Reconstitution of an episomal mouse \textit{aprt} gene as a consequence of recombination

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Summary. When a functional murine adenine phosphoribosyltransferase (\textit{aprt}) gene linked to bovine papilloma virus (BPV) DNA is transfected into \textit{Aprt}− L cells, the cells are rendered \textit{Aprt}+ and the \textit{aprt} gene persists as an episome.

Cotransfection with two BPV vectors, one containing the 5′ half of the \textit{aprt} gene and the other the 3′ half of the gene, that share about 300 bp of common sequence in intron 2, produces \textit{Aprt}+ cells with functional \textit{aprt} as an episome. Southern blot analysis of low molecular weight DNA derived from Hirt extracts revealed the regeneration of a diagnostic \textit{SmaI} fragment, consistent with establishment of an episome with functional \textit{aprt} that was reconstituted as a consequence of recombination. To establish cells with an episomal target for recombination, BPV vectors containing a G418 resistance marker and either the 5′ half or 3′ half of \textit{aprt} were transfected into \textit{Aprt}− L cells. Stably transfected cells, selected by their growth in G418, were in turn transfected with DNA containing the other half of the \textit{aprt} gene. Following selection of \textit{Aprt}+ cells, Southern blot and polymerase chain reaction (PCR) analysis of low molecular weight DNA confirmed the presence of a complete episomal \textit{aprt} gene. The region of DNA shared by the episomal \textit{aprt} fragment and the transfected \textit{aprt} half was sequenced after PCR amplification of the reconstituted, episomal gene and was found to be wild type. The region of overlap that serves as the substrate for recombination lies entirely within an intron and can, therefore, tolerate nucleotide substitutions and deletions. The absence of such errors in the sequences examined is consistent with recombination events that are not error prone.

Key words: APRT – BPV – Episome – Recombination – Gene targeting

Introduction

The exchange of genetic information between DNA strands sharing sequence homology occurs with varying degrees of efficiency in procaryotic and eucaryotic organisms. Recombination processes, including homologous recombination in procaryotes and lower eucaryotic organisms are, in some cases, fairly well understood and have provided a starting point for understanding mammalian recombination events. However, analysis of recombination in higher eucaryotic organisms has been complicated by the size and complexity of the mammalian genome, and the number and types of participating enzymatic activities.

We have examined homologous recombination within episomes in mammalian cells using incomplete but overlapping portions of a selectable mammalian gene as recombination substrates. The experimental approach is based on the properties of the bovine papilloma virus (BPV-1) vectors and the murine adenine phosphoribosyltransferase (\textit{aprt}) gene. Vectors containing an entire BPV genome, or a defined fragment thereof, persist as multicopy episomes when transfected into some murine cell lines (Lowy et al. 1980; Sarver et al. 1982; Karin et al. 1983; Law et al. 1983; Pavlakis and Hamu 1983). They replicate only once per cell cycle (Berg et al. 1986), and are maintained as mini-chromosomes (Rössl et al. 1983, 1986). Thus, recombinant episomal BPV DNAs, maintained in cultured cells, can serve as informative recombination substrates (Kitamura et al. 1990).

The mouse \textit{aprt} gene was chosen as a recombination target for these experiments for several reasons. First, one can select cells that either express adenine phosphoribosyltransferase (APRT) activity, or that lack a functional gene product, by culture in appropriate selective medium. Second, this gene is a mammalian gene in which introns can be utilized as the intragenic site for recombination. Third, \textit{aprt} is a particularly attractive selectable marker because its small size (less than 3 kb) allows it to be easily manipulated. Fourth, \textit{aprt} encodes an enzyme involved in purine salvage, is expressed at...
low levels in all cell types tested, and represents a typical mammalian housekeeping gene. Fifth, the gene has been cloned, sequenced, and characterized (Dush et al. 1988; 1985; Sikela et al. 1983) and found to be highly conserved in evolution (Broderick et al. 1987; Dush et al. 1985).

This report describes the use of episomes and plasmid DNAs to study recombination in mammalian cells. Recombination frequencies between cotransfected DNAs ranged from 1–12%, which is consistent with previous reports (Folger et al. 1984; 1985; Pomerantz et al. 1983; Small and Scangos 1983; Shapira et al. 1983). However, in contrast to other studies, the preferred substrates were supercoiled molecules rather than molecules linearized within the region of homology. To expand on this approach, we have transfected cell lines harboring an established episome containing one half of the aprt gene, with plasmids carrying the other half of the gene. These transfections also generated Aprt + colonies at high frequencies as a consequence of targeted recombination.

