Genet. Res., Camb. (1989), **53**, *pp.* 201–206 *With* 3 *text-figures Printed in Great Britain*

Cytogenetic manifestations associated with the reversion, by gene amplification, at the HGPRT locus in V79 Chinese hamster cells

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(*Received 20 June 1988 and in revised form 5 December 1988*)

Summary

Some HGPRT spontaneous revertants were isolated from a mutant line (E2) of V79 Chinese hamster cells and phenotypically characterized. Dot–Blot hybridization with a ^{32}P-labelled HGPRT probe revealed an increase in the number of HGPRT sequences in some of these revertants, suggesting the occurrence of gene amplification. Cytogenetic analysis performed in three of these revertants showed a characteristic abnormally banding region (ABR) on the elongated p arm of the *X* chromosome. *In situ* hybridization in one revertant (RHE2) showed that the amplified sequences reside on the p^+ arm of the *X* chromsome in two different localizations. Because of the very probable clonal origin of the revertant, these features indicate that the amplified sequences might rearrange after their integration into the chromosome.

1. Introduction

In recent years gene amplification has been considered a mechanism for adaptation of eukaryotic cells to a variety of selective conditions (Stark, 1986) and it has been indicated as a reversion mechanism at different loci in mammalian cells (Patterson *et al.* 1985; Steglich *et al.* 1985). While there are many examples in which amplified DNA sequences are associated with the presence of chromosomal abnormalities, such as homogeneous staining regions (HSRs), abnormally banding regions (ABRs) or double minutes (DMs) (Cowell, 1982; Lewis *et al.* 1982; Hamlin *et al.* 1984), cytogenetic analysis in HGPRT spontaneous revertants, carrying the altered gene amplified, did not identify some of these manifestations or differences in the number of *X* chromosomes (Zownir *et al.* 1984; Fenwick *et al.* 1984; Fuscoe *et al.* 1983), where the HGPRT gene has been localized (Farrel & Worton, 1977). The only example of chromosomal alteration was provided by Melton *et al.* (1981), in the HGPRT revertant mouse cell line NBR4 carrying a triplicate *X* chromosome. We have isolated some HGPRT spontaneous revertants from a mutant line (E2) of V79 Chinese hamster cell. After their phenotypic characterization dot–blot analysis was performed to establish the number of HGPRT alleles. Cytogenetic studies were carried out to verify changes in the *X* chromosome, and *in situ* hybridization was accomplished to assess the chromosomal localization of HGPRT amplified sequences. Our results, confirming gene amplification as a mechanism for spontaneous reversion at the HGPRT locus, show that the amplified sequences are associated with an abnormally banding region (ABR) on the *X* chromosome's p arm.

This work is dedicated to Rosalba Randazzo, always in our mind.
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2. Materials and methods

(i) *Cells and culture conditions*

6-thioguanine (6-TG)-resistant cell line E2, used in this work was isolated after 30 mM-EMS treatment of a V79 Chinese hamster cell line. Cells were grown in Dulbecco modified MEM (D-MEM Flow) supplemented with 5% fetal calf serum (Flow) in a CO_2 incubator at 37 °C. Revertant clones were selected seeding 5×10^5 cells/100 mm plate in medium containing 10^{-4} M hypoxanthine, $3{\cdot}2 \times 10^{-7}$ M aminopterin, 10^{-5} M thymidine (HAT medium).

(ii) *DNA isolation and dot–blot hybridization*

Genomic DNA from cultured cells was essentially isolated by the method of Gusella *et al.* (1979). For blotting the DNA was denatured at 100 °C for 5 min,

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cooled and serial diluitions were bound to nitrocellulose filter with a Bio-dot apparatus (Bio-Rad). Filter was air dried and baked under vacuum for two hours at 80 °C. The insert of the plasmid pHPT12, generously provided by Professor C. T. Caskey (Howard Hughes Medical Institute, Houston, Texas, USA), was used as a probe for the HGPRT gene, 200 ng of which was nick-translated, in presence of 60 μCi [^{32}P]dCTP (3000 Ci/mmol, Amersham) to a level of 10^8 cpm/μg DNA. The plasmid p102 containing the 3′ region of CAD gene was ^{32}P-labelled and used as a control probe. This plasmid was a generous gift from Dr G. M. Wahl (Salk Institute, La Jolla, California, USA). Nitrocellulose filters were hybridized as described by Maniatis *et al.* (1982) with 2×10^6 cpm/ml of ^{32}P-labelled probes.

