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ORIGINAL ARTICLE

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A novel expression system for genomic DNA loci using a human artificial chromosome vector with transformation-associated recombination cloning

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Abstract Following the recent completion of the human genome sequence, genomics research has shifted its focus to understanding gene complexity, expression, and regulation. However, in order to investigate such issues, there is a need to develop a practical system for genomic DNA expression. Transformation-associated recombination (TAR) cloning has proven to be a convenient tool for selective isolation of a genetic locus from a complex genome as a circular YAC using recombination in yeast. The human artificial chromosome (HAC) vector containing an acceptor *loxP* site has served as a platform for the reproducible expression of transgenes. In this study, we describe a system that efficiently expresses a genetic locus in mammalian cells by retrofitting a TAR-YAC with the donor *loxP* site and loading it onto the HAC vector by the *Cre*/*loxP* system. In order to demonstrate functional expression of genomic loci, the entire human hypoxanthine phosphoribosyl transferase (*HPRT*) locus contained in a 100 kb YAC was loaded onto the HAC vector and was shown to complement the genetic defect in *Hprt*-deficient CHO cells. Thus, the combination of TAR cloning and the HAC vector may serve as a powerful tool for functional genomic studies.

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Introduction

Spaciotemporal regulation of gene expression in cells is achieved by complex mechanisms such as alternative splicing (Faustino and Cooper 2003; Graveley 2001; Wen et al 2004), alternative promoter–enhancer usage (Landry et al 2003), intronic gene expression (Conrad et al 2002), and expression of inhibiting RNAs (Tufarelli et al 2003). DNA polymorphisms affect these regulatory systems, and the composition of these polymorphisms produces a haplotype which represents the phenotype of cells in an individual (Hoehe et al 2003). Most of the conventional gene expression studies employ cDNA or minigene constructs. However, they cannot easily recapitulate the context of a genomic locus (Garrick et al 1998; Whitelaw et al 2001). The delivery of a gene as a genomic locus would enable control of gene expression by a native promoter and regulation of endogenous enhancer and silencer elements.

A new approach has recently emerged that allows entire genes and large chromosomal regions to be selectively and accurately isolated from complex genomes (Kouprina et al 1998). This system, called TAR cloning, employs in vivo recombination in yeast in order to clone genomic DNA. Yeast spheroplasts are transformed with genomic DNA along with a linearized TAR cloning vector, which contains yeast centromere, auxotrophic selection marker, and unique 5′ and 3′ sequences specific to the gene of interest. Linearization of the vector bears the recombinogenic target sequence at both ends. Transformation-associated recombination between the gene-specific sequences in the vector and the gene-containing genomic fragment

leads to the establishment of a circular YAC. Propagation of the YAC in the yeast cell depends on the acquisition of autonomously replicating sequences in the cloned genomic segment. This allows an entire gene to be isolated without interruption by restriction digestion, which is inevitable in conventional in vitro cloning methods (Kouprina and Larionov 2003). Since TAR cloning eliminates laborious steps such as constructing a new genomic library and screening to obtain the desired clones, it is applicable for isolating haplotype alleles from an individual (Kim et al 2003; Leem et al 2002).

Gene delivery into cells for functional expression is sometimes hampered by problems such as irreproducible expression levels among transfectants. This is thought to result from the highly variable number of copies integrated into the host genome and from position effects on gene expression due to random integration (Garrick et al 1998; Whitelaw et al 2001). To overcome these problems, we have established a HAC vector system (Katoh et al 2004). The HAC vector, produced by truncating the distal parts of both p- and q-arms from human chromosome 21 using chromosome engineering, is equipped with an acceptor *loxP* site at which circular DNA can be loaded by the *Cre*/*loxP* system. In this study, we tested the loading of a genomic clone isolated by the TAR method into a HAC vector and verified the functional expression of a gene from the genomic locus introduced, utilizing the well characterized human *HPRT* gene as a model.

