Structure of an inverted duplication formed as a first step in a gene amplification event: implications for a model of gene amplification

Claudio Passananti¹, Brendan Davies, Miriam Ford and Mike Fried

Department of Tumour Virus Genetics, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK ¹On leave of absence from Università Degli Studi di Roma, Istituto di Biologia Cellulare, Policlinico Umberto I°, 00161, Roma, Italy

Communicated by M.Fried

Inverted duplications have been observed to be a common feature of gene amplification in mammalian cells and appear to be generated as a primary event in the amplification process (Ford *et al.*, 1985; Ford and Fried, 1986). The structural features of the amplified inverted duplication, containing the polyoma virus oncogene middle T-antigen, were analysed in transformed 3B rat cells. No unusual sequences such as transposition elements were detected at the site of the inversion. The inversion was generated by a simple illegitimate recombination event in which only a single nucleotide directly at the point of the inversion cannot be accounted for from the sequence of the two parental strands. Possible structural (hairpin formation) and sequence (rich AT) features may have been involved in the illegitimate recombination event at the inversion join. In the cellular DNA near one of its joins with polyoma virus DNA an unusual sequence of 198 bp composed of 99 consecutive purine–pyrimidine pairs has been detected. A model for the generation of amplified DNA containing inverted duplications is proposed.

Key words: gene amplification/inverted duplication/recombination/Z DNA

Introduction

Gene amplification is a common phenomenon in eukaryotic cells. In addition to certain genes which can be amplified as part of the normal developmental process (e.g. ribosomal, chorion), oncogenes (e.g. *myc*, *sis*, *ras*) have been found to be amplified in a number of tumour cells and genes encoding enzymes and glycoproteins have been found amplified in cells resistant to agents that cause metabolic stress (e.g. drugs) (for reviews see Hamlin *et al.*, 1984; Schimke, 1984a,b; Stark and Wahl, 1984; Stark, 1986).

In a previous communication we described the transformed 3B rat cell line which was isolated after DNA transfection of Rat-1 cells with a ligated mixture of restricted enhancerless polyoma virus (Py) transforming region DNA and restricted mouse cellular DNA (Ford *et al.*, 1985). The 3B cell line was found to contain a large inverted duplication which was also amplified 20- to 40-fold. A study (Ford and Fried, 1986), based on our initial findings with the 3B cell line, indicated that inverted duplications are a general feature of DNA amplification being associated with a number of independent amplifications of both the *myc* and *CAD* genes. These findings suggest that the formation of inverted duplications relates to the mechanism by which DNA amplification occurs.

The DNA containing the join at which the inversion took place in the 3B cells has been molecularly cloned and partially characterized (Ford *et al.*, 1985). As the inversion is amplified 20- to 40-fold its generation must have preceded the DNA amplification. This suggests that the formation of the inverted duplication is a primary event in the amplification process. Thus it would be of great interest to determine the detailed structure of the inverted duplication as it may give some insight into a mechanism of DNA amplification. In this report we present a number of structural features of the recently generated amplified inverted duplication present in 3B cells and suggest a model for the generation of amplified DNA-containing inverted duplications.

Results

The large inversion found in the 3B cells could have arisen extra-chromosomally, directly after DNA transfection, or occurred

Fig. 1. Identification of the 3B sequences upstream from the Py insert as being derived from F9 mouse DNA. A Southern blot of *Hind*III-cleaved mouse F9, Rat-1 and 3B cellular DNA probed with the cloned 3B 1.3-*Eco*RI fragment (see Figure 2). The positions in the restricted mouse F9 DNA of the 9-kb pHF9 and 5.6-kb pUH3 positive fragments, whose restriction maps are shown in Figure 2, are indicated. The numbers on the right are the sizes in kb of the λ *Hind*III markers.

