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DNA amplification–deletion in a spontaneous mutation of the hamster *aprt* locus: structure and sequence of the novel joint

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Abstract

In a collection of spontaneous mutants of Chinese hamster ovary cells selected for deficiency in adenine phosphoribosyl transferase (aprt) activity, one was detected having not only a deletion of aprt coding sequences but also an apparent amplification of remaining sequences. The HindIII fragment bearing the novel joint was cloned and sequenced revealing a complex gene rearrangement. A deletion of at least 9 kb extending upstream from the aprt locus is accompanied by an inverted duplication of flanking sequences 672 bp downstream from the novel joint. This unit is amplified three to four times with the net result of some sequences being increased as much as eight fold in copy number because of the duplication. The fidelity of the sequences involved is preserved. We propose a model which could account for this inverted duplication.

INTRODUCTION

Gene amplification occurs in cells in vivo during the course of development (1,2) or tumour formation (3-5) and in vitro as detected in drug resistant cell populations (6-8). Despite the widespread occurrence of these events, the mechanisms responsible for the generation of amplified genes are still poorly understood. In part, this is due to the vast amounts of DNA involved in each step of amplification (recent findings suggest up to 10,000 kb,(5,9)) making it difficult to resolve the novel arrays of genes and novel joints produced by the process.

Analyses of single step mutants in cell culture indicate that low level gene amplification is a randomly occurring spontaneous mutational event (9,10). Consequently, it was not particularly surprising that during our characterization of spontaneously occurring mutations at the nonessential adenine phosphoribosyl transferase (aprt) locus (11), we found one mutant in a collection of 120 which had amplified aprt sequences. However,

since the selection we applied to our cells was negative (i.e. for the loss of aprt activity), rather than a positive selection for increased gene expression, we anticipated that any amplification of sequences at this locus might allow a determination of the gene rearrangements associated with the generation of the rare novel joints. Here we report the properties of such a novel joint at the aprt locus. In this case, gene amplification is accompanied by deletion of aprt coding sequences as well as an inverted duplication of flanking sequences.

MATERIALS AND METHODS

Mutant selection

Cell culture techniques and mutant selections have been described previously (11). Spontaneous aprt$^-$ mutants were selected from the Chinese hamster ovary strain D422 which is hemizygous for aprt (11,12). An initial 100 cell inoculum was grown to 2×10^6 cells in α medium, 4μm thymidine, and 2.5% dialyzed fetal bovine/2.5% horse sera. All the cells from each replica were plated in 8-azaadenine-containing selective medium (11). To ensure that all the mutants obtained were truly independent, only one drug resistant colony was picked from each positive replica culture.

Southern blot analysis

Protocols used for extraction of high molecular weight DNA, restriction endonuclease digestion and Southern blot analysis have been described in detail (11). All probes were labelled to high specific activity (2×10^8 dpm/min/μg) by nick translation (13). Densitometric scans of autoradiograms were performed with an LKB Ultrascan. When measuring the amounts of aprt containing sequences in tracks, the measurements were normalized to the intensity of hybridization of non aprt bands in the same tracks produced after stripping the filters and rehybridizing to an appropriate labelled probe.

Cloning and sequencing

Mutant S70 was cloned into the HindIII site of the insertion vector λNM1149 (14). Genomic DNA (50 μg) was digested with HindIII (2 units/μg), dephosphorylated with bacterial alkaline phosphatase and size fractionated on agarose. Fragments of 2 to

3kb were isolated by electroelution and extracted twice with phenol/chloroform prior to ethanol precipitation. Ligations were carried out at $4^{o}C$ at a final DNA concentration of 50-100ng/µl. This resulted in a packaging efficiency of 1×10^{6} recombinants/µg vector when the packagings were plated out on the hfl strain NM514recA (14;recA strain constructed by J. Nalbantoglu).

Fragments of interest were subcloned directly from λ minipreps (15) into M13 vectors mp18 or mp19 (16). Sequencing was carried out by the dideoxy chain - termination method (17) using [α-^{35}S]dATP (18).

Wild type and mutant sequences were aligned precisely using the NUCALN programme of Wilbur & Lipman (19) and direct or inverted repeats were examined using the SEQ program (20).