Materials and methods

Cell culture and establishment of *Hprt*-deficient CHO cells

CHO cells carrying the 21ΔpqHAC vector were maintained in Ham's F-12 nutrient mixture (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS, USA) and 8 μg/ml blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). *Hprt*-deficient CHO cells were established by culturing the CHOHyg8 clone (Katoh et al 2004) in Ham's F-12 medium supplemented with 10% FBS, 8 μg/ml blasticidin S hydrochloride, and 15 μM 6-thioguanine (Kanto Chemicals, Tokyo, Japan).

Construction of the retrofitting vector

The retrofitting vector BRV1/hCMV-*loxP* was constructed as follows: a 0.8-kb hCMV-*loxP* fragment was obtained by digesting pBS226 (Invitrogen) (Fukushige and Sauer 1992) with *Spe*I and *Not*I, and then cloned into *Avr*II/*Not*I sites in the BRV1 vector (Kouprina et al 1998).

Retrofitting of the YAC and conversion to BAC

A standard lithium acetate transformation procedure (Ito et al 1983) was used to retrofit the circular *HPRT*-YAC isolated by TAR cloning (Kouprina et al 1998). Briefly, yeast containing the *HPRT*-YAC was treated with lithium acetate, then the linearized retrofitting vector was introduced, and it was plated on SD-URA plates. Ura + His + clones were isolated and propagated. The yeast chromosome-size DNA was prepared in agarose plugs, treated with agarase, and then electroporated with the linearized retrofitting vector into DH10B competent cells (Invitrogen) using Gene Pulser (Bio-Rad, Hercules, CA, USA). Transformants were selected on LB plates with chloramphenicol.

Pulsed field gel electrophoresis

Agarose plugs prepared from yeast transformants containing circular YACs were exposed to X-rays (100 Gy) for 30 min (Hitachi Medico, Tokyo, Japan). Chromosome-size DNA was separated using CHEF DR-II pulsed field gel electrophoresis apparatus (Bio-Rad), on 1% agarose gel in 0.5× TBE buffer at 14 °C. The run-time was 24 h at 6 V/cm with a 60–120 sec switch time ramp.

Southern blotting

After separation by agarose gel electrophoresis, DNA was transferred to Hybond N+ membranes (Amersham, Piscataway, NJ, USA) in 0.4 N NaOH solution. The membranes were hybridized overnight at 65 °C with ^{32}P-labeled probes and washed twice at 65 °C in 0.1× SSC and 0.1% SDS for 15 min. Radioactivity on the membrane was detected and visualized by an image analyzer, BAS2000 (Fuji Photo Film, Tokyo, Japan).

Restriction analysis of *HPRT*–BAC/YACs

The endonuclease restriction analysis was performed as follows. BACs were isolated using the standard method (Qiagen, Tokyo, Japan). Ten micrograms of BAC DNA were incubated overnight with 4 U of *Eco*RI per 1 μg DNA in 1× buffer (Wako, Osaka, Japan) at 37 °C. Digested fragments were separated by electrophoresis on a 0.8% agarose gel in 0.5× TBE.

Transfection of *HPRT*–BAC/YACs into CHOHyg8Δ*Hprt* cells

HPRT-loxP/BAC/YACs were isolated using a standard alkaline lysis procedure, purified on Qiagen columns, and transfected into *Hprt*-deficient CHOHyg8Δ*Hprt*. A total of 4×10^5 CHOHyg8Δ*Hprt* cells were transfected

with 5 μg of the *HPRT*-loxP/BAC/YACs and 1 μg of the *Cre* expression vector pBS185 (Invitrogen) by lipofection with 10 μl of Lipofectamine 2000 reagent (Invitrogen), following manufacturer's instructions. After being cultured for 24 h in basic growth medium, cells were then cultured in a medium containing 800 μg/ml G418 (Invitrogen). Fourteen days later, drug-resistant colonies were isolated and expanded for further analysis.

