
Rapid assay for detection of *Escherichia coli* xanthine-guanine phosphoribosyltransferase activity in transduced cells

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ABSTRACT

Cultured mammalian cells transduced with the *Escherichia coli* gene, *Ecogpt*, synthesize the bacterial enzyme xanthine-guanine phosphoribosyl transferase (XGPT) (1). This paper describes a method for measuring XGPT activity in crude cell extracts by following the conversion of ^{14}C -xanthine (X) to ^{14}C -xanthine monophosphate (XMP) and ^{14}C -xanthosine (XR) by thin layer chromatography. The method is rapid, easy to use, sensitive and linear over a wide range of XGPT activity and has been useful for detecting XGPT in cells that were transiently transfected or stably transformed with *Ecogpt*. During our studies, we have found that a human cell line (XP20S) converts xanthine to XMP. This activity is probably catalyzed by a variant hypoxanthine-guanine phosphoribosyltransferase (HGPT) since the low activity is readily inhibited by hypoxanthine. A low level of conversion of X to XMP may explain why some cell lines are not killed in a medium containing mycophenolic acid and X.

INTRODUCTION

The enzyme xanthine-guanine phosphoribosyltransferase (XGPT), encoded by the *Escherichia coli* gene, *Ecogpt* (hereafter referred to as *gpt*), converts xanthine (X) to xanthine monophosphate (XMP) in the presence of phosphoribosylpyrophosphate (PRPP) (2). There appears to be no mammalian enzyme with comparable activity (3).

Mycophenolic acid (MPA), an inhibitor of IMP dehydrogenase (4), prevents the formation of XMP, and therefore of guanosine monophosphate (GMP). Normal mammalian cells do not grow in medium containing MPA. However, cells that have been transduced with an expressible form of *gpt* can convert X to XMP and then to GMP, and, therefore, acquire the ability to grow in a medium containing MPA and X (5). The marker *gpt* has therefore been used as a dominant selectable marker to transform cultured mammalian cells.

A variety of methods (1, 6) have been used to detect and measure XGPT enzyme activity in extracts of transduced cells but these are time consuming or limited because of high background activities. This paper describes a method using thin layer chromatography (TLC) to separate the substrate and

products of the XGPT reaction. Because the method is sensitive, quantitative and rapid, the expression of gpt during transient transfection of mammalian cells can be readily detected and quantitated.

METHODS

Plasmids

The gpt gene in pSV2-gpt is transcribed from the SV40 early region promoter and the transcript is processed at SV40 splicing and polyadenylation signals located beyond the gpt segment (1). The plasmid pRSV-gpt is derived from pSV2-gpt by substitution of the long terminal repeat from Rous sarcoma virus for the SV40 promoter (7).

Plasmids were grown in *E. coli* strain HB101. DNA was prepared from bacterial cultures by established methods (8) with some modifications. The bacterial isolates were grown in one liter of culture to an optical density of 2 at 600 nm. After chloramphenicol (150 mg/ μ l) was added, the cultures were grown for 12-16 hours more, and the cells were pelleted at 4°C and resuspended in 12 ml of 25% sucrose, 50 mM Tris buffer, pH 8. Lysozyme was added to 14 mg/ml (2 ml of 100 mg/ml lysozyme) and after 5 minutes of incubation on ice, EDTA was added to 0.0625 M (2 ml of 0.5M EDTA). After 5 minutes more incubation on ice, 15 ml of a lysis mixture containing 0.5% Triton X100, 50 mM Tris buffer pH 8 and 0.0625 M EDTA was added and the mixture was incubated for 15 minutes more on ice. The crude lysate was centrifuged at 46,000 x g to remove the bulk of the chromosomal DNA and the supernatant was purified twice by centrifugation in cesium chloride density gradients containing ethidium bromide (9). This method routinely yielded 5-8 mg of plasmid DNA per liter of bacterial culture.

Cell Culture and DNA Transfection

Mouse fibroblasts (3T6) and 3T6 cells that had been stably transformed with pSV2-gpt (3T22) were grown in Dulbecco's modified Eagle's medium containing penicillin, streptomycin and 5% fetal calf serum. Normal human fetal lung fibroblasts immortalized by SV40 (WI-38 VA-13 subline 2RA, ATCC CCL 75.1) and human fibroblasts immortalized by SV40 from patients with xeroderma pigmentosum, XP20S (10) and XP2Y0 (11), were obtained from Errol Friedberg (Stanford University) and grown in the same medium but supplemented by 10% fetal calf serum.