(iii) *Preparation of cell extracts and enzyme assay*

Cell free extracts were prepared as described by Fuscoe *et al.* (1982). Protein concentrations were determined using bovine gamma globulin as a standard (Bio–Rad protein assay). About 1–2 mg of proteins were obtained from 1×10^8 cells. HGPRT activity was measured as described by Fuscoe *et al.* (1982), using 4 μCi [^{3}H]hypoxanthine (10 Ci/mmol, NEN). The enzyme activity was calculated from the linear portion of the reaction and activities were expressed per milligram of soluble protein.

(iv) *Preparation of chromosome for cytogenetic analysis and* in situ *hybridization*

Cells were seeded in 75 cm^2 flasks (Sterilin) at a density of 2×10^6 cells/flask. Before cultures reached confluence 0·1 μg/ml of Colcemid (C. Erba) was added for 2 h and mitotic cells were harvested by shaking the flasks. Metaphase spreads were prepared by standard techniques. Air-dried slides were C- and G-banded according to Sumner (1972) and Seabright (1971) respectively, with minor modifications. For *in situ* hybridization the Harper & Saunders method (1981) was essentially used. The probe was ^{3}H-labelled by nick translation to specific activity of 1×10^7 cpm/μg using 6 μM each of the three ^{3}H-labelled nucleotides ([^{3}H]dCTP, 54·3 Ci/mmol; [^{3}H]dATP, 49·5 Ci/mmol; [^{3}H]dTTP 93·5 Ci/mmol) (Zabel *et al.* 1983). Fifty microlitres of hybridization mixture containing $2·5 \times 10^5$ cpm were placed on each slide. After hybridization the slides were dipped in Kodak NTB2 emulsion and exposed for 20–30 days, developed in Kodak Dektol, fixed and stained with Wright Giemsa diluted 1/3 with 0·06 M phosphate buffer pH 6·8 for 5–8 min.

3. Results

Thirteen spontaneous revertant clones derived from the HGPRT$^-$ mutant line (E2), were stabilized from separated colonies selected in HAT medium and

Table 1. *Phenotypic characterization of the revertant clones derived from E2*

	Cloning efficiences	
Cell line	HAT ([a])	6-TG ([b])
V79	N.T.	$5·7 \times 10^{-6}$
E2	$2–6 \times 10^{-6}$	N.T.
5R	1·3	$1·6 \times 10^{-3}$
6R	1·3	$2·9 \times 10^{-3}$
DR	1·1	$4·0 \times 10^{-4}$
7R	1·0	$1·0 \times 10^{-3}$
8R	1·0	$1·7 \times 10^{-3}$
AR	1·7	$1·3 \times 10^{-3}$
3R	0·9	$5·0 \times 10^{-3}$
9R	1.0	$8·7 \times 10^{-3}$
CR	0·8	$9·7 \times 10^{-3}$
1OR	1·0	$5·2 \times 10^{-3}$
2R	0·7	$1·0 \times 10^{-2}$
BR	0·9	$1·2 \times 10^{-3}$
RHE2	1·0	$2·5 \times 10^{-4}$

[a] The frequency of colony formation in HAT medium is expressed relative to that in non-selective medium (D-MEM).
[b] The frequency of colony formation in medium containing 6-TG (5 μg/ml) is determined comparing the number of colonies at density of 200, 1×10^3 or 1×10^4 cells per dish (triplicate plates).
N.T., not tested.