Fig. 2. Maps of the cloned mouse pHF9 and pUH3 *Hind*III fragments and relationship to 3B DNA. (A) At the top is shown the restriction enzyme cleavage site map of the previously cloned 3B λ , containing the Py insert and an end of the inverted duplication, above which the sizes of the *Eco*RI fragments in kb are indicated (Ford *et al.*, 1985). The 3B 1.3-kb *Eco*RI fragment used as the probe in Figure 1 is starred. The long arrows at the very top indicate the extent of the inverted duplicated sequences. Beneath is shown the 9-kb *Hind*III pHF9 clone derived from F9 mouse DNA (see Figure 1). Regions of sequence homology between pHF9 and 3B are indicated by parallel lines of the two maps and a number of common restriction enzyme cleavage sites. The amount of homology between pHF9 and 3B is slightly greater on the left side than the right side. This extra amount of homologous pHF9 sequence on the left side is not in the inverted duplicated region and indicates that the point of inversion which occurs in 3B at the end of the region of its homology with pHF9 sequence is at the right side (see Figures 5 and 6). On the left side the homology between pHF9 and 3B DNA ends after the *Xba*I site but before the *Sst*I site. (B) At the top is shown the restriction enzyme cleavage site map of the previously cloned 3B λ , containing the Py insert and an end of the inverted duplication, above which the sizes of the *Eco*RI fragments in kb are indicated (Ford *et al.*, 1985). The 3B 1.3-kb *Eco*RI fragment used as the probe in Figure 2 is starred. Regions of repetitive sequence detected with both nick translated total mouse and rat cellular DNAs are indicated by unshaded boxes. The long arrows at the very top indicate the extent of the inverted duplicated sequences. Beneath is shown the 5.6-kb *Hind*III pUH3 clone derived from F9 mouse DNA (see Figure 1). Regions of sequence homology between pUH3 and 3B are indicated by parallel lines of the two maps and the common *Sst*I and *Bam*HI restriction enzyme cleavage sites. The part of the pUH3 sequence which is homologous to the 3B sequence is not in the inverted duplicated region. The joins between the Py sequences and rat DNA (J1), Py DNA and mouse DNA (J2), the linking join between pHF9 and pUH3 (J3) and the inversion join between pUH3 (see Figure 6) are indicated by unshaded triangles under the 3B map. The linking join of pUH3 with the pHF9 sequence (J3) is thought to have been formed extrachromosomally by recombination between the two mouse sequences after transfection whereas the inversion join of pUH3 with pHF9 sequence (J4) was generated within the 3B chromosomal DNA (see Figure 5).

after integration of the transfected DNA into the rat chromosome. If the inversion arose extrachromosomally before integration, the inverted sequences should only contain the transfected mouse

DNA and not the recipient rat cellular sequences. If, on the other hand, the inversion occurred after integration, rat DNA should be found within the inverted sequences. The origin of the cellular

Fig. 3. Identification of the 3B sequences downstream from the Py insert as being derived from rat DNA. (A) On the top is shown the previously described restriction map of the amplified cellular DNA surrounding the Py insert in 3B cells (Ford *et al.*, 1985). Only one half of the inverted duplication is shown. The long arrow indicates the inverted duplicated sequences which extend at least 28 kb (see text). Beneath the 3B map is shown the 11.5-kb 3B *Bcl*I λ isolated using Py DNA as a probe which shows exact identity with the 11.5-kb *Bcl*I fragment of the 3B genomic DNA. The 2.5-kb *Bgl*II fragment used as a probe in (B) is shown. (B) A Southern blot of Rat-1, mouse F9 and 3B cellular DNA restricted with *Eco*RI, *Bam*HI or *Hind*III using the 2.5-kb *Bgl*II fragment of 3B *Bcl*I λ (see A) as a probe. The positive bands found in the Rat-1, but not the mouse F9 DNA co-migrate with the 3B positive bands. The lower intensities of *Bam*HI and *Hind*III bands is mainly due to the decreased efficiency of transfer of these larger fragments. The numbers on the right are the sizes in kb of the λ *Hind*III markers.

sequences in the previously isolated 3B λ clone could not be distinguished when restriction enzyme digests of 3B λ were probed with either nick translated mouse or rat total cellular DNA. This type of probe will detect highly repetitive sequences. Highly repetitive sequences were detected in the same two regions of

the cloned 3B DNA with each of the cellular probes (see Figure 2B).