RESULTS

To determine the nature of gene structural alterations causing deficiencies of aprt activity in CHO cells, we have been analyzing aprt sequences in DNA purified from a collection of 120 spontaneous mutants (11). Mutant DNAs digested with various restriction endonucleases were fractionated on agarose gels and transferred to nitrocellulose by Southern blotting. Aprt containing fragments were then visualized by hybridization with the unique 3.9kb BamHI probe containing all the coding information for aprt (Fig.1). In one spontaneous mutant, S70, the restriction pattern observed indicated a large deletion of aprt-homologous sequences (Fig. 2). Southern blotting of S70 genomic DNA digested with MspI revealed only the 4.5kb fragment indicating that all MspI sites in the aprt gene up to the one at map position 2.7 had been eliminated (Fig. 1). Double digestion with KpnI/EcoRI confirmed this as the 3.8kb KpnI fragment remained intact although most sequences 5' to this site were absent. The size of the deletion was estimated to be larger than 9kb since we were unable to detect sequences homologous to the upstream 1.0kb BclI-Hind III fragment (Fig.1, data not shown). But the unusual feature of this deletion mutant was the intensity of the remaining aprt fragments (Fig. 2) - - residual homologous fragments were more intense than would be expected in such a large deletion. This suggested that the sequences remaining had been amplified during the deletion event.

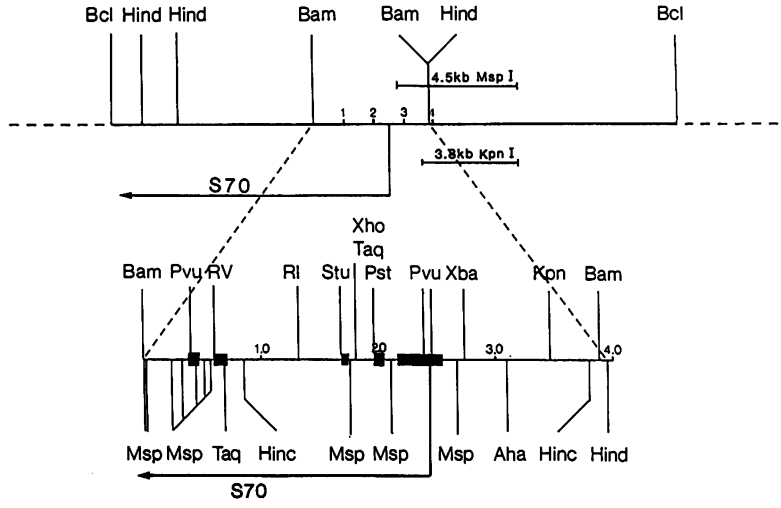

Figure 1
Restriction map of the aprt locus of CHO cells showing the region deleted in S70. Top portion represents the 18kb Bcl I fragment bearing aprt with a more detailed map of the 4.0kb fragment coding for the structural gene. Restriction sites of the 4.0kb fragment have been assigned on the basis of our DNA sequence of this fragment (26). Boxed regions represent exons determined by homology (87%) with the mouse cDNA sequence (27). The deletion terminus for S70 occurring within aprt is indicated, as are the MspI and KpnI genomic fragments left intact by the deletion.

To test this possibility further and to characterize the deletion junction formed, we cloned the genomic 2.3kb Hind III fragment of S70 having sequences homologous to the wild type BamHI aprt probe. An unusually high frequency of positives was obtained upon screening our λ genomic library (15 from $5x10^5$ recombinant phage). The deletion junction which had been mapped by Southern blotting, was subcloned into M13 and sequenced (Fig. 3a). The S70 sequence to the right of the junction is identical to that of wild type from nucleotide 2454 to the 3' HindIII site at 3960. Further examination revealed that the sequence to the left of the junction was also aprt, although of the complementary strand beginning at nucleotide 3126 and extending in the 3' direction. Thus the deletion of aprt coding sequences is accompanied by an inverted duplication of sequences 3' to nucleotide

