Cloned cDNA sequences of the hypoxanthine/guanine phosphoribosyltransferase gene from a mouse neuroblastoma cell line found to have amplified genomic sequences

(recombinant DNA/nucleic acid hybridization/somatic cell mutant/protein overproduction)

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ABSTRACT Cloned cDNA sequences of the murine hypoxanthine/guanine phosphoribosyltransferase (HPRT; EC 2.4.2.8) gene have been isolated by using a mouse neuroblastoma cell line containing increased levels of a variant HPRT protein. We have used these sequences as probes to demonstrate that protein overproduction in this cell line is a consequence of at least a 20-fold increase in HPRT mRNA levels resulting from approximately 50-fold amplification of HPRT genomic sequences. The largest cDNA insert so far characterized represents about 70% of the HPRT mRNA sequence. This cDNA is shown to possess regions of homology with mRNA and DNA from Chinese hamster, baboon, and human, thus facilitating detailed analysis of this locus in these four species.

Hypoxanthine/guanine phosphoribosyltransferase (HPRT; EC 2.4.2.8), a multimeric enzyme catalyzing the conversion of hypoxanthine or guanine to its 5′ mononucleotide, is essential for normal purine metabolism in man. A deficiency of it leads to the clinical disorders of Lesch-Nyan syndrome and gouty arthritis (for review, see ref. 1). Somatic cell hybridization studies have localized the HPRT gene to regions of the X chromosome of human (2), hamster (3), and mouse (4) and the locus has been extensively studied in somatic cell systems because of the ease of selection for both forward and reverse mutations (1). Several laboratories have identified structural mutations within this gene in cultured cells by peptide mapping, kinetic analysis, thermal sensitivity studies, and immunological studies, and in the HPRT protein (5–11). The isolation of cloned sequences of this gene should facilitate the examination of mutations that lead to alterations in gene expression.

The highest tissue levels of mammalian HPRT occur in brain where it represents 0.2% of the total soluble protein (12), in cultured rodent fibroblasts it represents ≈0.05% of the protein (13). A mouse neuroblastoma HPRT revertant cell line (NBR4) has been characterized recently (13, 14) which produces 20- to 50-fold increased levels of an altered HPRT protein. In vitro translation of NBR4 mRNA (13) suggested that its HPRT mRNA level was also increased relative to the wild type. We now report the isolation of cDNA sequences of the HPRT gene from this cell line and demonstrate that these cells contain amplified HPRT genomic sequences and increased levels of HPRT mRNA.

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EXPERIMENTAL PROCEDURES

Mammalian Cell Lines. The origin and characterization of the mouse neuroblastoma wild type (NB’), 6-thioguanine-resistant (NB’), HPRT-overproducing derivative (NBR4), and wild-type Chinese hamster lung fibroblast (RJKO) cell lines have been reported (8, 13, 14).

Bacterial Strain and Plasmid. The Escherichia coli K-12 derivative RR1 (F’ pro leu thi lacY rpsL hsdR7 hsdM− endA−) (15) and the plasmid pBR322 were used. Construction of recombinant plasmids was performed in accordance with the National Institutes of Health guidelines for recombinant DNA research.

Enzymes. Avian myeloblastoma virus reverse transcriptase was a gift from J. Beard (Life Sciences, St. Petersburg, FL). Aspergillus oryzae S1 nuclease was purchased from Miles, terminal deoxynucleotidyltransferase was from Bethesda Research Laboratories, and all restriction endonucleases were from New England BioLabs.

HPRT mRNA Detection and Purification. The mRNA used in these studies was extracted from cultured cells or tissues and partially purified by two cycles of oligo(dT)-cellulose column chromatography (13). Sucrose gradient-fractionated mRNA containing optimal HPRT mRNA activity (13) was used where stated. Cell-free mRNA translation and radioimmune detection of [35S]methionine-labeled HPRT protein have been reported (13).

cDNA Cloning. Construction of Recombinant Plasmids, and Transformation. Double-stranded cDNA was synthesized by reverse transcription of 75 μg of sucrose gradient-purified NBR4 mRNA, essentially as described by Richards et al. (16). The cDNA was rendered blunt ended by treatment with S1 nuclease (60 units/μg of cDNA). Double-stranded cDNA was hybridized to Pst I-cleaved pBR322 after homopolymeric G-C “tailing” (17, 18). Recombinant plasmids were used to transform CaCl2-treated bacterial cells (19). Transformants were selected for their resistance to tetracycline, and recombinants were identified by loss of ampicillin resistance.