PCR analysis of genomic DNA

DNA from cell lines was prepared with Puregene solution (Gentra Systems, Minneapolis, MN, USA). Four pairs of primers were used for PCR:

- IN1L (5′-CCCCATCAGCCTCTGGTATCTTAGC-3′) and IN1R (5′-AGCCAGCACCTCAGATATACA-3′), specific for the intron 1 sequence (516-bp)
- 46L (5′-TGCTGGGATTACACGTGTGAACC-3′) and 47R (5′-GACT CTGG CTAGAGTT CCTTC TTCC-3′), specific for exon 2 (575-bp)
- exon9F (5′-ATTAAACTAATGTGATAGACTAC T GCTTTG-3′) and exon9R (5′-TTCAATGTTTCACT CAATAGTGCTGTGG-3′), specific for exon 9 (530-bp) of the *HPRT* gene, and CMVneo586 (5′-CGT AACAACTCCGCCCCATT-3′) and CMVneo972 (5′-CGGACAGGTCGGTCTTGACA-3′) for junction with *Neo* reconstruction (387-bp).

RNA expression analysis of *HPRT*

Cytoplasmic RNA was extracted from 5×10^6 cells using the NucleoSpin RNAII kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. Human *HPRT* RNA was detected by RT-PCR using the primers

- *HPRT*5′L(5′-TCCTCCTCCTGAGCAGTCA-3′) and
- *HPRT*exon3R (5′-CATCTCGA GCAAGACGT TC A-3′).

Fluorescence in situ hybridization analysis

Metaphase chromosomes were prepared from the exponentially growing cells in cultures, and fluorescence in situ hybridization (FISH) was carried out by standard methods (Kugoh et al 1999). The probe used was digoxigenin (Roche, Basel, Switzerland)-labeled human Cot-1 DNA (Invitrogen). The digoxigenin signal was detected by an anti-digoxigenin–rhodamine complex (Roche). The chromosomes were counterstained with DAPI (Sigma, St. Louis, MO, USA). Images were captured using a microscope (Nikon, Japan) equipped with a photometric CCD camera, digitally processed, and visualized with the Argus system (Hamamatsu Photonics, Hamamatsu, Japan).

Results

Retrofitting of the *HPRT*–YAC with loxP

The *HPRT1* gene is located on human chromosome Xq26.2 and encodes a purine salvage enzyme. Mutations in this gene result in Lesch–Nyhan syndrome (MIM 300322), a neurodevelopmental disorder characterized by self-injurious and abnormal motor behavior (Caskey and Kruh 1979). In our previous study, the *HPRT1* locus was selectively isolated as a circular YAC from a normal human fibroblast cell line MRC5 using the TAR cloning method (Kouprina et al 1998). A 381-bp genomic DNA sequence approximately 12 kb downstream of the last exon (exon 9) of the *HPRT* gene was used as a specific hook for recombination at the 3′ end of the locus. An 189-bp Alu-consensus sequence was used as the other hook for recombination at an upstream region of the locus. The size of the isolated circular YAC was ~100 kb, suggesting that the 40 kb sequence upstream of exon 1 was included in the *HPRT*-YAC. We first constructed the retrofitting vector BRV1-hCMV/*loxP* by modifying a BAC vector BRV1 using homologous recombination in yeast (Kouprina et al 1998) in order to equip the *HPRT*-YAC with a hCMV promoter and *loxP* site (Fig. 1a, b). A selectable marker URA3 was introduced into BRV1-hCMV/*loxP* for selection in yeast after transformation (Fig. 1a). BRV1-hCMV/*loxP* was linearized with *Bam*HI, leaving free ends homologous to the circular YAC vector, and used for transformation of yeast VL6-48 containing *HPRT*-YAC, 13-2. Transformants were selected on SD-URA plates and replica plated on SD-HIS and SD + 5FOA plates. Successful transformants grew on SD-HIS but not on SD + 5FOA.

