The chicken HPRT gene: a counter selectable marker for the DT40 cell line

Tatsuo Fukagawa, Neil Hayward, Jian Yang, Claus Azzalin, Darren Griffin¹, A. Francis Stewart² and William Brown*  

Biochemistry Department, Oxford University, South Parks Road, Oxford OX1 3QU, UK, ¹Department of Biochemistry and Biology, Brunel University, Uxbridge, Middlesex UB8 3PH, UK and ²European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

Received February 9, 1999; Revised and Accepted March 17, 1999

ABSTRACT

We describe the cloning, characterisation and chromosomal mapping of the chicken hprt gene together with the construction of two counter selectable hprt−− DT40 derived cell lines. One of these cell lines contains a stably integrated gene encoding a conditionally active cre recombinase and thus allows efficient manipulation of targeted loci by site-specific recombination. These cell lines will enhance the utility of the hyper-recombinogenic DT40 cell line as a system for the genetic analysis of cell autonomous functions in vertebrates and as a tool for mammalian chromosome engineering.

INTRODUCTION

DT40 is an avian leukemia transformed chicken B cell line which efficiently integrates transfected DNA at homologous loci (1). Human chromosomes can be transferred into DT40 by somatic cell genetics techniques and can also be efficiently integrated in the resulting hybrid cells (2). The efficiency of sequence targeting in DT40 and the ease with which DT40 cells can be maintained have led to DT40 cells being widely used for studies of recombination (3), intracellular signalling (4), chromosome segregation (5) and for mammalian chromosome engineering (6). Although seven selectable marker systems (neomycin, hygromycin, blasticidin, puromycin, histidinol, zeocin/bleomycin and ecogpt) are currently in use, there exists no counter selectable marker gene that can be used in DT40 cells. In both budding yeast and in mammalian cells, counter selectable marker genes, either URA3 or hprt (hypoxanthine guanine phosphoribosyl transferase), respectively, have facilitated the introduction of precise mutations into endogenous genes by either pop-out or double replacement strategies (7), the study of the mechanism of homologous recombination (8) and chromosome engineering (9). Two features of the mammalian hprt gene have led to it being so widely used (10). First, it is encoded on the mammalian X chromosome and consequently it is very easy to select for loss of function mutants in cells derived from males which in mammals is the heterogametic sex. Secondly, the biochemical selection systems used to isolate either loss of function (6-thioguanine or 6-azaguanine) or gain of function mutants (hypoxanthine, aminopterin and thymidine) are simple and effective (11). Avian and mammalian sex chromosomes differ in their respective origins (12), and the heterogametic sex in birds is the female. Thus the avian hprt gene is probably autosomal and, as far as we are aware, no avian hprt mutants or genes have been described. Here we describe the cloning and organisation of the chicken hprt gene and the construction of two hprt−− DT40 cell lines that will enable genetic manipulations and analyses that require a counter selectable marker gene. One factor which currently restricts the utility of the DT40 cell line is that cell lines containing multiple mutations are difficult to construct because of the finite number of selectable marker genes. Therefore, one of the DT40 cell lines that we describe contains a stably integrated conditionally active cre recombinase (13,14). This cell line will allow the recycling of marker genes as well as the engineering of chromosomal translocations (9), deletions and other complex manipulations.

MATERIALS AND METHODS

Codon usage optimised degenerate oligonucleotides (15) corresponding to amino acid residues 52–59 and 151–159 of mammalian hprt were used to amplify a segment of the chicken cDNA using the RNA PCR kit of Perkin-Elmer (N808-0017). Total DT40 RNA (1 µg) was reverse transcribed with MuLV reverse transcriptase primed with random hexamers according to the manufacturer’s instructions. The cDNA was then amplified using the degenerate primers using 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. The product of this reaction was then cloned into pCRII (Invitrogen) and sequenced. The fragment was then used to screen a chicken macrophage cDNA library (a gift of John Young, Institute of Animal Health, Compton, Berkshire), and partial MboI digest libraries of DT40 genomic DNA in both lambda fix and Superco (both vectors from Stratagene). Cloned DNA was then mapped and sequenced according to standard procedures.