Subconfluent cultures of these cells on 85 mm plates were transfected with 10 μ g of pSV2-gpt or pRSV-gpt DNA by coprecipitation with calcium phosphate in 0.5 ml (12) followed by a one minute 25% glycerol shock (13, 14).

Two days following transfection, cell monolayers were washed twice with ice cold tris-saline, covered with 1 ml of tris-saline, scraped into a microfuge tube with a rubber policeman and centrifuged in an Eppendorf centrifuge for 30 seconds at 4°C. After removal of the supernatant, the cells were gently resuspended in 0.1 ml of ice cold extraction buffer consisting of 15% glycerol, 20 mM Tris pH 7.5, 10 mM dithiothreitol (DTT) and then disrupted by sonication for 2 minutes. The cell debris was pelleted in an Eppendorf centrifuge for 10 minutes at 4°C and the supernatant was used for the enzyme assay or stored at -20°C.

Enzyme Assay

Reaction mixtures contained 50 mM Tris buffer pH 8.5, 100 mM KCl, 5 mM MgCl₂, 2 mM DTT, 1 mM PRPP, 100 µg/ml bovine serum albumin and 180 µM ¹⁴C-labelled X (55 mCi/mole, ICN) with the pH chosen to optimize XGPT activity (6). A five-fold concentrate of buffer without ¹⁴C-X was stored in aliquots at -70°C.

Cell extracts (5-20 µl) were added last to make a 50 µl reaction mixture, which was incubated at 37°C, usually for 2 hours. After a single extraction with phenol/chloroform (1:1) to remove proteins which interfere with the resolution during TLC, the sample was applied to TLC plates (Polygram CEL 300 PEI, Brinkman Instruments), the spots were allowed to dry, and the plates were soaked in methanol for 5 minutes. The methanol extraction removes much of the unconverted X as well as impurities in the TLC plates which interfere with migration of the solvent front (15), but does not affect XMP. After drying, the plates were chromatographed in 0.9 M guanidine-HCl (Ultrapure, Schwarz-Mann) until the solvent front traveled 8-10 cm (15), marked with labeled India ink for orientation, dipped in 2-methylnaphthalene containing 0.4% PPO (New England Nuclear), wrapped in Saran wrap, and exposed to X-ray film (XAR-5, Kodak). For quantitation, the spots were cut out, immersed in 10 ml scintillation fluid (ACS II Aqueous Counting Scintillant, Amersham) and counted in a scintillation counter (Beckman LS 6800).

RESULTS

Extracts prepared from untransformed mouse cells and from cells that had been stably transformed with pSV2-gpt (3T6 and 3T22, respectively) were incubated with ¹⁴C-X and PRPP and analyzed by TLC as described in the Methods section. There was no detectable conversion of X to XMP by the untransformed cell extracts. However, appreciable amounts of XMP and XR

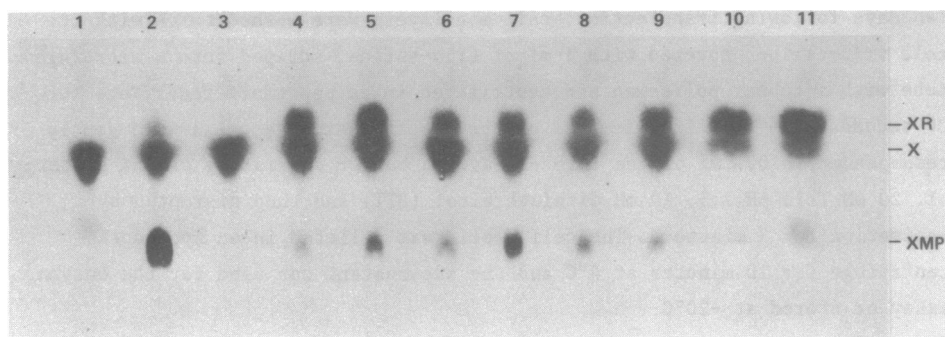


Figure 1. Thin layer chromatography for XGPT activity.

The positions for XMP, X and XR are marked on the right. Wider separation between XR and X may be obtained by more extended chromatography.

Lanes 1 and 2 are from cell extracts of mouse cell lines, 3T3 and 3T22, respectively. The latter is derived from 3T3 by stable transformation with pSV2-gpt.

Lanes 3 and 4 are from mock transfected and pRSV-gpt transfected VA-13 cells, respectively.

Lane 5 is activity from a 1:1 mixture of the cell extracts from 3T22 cells and pRSV-gpt transfected VA-13 cells.