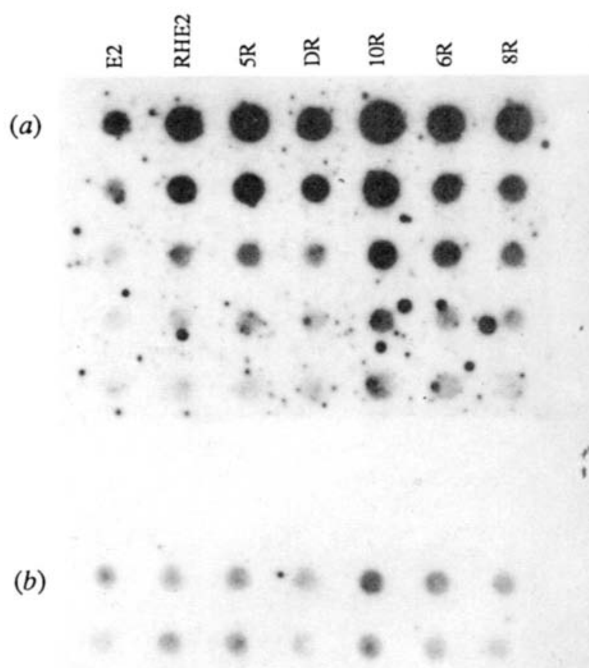

Fig. 1. Reproduction of the dot–blot autoradiogram. Letters above columns refer to the clone tested. (*a*) On the filter hybridized with hamster HGPRT cDNA, the amount of DNA spotted is, from top to bottom: 1, 0·5, 0·2, 0·1, 0·05 μg. (*b*) To normalize results, for variations in DNA concentration, dots with (top) 0·5 μg or (bottom) 0·2 μg DNA from each clone were hybrized with a probe for the CAD gene.

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Table 2. *HGPRT activities of E2 and its phenotypic revertants arising from gene amplification*

Cell line	HGPRT activity in extracts[a]
V79	0·50
E2	0·01
RHE2	0·24
DR	0·48
5R	0·17
6R	0·25

[a] Values are expressed as nanomoles of IMP formed per minute per milligram of protein.

maintained under selection. To characterize the phenotype of these revertants, the cloning efficiency was estimated either in HAT medium or in presence of 6TG. The cloning efficiency in 6TG medium was much higher than that of wild type cells, suggesting that these revertants have an unstable phenotype (Table 1). In some reports (Kaufman *et al.* 1979; Zownir *et al.* 1984) phenotypic instability has been associated with gene amplification at the selected locus. To test this hypothesis we have performed dot–blot hybridization of genomic DNA isolated from revertants indicated in Table 1. As can be seen in Fig. 1 some of these revertants (6/13) gave an hybridization signal stronger than the control one, indicating an increased copy number of the altered HGPRT gene. Using as internal standard (Sager *et al.* 1985) the probe for the CAD gene, that does not amplify under the selective conditions used, the copy number of HGPRT gene in each revertant were calculated by densitometry and resulted to be 4–8. In Table 2 is shown that the increase in the HGPRT copy number is accompained by a relative enhancement in HGPRT activity, with respect to the mutant level, as revealed by the enzyme assay. The observed phenotypic instability, increased number of the HGPRT sequences and partial recovery of HGPRT enzyme activity, all together suggest gene amplification as responsible for the reversion in these clones; although it is difficult rigorousely to rule out the possibility that some of these amplified clones have undergone also to a second site mutation in one of these extracopies. Cytogenetic studies, of more than one hundred metaphases for each revertant clone, revealed the presence of *X* chromosomes with an unusual elongated p arm and some dicentrics involving the *X* chromosome. The elongated Xp arm, after G-banding, appared with a discernibile banding pattern, rather than with an homogeneous staining pattern (Fig. 2), resembling the ABRs detected in MTX-resistant lines carrying a low degree of DHFR gene amplification (Lewis *et al.* 1982). The same investigation in revertant clones that did not show amplification of the HGPRT gene, did not reveal

