Proc. Natl. Acad. Sci. USA
Vol. 78, No. 11, pp. 6977–6980, November 1981
Cell Biology

In vitro translation of hypoxanthine/guanine phosphoribosyltransferase mRNA: Characterization of a mouse neuroblastoma cell line that has elevated levels of hypoxanthine/guanine phosphoribosyltransferase protein

(immunological detection/protein overproduction/chromosome alteration)

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Communicated by James B. Wyngaarden, August 7, 1981

ABSTRACT Antibody specific for the native form of Chinese hamster hypoxanthine/guanine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) was used to detect the synthesis of HPRT protein in a rabbit reticulocyte lysate translation system primed with mRNA from Chinese hamster tissues and cultured cells. Electrophoretic analysis of the immunopurified products from the translation of mRNA from wild-type and a series of mutant Chinese hamster cells indicated that HPRT synthesis *in vitro* qualitatively and quantitatively corresponded to synthesis *in vivo*. The translation system was used to identify two mRNA sources producing high levels of HPRT protein: Chinese hamster brain and a mouse neuroblastoma HPRT revertant cell line, NBR4. Translation of NBR4 mRNA generated 25–50 times more HPRT protein than mRNA from wild-type cells. The basis for HPRT overproduction is considered in view of an X chromosome alteration found in NBR4 cells.

Mutational and regulatory events at the X-linked hypoxanthine/guanine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) locus in cultured animal cells have been studied intensively only at the level of the protein itself (1). Specific antisera recognizing the native form of the enzyme have facilitated the characterization of a range of cultured mammalian cells lacking, or having an altered, enzyme activity (2–4). Immunological detection of HPRT protein in a rabbit reticulocyte lysate translation system primed with mRNA prepared from Chinese hamster brain tissue (5) suggests an additional analytical method for mutant study. The *in vitro*-synthesized HPRT was recognized preferentially by antiserum to native, rather than denatured, Chinese hamster enzyme, suggesting that the *in vitro* system is capable of faithful translation of HPRT mRNA and protein assembly. We have investigated the fidelity of this translation system by analyzing the translation products of mRNA prepared from a series of mutant Chinese hamster cells. The relative abilities of mRNA from Chinese hamster brain, liver, and testis to direct HPRT protein synthesis are compared. Translation of twice-purified [oligo(dT)] mRNA from a mouse neuroblastoma HPRT revertant cell line, NBR4, that has elevated levels of a variant protein (6) generated 25–50 times as much HPRT protein as equivalent amounts of mRNA from wild-type neuroblastoma cells. An X chromosome alteration in NBR4 cells is described and the basis for overproduction is considered.

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MATERIALS AND METHODS

Adult male Chinese hamsters were obtained from Chick Line Company (Newfield, NJ). All Chinese hamster cell lines used were derived from a clone of the male Chinese hamster V79 line. The characterization of the wild-type line, RJK0, and the isolation of 8-azaguanine-resistant mutants (RJK3, 36, and 39) has been described by Gillin *et al.* (7). Previously, the mutant cell lines were referred to as A3, E36, and E39. The isolation and characterization of wild-type (NB^+), 6-thioguanine-resistant (NB^-), and HPRT revertant (NBR4) mouse neuroblastoma cell lines has been described (6). Conditions for cell growth, maintenance, and *in vitro* determination of HPRT specific activity have been described by Fenwick and Caskey (8).

Total cellular RNA, isolated from freshly harvested cultured cells or tissues stored in liquid nitrogen since dissection by the method of Przybla *et al.* (9), was subjected to two oligo(dT) cellulose (type 2, Collaborative Research, Waltham, MA) chromatographic fractionations as described by Aviv and Leder (10). Further purification of polyadenylylated mRNA was accomplished by centrifugation on linear 10–30% sucrose gradients in 10 mM Tris·HCl, 1 mM EDTA/0.5%$NaDodSO_4$, pH 7.6, for 17.5 hr at 20°C at 35,000 rpm in a Beckman SW 41 rotor. Sucrose gradient-fractionated mRNA was concentrated by ethanol precipitation, dissolved in Milli-Q (Millipore) deionized water, and stored at −80°C.