The chicken chromosome 4 paint (Griffin,D.K. et al., submitted) was labelled by incorporating biotin using degenerate oligonucleotide PCR and used in conjunction with hprt cosmid 4 DNA (Fig. 2) labelled with digoxigenin by nick translation to localise the hprt gene on spreads of mitotic DT40 chromosomes prepared by conventional techniques. The PCR labelling and fluorescent in situ hybridisation (FISH) were as described in Griffin et al. (submitted).

The two alleles of the hprt locus in DT40 cells were disrupted by two rounds of sequence targeting. In the first, an allele 2 specific construct was assembled using the Expand PCR kit (Boehringer),

*To whom correspondence should be addressed. Tel: +44 1865 275225; Fax: +44 1865 275259; Email: wrab@bioch.ox.ac.uk
primers specific for exons 3 and 4, 4 and 7 and a histidinol resistance gene. Oligonucleotides were designed to incorporate a loxP site on each side of the resistance gene and also included restriction enzyme sites for the plasmid constructions. The sequences of the oligonucleotides used to construct the 5′ targeting sequence were GAA GGA AAA AAG CGG CCG CTT GTA GCT CTC TGT GTA CTC and ACC TGG ATC CAT AAC TTC GTAT AGC ATA CAT TAT ACG AAG TTA TCT CCA GTT GAC TGA TCA TT, and for the 3′ targeting sequence ACC TGG ATC CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT AAT CTC AAC CT T and GAA GGA AAA AAG CGG CCG CTT GTC TGG CAC TTC AAA TCC. Thirty stably transfected clones were assayed for targeting of which 17 were targeted to the isogenic allele and three to the allogenic allele. Subsequently, an allele 1 targeting construct was assembled using an approach identical to the one above and a gene conferring resistance to hygromycin. Seven targeting sequence were GAA GGA AAA AAG CGG CCG CTT GAC TGA TCA TT, and for the 3′ sequences of the oligonucleotides used to construct the 5′ restriction enzyme sites for the plasmid constructions. The loxP site on each side of the resistance gene and also included restriction enzyme sites for the plasmid constructions. The sequences of the oligonucleotides used to construct the 5′ targeting sequence were GAA GGA AAA AAG CGG CCG CTT GTA GCT CTC TGT GTA CTC and ACC TGG ATC CAT AAC TTC GTAT AGC ATA CAT TAT ACG AAG TTA TCT CCA GTT GAC TGA TCA TT, and for the 3′ targeting sequence ACC TGG ATC CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT AAT CTC AAC CT T and GAA GGA AAA AAG CGG CCG CTT GTC TGG CAC TTC AAA TCC. Thirty stably transfected clones were assayed for targeting of which 17 were targeted to the isogenic allele and three to the allogenic allele. Subsequently, an allele 1 targeting construct was assembled using an approach identical to the one above and a gene conferring resistance to hygromycin. Seven stably transfected clones were analysed, of which four were targeted at allele 1. One of these clones was picked and co-transfected with a gene conferring resistance to zeocin (16) and a plasmid capable of expressing a fusion (13) between the cre recombinase and a mutant (G521R) human oestrogen receptor (pNPKcreER1cΔXholSalI) that was selectively sensitive to the steroid agonist 4-hydroxytamoxifen (4-OHT). Stably transfected clones were selected for the uptake of the cre oestrogen receptor (cre–ER) fusion plasmid, expanded and then cultured in 100 nM 4-OHT for 48 h. The excision reaction was monitored at 2, 6, 12, 24 and 48 h and found to be 50% complete at 24 h. The cells were then cloned by limiting dilution and checked for marker excision by restriction analysis of the hprt locus. Two clones in which both markers had been excised were picked and loss of the markers confirmed by PCR with reactions designed to separately detect the histidinol and hygromycin resistance genes. One of these two clones, referred to as DT4032zeocre, is presented below. The plasmid encoding the bacterial guanine phosphoribosyl transferase (gpt) gene used in the experiments to confer HAT resistance on the hprt−/− cell lines contained the Escherichia coli gpt gene coding sequence driven by the chicken β-actin promoter and was a kind gift of J. M. Buerstedde.