Lanes 6-9 show activity from pRSV-gpt transfected VA-13 cell extracts incubated in the presence of various inhibitors of alkaline phosphatase, 10 mM sodium phosphate, 10 mM sodium fluoride, 5 mM adenosine monophosphate and 5 mM β -glycerolphosphate respectively.

Lanes 10 and 11 show the result of treatment with bacterial alkaline phosphatase after completion of reactions with cell extracts from pRSV-gpt transfected VA-13 cells and from 3T22 cells, respectively.

were produced from X by extracts from the pSV2-gpt-transformed cells (Figure 1, lanes 1 and 2).

XGPT activity was also measured in transient transfections of the human cell line VA-13 with pRSV-gpt DNA. Thus, 48 hours after transfection with pRSV-gpt DNA, there were substantial amounts of XR formed from X but there was no detectable activity in the mock-transfected cells (Figure 1, lanes 3 and 4).

We surmise that the accumulation of XR rather than XMP with VA-13 transfected cell extracts is a consequence of the presence of phosphatase. Thus, incubations with a mixture of 3T22 and VA-13 extracts produced more XR and less XMP than with 3T22 extract alone (Figure 1, lane 5). Also, the addition of alkaline phosphatase (15 units, Sigma P4252) following reaction of $^{14}\text{C-X}$ and PRPP with extracts of 3T22 cells caused a decrease in the amount of XMP and a concomitant increase in the level of XR (Figure 1, lane 11. Consistent with the likelihood of phosphatase activity in the VA-13 cell

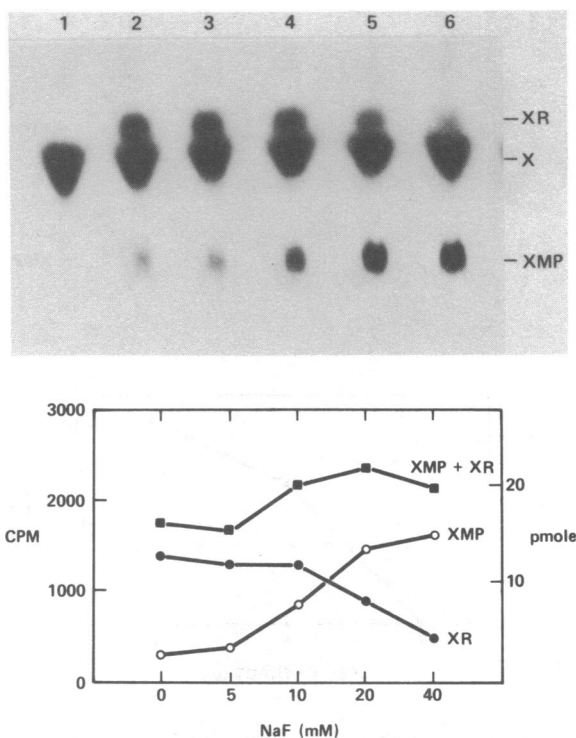


Figure 2. Inhibition of phosphatase activity with sodium fluoride.

The upper part of the figure shows the TLC patterns for XGPT activity in VA-13 cells. Lane 1 is from a mock transfection and lanes 2 to 6 are from transfections with pRSV-gpt. The levels of sodium fluoride in the various reaction mixtures shown in lanes 2 to 6 were 0 mM, 5 mM, 10 mM, 20 mM and 40 mM, respectively. Each lane was loaded with 6 μ l from a 50 μ l reaction.

The lower part of the figure shows the activity for XR, XMP, and the sum of XR and XMP, measured by cutting the corresponding spots from the PEI plate.

extracts is the restoration of XMP formation at the expense of XR by phosphatase inhibitors; the addition of 10 mM sodium phosphate, 10 mM sodium fluoride, 5 mM adenosine monophosphate or 5 mM β -glycerol phosphate produced increased amounts of XMP at the expense of XR (Figure 1, compare lane 4 with lanes 6 to 9). Because sodium fluoride appeared to be the most effective inhibitor, we tested various levels to determine conditions for maximal accumulation of XMP. Figure 2 shows that as the level of sodium fluoride is increased there is a substantial reduction in the amount of XR and a corresponding increase in XMP. Because of the possibility of phosphatases in