Cell-free translation was carried out by the method of Pelham and Jackson (11), using a [^{35}S]methionine translation kit (New England Nuclear). Reactions were terminated by addition of 0.2 vol of 100 mM EDTA, pH 7.0, containing pancreatic RNase A at 1.0 mg/ml (Worthington) and incubation for 10 min at 37°C. Total protein synthesis was measured by determining the amount of radioactivity insoluble in 10% trichloroacetic acid. Immunopurification and $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of [^{35}S]methionine-labeled HPRT were as reported by Fenwick *et al.* (4) with minor modifications: The excess of cold protein present during immunoadsorption was derived from 85% ammonium sulfate precipitation of postribosomal supernatant from *Artemia salina* (12), the buffer used for final elution of the sample from the antibody-linked Sepharose beads did not contain urea, the polyacrylamide gel was treated

Abbreviations: CRM, crossreacting material; HPRT, hypoxanthine/guanine phosphoribosyltransferase.

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with EN^3HANCE (New England Nuclear), and the radioactive proteins were located by exposing the dried gel to Kodak X-Omat AR film at −80°C in the presence of Dupont Lightning Plus intensifying screens. X-ray fluorographs were scanned using a Helena Auto Scanner densitometer (Helena Laboratories, Beaumont, TX). Unless otherwise stated, all immunopurifications were carried out using antibody to native Chinese hamster HPRT. The specificity of this antibody, which was raised in a sheep immunized with purified native Chinese hamster HPRT protein, has been described (4). The antibody crossreacts with mouse HPRT protein (6).

Cytogenetic analysis was carried out on the NB^+, NB^-, and NBR4 cell lines by standard techniques. Standard nomenclature for mouse chromosomes was used (13).

RESULTS

Fidelity of the Translation System. $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of the products from the *in vitro* translation of mRNA prepared from a series of Chinese hamster cell lines is shown in Fig. 1. Purification of the translation products from RJK0 mRNA with anti-HPRT antibody-linked Sepharose beads resulted in a single major band on the fluorogram (lane B) that was not present when preimmune serum-linked beads were used (lane D). The band was considerably reduced in intensity when an excess of unlabeled purified Chinese hamster HPRT protein was present during immunoadsorption (lane C). We conclude that the band corresponds to HPRT subunit protein. HPRT subunits synthesized *in vitro* from RJK0 mRNA had the same mobility relative to the molecular size marker protein triose phosphate isomerase (26,600 daltons) as subunits made *in vivo* (4). Translations primed with mRNA from RJK36 cells made no detectable HPRT protein (Fig. 1, lane E), nor were HPRT protein fragments detected using antibody to denatured HPRT subunits (data not shown). Previous *in vivo* studies have identified this cell line as a nonreverting $HPRT^-$ crossreacting material (CRM)-lacking mutant

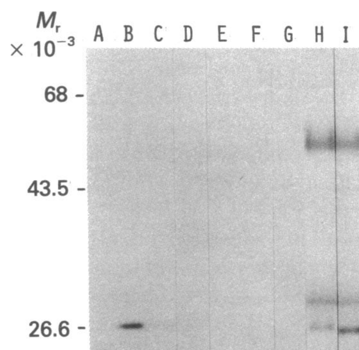

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of products from *in vitro* translation of twice oligo(dT)-purified mRNA from Chinese hamster cells. Translations were carried out in a total volume of 250 μl for 1 hr at 37°C, and [^{35}S]methionine-labeled HPRT protein was immunoadsorbed to either anti-HPRT antibody-linked Sepharose beads or preimmune serum-linked beads. Material that eluted from the beads was analyzed. Lanes: A, no mRNA, immune serum; B–D, RJK0 (12.4 μg of mRNA)—B, immune serum; C, immune serum, immunoadsorption carried out in the presence of 10 μg of unlabeled Chinese hamster liver HPRT protein (purity, >90%); D, preimmune serum; E–G, RJK36 (15.1 μg)—E, immune serum; F, immune serum, immunoadsorption in the presence of 10 μg of unlabeled Chinese hamster HPRT; G, preimmune serum; H, RJK39 (25.8 μg), immune serum; I, RJK3 (14.6 μg), immune serum. M_r marker proteins: bovine serum albumin (68,000), ovalbumin (43,500), and triosephosphate isomerase (26,600).