RESULTS AND DISCUSSION

We used degenerate primers and reverse transcriptase PCR to isolate a 321 bp fragment of the chicken hprt cDNA. We then used this cDNA to screen a chicken macrophage cDNA library, and this in turn led to the isolation of a 1585 bp cDNA which incorporated the entire hprt coding region of 218 amino acids. With the exception of non-conservative substitutions at residues 15, 56 and 214, the amino acid sequence of the chicken hprt enzyme is identical to or differs from the mammalian enzymes by no more than conservative substitutions (Fig. 1). We also used the original cDNA fragment to screen DT40 phage lambda and cosmid libraries, and this enabled us to isolate the chicken hprt gene and flanking DNA (Fig. 2). We had anticipated that the hprt gene would be autosomal and consistently clones corresponding to two alleles were isolated in our screen. Restriction analysis and filter hybridisation confirmed that these clones reflected the organisation of the genomic DNA and that DT40 is heterozygous at the hprt locus (not shown). Analysis of DNA isolated from chicken strains 6, 7, 15 and N demonstrated that the polymorphisms detected at the DT40 hprt locus were also segregating in free living birds (not shown). We assumed an identical intron–exon structure to the human locus and then used PCR and sequencing to map the nine exons encoding chicken hprt. Together this demonstrated that the hprt gene extended for 16 kb and it is therefore approximately one-third the size of the corresponding human gene. Although the intron–exon organisation of the gene is conserved between human and chicken, the rank order of intron sizes is not; thus in humans the largest intron is the first which is 13.3 kb (17) in size, while the corresponding chicken hprt intron is only 2.3 kb long.

The identification of two alleles in the chicken genomic DNA suggested that the hprt gene was autosomal and this was confirmed by multi-colour FISH, which mapped the chicken hprt locus to the tip of the short arm of chromosome 4 (Fig. 3). It is notable that chicken PGK 1 also maps to the tip of the short arm of chromosome 4 (18) and, like hprt, maps to the long arm of the human X chromosome (19). However, in humans, PGK1 and hprt map far apart on Xq with hprt mapping to Xq26 and PGK1 to Xq13, suggesting that if the synteny of the genes in the two species is due to synteny in a common ancestor then this relationship has been extensively disrupted in the estimated 300 million years (20) since the two species diverged. It is possible that chicken chromosome 4 and the mammalian sex chromosomes share a common ancestor, and it would be interesting to address this question by mapping the location in the chicken genome of other genes which in humans are located on the X chromosome.
Figure 2. Physical organisation of the chicken hprt locus. Cosmid and phage lambda clones of DT40 genomic DNA were mapped and aligned by restriction mapping and then sorted into two groups according to the presence of polymorphic enzyme sites. λ33 lies outside the polymorphic region and may derive from either allele. Exons were mapped by PCR and limited genomic sequencing.

Figure 3. Chromosomal localisation of the hprt gene by FISH. DT40 metaphase chromosomes were hybridised with a chromosome paint probe for chromosome 4 (red) and a probe derived from hprt-cos 4 (green). The respective probes were labelled with biotin and digoxygenin and detected with CY3 labelled avidin and fluorescein labelled antibodies.