Identification of Recombinants of Interest. Plasmid DNA from 200 recombinant clones was isolated (20), spotted onto duplicate nitrocellulose membranes (Schleicher & Schuell, BAS5/20), and probed with [32P]labeled cDNA synthesized from the sucrose gradient fractions of NBR4 or NB’ containing max-
imal HPRT mRNA (13). Autoradiography was at -80°C with Kodak XAR5 or RP1 x-ray film in the presence of Du Pont Lightning Plus intensifying screens.

Selection of HPRT mRNA by Hybridization to Recombinant DNA. Recombinant plasmid DNAs were isolated (21) and bound, in groups of four or individually, to nitrocellulose (Schleicher & Schuell, BA85) (22). mRNA from NBR4 was hybridized to the membrane-bound DNA, eluted for 3 min at 70°C, and translated in vitro as described (13, 22).

Analysis of Cellular mRNAs. mRNAs were glyoxylated (23), fractionated in 1% agarose gels, and transferred to nitrocellulose (Schleicher & Schuell, BA85) (24). The conditions of hybridization to 32P-labeled plasmid DNAs have been reported (25).

Analysis of Genomic DNAs. High molecular weight cellular DNA was extracted (26), digested to completion with HindIII, and fractionated in 1% agarose gels. DNA fragments were transferred to nitrocellulose (27) and probed with 32P-labeled plasmid DNA (25).

Identification of Additional HPRT cDNA Recombinants. The recombinant clones were screened by colony hybridization (25) with the nick-translated 540-base-pair (bp) Pst I/HindIII fragment isolated (28) from the plasmid pHPT1 (described in Results).

RESULTS

Construction of Recombinant Clones. A yield of 4 μg of double-stranded cDNA was obtained from 75 μg of NBR4 mRNA. A total of 1619 tetracycline-resistant colonies were obtained after the annealing of 0.05 μg of cDNA with 1 μg of G "tailed" pBR322. Only two tetracycline-resistant colonies were obtained from the same amount of tailed pBR322 alone. Of 200 transformants examined, all exhibited ampicillin sensitivity and therefore contained recombinant plasmids.

Selection of Recombinants for Analysis. We used a screening method which utilized differential hybridization of recombinants to cDNAs prepared from NBR4 (HPRT overproducer) or NB- (HPRT-) mRNA (Fig. 1). The sucrose gradient fractions containing the highest concentrations of HPRT mRNA were identified by translation assay and used for cDNA preparation. Several recombinants appeared to hybridize more strongly to the cDNA prepared from NB- mRNA. This probably reflects some inequality in the mRNA populations resulting from sucrose gradient fractionation. A number of recombinants hybridized equally to both probes and were therefore not considered strong candidates for HPRT recombinants. The recombinant indicated by the arrow hybridized more strongly to NBR4 cDNA and thus was an example of a potential HPRT recombinant. Weaker differential hybridizations were observed in this and earlier studies. On the basis of these studies, 33 recombinants were selected and assayed for their ability to hybridize to HPRT mRNA.

HPRT mRNA Selection. We used the method of Parnes et al. (22) to identify recombinants that hybridize to HPRT mRNA. One membrane containing recombinant DNA from cDNA clones 205, 209, 214, and 242 selected HPRT mRNA (Fig. 2, lane D). When examined individually, one selected HPRT mRNA, and therefore we concluded that it was an HPRT cDNA recombinant (Fig. 2, lane F). This recombinant, 209, also exhibited the strongest differential hybridization between NBR4 and NB- cDNA (indicated by the arrow in Fig. 1) and henceforth will be referred to as pHPT1. Recombinants 205, 214, and 242 were devoid of HPRT mRNA selection capacity (Fig. 2, lanes E, G, and H).