Transformation of 13-2 yielded 17 colonies that grew on SD-URA and SD-HIS but not on SD + 5FOA. Retention of intron 1, exon 2, and exon 9 of the *HPRT* gene was confirmed by PCR analysis (data not shown). The *HPRT*-YACs were linearized by exposure to X-rays and separated by pulsed field gel electrophoresis, followed by Southern blotting with a human Cot-1 probe (Fig. 2b). Replacement of the ColE1 replication origin and Ampicillin resistance gene with the retrofitting vector was expected to increase the YAC size (Fig. 1a). Slightly shifted bands were detected in the retrofitted YACs, compared to the original *HPRT*-YAC 13-2, with one exception (clone#45). High molecular weight (HMW) DNA from the transformants was digested with *Eco*RI and subjected to Southern blotting with the *HPRT* cDNA probe (Fig. 2b). The DNA sequence information predicted that the nine exons are dispersed in four *Eco*RI fragments (Fig. 2a). Southern blotting showed the expected fingerprinting pattern of restriction fragments in 15 out of 17 transformants. Restriction fragments detected in the retrofitted YACs were identical to those in the

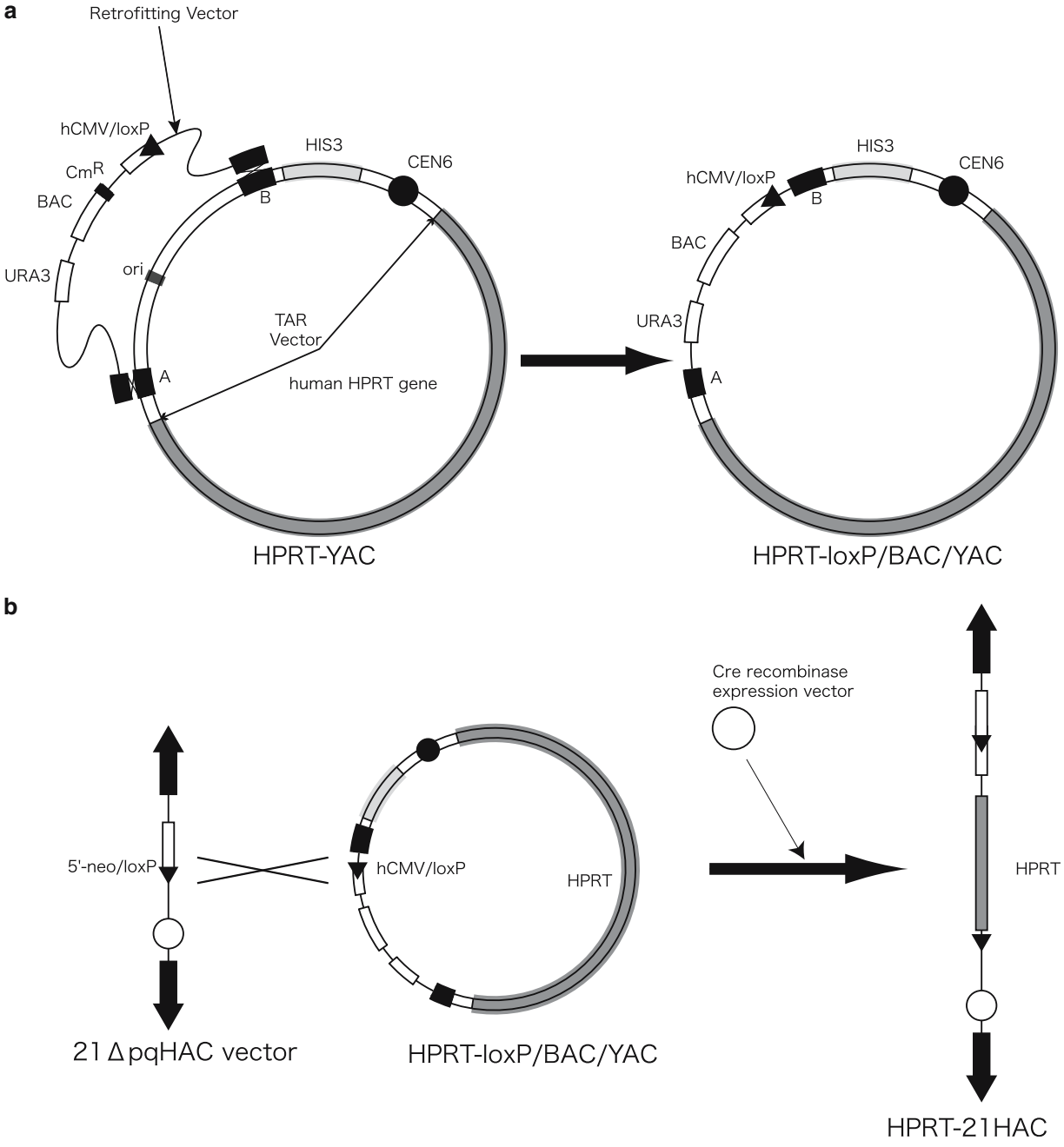