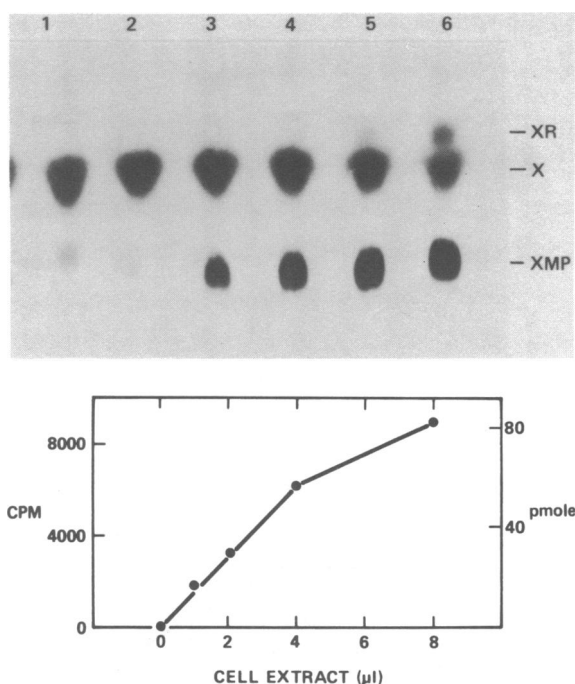


Figure 3. Xanthine conversion as a function of cell extract.

The upper part of the figure shows the result of TLC for XGPT activity in cell extract from 3T6 cells (lane 1) and varying amounts of cell extract in 3T22 cells (lanes 2-6). In lane 1, the reaction mix contained 5 µl of cell extract from 3T6 cells. In lanes 2-6, the reaction mix contained 0 µl, 1 µl, 2 µl, 4 µl and 8 µl respectively of cell extract from 3T22 cells.

The lower part of the figure shows the activity for the sum of XR and XMP, measured by cutting the corresponding spots from the PEI plate.

various extracts, the XGPT reaction mixtures routinely included 40 mM sodium fluoride and the cell extracts were added last to the reaction in order to minimize the destruction of PRPP.

A measure of the sensitivity of the assay can be obtained from the experiment shown in Figure 2. Approximately 5×10^6 VA-13 cells (from one 85 mm plate) were used to make 100 µl of crude cell extract; 5 µl of this extract were added to a 50 µl reaction and 6 µl of the reaction contents were loaded into a lane for the TLC separation. Therefore, the signal of approximately 2000 cpm or 20 pmole (Figure 2) represents 0.6% of the activity in an 85 mm plate of transiently transfected cells. The XGPT activity was readily detectable in an overnight exposure of the X-ray film.

With stably transformed cells (e.g. 3T22) the signal is even more pronounced (Figure 1).

The conversion of X to XR and XMP is a linear function of the amount of XGPT in the cell extract over a wide range of activity. Figure 3 shows the result with 3T6 cell extract (lane 1) and with increasing amounts of 3T22 cell extract (lanes 2 to 6). The formation of XR plus XMP from X is a linear function of the amount of cell extract although activity falls off at high levels of extract. Note that the untransformed 3T6 cell extract produces a product that migrates somewhat faster than XMP, but we have not identified its nature.

We have not detected any apparent XGPT activity in mock transfected extracts from a number of cell lines, including mouse 3T6, human VA-13 and human XP2YO cells (data for XP2YO not shown). However, there is a small, but significant, background conversion of xanthine to XMP in XP2OS cells. Thus, when extracts from XP2OS cells were assayed under standard conditions there was a detectable production of XMP (Figure 4A, lane 2); XMP production was markedly increased with extracts from pRSV-gpt transfected cells (Figure 4A, lane 6). The activity in the XP2OS extracts amounted to 1.5 pmoles or about 6% of the activity found in the pRSV-gpt transfected extracts. Since the bacterial XGPT utilizes hypoxanthine (HX) much less efficiently than does the mammalian HGPT, we tested the effect of HX on XMP production from X. Although the activities of untransfected and transfected cell extracts are both inhibited by unlabelled HX, there is nearly 80% inhibition of activity in the untransfected extract with 80 μ M HX but only about 10% inhibition of the activity in the transfected extract. Since the XGPT reaction occurs in the presence of 180 μ M X, the differential inhibition of background activity with HX is highly significant. The inhibition of bacterial XGPT activity becomes appreciable above 160 μ M HX. (See Figure 4B for a graphic presentation of the data).

Figure 4B indicates the Michaelis-Menton constants (K_m) of HGPT and XGPT for HX; 17 μ M and 167 μ M, respectively (6, 16). Since the activity in untransfected cell extracts is strongly inhibited by HX at concentrations much lower than that of the labelled X, but not much higher than the K_m of HGPT for HX, we conclude that the low level conversion of X to XMP in XP2OS cells is very likely catalyzed by the cellular HGPT.