HPRT mRNA Analysis. pHPT1 was used as probe to characterize mRNA isolated from NBR4 (HPRT overproducer), NB+ (wild type), and NB- (HPRT-) cells (Fig. 3). pHPT1 hybridized to a mRNA species present in greater amounts in NBR4 compared to NB+ and NB- . No difference in the levels of HPRT mRNA was apparent between NB+ and NB- . The predominant mRNA species in all three cell lines was around 1600 nucleotides long. An additional RNA species around 6500 nucleotides long also was evident in the three cell lines. Both RNA species were more abundant in NBR4, and quantitation by liquid scintillation counting of the membranes, in the region of the smaller mRNA species, suggested a 20-fold greater level in NBR4 than in wild type. Clones 205, 214, and 242 hybridized to mRNAs of dif-

![Fig. 1. Hybridization of recombinant plasmid DNA to NBR4 or NB- cDNA. Each membrane, containing DNA (~0.25 μg) from 200 recombinant plasmids, was hybridized to ~4 × 10^6 cpm of 32P-labeled cDNA prepared from 2 μg of mRNA purified by sucrose gradient fractionation. The autoradiogram was developed after exposure for 24 h. Recombinant plasmid 209 is indicated by the arrow.](image-url)

![Fig. 2. In vitro HPRT synthesis from mRNA selected by recombinant DNAs. The mRNA eluted after hybridization to membrane-bound recombinant DNA was translated in vitro, and [35S]methionine-labeled HPRT was detected by radioimmunoassay. Lanes: A, no mRNA; B, 2 μg of mRNA from NBR4; C, 2 μg of unbound mRNA recovered from the hybridization mixtures; D, mRNA eluted from recombinants 205, 209, 214, and 242 bound to 1 membrane; E, F, G, and H, mRNA eluted from membranes containing DNA from individual recombinants 205, 209, 214, and 242, respectively. The autoradiogram was developed after exposure for 240 h. Positions of bovine serum albumin (68,000), ovalbumin (43,500), triose phosphate isomerase (36,600), and myoglobin (17,200) molecular weight marker proteins are shown.](image-url)
Different molecular weights in equal abundance in all three cell lines.

**HPRT Gene Analysis.** In order to determine the mechanism of HPRT protein and mRNA overproduction in NBR4 we performed Southern analysis of HindIII-digested DNA from NBR4, NB+, and NB- with pHPT1 as probe (Fig. 4). This plasmid hybridized preferentially to a 900-bp fragment of DNA present in all cell lines. Hybridization to a 13-kilobase-pair (kb) fragment was also observed in NBR4 but was not detectable for NB+ or NB- in this study because of high background. The hybridization of pHPT1 to the smaller fragment of NBR4 with 1 μg of DNA greatly exceeded that with 10 μg of NB+ or NB- DNA. On the basis of these studies and quantitation by densitometer scanning of the x-ray film, we estimate that the copy number of this 900-bp sequence is about 50-fold greater in NBR4 than in NB+ or NB-. Liquid hybridization studies are required for a more accurate quantitation of the gene copy number.

There also was a greater (≈3-fold) hybridization of NBR4 DNA sequences (compared to NB+ and NB-) with 205 plasmid DNA. The high level of hybridization in all three cell lines suggests that the sequence is present in more than a single copy. The possibility that 205 corresponds to a repeated DNA sequence present on the mouse X chromosome, triplicated in NBR4 (13), must be considered. No differences in the hybridization pattern and intensity between cell lines were evident with the 214 and 242 probes, but recombinant 242 probably detects repeated sequences whereas 214 detects low- or single-copy sequences.

**Identification and Characterization of Other pHPT Recombinants.** Restriction endonuclease analysis of pHPT1 (see below) indicated that it contained an insert of 640 bp and that only one Pet I site was regenerated by the cloning procedure. A HindIII site was identified within the inserted sequence 540 bp from the intact Pet I site. This 540-bp Pet I–HindIII fragment was isolated, nick-translated, and used to identify other pHPT recombinants. Additional pHPT recombinants were identified within the cDNA recombinant library at a frequency of ≈1%.