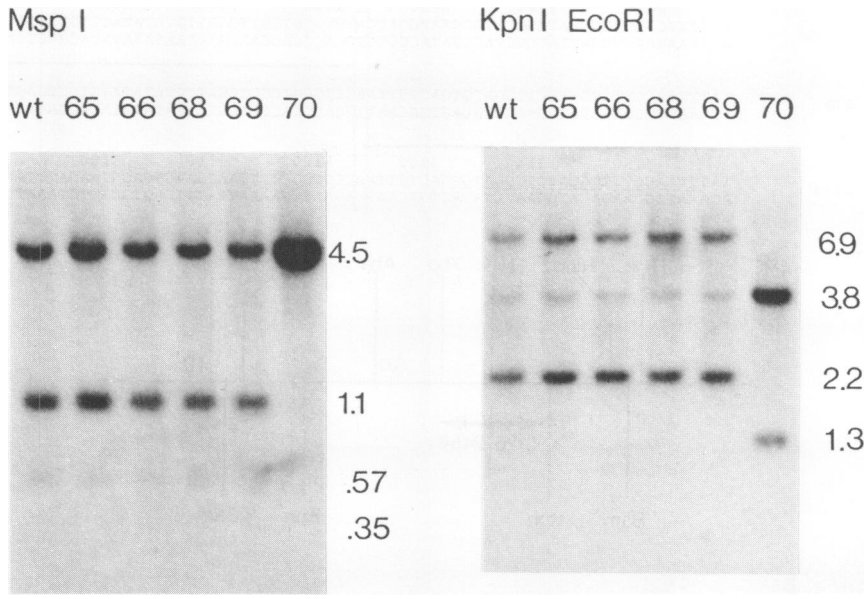

Figure 2
Southern blot analysis of wild type and mutant aprt alleles. 12µg of DNA from each strain (identified by the number above each lane) was digested with the indicated restriction endonuclease(s) before fractionation on agarose gels and Southern blotting as described in Materials and Methods. Band sizes are indicated in kb; the .35kb MspI fragment did not reproduce in these photographs.

3126. This is indicated in the restriction map presented in Fig. 3b which was compiled from the sequence of the entire 2.3kb Hind III fragment. Sequences from 2454 to 3126 are not duplicated but are flanked by the inverted duplication which begins at nucleotide 3126. The deletion/duplication process has also produced a novel Hinc II site at the junction (Fig.3a).

To rule out the possibility that this structure arose as an artifact of the cloning procedure, we examined genomic digests of S70 DNA for the presence of fragments predicted from the sequence. When Hinc II digests of S70 were probed with wild-type 0.8 kb AhaIII/BamH1 fragment, novel fragments of 1.3kb and 0.7kb were detected as well as the wild type 1.7kb fragment, confirming the presence of the novel Hinc II site (Fig.4). Similarly, in XbaI/KpnI genomic digests of S70, 3 fragments of 3.9,

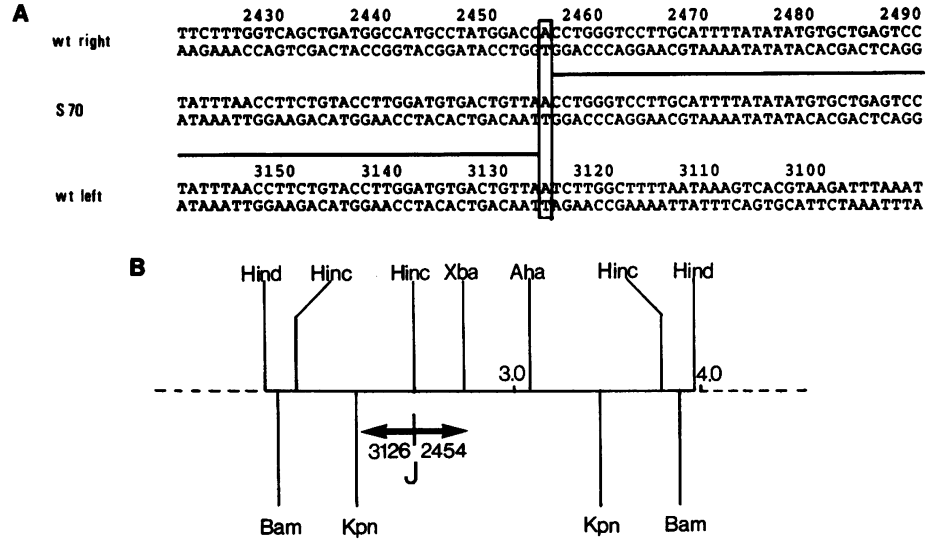

Figure 3
Molecular analysis of the novel joint occurring at the aprt locus of mutant S70: A. Nucleotide sequence at the novel joint and the corresponding wild type aprt sequences (the sequence to the left is the complement of the wild-type 5' to 3' sequence beginning at 3156) and B. The restriction map obtained from the sequence of the entire 2.3kb HindIII fragment of S70 bearing the novel joint. Numbering represents position of the sequences in the wild type gene (26).

0.7 and 0.6 kb were seen in accordance with the restriction map of Fig.3b.

Densitometric scanning of aprt bands on autoradiographs also confirmed the map and the nature of the amplification. In the XbaI/Kpn I double digests, there was a 3 to 4-fold increase in the hybridization signal of the 0.7kb XbaI/KpnI fragment which was not duplicated by the inversion event. The intensity of the 3.8kb KpnI fragment on the other hand was increased 6-8 fold as a result of the inverted duplication. Thus in what is likely to be a single step mutational event, the deletion of aprt coding sequences is accompanied by a 3-4 fold amplification of a unit produced by an inversion resulting in as much as an eight fold increase in copy number for certain sequences.