Materials and methods

Plasmid constructions. The parental vector for the constructs described below is pS5, a derivative of pCGBPV9A5 (Matthias et al. 1983). This vector contains the full-length BPV-1 genome, the neo gene driven by the HSVtk promoter, and a unique SalI cloning site. The plasmid p3.1 is a derivative of pS5 and contains the full-length BPV-1 genome, the aprt gene cloned into the unique SalI site. Both lines were G418 resistant and Aprt-. Cell lines produced as a consequence of transfection of the cell line. For example, cell line RB7/HS3 is an Aprt+ cell line produced by cotransfecting LS-24b cells with plasmids pRB7 and pHS3.

Recombination substrates

1. Plasmids pRB6a and pRB7. The pRB plasmids contain the 5' portion of the mouse aprt gene cloned into the SalI site of pS5. The 1.9 kb EcoRI-BamHI fragment, containing the first two exons and 5' flanking sequences of the aprt gene, was isolated from pSAM3.1 (Sikela et al. 1983), cloned into the EcoRI/BamHI sites in the polylinker of pUC19 to generate pUCRB, which was then linearized with EcoRI, blunt-ended, and ligated to phosphorylated SalI linkers. The 1.9 kb 5' aprt fragment, flanked by SalI sites, was cloned into the SalI site of pS5 to generate pRB6a and pRB7 (see Fig. 1B, a and b).

2. Plasmids pHS1 and pHS3. The pHS plasmids contain the 3' portion of the aprt gene, cloned into the SalI site of pS5. The 1.4 kb HindIII-SalI fragment, containing the last three exons of the aprt gene, was isolated as follows. The plasmid pSAM3.1 was linearized with HindIII, within the second intron of the aprt gene, blunted, and ligated to phosphorylated SalI linkers. Digestion with SalI produces the 3' fragment of the aprt gene plus about 100 bp of pBR328 sequence which was then cloned into the SalI site of pS5 to generate pH5S1 and pH5S3 (see Fig. 1B, c and d), and into the SalI site of pUC19 to generate pUCHS (not shown). All plasmids were propagated in recA- bacterial strains HB101 or DH5α.

Cell culture. An Aprt- mouse L cell line, LS-24b (Tischfield et al. 1982), was used as the recipient cell line for the DNA transfections. Although LS-24b cells have a negligible spontaneous reversion rate (Tischfield et al. 1982), they were grown in the presence of 2,6-diaminopurine (DAP, 100 mg/ml, Sigma) for several passages prior to transfection to eliminate potential spontaneous Aprt+ revertants.

Transfections were performed by the calcium phosphate method (Graham and van der Eb 1973; Wigler et al. 1978) using 5 μg of each plasmid DNA, without carrier DNA, per 2 × 10^5 cells per 100 mm plate. Selection was applied 24 h post-transfection by adding medium containing either AAA (4 μM azaserine, Sigma; 4 μg/ml alanosine, NCI or Le Petite, Italy; and 0.1 mM adenine, Sigma) to select for Aprt+ colonies or G418 (400 μg/ml; Sigma) to select for G418 colonies. Aprt+ and G418 colonies were visible within 2 weeks and were either isolated and expanded into cell lines for analysis or stained with Giemsa and counted.

Recombinant cell lines were designated as follows. One set of cell lines, established by cotransfection of LS-24b cells with either supercoiled (S) or linear (L) plasmids pRB7 or pH5S1 was designated RBHS and RBLHS, respectively. The RBLHS cell lines were produced by cotransfection with pRB7 (RB) linearized (L) at the BamHI site and supercoiled plasmid pH5S1 (HS). Similarly, cell line RBHSL was derived by a cotransfection with supercoiled plasmid pRB7 (RB) and with plasmid pH5S1 (HS) linearized (L) at the XhoI site. Subscripts identify different, independent cell lines from the same combination of cotransfected vectors. Cell lines derived from cotransfection with different combinations of plasmids are identified by the two plasmids used to establish the cell line. For example, cell line RB7/HS3 is an Aprt+ cell line produced by cotransfecting LS-24b cells with plasmids pRB7 and pH5S3.

Parental cell lines used for targeting of episomes were RB6a, produced by transfection of LS-24b cells with the plasmid pRB6a (containing the 5' aprt fragment), and cell line HS21, derived by transfection of LS-24b cells with the plasmid pH5S1 (containing the 3' aprt fragment). Both lines were G418 resistant and Aprt-.

Cell lines produced as a consequence of transfection of RB6a or HS21 with a vector containing the other half of the aprt gene, were represented as follows: the parental cell line (RB6a or HS21) plus the plasmid used for transfection (e.g. RB6a plus pH5S1 is designated RB6a/pHS1).

DNA Isolation. Total genomic DNA was isolated from cultured cells or frozen cell pellets as previously described (Stambrook 1974). Low molecular weight DNA was extracted by the method of Hirt (1967).

Southern blotting and hybridization. Electrophoretically fractionated DNA was transferred to Gene Screen Plus membranes by the method of Southern (1975). Prehybrid-