We have studied nine other recombinants in detail. All had the capacity to select HPRT mRNA and therefore correspond to HPRT cDNA recombinants (Fig. 5). Insert sizes ranged from 150 to 1100 bp, and the largest recombinant, pHPT2, selected HPRT mRNA most efficiently (Fig. 5, lane K). Partial restriction enzyme maps of pHPT1 and pHPT2 are shown in Fig. 6. Neither cDNA insert could be excised by Pet I digestion of the plasmids although each could be recovered intact, with only a small amount of contaminating vector sequences, after Hpa II digestion.

**Homology of Mouse HPRT cDNA to Nucleic Acid Sequences from Other Species.** The cross-hybridization of mRNA from Chinese hamster cells and baboon brain to the intact cDNA insert, isolated after Hpa II digestion of pHPT2 DNA, is shown in Fig. 7 Left. The lower molecular weight mRNA band

![Fig. 3. Characterization of mRNA from mouse cell lines NB+, NB-, and NBR4. mRNA (4 μg) from NB+ (lanes A), NB- (lanes B), and NBR4 (lanes C) was denatured by glyoxal, fractionated in 1% agarose gels, and transferred to nitrocellulose prior to hybridization with plasmid DNAs (pHPT1, 205, 214, and 242) labeled with 32P by nick-translation to a specific activity of 3 × 107 cpm/μg. Autoradiogram exposure times were: pHPT1, 17 hr; 205, 14 hr; 214, 8 hr; and 242, 2 hr. The positions of 4 of the 19 fragments (sizes in kb) obtained after Hae III digestion of φX174 DNA and HindIII digestion of A DNA, in the same gel, are indicated. (Glyoxal-denatured DNAs and RNAs of the same size migrate identically (23)).](image)

![Fig. 4. Southern analysis of genomic DNA from mouse cell lines NB+, NB-, and NBR4. DNA was completely digested with HindIII, fractionated in 1% agarose gels, and transferred to nitrocellulose. Lanes A, B, and D contained 10 μg of DNA from NB+, NB-, and NBR4, respectively. Lane C contained 1 μg of NBR4 DNA. Plasmid DNAs, nick-translated to a specific activity of 2–5 × 107 cpm/μg with 32P, were used as probes. The autoradiogram exposure time was 72 hr. The mobilities of suitable nucleic acid fragments of known molecular sizes (kb) (see Fig. 3) are shown.](image)

![Fig. 5. In vitro HPRT synthesis with mRNA selected by HPRT cDNA recombinants. The method was as in Fig. 2. Lanes: A, 2 μg of NBR4 mRNA; B–L, mRNA recovered after hybridization of NBR4 mRNA to nitrocellulose membranes containing DNA from recombinants pHPT1 (B), 205 (non-HPR1 cDNA sequence) (C), 419 (D), 533 (E), 692 (F), 1023 (G), 1139 (H), 1191 (I), 1290 (J), pHPT2 (K), and 1339 (L).](image)
was detected in all extracts although it appeared to be a slightly larger molecule in the baboon. The extent of hybridization of pHPT2 to this mRNA sequence was greatest in NBR4 and least in baboon. The high molecular weight RNA sequence was not detectable in the sucrose gradient fraction of NBR4 mRNA maximal for in vitro HPRT synthesis (lane C). Thus, the smaller (1600-nucleotide) mRNA sequence represents HPRT mRNA; the possibility that the larger RNA molecule represents a precursor of the smaller is yet to be resolved. The hybridization of HindIII-digested mouse, Chinese hamster, and human lymphocyte DNA with the same Hpa II fragment of pHPT2 is shown in Fig. 7 Right. The major difference when pHPT2, rather than pHPT1 (Fig. 4), was used as probe is the appearance of three additional bands in the NBR4 DNA and the appearance of all five bands in NBR+ DNA. The approximate sizes of these fragments are 13, 9.3, 6.4, 1.1, and 0.9 kb. The banding patterns obtained with DNA from species other than mouse were different. Only three bands (17.5, 11.3, and 5.5 kb) were detected in Chinese hamster (lane I) and five bands (17.5, 6.5, 6.0, 4.6, and 3.5 kb) in human (lane H). Furthermore, the intensities of the signals were similar for NB+ (mouse), RJKO (hamster), and human DNAs, demonstrating the ability of this probe to detect single-copy HPRT sequences in these species.