We were also able to estimate a minimum size of 9kb for the

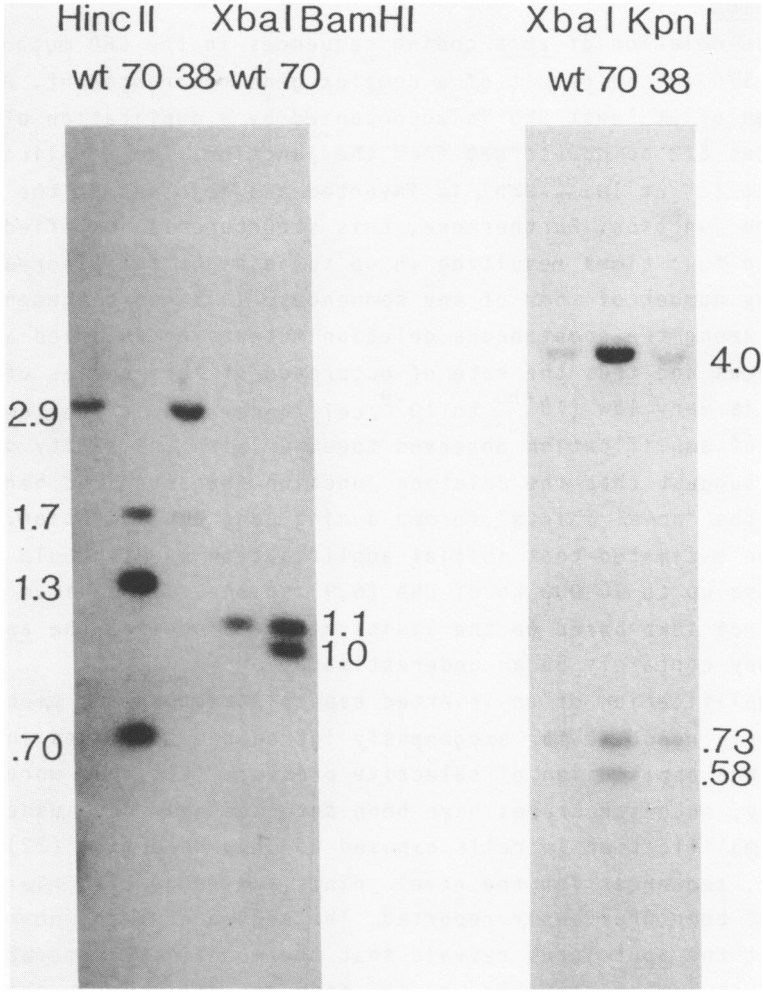

Figure 4
Southern blot analysis of wild type DNA and DNA of mutants S70 and S38 (11) by various restriction endonucleases. The DNA digested is indicated by the number over the track. The nick translated probe used in this experiment was the 0.8 kb AhaIII - BamHI fragment (Fig.1) instead of the usual full length BamHI probe (3.9 kb).

duplicated and inverted region on the basis of sites downstream from the junction of the inverted repeat which remain unaltered (data not shown). This in turn indicates a minimum size of 18 kb for the amplified unit.

DISCUSSION

The deletion of aprt coding sequences in the CHO mutant strain S70 is the result of a complex gene rearrangement. A deletion of at least 9kb is accompanied by a duplication of sequences 672 bp downstream from the junction. The duplicated fragment (of at least 9kb) is inverted and rejoined at the deletion junction. Furthermore, this structure is amplified three to four times resulting in up to an eight fold increase in the copy number of some of the sequences. This rearrangement is unique among the spontaneous deletion mutants we analyzed at the aprt locus and thus the rate of occurence of this series of events is very low (10^{-10} to 10^{-9}/cell/generation). The low degree of amplification observed together with the rarity of the events suggest that the deletion junction characterized here is one of the "novel joints" formed during gene amplification. It has been estimated that initial amplification events could encompass up to 10,000 kb of DNA (5,9) so the size minimum established of 18kb based on the limits of our probes of the aprt locus may certainly be an underestimate.

Amplification of an inverted repeat structure has been previously reported for exogenously introduced sequences in a cell after application of selective pressure (21) and, more recently, such structures have been detected near loci undergoing amplification in cells exposed to drug selection (22). However, sequences for the novel joints formed by the inversion have not been previously reported. The sequence of the novel joint at the aprt locus reveals that the mechanisms generating the structure are accurate, as the fidelity of the sequences undergoing rearrangements is maintained. No deletions, insertions, or base pair changes were detected within this region of the amplified unit. The sequences at the novel joint do not resemble those at deletion junctions of the aprt locus previously reported (23) in that there do not appear to be short direct repeats at the two termini, nor is there any indication of significant secondary structure which might play a role in the generation of the inverted repeat.