In order to determine the derivation of the cellular sequences in the inverted duplication an analysis was performed using cloned unique probes from both upstream and downstream of the Py insert. When the cloned 1.3-kb *Eco*RI fragment at the centre of the inverted duplication and which is only partly in the inverted sequences (see Figure 2), was used to probe *Hind*III-cleaved rat and F9 mouse DNA, no strongly positive fragments were detected in the rat DNA but two strongly positive fragments (9 and 5.6 kb) were detected in the *Hind*III-cleaved F9 mouse DNA (Figure 1). Neither of these fragments co-migrated with the 10-kb *Hind*III fragment found in 3B DNA using this probe (Figure 1). The 9-kb (pHF9) and 5.6-kb (pUH3) *Hind*III fragments were cloned into λ NM1149 (Murray, 1983) from size-fractionated *Hind*III-cleaved F9 DNA and further subcloned into pXF3 plasmids. Over 5-kb of its length the 9-kb pHF9 clone has a restriction map identical to that of the 3B inverted DNA upstream from the Py insert (Figure 2A). The remainder of the 3B DNA in this region contains perfect homology with part of the 5.6-kb pUH3 clone isolated from the F9 DNA (Figure 2B). From their intensities both the pHF9 and pUH3 positive bands (Figure 1) appear to be present in only one copy per haploid mouse genome. These results show that the inverted cellular sequences upstream from the Py insert in 3B DNA were derived from mouse and not rat DNA.

An 11.5-kb *Bcl*I fragment (3B *Bcl*I) containing part of the Py DNA and downstream adjacent cellular sequences was also cloned from 3B cell DNA into the *Bam*HI site of λ EMBL3 (Frischauf *et al.*, 1983) (Figure 3A). Probes containing unique DNA sequences derived from this clone were used to determine the origin of the cellular sequence downstream from the Py insert (Figure 3B). Using these unique probes it was found that both the size and the organization of the restriction fragments of the cellular sequences in the cloned 3B *Bcl*I fragment and the inverted cellular DNA downstream from the Py insert in 3B cellular DNA were identical with rat DNA (see Figure 3A). Thus the presence of rat DNA within the inverted duplication shows that the inversion present in 3B cells took place after integration of the transfected DNA into the rat chromosome. In addition the identification of a 15.5-kb *Hind*III fragment and a 15-kb *Bam*HI fragment in both rat and 3B DNA by Southern blot analysis (Figure 3B) demonstrates that the inverted duplication extends at least 19.5 kb (rat DNA sequences) downstream from the Py DNA and in total is at least 28 kb in length.

The recombinant joins of the Py DNA with the mouse and rat cellular DNAs were determined from the cloned 3B DNA by DNA sequencing. The sequence at the recombinant join between the rat cellular and Py DNAs (J1) is shown in Figure 4A. This recombinant event, which occurred at or after integration of the *in vitro* ligated Py-mouse DNA into the rat chromosome, has resulted in the truncation of the Py early region. The join occurs at Py nt 2807 in the viral early region (Figure 4A). The recombination with the Py sequences has taken place in a CT-rich region of the rat DNA. Otherwise no special structural or sequence features are evident at the viral-host join and no large regions of homology are seen between the missing (displaced) viral DNA and the rat cellular sequence at and around the illegitimate recombinant join (Figure 4A).

The join between the mouse and Py sequences (J2) is as predicted for the *in vitro* ligation join (Ford *et al.*, 1985) between *Bgl*II cleaved mouse cellular DNA and the *Bam*HI cleavage site of the Py vector DNA (Figure 4B). Upstream from J2 in

Fig. 4. DNA sequences around the joins between the Py insert and cellular DNA in 3B cells. (A) The sequence of the recombinant join (J1) between the Py early region sequence and Rat-1 cell DNA (see J1 in Figure 2B) is shown. The displaced Py sequence (Py sequences lost as a result of recombination with the rat cellular DNA) are indicated. The vertical lines indicate the Py sequences in the 3B DNA. The Py nucleotide numbers are shown (see Tooze, 1981). (B) The ligation join (J2) between the *Bam*HI cleavage site of the Py vector and the *Bgl*II cleavage site of the mouse F9 DNA is indicated (see J2 in Figure 2B). Eighty-four base pairs upstream from this join is found the mouse sequence containing 199 alternating purine and pyrimidines (boxed). Arrows beneath the sequence indicate the 62-bp direct repeat within this alternating purines and pyrimidine sequence.