Table 1. HPRT quantitation in cultured cells and Chinese hamster tissues

Cell or tissue source	HPRT enzyme activity*	HPRT immunoprecipitable radioactivity†	
		In vivo‡	*In vitro*
RJK0	1.55	1.80	1.35
RJK3	0.32	ND	1.12
RJK39	<0.01	ND	0.89
RJK36	<0.01	Not detectable	Not detectable
NB^+	0.83	0.98	0.96
NB^-	<0.01	Not detectable	0.67
NBR4	0.74	4.88–9.33	26.20–47.40
Brain	6.80	ND	4.08
Liver	0.03	ND	0.61
Testis	0.04	ND	0.67

ND, not determined.
* Expressed as nmol of IMP formed min^{-1} mg of protein $^{-1}$.
† Expressed as percentage × 10^2 of total Cl_3CCOOH-precipitable radioactivity.
‡ Taken from Melton (6).

(2). HPRT subunits translated from mRNA extracted from structural mutant cell lines RJK39 and RJK3 (Fig. 1, lanes H and I, respectively) showed the characteristic faster migration found with enzyme synthesized *in vivo* (4). An estimate of *in vitro* synthesis of HPRT as a percentage of total mRNA-directed protein synthesis was obtained as follows: Material eluted from the antibody-linked Sepharose beads was analyzed by $NaDodSO_4$/gel electrophoresis, and the fluorogram obtained was scanned in a densitometer. Radioactivity in the HPRT band was estimated by expressing the area under the HPRT peak on the densitometer trace relative to the area under all peaks along one channel and multiplying by total radioactivity eluted from the beads. HPRT-specific radioactivity was expressed as a percentage of total Cl_3CCOOH-precipitable material synthesized in each translation (Table 1). Inherent in these estimates are difficulties of precise translational optimization for mRNAs of low abundance and detection of weakly radiolabeled protein bands. Nevertheless the value obtained for RJK0 (0.0135%) was in close agreement with an *in vivo* determination described previously (6).

Chinese Hamster Tissue Sources of HPRT mRNA. HPRT specific activities in tissues from which mRNA was extracted are shown in Table 1. Enzyme activity in brain was much higher than that in liver or testis. Previous reports have also identified mammalian brain as the richest tissue source of HPRT activity (14). Fig. 2 shows the relative abilities of tissue mRNAs to direct HPRT protein synthesis. This ability was correlated with tissue HPRT activity (Table 1) and suggests tissue specificity for HPRT mRNA levels.

Translation of HPRT mRNA from Mouse Neuroblastoma Cell Lines. The amount of HPRT protein relative to total pro-

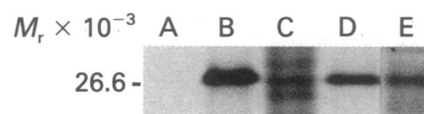

FIG. 2. *In vitro* translation of mRNA from Chinese tissues. Translations were carried out in a total volume of 250 μl for 1 hr at 37°C and products were immunopurified with anti-HPRT antibody-linked Sepharose beads. Lanes: A, no mRNA; B, sucrose gradient fraction of twice oligo(dT)-purified Chinese hamster brain mRNA enriched for HPRT mRNA (11.5 μg of mRNA); C–E, twice oligo(dT)-purified mRNA from Chinese hamster testis (18.5 μg), brain (10.0 μg), and liver (42.2 μg), respectively. M_r marker protein: triosephosphate isomerase (26,600).

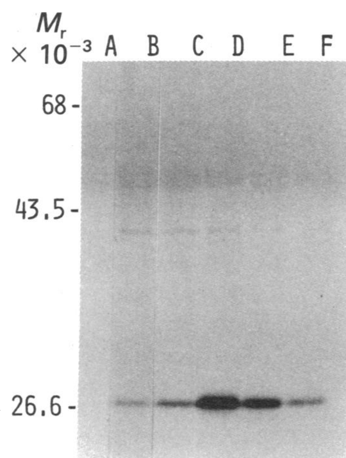

FIG. 3. *In vitro* translation of twice oligo(dT)-purified mRNA from mouse neuroblastoma cells. Translations were carried out in a total volume of 25 μl for 1 hr at 37°C and the products were immunopurified. Lanes: A, no mRNA; B, NB^- (3.7 μg of mRNA); C, NB^+ (4.6 μg); D–F, NBR4 (0.06, 0.03, and 0.015 μg of RNA, respectively).