Fig. 1a,b Diagram of *HPRT*-YAC retrofitting and loading to the 21ΔpqHAC vector. A circular YAC carrying the human *HPRT* locus was first retrofitted by recombination with the retrofitting vector derived from BRV1. It contains the F-factor (BAC) origin of replication, the chloramphenicol acetyltransferase (Cm^R) gene, the URA3 yeast-selectable marker, two targeting sequences, A and B, and the hCMV-*loxP* sequence (**a**). The retrofitted YAC converted to BAC was isolated and transfected into *Hprt*-deficient CHO cells along with the *Cre* recombinase expression vector. Site-specific insertion was verified by reconstructing the functional Neo^R gene, which confers resistance to G418 (**b**)

parental YAC, suggesting that the *HPRT* locus in the YACs maintained genomic integrity. Arbitrarily chosen *HPRT*-hCMV/*loxP* YAC/BAC was transferred into *E. coli* cells by electroporation for further BAC isolation. Retention of intron 1, exon 2, and exon 9 of the *HPRT* gene in isolated BACs was confirmed by PCR analysis (data not shown).

Isolation of *Hprt*-deficient derivatives from CHO cells containing the HAC

The 21ΔqHAC, established in our previous study, is maintained in CHO cells (Katoh et al 2004), and possesses endogenous *Hprt* loci. To facilitate the detection of functional expression of the exogenously introduced