the mouse DNA an interesting sequence of 199 alternating purine and pyrimidines was detected (Figure 4b). Within this region a perfect tandem repeat of 62 bp is found. In the 199 bp each of the 99 purine residues is an adenine and is preceded by either a cytosine or thymidine pyrimidine residue [(C/TA)⁹⁹]. The major dinucleotide pair CA occurs 85 times. To our knowledge this region is the largest region of naturally occurring sequence with the potential to form a Z DNA configuration (Haniford and Pulleyblank, 1983; Nordheim and Rich, 1983; Singleton *et al.*, 1984) so far described. Numerous stretches of alternating purine and pyrimidine dinucleotide pairs are found in different eukaryotic genomes as repetitive elements (Miesfeld *et al.*, 1981; Hamada *et al.*, 1982; Bennett *et al.*, 1984; Sun *et al.*, 1984; Tautz and Renz, 1984). This alternating purine and pyrimidine sequence appears to account for the repetitive DNA detected in this region with both mouse and rat total DNA probes (see Figure 2B). The biological relevance of this sequence is unknown.

The recombinant join between the pHF9 and pUH3 sequences (J3) (linking join), that is thought to have occurred during the transfection, as well as the recombinant join between the pHF9 and pUH3 sequences at the point of inversion (J4) (inversion join), that appears to have been created as a first step in the amplification process, were analysed. The 3B DNA sequences through J3 and J4 were determined and compared with the corresponding sequence from the pHF9 and pUH3 cloned DNAs (Figure 5). The sequence data indicate that a simple illegitimate recombination event has taken place to link the two noncontiguous regions (pHF9 and pUH3) of mouse F9 DNAs at J3 (Figure 5). Three bases of homology, which could have been derived from

Fig. 5. The DNA sequence at the linking (J3) and inversion (J4) recombination joins between the pHF9 and pUH3 sequences in 3B cellular DNA. At the top is shown the restriction map of the 3B 1.3-kb *Eco*RI fragment which contains both the linking join, J3, and the inversion join, J4, between the pUH3 (black) and pHF9 (white) sequences (see Figures 2B and 6). Below the map on the left is shown the DNA sequence at the linking join (J3) and on the right the DNA sequence at the inversion join (J4) (see J3 and J4 in Figure 2B). At each join the 3B DNA sequence is shown in the middle with the sequence of pUH3 above and that of pHF9 beneath. Homologies between the recombinant 3B and parental pUH3 and pHF9 sequences are indicated by vertical lines. At the linking join, J3, the three bases (ATC) at the site of recombination which could be derived from either of the parental sequences are boxed. At the inversion join, J4, the G residue found at the point of inversion cannot be accounted for by either of the parental sequences. Beneath the inversion join, J4, is shown two possible hairpin structures of the parental pUH3 sequence around the site of inversion. The arrows in the hairpin structures indicate the point of recombination within the pUH3 sequence. The left-hand palindromic structure is energetically less stable than the one on the right.

either parental DNA, are found at the recombination join (Figure 5). Similar small amounts of homology at illegitimate recombination joins are not unusual (see Williams and Fried, 1986, and references therein). As a large amount of recombination among the incoming DNA molecules has been reported to take place during DNA transfection (Perucho *et al.*, 1980), the recombination event between these noncontiguous regions of mouse DNAs apparently occurred during the DNA transfection step used in the generation of the 3B cell line (Ford *et al.*, 1985).