tein synthesis directed by mRNA prepared from the wild-type, NB^+, cell line is shown in Table 1. This is comparable with the *in vivo* determination described previously (6). The 6-thioguanine-resistant NB^- line has been identified as CRM^- from *in vivo* studies (6), yet the presence of CRM was clearly visible after translation of NB^- mRNA (Fig. 3, lane B). In a series of experiments, translation of mRNA prepared from NBR4, a hypoxanthine/aminopterin/thymidine-resistant revertant of NB^- with elevated levels of a variant protein resulted in a 25- to 50-fold overproduction of HPRT protein relative to wild type (Table 1). Fifty-fold overproduction was calculated from the mRNA titration shown in Fig. 3, lanes D–F. NBR4 mRNA was separated on a sucrose gradient and HPRT mRNA-containing fractions were identified by their ability to direct HPRT protein synthesis *in vitro* (Fig. 4). The HPRT mRNA peak was located in fraction 10, corresponding to an estimated S value of 12.5. HPRT-specific radioactivity in translations primed with mRNA from the peak fraction was estimated to be 2%. These studies suggest that NB^- cells synthesize HPRT protein that is unstable *in vivo* but detectable *in vitro* and that mRNA from one of its

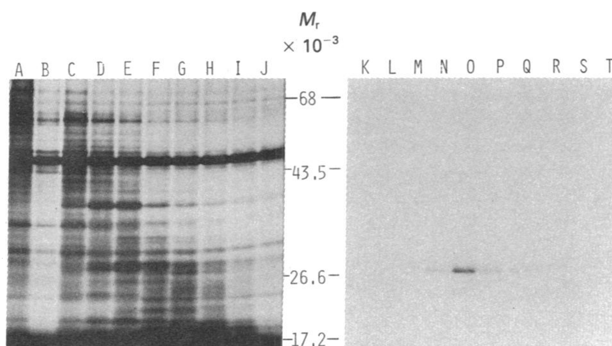

FIG. 4. *In vitro* translation of sucrose gradient-fractionated mRNA from mouse neuroblastoma HPRT revertant NBR4. Two hundred micrograms of twice oligo(dT)-purified NBR4 mRNA was separated on a linear 10–30% sucrose gradient and 19 fractions were collected from the bottom. Translations were carried out with 2% of the material from each fraction (total volume, 25 μl) for 1 hr at 37°C. Lanes: A–J, total translation products from fractions 6–15, respectively; K–T, immunopurified translation products from fractions 6–15, respectively. M_r markers: bovine serum albumin (68,000), ovalbumin (43,500), triosephosphate isomerase (26,600), and myoglobin (17,200).

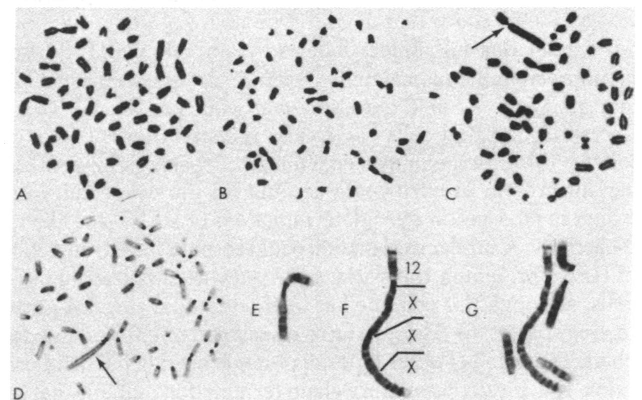

FIG. 5. Chromosome analysis of neuroblastoma cells. (*A*) Giemsa-stained metaphase of NB^+. (*B*) Giemsa-stained metaphase of NB^-. (*C* and *D*) Giemsa-stained and C-banded metaphase of NBR4, respectively. →, Elongated marker chromosome. (*E*) G-banded marker 3 from NB^+, comprising a centric fusion of chromosome 12 and the X. (*F*) G-banded elongated marker of NBR4. The p arm corresponds to chromosome 12, while the q arm consists of three copies of the X with the middle segment inverted. Note the partial constrictions separating the three segments. (*G*) Reconstruction of the NBR4 marker shown in *F*; the X;12 translocation from NB^+ (*E*) has been cut at the centromere and three copies of the q arm are aligned to demonstrate homology with the NBR4 marker.

revertants, NBR4, has enhanced capacity to direct HPRT synthesis *in vitro*.