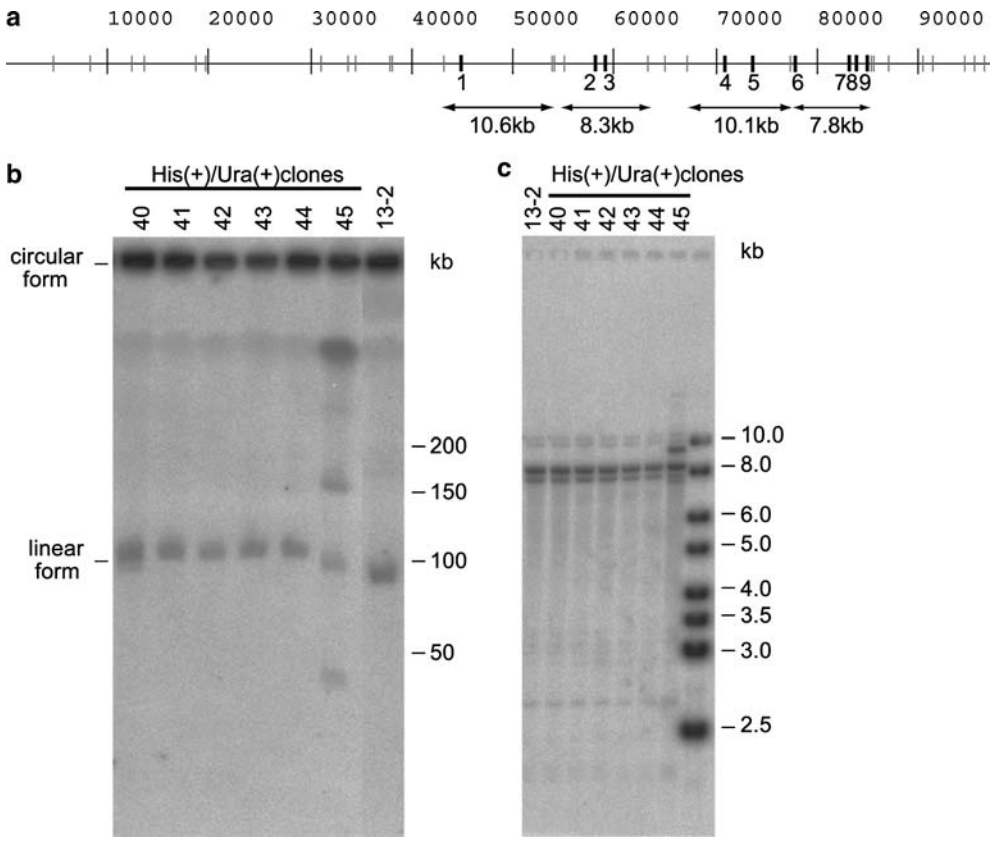

Fig. 2a–c Characterization of retrofitted *HPRT-loxP*/BAC/YACs by Southern blotting. **a** Restriction map of the 100 kb *HPRT*-YAC isolated by TAR cloning. Nine exons disperse in four *Eco*RI fragments. **b** Chromosomal-size DNA was prepared in agarose plugs from independent TAR YAC isolates containing the *HPRT* gene, exposed to X-rays, separated by pulsed field gel electrophoresis, and blot-hybridized with human Cot-1 DNA. The strong signals at the wells correspond to non-migrating circular molecules. The 100-kb sized bands correspond to linearized molecules containing the gene and the TAR vector. **c** The *Eco*RI fragment

human *HPRT* allele, *Hprt*-deficient CHO derivatives were isolated. Null mutations in the *Hprt* gene were identified by the phenotype resistance to a purine analog 6-thioguanine (TG). Sixteen TG-resistant colonies were obtained out of 5×10^5 cells after drug selection; these were isolated and expanded for the following analysis. All of the clones were sensitive to HAT selection, suggesting that the endogenous *Hprt* loci were spontaneously disrupted. Two clones named ΔH1 and ΔH2 were arbitrarily selected and subjected to FISH analysis (Table 2). In both clones, it was observed that one copy of the independent HAC was not integrated into the host chromosomes in almost all of the metaphase spreads. This result indicated that the selection of *Hprt*-deficient derivatives did not affect the retention and integrity of the HAC vector.

Introduction of the *HPRT*-hCMV/loxP YAC/BAC into the 21HAC vector

The CHO H8ΔH1 cells were transfected with *HPRT*-hCMV/*loxP* YAC/BAC and the *Cre* expression vector pBS185, followed by G418 selection. The *loxP* site-specific insertion of *HPRT*-BAC into the HAC vector was selected by reconstructing the functional *Neo* gene that confers G418 resistance (Fig. 1b). Thirty-two G418 resistant transfectants were obtained and propagated for the following analyses. PCR was performed in order to test the retention of the reconstructed *Neo* gene, intron 1 and exon 9 of the *HPRT* gene (Table 1). Although the presence of the *Neo* junction was detected in almost all of the clones, the deletion of the 5′- and/or 3′-ends of the *HPRT* locus was observed in just a few clones. Retention of the functional *HPRT* locus was tested by culturing the cells in HAT containing medium. While 18 clones (56%) retaining intron 1 and exon 9 were resistant to HAT selection, the remaining clones showing a deletion were HAT-sensitive (Table 1). Expression of the *HPRT* gene was tested by RT-PCR in the selected seven clones (Fig. 3). The transcripts were detected in the clones that retained the *HPRT* locus and exhibited a HAT^R phenotype. The integrity of the *HPRT*-HAC was also assessed in the three representative clones by FISH analysis. An independent HAC was observed, without any aberration such as translocation, amplification, or integration into a host chromosome (Fig. 4). One copy of the HAC was detected in the majority of the meta-