The amplified inversion join, J4, involves sequences from both the pHF9 and pUH3 derived DNAs (Figure 5). The inversion appears to have been generated by a simple illegitimate recombination event. Except for a single nucleotide directly at the point of inversion all the nucleotides can be accounted for by the two pUH3 and pHF9 parental DNA strands involved in the recombination event (Figure 5). The site of recombination in the pUH3 sequence occurs within a region which contains dyad symmetries (two possible dyads) which could form hairpin structures, while in the pHF9 sequence the site of recombination has occurred within an AT-rich region (Figure 5). No positive bands were detected when a unique sequence derived from pUH3 DNA upstream from the point of inversion was used to probe 3B DNA (data not shown) indicating that these sequences had been lost upon the generation of the inversion.

Discussion

Recent observations have indicated that inverted duplications are a common feature of amplified DNA and are formed as a primary event in the amplification process (Ford *et al.*, 1985; Ford and Fried, 1986; Saito and Stark, 1986). The amplified inverted

Fig. 6. A model for the generation of the structure of the amplified DNA found in 3B cells. The linear enhancerless Py vector (shaded) containing a *Bam*HI cleavage site at the end of the early region is ligated to mouse F9 *Bgl*II-cleaved cellular DNA *in vitro* and the ligated mixture is transfected into Rat-1 cells. A hybrid molecule containing the Py vector ligated at its *Bam*HI site to a *Bgl*II mouse DNA fragment (upper case letters) recombines illegitimately with another restricted mouse DNA fragment (lower case letters) after transfection into the Rat-1 cells. The resulting recombinant molecule is composed of the Py vector and two noncontiguous mouse DNA sequences integrated into the rat chromosomal DNA (numbers). As a result of the integration event part of the Py sequences and part of the mouse sequences (lower case letters) are lost. As a first step in the DNA amplification process an inverted duplication (arrow) is generated as a result of an illegitimate recombination event between different mouse sequences with the duplication consisting of mouse, Py and rat sequences. Subsequently the sequence containing the inverted duplication is amplified (n) times.

duplication found in 3B cells has been analysed in order to determine if any special features associated with its generation could be detected. In Figure 6 is presented a scheme for the generation of the amplified inverted duplication in 3B cells consistent with the results presented here. The 3B inversion appears to have been generated within the rat chromosome and no unusual sequences like transposition elements were detected at the site of the inversion. A simple illegitimate recombination event appears to have taken place in the formation of the inversion in which only a single nucleotide directly at the recombination join cannot be accounted for from the sequence of the two parental DNA strands (Figure 5). Possible structural (hairpin formation) and sequence (AT-rich region) features (Figure 5) may have been involved in the recombination event at the inversion point. The mouse sequences on one side of the point of inversion are lost upon the generation of the inversion.

Why inverted duplications should be associated with gene

amplification is not clear. It may be a matter of stability as the loss of amplified DNA by homologous recombination is less likely to occur when the amplified DNA is in the form of inverted arrays as opposed to tandem arrays. A number of mechanisms have been proposed to explain the generation of amplified DNA (for reviews see Hamlin *et al.*, 1984; Schmike, 1984a,b; Stark and Wahl, 1984; Stark, 1986). In none of these mechanisms is the presence of an inverted duplication a predominant feature. In neither unequal crossing over nor disproportionate replication, which are the two favoured models to explain the generation of amplified DNA, is the presence of inverted duplications obligatory. However, the presence of an inverted duplication is thought to be a prerequisite for one form of DNA amplification in lower eukaryotes. This is the amplification in the yeast *Saccharomyces cerevisiae* of the 2μ circle plasmid, which contains an inverted duplication as part of its genomic structure.

Normally the replication of the 2μ circle is under the same

Fig. 7. A model for DNA amplification of mammalian cell DNA involving inverted duplications based on the Futcher model (Futcher, 1986) for amplification of the 2μ circle of yeast. A circular molecule (2) containing an inverted duplication (rectangle) harbouring the gene to be amplified (dot) is excised from the chromosome possibly by illegitimate recombination between newly replicated DNA strains (1). Replication is initiated from a sequence within the circle that can act as an origin of DNA replication. After the inverted sequence containing the target gene is replicated once (3), an homologous recombination event takes place between a copy of the newly replicated inverted sequence and the copy of the inverted sequence that has yet to be replicated (4). This results in a flip of the replicating forks (small arrows) so that they are not chasing each other in the form of a double rolling circle molecule (5). Further replication results in the generation of amplified arrays of inverted duplications (6,7) derived from a single initiation of DNA replication during a single S phase. The amplified forms could either remain extrachromosomal (double minutes) (7) or could become stabilized by integrating into the chromosomal DNA (8). In the model presented here five copies of the amplified inverted sequence are shown to become integrated into the chromosomal DNA (8).