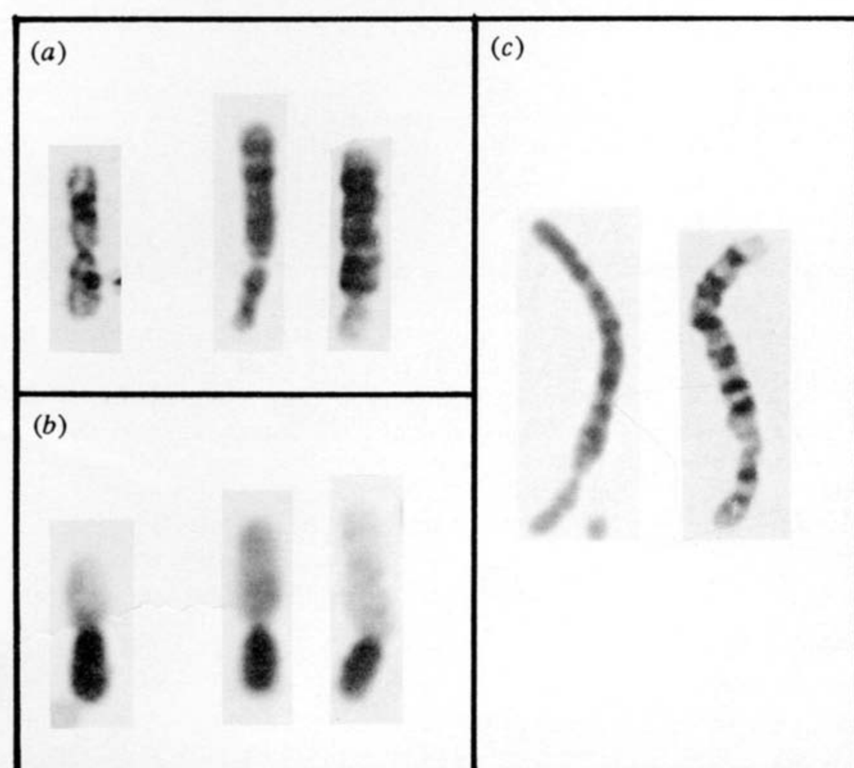

Fig. 2. Comparison between the *X* chromosome, G- and C-banded, of the parental mutant line E2 (*a–b*, on the left) and representative *X* chromosomes with an abnormally elongated p arm (*a–b*, on the right), from different metaphase spreads of DR clone. (*c*) *X* chromosomes with an extremely elongated p arm, probably derived by *X* dicentric chromosome breakage (see text for details) observed in some metaphase spreads of 5R clone. Similar Xp^+ chromosomes were present in all the revertant clones examined.

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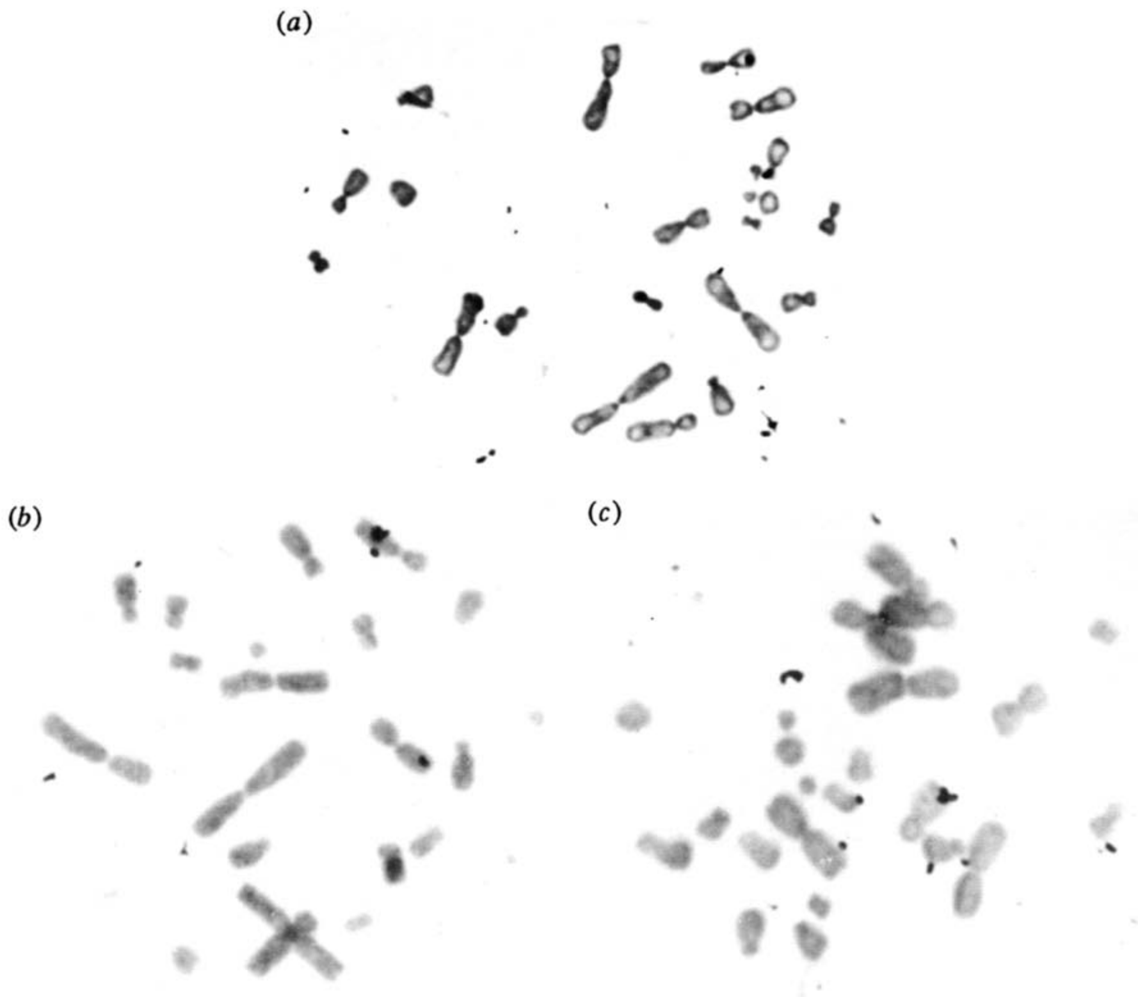