Cytogenetics. Modal chromosome numbers for the NB^+, NB^-, and NBR4 cell lines were 59, 57, and 56, respectively. Conventional Giemsa staining consistently showed a large biarmed marker chromosome in NBR4 cells (Fig. 5*C*) that was present in neither the NB^+ (Fig. 5*A*) nor the NB^- (Fig. 5*B*) line. The marker had only a single C-band located at the centromere (Fig. 5*D*). G-banding showed the marker chromosome to be partially homologous with the third largest biarmed chromosome (marker 3) of NB^+ and NB^-, which is the product of a centric fusion between chromosomes 12 and X (Fig. 5*E*). The p arm of the NBR4 marker is homologous to chromosome 12 (Fig. 5*F*), while the elongated q arm consists of three copies of the X chromosome (less two centromeres) with the middle segment inverted relative to the other two. No free X chromosomes were found in any of the neuroblastoma lines and there was no evidence for either homogeneously staining regions or double minute chromosomes in the NBR4 karyotype.

DISCUSSION

The qualitative and quantitative data reported here for wild-type and mutant Chinese hamster cell lines suggest that HPRT synthesis *in vitro* accurately reflects the *in vivo* situation. HPRT subunits synthesized by wild-type Chinese hamster cells had the same molecular weight as subunits made by *in vitro* translation of RJK0 mRNA. Thus, there was no evidence for a naturally occurring HPRT precursor analogous to those identified for some proteins by *in vitro* translation (15–17).

The translation system has been used to identify both tissue and cultured cell sources producing high levels of HPRT protein. HPRT activity in mammalian brain is known to be higher than that in other tissues (14). This study, which has shown that mRNA from Chinese hamster brain directs the synthesis of seven times more HPRT relative to total protein than Chinese hamster liver mRNA, suggests tissue-specific regulation of HPRT mRNA levels. Greatest overproduction was observed with mRNA from the mouse neuroblastoma HPRT revertant cell line NBR4: HPRT-immunoprecipitable radioactivity *in vi-*

tro was 25–50 times that in wild-type neuroblastoma cells and 6–12 times that in Chinese hamster brain. *In vivo* HPRT-immunoprecipitable radioactivity determinations have previously indicated 5- to 9.5-fold elevation over wild type (6). HPRT extracted from NBR4 cells has greatly reduced thermal stability relative to wild-type mouse enzyme (6). Greater stability of the variant protein *in vitro* could account for the discrepancy between *in vivo* and *in vitro* determinations of HPRT-specific radioactivity. A similar explanation could account for the detection of HPRT protein in translations primed with mRNA from NB^- cells, although this cell line has been identified as CRM^- from *in vivo* studies (6). Thus, *in vitro* translation might facilitate the characterization of other types of mutants making unstable proteins, specifically premature chain termination mutants that are thought to be difficult to detect *in vivo* because the incomplete polypeptide chains are rapidly degraded (1).

HPRT extracted from NBR4 cells has reduced catalytic activity and thermal stability relative to wild-type mouse enzyme (6). NBR4 cells achieve near normal apparent HPRT specific activity and survive in hypoxanthine/aminopterin/thymidine medium by overproducing the variant protein. The experiments reported here cannot discriminate between increased HPRT mRNA levels and increased translational efficiency as the basis for HPRT overproduction. We believe that the presence of an X chromosome alteration in NBR4 cells favors the first hypothesis. Although there are three copies of the X chromosome in NBR4 cells, this alone cannot explain the observed 25- to 50-fold overproduction of HPRT protein. There was no evidence for the presence of homogeneously staining regions or double minute chromosomes in the NBR4 karyotype. Both have been associated with dihydrofolate reductase (tetrahydrofolate dehydrogenase, 7,8-dihydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) gene amplification in cultured cells selected for resistance to progressively increasing concentrations of methotrexate (18, 19). The possibility cannot be excluded that small regions of amplified HPRT genes exist, either on the marker or scattered amongst other chromosomes. However, since HPRT has been mapped to the proximal region of the X chromosome (20), the cytogenetic data are also compatible with a position effect altering the regulation of one or more of the *HPRT* loci. The isolation of nucleic acid probes for the *HPRT* gene should resolve the molecular basis for the HPRT overproduction.

We thank Susan Airhart for technical assistance and Drs. R. H. Geller, J. E. Reichenbecher, Jr., and R. G. Fenwick, Jr., for critically reviewing this manuscript. D.W.M. gratefully acknowledges a long-term fellowship from the European Molecular Biology Organization. This investigation was supported by the Howard Hughes Medical Institute.

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