Our strategy for disrupting the two hprt alleles in DT40 is illustrated in Figure 4A. We used PCR from cloned DNA to assemble allele-specific targeting constructs in which the 5' targeting sequence extended from exon 3 to exon 4 and the 3' targeting sequence extended from exon 4 to exon 7. Targeting led to replacement of nucleotides encoding amino acid residues 114–124 by either a hygromycin resistance gene (allele 1) or a histidinol resistance gene (allele 2). The markers were flanked by loxP sequences which also led to the introduction of an in frame stop codon between the marker and the 5' segment of exon 4. The cell line in which both alleles were disrupted by targeting was designated DT4032. As expected, this cell line proliferated in the presence of 6-thioguanine at 1.3 μg/ml but not in hypoxanthine, aminopterin and thymidine (HAT), and was thus phenotypically hprt deficient (not shown). The loxP sites were incorporated into the constructs in order to excise the antibiotic resistance marker genes subsequent to targeting. We were unable, however, to use transient expression of cre recombinase to remove either marker, and so we stably co-transfected DT40 cells with a gene encoding a conditionally active cre recombinase–ER fusion protein (13) and a construct conferring resistance to zeocin. Stably transfected zeocin resistant clones containing the cre recombinase gene were then grown for 2 days in the presence of the steroid agonist 4-OHT and analysed at the hprt locus by gel electrophoresis and filter hybridisation. This demonstrated that in several clones the markers had been lost from both loci to ~80% efficiency. We therefore further cloned one of these cell lines to generate an zeocin resistant hprt−/− DT40 cell line free of histidinol and hygromycin marker genes, but which contains a conditionally active cre recombinase which we refer to as DT4032zeocre. This cell line allows both the use of the counter selectable hprt marker system and the conditionally active cre recombinase. Restriction analysis of the hprt locus in the DT40, DT4032 and DT4032zeocre confirmed the sequence of targeting reactions and marker excision (Fig. 4B). Fragments other than the hprt genes were detected in the DT4032zeocre by filter hybridisation; these originate from hybridisation of vector fragments in the probe to the zeocin and cre–ER constructs present in this line. We have also confirmed that both the DT4032 and DT4032zeocre cell lines can be transfected to HAT resistance by a plasmid encoding bacterial guanine phosphoribosyl transferase driven by a chicken β-actin promoter.

Although both cell lines described above are hprt−/−, they have different merits and should be useful for different types of experiments. The DT4032 cell line is particularly suited for studies of the mechanism of homologous recombination in which it is necessary to be confident that the resident locus is not reverting. An example of such a study would be one designed to measure the frequency of repair of an ectopically located sequence by double strand break initiated gap repair. The DT4032zeocre cell line allows marker genes to be recycled and thus it should be possible to construct cell lines containing combinations of different mutations by multiple rounds of targeting and marker excision in this line. The use of zeocin or bleomycin in this line is, of course, precluded by the presence of the stably integrated zeocin resistance gene. The DT4032zeocre line will also be useful as a reagent for engineering deletions
Figure 4. (A) Disrupting the chicken hprt gene by gene targeting and excision of selectable marker genes using cre recombinase. The targeting reactions were carried out in two steps with the allele 2 being targeted first. (B) Targeting to the indicated cell lines was monitored by EcoRI digestion, gel electrophoresis and filter hybridisation using the probe indicated in (A).

within and translocations between mammalian chromosomes and as a system for conditional gene expression (21). One potential problem of the use of cre recombinase to recycle markers is that the genome may become littered with potential target sites. However, this problem might be easily solved by using as substrates loxP sites which include a half site mutation to effectively inactivate the product of any cre mediated reaction (22).

In addition to the hprt+/– cell lines described above, we engineered cell lines in which either allele 1 or allele 2 of the hprt locus was disrupted with genes conferring hygromycin resistance or histidinol resistance, respectively. These hprt+/– cell lines should be of value in the study of DNA mutation in DT40 cells.

ACKNOWLEDGEMENTS

We thank Dave Sherratt for advice about the cre recombinase, Nat Bumstead for chicken genomic DNA, Michelle Debatisse and members of her laboratory for hosting the stay of N.H. at the Pasteur Institute where the initial cloning was done. We also thank Elena Giulotto for discussion. The project was supported by the European Union as a concerted action and by the UK Cancer Research Campaign. T.F. was a JSPS fellow during part of the work.

REFERENCES

18 http://www.ri.bbsrc.ac.uk
19 http://www.sanger.ac.uk