Fig. 3. *In situ* hybridization with an [^{3}H]cDNA HGPRT probe of representative metaphases of (*a*) the parental mutant line E2 (30 days of exposure) and (*b*–*c*) the revertant clone RHE2 (20 days of exposure). In the RHE2 clone grains are localized in two different regions mutually exclusive of the Xp$^+$ arm.

alterations in the *X* chromosome. To ensure that ABR-carrying *X* chromosome was a feature of the amplification event, *in situ* hybridization was carried out on the revertant clone RHE2 and on the parental mutant line E2. A total of 24 well discernible metaphase spreads of the RHE2 clone were examined, in which 130 grains were on chromosomes; of these 130 grains 71·5% (93/130) were located on the p$^+$ arm of the *X* chromosome. Grains at sites other than those on the *X* chromosome were distributed randomly over the rest of the chromosomes. As shown in Fig. 3 grains are localized on the p$^+$ arm of the *X* chromosome either distally or closer to the centromere, the two localization being mutually exclusive; in the clone E2 grains are localized in the terminal region of the Xp arm.

4. Discussion

Gene amplification in cultured mammalian cells has been associated with the reversion of a variety of mutant phenotypes (Roberts & Axel, 1982). Although phenotypic reversion might occur by other mechanisms, such as a second site mutation, gene amplification has been indicated as a mechanism mediating reversion of HGPRT genes encoding kinetically altered (Fenwick *et al.* 1984) or thermosensitive enzyme (Fuscoe *et al.* 1983; Melton *et al.* 1981; Brennand *et al.* 1982; Zownir *et al.* 1984).

At present time studies, about gene amplification as a mechanism for reversion at the HGPRT locus, have not shown cytogenetic manifestations typically associated with many examples of drug resistant cell lines containing multiple copies of the target gene. In this study chromosomal abnormalities associated with the *X* chromosome were shown by C- and G-banding in the revertant clones RHE2, DR and 5R. These abnormalities revealed themselves as disruptions in the normal G-banding pattern of the *X* chromosome, replaced by an abnormally banding region (ABR), or as a dicentric *X* chromosome, or as a very elongated Xp arm. The formation of an *X* dicentric chromosome and the subsequent bridge–breakage–fusion events (McClintock, 1941) might

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account for the extremely elongated Xp arm. Dicentric *X* chromosomes have been detected in diploid metaphases and because V79 cells contain only one *X* chromosome they might be derived either from sister chromatid fusion (Kaufman *et al.* 1983), accompanied by centromere inactivation, or from a switch of the replication fork that generates an entire duplication of the chromosome (Cowell & Miller, 1983).

Since in the parental mutant line E2 X dicentric chromosomes have not been detected, they must be correlated with the presence of amplified DNA sequences. Until now, whether chromosomal alterations underlie gene amplification is an open question. In recent reports Hahn *et al.* (1986) and Morgan *et al.* (1986) have shown chromosomal alterations to occur immediately after HU-treatment suggesting these as responsible for gene amplification. On the other hand Schimke *et al.* (1986) proposed a model that explains the generation of a variety of chromosomal rearrangements resulting from different manners in which the overreplicated strands can recombine.

Our *in situ* hybridization results indicate that in the revertant clone RHE2 the amplified sequences are localized either distally or proximally to the centromere of the Xp^{+} chromosome arm. An explanation for this result might be the possibility for the amplified sequences to rearrange in a different way. However, because of the very probable clonal origin of the revertant, the different localization of HGPRT amplified sequence is likely due to chromosomal rearrangements following the integration of amplified sequences in the *X* chromosome. These results suggest that the observed chromosomal abnormalities-rearrangements have been provoked by the presence of amplified sequences and that similar chromosomal anomalies associated with gene amplification as a mechanism for drug resistance can be correlated with gene amplification mediating reversion in the HGPRT system. We are now investigating, by *in situ* hybridization, if in the other two revertant clones, 5R and DR, the amplified sequences reside in alternative regions of the Xp^{+} chromosome arm.

We wish to thank Professor A. Abbondandolo for his encouragement to publish our data, and A. Polisano for his photographic assistance. This work was partially supported by a grant '40%' of the MPI.

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