT revertants.

We thank Susan G. Cummings for technical assistance. This work was supported by USPHS Grant CA-12308 from the National Cancer Institute, Grant DRG-8-F from the Damon Runyon Memorial Fund for Cancer Research, and Grant GM-38658 from the National Science Foundation.

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