Table 1 PCR analysis and HAT selection of *Neo* resistant clones

HPRT			HAT resistance	Number of *Neo*^{r} transfectants (%)
Neo junction	5′-intron 1	3′-exon 9		
+	+	+	+	18 (56)
+	+	+	–	4 (12)
+	+	–	–	3 (9)
+	–	+	–	4 (12)
–	–	–	–	2 (6)
–	+	+	+	1 (3)

phase spreads and interphase nuclei (Table 2), suggesting that the HAC is correctly maintained throughout mitotic division. These results indicate that the *HPRT* locus loaded into the HAC vector successfully achieved functional expression through transcription, utilizing its own promoter with the correct splicing of the transcript.

Discussion

The HAC vector described here has several advantages. It has the capacity to house large inserts, can be introduced into many types of mammalian cells, and is stably maintained as an independent chromosome in these cells (Kakeda et al 2005; Katoh et al 2004). It also provides a defined acceptor site for site-specific integration of a transgene, which assures reproducibility in the expression level of transgenes (Otsuki et al 2005). We used this HAC vector to introduce the native human *HPRT* locus into *Hprt*-deficient CHO cells, and the gene expression was demonstrated on a transcriptional level and by functional complementation of the HAT-sensitive phenotype. These results are the first demonstration that a genomic locus carried on the HAC vector can correct a genetic defect of cells in vitro through production of an adequate level of the required protein.

On the 21Δpq HAC vector, the acceptor *loxP* site is surrounded by viral promoters that drive drug-resistant genes. The EGFP reporter gene driven by the CMV promoter was efficiently expressed from the 21Δpq HAC vector accommodated in an HT1080 hybrid (Katoh et al 2004). In contrast, the EGFP reporter gene driven by a minimal promoter with tissue specificity showed appropriate expression regulation in human mesenchymal stem cells during in vitro differentiation into osteoblasts only when the expression unit was surrounded by the insulators (Ren et al 2005). In this study, site-specific insertion of *HPRT* locus into the acceptor loxP site on the HAC vector showed functional expression of the *HPRT* gene. The *HPRT*-YAC/BAC used in this study has ~40 kb upstream and ~10 kb downstream regions around the substantial *HPRT* locus that include all regulatory elements needed for proper expression. Presence of these intervening sequences on both sides might ensure that the expression unit is insulated from being affected by the surrounding sequence at the acceptor *loxP* site.

Although the site-specific insertion of a genomic locus by *Cre*/*loxP* system has been reported by a few groups, the acceptor *loxP* sites were introduced in host chromosomes by random integration (Voet et al 2003). Call et al (2000) produced mouse ES cells carrying the acceptor *loxP* site and introduced a circularized linear YAC containing the *AML1* and *KCNE1* locus from the human chromosome 21q22.3. Random integration of the acceptor *loxP* site led to the insertion of multiple copies of the *loxP* sites in the majority of transfectants. In a selected transfectant showing a single *loxP* site, correct insertion of a full-length intact YAC was detected in a relatively small fraction (21%) of lox rec-