We considered the possibility that the activity detected in XP2OS cells was due to contamination of the 14 C-X by 14 C-guanine (G); conversion of G to GMP by cellular HGPT would be expected to be inhibited by HX. But, this is

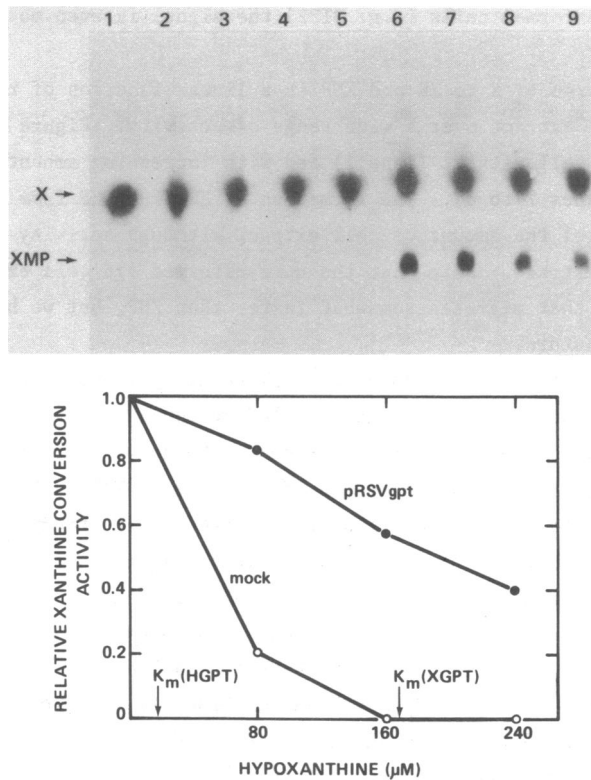


Figure 4. Background activity of X conversion in XP20S cells and inhibition by HX.

The upper part of the figure shows the result for the conversion of X in cell extracts from XP20S cells. Lane 1 is a mock reaction with no cell extract. Lanes 2-5 are from mock transfected XP20S cell extracts incubated in the presence of 0, 80, 160, 240 μM unlabelled HX, respectively. Lanes 6-10 are from pRSV-gpt transfected XP20S cell extracts incubated in the presence of 0, 80, 160, 240 μM unlabelled HX, respectively.

The lower part of the figure shows the result of measuring the amount of XMP in a scintillation counter and plotting relative conversion of X as a function of HX concentration. The curves for both mock and pRSV-gpt transfected cell extracts are normalized to the respective activities at zero HX concentration.

unlikely because GMP migrates significantly faster than XMP under the conditions of TLC used here.

DISCUSSION

We have described a new method for detecting and quantitating XGPT activity in crude cell extracts after the introduction of an expressible

form of the E. coli gene, *gpt*, into mammalian cells. The method measures the conversion of $^{14}\text{C-X}$ to $^{14}\text{C-XMP}$ in the presence of PRPP by the rapid separation of the labelled substrate and product by thin layer chromatography. The assay is easy to perform, sensitive and linear over a range of XGPT activity; these attributes make possible the detection and measurement of XGPT activity in cells that are transiently transfected even with non-replicating plasmids or in cells that are stably transformed with *gpt*. The same reaction mixture and TLC system can be modified to detect HGPT activity in transiently transfected HGPT negative cell lines, by substituting $^3\text{H-hypoxanthine}$ for $^{14}\text{C-xanthine}$ (H. Hayakawa, private communication).

In some cell lines, the product produced in the incubation is XR; this occurs where endogenous cellular phosphatases convert XMP, the primary product of XGPT, to the ribonucleoside. The phosphatase activity can be effectively inhibited by including 40 mM sodium fluoride in the reaction so that most of the product appears as XMP.

In one cell line studied, XP20S, a human line established from a female patient with xeroderma pigmentosum, the extracts can convert X to XMP at a low level. These cells do not have a defective HGPT since they grow well in HAT (HX, aminopterin and thymidine) medium. (Data not shown.) This low level activity is not characteristic of human lines, since neither VA-13 nor XP2Y0 cells (another human xeroderma pigmentosum cell line) show the same activity. Since the activity is strongly inhibited by unlabelled HX at concentrations which have little effect on the activity of XGPT, this activity can be specifically suppressed. The assay thus retains nearly full sensitivity in such variant cell lines.

The finding mentioned above agrees with an earlier report that HGPT can convert X to XMP, albeit with a low efficiency (3). Since this capability was not detected in all cells, we suggest that there is genetic variation in the affinity of HGPT for X. Thus, the XP20S cell line may represent a variant, but otherwise normal, HGPT which is capable of converting X to XMP. Such variation in activity of HGPT may explain why some cell lines are not efficiently killed in medium containing mycophenolic acid and X.

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