Most current models of gene amplification involve multiple initiations of replication of a portion of the genome in a

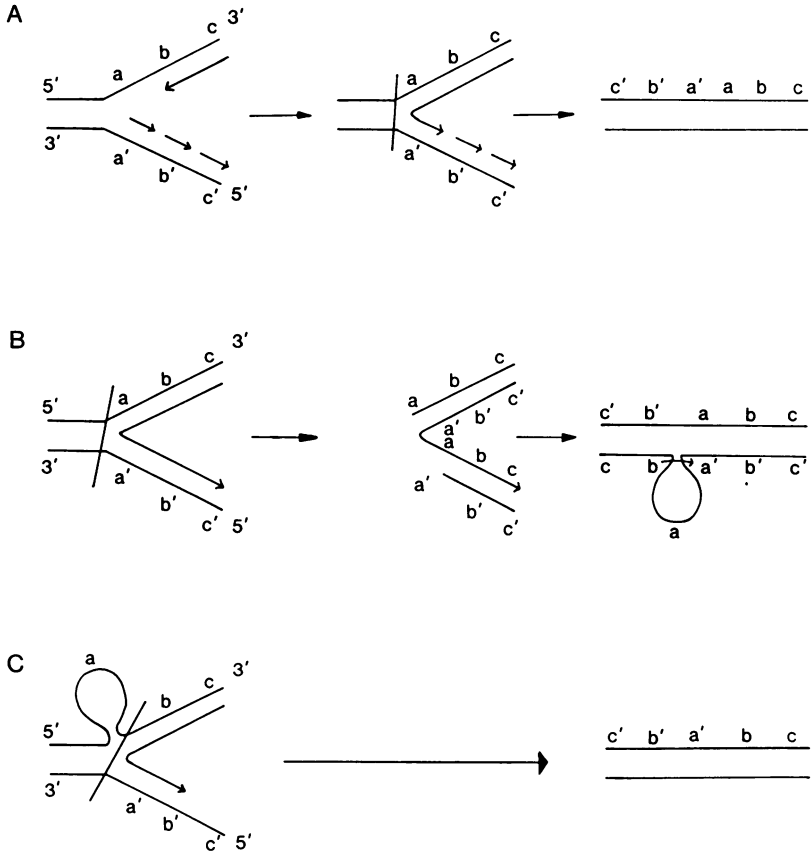

Figure 5
Models proposed to explain the generation of the inverted duplication of the aprt locus characterized in mutant S70 (see discussion in text).

single cell cycle followed by resolution of the resulting "onion-skin" structure by recombination to produce multiple arrays of genes (6-8). The recurrence of inverted repeat structures at arrays of amplified genes (21, 22 & 24) raises the possibility that these rearrangements play a role or are intermediates in the process of spontaneous gene amplification. This is not an obvious prediction of the current models, although we can propose a series of events which could generate an inversion during DNA replication. We assume that, in a rare event, replication switches strands and proceeds around the replication fork rather than in a bidirectional manner, as in figure 5a, produc-

ing an inverted repeat in the two arms of the fork. If this structure were resolved by a double strand break in the unreplicated parental molecule, the resultant DNA would bear an inverted repeat. This type of event is not necessarily lethal to mammalian cells as evidence is accumulating for the occurrence and repair of double strand breaks during processes such as recombination (25). The non duplicated region (found in several of these structures (21, 24)) could be produced by exonucleolytic digestion of one of the strands during repair of the double strand break followed by rejoining of the parental (digested) strand leaving the unduplicated sequence looped out to be subsequently degraded or lost in the next round of replication (Fig.5b). An alternative is a loopout of single strand DNA sequences in advance of the replication complex before the strand switch event (Fig. 5C). This loopout could be stabilized by inverted repeat sequences which in aprt are close to each junction of S70. Resolution of this structure by a double strand break again could lead to the inverted repeat we characterized here.

If the amplified unit is part of a precise linear, tandemly arranged array, one other novel joint will have been created during the amplification event; additional ones will be present if the unit is also transposed or rearranged. Due to the nature of the selection employed, we were able to characterize the novel joint occuring in the aprt locus. It will certainly be more difficult to identify the other novel joints in the large segments of DNA amplified. However, the event described here clearly establishes the capacity of cultured cells to produce complex gene rearrangements at chromosomal gene loci in the absence of selective pressure for these modifications.

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