stringent control as the chromosomal DNA being replicated only once, semi-conservatively, during the S phase of the cell cycle. Under certain circumstances the copy number of the 2μ circle can become quite low (e.g. unequal partitioning between mother and daughter cells). Under conditions of low copy number the 2μ circle has the ability to overcome the cellular control of DNA replication by amplifying its copy number to a high level within a single S phase. Recently Futcher (1986) has proposed a model for amplification of the 2μ circle which avoids the need for multiple initiations at the origin of DNA replication which is a facet of the disproportionate replication model for DNA amplification in mammalian cells (Bullock and Botchan, 1982; Roberts *et al.*, 1983). Futcher (1986) postulates that after replication has been initiated an homologous recombination event takes place between one copy of the 2μ circle inverted sequence in the newly replicated DNA and another copy of the inverted sequence which has yet to be replicated. This results in a flipping of a region of the 2μ genome so that the two replication forks, which were previously proceeding around the 2μ circle towards each other and eventual termination and resolution of the two daughter DNA molecules, are now both proceeding in the same direction, as if one is chasing the other, and are unable to terminate in the normal fashion. The consequence of this replicating structure (double rolling circle) is the production of a long array of amplified DNA from a single initiation event at the 2μ circle origin of DNA replication. Eventually another recombination event occurs between the inverted sequences so that the replication forks are returned to their original position and replication is terminated by the two forks meeting. Single monomer 2μ circles are generated from the amplified array by homologous recombination. The 2μ circle encodes a site-specific recombinase whose substrate is within the inverted sequence and recombination bet-

ween the inverted repeats is essential for amplification to occur (Broach *et al.*, 1982; Andrews *et al.*, 1985; Volkert and Broach, 1986).

In Figure 7 is shown a diagrammatic representation of how this model may be envisioned for DNA amplification in mammalian cells. By illegitimate recombination events a circular form, containing a structure that acts as an origin of DNA replication and the gene to be amplified in a large inverted duplication, is excised from the chromosomal DNA. The large inverted duplicated sequences are required to provide a large target area for an homologous recombination event (not site specific) during replication to generate a double rolling circle molecule in which one of the replicating forks has flipped. This results in the synthesis of an amplified array of inverted duplications. These amplified forms could either remain extrachromosomal (double minutes) in an unstable form or could become stabilized by integrating into the chromosomal DNA. We are presently attempting to test some of the consequences of this model on amplified DNA.

Materials and methods

The isolation of cellular DNA and analysis by Southern blotting were performed as previously described (Ford and Fried, 1986). *Hind*III fragments of 9 and 5.6 kb (from mouse F9 teratocarcinoma cellular DNA) and a 11.5-kb *Bcl*I fragment (from 3B rat cellular DNA) were cloned into λ vectors NM1149 (Murray, 1983) and EMBL3 (Frischauf *et al.*, 1983) respectively from appropriate size fractions separated by sucrose gradient centrifugation as previously described (Ford *et al.*, 1985). Either the entire cloned fragment or subfragments were transferred from phage to pxf3 plasmids (Hanahan, 1983) for restriction mapping and for use as probes. DNA sequences were determined using the dideoxy chain termination method of Sanger *et al.* (1977) using fragments subcloned into M13mp18 and M13mp19 (Norlander *et al.*, 1983). The copy number of a sequence in genomic DNA was estimated from blots by the relative signal to known single copy sequences taking into account the amount of homology with the probe used.

Acknowledgements

We would like to thank Moira Griffiths for her excellent technical assistance and Clive Dixon, Nic Jones, Chris Norbury and Paul Nurse for their advice and helpful discussion in the preparation of this manuscript.

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Received on January 26, 1987; revised on March 9, 1987