**DISCUSSION**

This communication describes the successful cloning of cDNA sequences of a mammalian HPRT gene. One recombinant, pHPT2, contains an 1100-bp insert corresponding to ~70% of the HPRT mRNA. Because the 1.6-kilobase mRNA species exceeds the requirement (~700 nucleotides) for encoding the HPRT subunit, we anticipate that ~50% of the HPRT mRNA is noncoding and that pHPT2 contains a significant amount of HPRT-encoding sequence. The reported similarities in HPRT subunit molecular weights and tryptic peptides of mouse (5), Chinese hamster (30), and human (6) suggest that these species possess regions of nucleic acid homology. Our studies provide evidence of such homology among mouse, Chinese hamster, baboon, and human (Fig. 7). The availability of these HPRT cDNA sequences should facilitate the isolation of full-length cDNA and genomic DNA sequences from these species.

The NBR4 cell line was originally selected (14) as an HPRT+ revertant of an HPRT- mutant cell line, NB−. It achieves near-normal HPRT specific activity by producing increased levels of variant protein with altered kinetic constants and thermal stability relative to wild type. The subunit molecular weight of this protein is indistinguishable from that of wild type (13), suggesting that the mutation is probably missense resulting from a small alteration in the DNA sequence. The nucleic acid hybridization studies reported here demonstrate that NBR4 contains markedly enhanced levels of HPRT mRNA and amplified genomic sequences. The NB− cell line has no detectable in vivo enzymic or immunological activity (14) but in vitro transfection studies (13) identified the presence of HPRT mRNA. The data presented here show that NB− is indistinguishable from NB+ with respect to mRNA properties and genomic DNA sequences, and it probably synthesizes wild-type levels of variant enzyme.

Karyotypic analysis of NBR4 (14) revealed the presence of an X-autosome translocation with triplication of the X chromosome. Our data demonstrate that HPRT genomic sequences are about 50-fold amplified in NBR4 but their precise molecular configuration cannot be elucidated at present. No homogeneously staining regions or double minute chromosomes were identified in NBR4 (14). These have been correlated with gene amplification in other systems (31–33). We have shown that reversion of NB− to NBR4 was achieved by an increase in gene copy number and not by intragenic reversion events. We propose that a similar approach with other cell lines containing a similar type of mutation in HPRT, or another selectable gene,
might afford a general method for amplifying specific genomic sequences.

Analysis of the partial restriction map of pHPT2 (Fig. 6) leads to the prediction that, if the mRNA and genomic DNA sequences are co-linear, then Southern analysis of HindIII-digested NBR4 DNA should reveal three bands—one 275 bp, and the other two should equal or exceed 340 and 520 bp. The 275-bp fragment was not observed under the conditions used in these experiments but the existence of five bands of higher molecular weight (Fig. 7 Right) suggests the presence of at least one intervening sequence in the HPRT gene. Furthermore, Southern analysis of NBR4 DNA with pHPT2 after digestion with several restriction endonucleases that do not cleave the cDNA insert reveals the presence of more than one band (data not shown). This is conclusive evidence for the presence of at least one intervening sequence in the HPRT gene of NBR4. The presence of one or more introns within the gene would lead to the production of high molecular weight precursor mRNA and this may have been detected in this study (Figs. 3 and 7 Left).

These cloned unique X-linked cDNA sequences will permit detailed analysis of mutations in the structural and regulatory elements of the HPRT gene in a number of species, providing a molecular view of mutational targets within single-copy genes in cultured mammalian cells. Furthermore, we now have a probe corresponding to a unique site on the human X chromosome facilitating the study of Lesch–Nyhan syndrome and possibly other human disease genes mapped to the X chromosome.

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