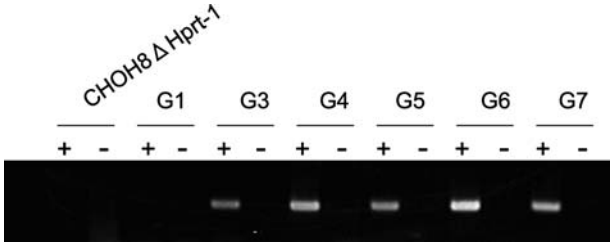

Fig. 3 RT-PCR analysis of 21ΔpqHAC vectors containing *HPRT*-*loxP*/BAC/YACs in *Hprt*-deficient CHO hybrid cells. RT-PCR was performed using a pair of primers specific to parts of exons 1–3 of the human *HPRT* gene. PCR products were detected in clones retaining the genomic locus that exhibited the HAT^R phenotype (clones G3–G7). *Plus* and *minus* signs indicate the presence or absence of reverse transcriptase

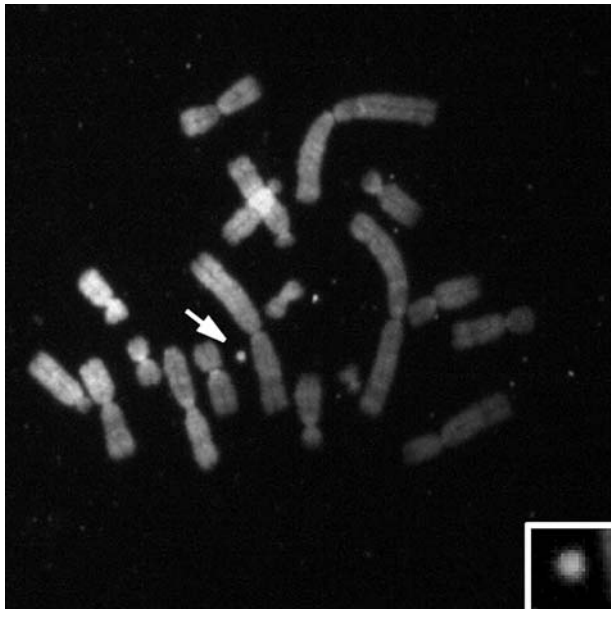

Fig. 4 FISH analysis of the 21ΔpqHAC vectors containing *HPRT*-*loxP*/BAC/YACs in *Hprt*-deficient CHO hybrid cells. Shown are photomicrographs of representative metaphase spreads of hybrid cells containing a HAC vector in CHO background. The human chromosome fragment was detected by the human Cot-1 DNA probe as an independent copy without any chromosomal aberration such as amplification or translocation (*arrow*). *Inset* shows a clipping of the human chromosome fragment

Table 2 FISH analysis of 21ΔpqHAC vectors in CHO cells

Clone	Number of analyzed nuclei metaphase/interphase	Number of independent Cot1 signals per nucleus metaphase/interphase			Retention rate of the HAC in interphase (%)
		0	1	2	
ΔH1[a]	50/100	0/1	48/96	1/3	99
ΔH2[a]	50/100	1/5	48/94	0/1	95
G1	50/100	8/11	40/85	2/4	89
G2	50/100	5/12	44/88	1/0	88
G4[a]	50/100	6/5	22/89	0/6	95

[a]Integration of the HAC in a host chromosome was observed in a fraction of metaphase spreads

ombinants. This might be partly due to inefficiency in isolating intact circular YAC molecules by fractionation in gel electrophoresis (Compton et al 1999). In contrast to this report, the copy number of the acceptor *loxP* site is strictly regulated in our HAC vector, and the HAC carrying the gene of interest can be transferred to other cells such as mouse ES cells by microcell-mediated chromosome transfer (Shinohara et al 2001). In addition, conversion of the circular YAC to BAC by retrofitting facilitated subsequent isolation of the BAC and efficient loading of the genetic locus into the HAC vector in intact form. Thus, improvement in some aspects of site-specific insertion provides a more practical tool for functional analysis of genomic loci in the isogenic cell system.

In conclusion, combination of the HAC vector and TAR cloning system provides a practical system for the functional expression of a gene from the genomic locus. It might potentially offer a straightforward approach to testing the functional significance of a large number of gene-associated haplotypes by direct isolation of a genetic locus from individuals followed by transfer to an appropriate in vitro cell culture.

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