Further characterization of the gapped DNA intermediates of human spumavirus: evidence for a dual initiation of plus-strand DNA synthesis

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We recently reported the presence of linear duplex DNA intermediates with a gap in the middle of the molecules in the replicative cycle of human (HSRV) and simian (SFV1) spumaviruses. The polypurine tract (PPT), at the 5' boundary of the 3' long terminal repeat, was found to be duplicated in the gap region. By molecular analysis of HSRV proviral DNA with region- and strand-specific probes, we have now determined that the gap is located on plus-strand DNA and that it is 120 bases long with the 3' end mapping at the duplicated PPT site. Kinetic analysis of proviral DNA provided evidence that the gap did not result from processing of a complete, full-length DNA molecule. These data strongly suggest that plus-strand DNA synthesis is initiated at both PPT sites.

Introduction

Spumaviruses (foamy viruses) are a subfamily of the Retroviridae which have been isolated from an extensive range of animal species (Hooks & Gibbs, 1975). No proven pathogenic potential has been clearly demonstrated for any of the virus strains; however, spumavirus isolations from De Quervain thyroiditis (Stancek et al., 1975) and induction of immunosuppression by simian foamy virus in rabbits (Hooks & Detrick-Hooks, 1979) have been reported.

We have previously demonstrated the existence of linear DNA forms with a gapped region located approximately in the middle of the human spumavirus (HSRV) and simian foamy virus type 1 (SFV1) DNA molecules (Kupiec et al., 1988). This gapped structure was recently confirmed for another SFV, the LK3 isolate (Schweizer et al., 1989). The aim of the present study was to gain more insight into the structure and function of these replicative DNA intermediates. Plus-strand synthesis of retroviral DNAs is usually initiated at the 5' boundary of the 3' long terminal repeat (LTR) at the polypurine tract (PPT) (Varmaus & Swanstrom, 1982). The fact that this conventional PPT is duplicated and maps in the same central region as the gap led to the suggestion that plus-strand DNA synthesis is initiated at both PPT sites, thus generating the gap. Molecular analysis of HSRV proviral DNA with region- and strand-specific probes allowed us to determine the size and precise localization of the gap and the process by which it is formed. The data suggest a dual mode of initiation of plus-strand synthesis.

Methods

Cells and virus. The human U373-MG neural cell line (ATCC, Rockville, Md., U.S.A.) was maintained in Eagle's MEM supplemented with 10% foetal calf serum, non-essential amino acids and sodium pyruvate. A stock of the Epstein–Achong isolate of HSRV (Achong et al., 1971) was propagated in these cells (Benzair et al., 1982).

Infection with HSRV was at an m.o.i. of 1. For the viral DNA synthesis time-course experiment, cells were removed from culture at the designated times after infection for preparation of low Mr DNA. For other experiments, U373-MG cultures inoculated with HSRV were split 72 h post-infection (p.i.) and harvested 72 h later.

Preparation of viral DNA. Low Mr DNA was prepared from infected cells by the Hirt procedure (Hirt, 1967). After precipitation of chromosomal DNA, the supernatant was digested with proteinase K, extracted with phenol-chloroform and precipitated by ethanol. The DNA pellet was dissolved in 10 mM-Tris–HCl pH 8.0, 1 mM EDTA and treated with pancreatic RNase (50 µg/ml) for 1 h at 37°C.

Probes. Virus-specific DNA fragments were prepared from recombinant clones pHSRV-H-C55 and pHSRV-E-D2 (Flügel et al., 1987; Rethwilm et al., 1987). These fragments were C55 (the HindIII insert covering the 3' part of the pol, env and bel genes), C2-hh (the 688 bp HinfI fragment schematically shown in Fig. 2) and LTR (the 700 bp HindIII–HinfI fragment of clone D2 DNA covering about half of U3 and R plus the U5 region).

Three oligonucleotides of 20 bases were synthesized on an automatic DNA synthesizer, Applied Model 380B (Organic Chemistry Unit, Pasteur Institute, Paris, France). Their sequences are 5' TTGTAGTTAAGTACAGGTGA 3' (Belm), 5' GATGAGAGAAGGTTGTTGAGAT 3' (Belp) and 5' ACCTTAAGTACAGTAGCGG 3' (Sm).
Belm and Belp were derived from the Bel gene and correspond to sequences between nucleotides 5345 and 5364 and nucleotides 5066 and 5085. They hybridized to plus- and minus-strand DNA respectively. Sm was derived from the $1$ gene and corresponds to a sequence between nucleotides 1050 and 1069. Positions are given according to Flügel et al. (1987).

DNA fragments were labelled with $[^{32}P]dCTP$ by the method developed by Feinberg & Vogelstein (1983), using a random primed DNA labelling kit (Boehringer Mannheim). Synthetic oligonucleotides were end-labelled with $[^{32}P]ATP$ and T4 polynucleotide kinase and separated from unincorporated dNTP by ethanol precipitation in the presence of 2.5 M-ammonium acetate.

**Blot hybridization.** DNA was digested with nuclease S1, or heat-denatured as described previously (Kupiec et al., 1988), or HinpI-restricted according to the conditions recommended by the supplier (Biolabs). Southern blots were performed on agarose gels using nitrocellulose.

For single-stranded DNA analysis, DNAs denatured with glyoxal were subjected to electrophoresis in 1:1% agarose. After 2 h at 80 °C, the blot was treated with 20 mM-Tris–HCl pH 8.0 at 100 °C for 5 min to remove residual glyoxal (Thomas, 1983).

Prehybridization and hybridization with subcloned probes were carried out in 50% formamide at 42 °C (Meinkoth & Wahl, 1984). Filters were washed twice for 5 min in 2 x SSC, 0.1% SDS at room temperature and twice for 15 min in 0.1 x SSC, 0.1% SDS at 50 °C. With oligonucleotide probes, prehybridization and hybridization were performed in 5 x SSC, 0.5% SDS at 46 °C. The filters were washed at the hybridization temperature in 1 x SSC, 1% SDS.

**Results**

**Localization of the gap on plus-strand DNA**

We have previously reported that the majority of free proviral HSRV DNA is a gapped linear duplex structure (Kupiec et al., 1988). To determine on which strand the gap is located, HSRV DNA was heat-denatured and analysed by Southern blot hybridization either with a double-stranded probe or with two strand-specific oligonucleotide probes. As shown in Fig. 1, hybridization with probe C55 (panel a) revealed in the heat-denatured DNA sample three single-stranded fragments which had been previously identified as the full-length strand (Fss), the right strand (Rss) corresponding to the 3' side of the gap, and the left strand (Lss) corresponding to the 5' side of the gap.

Hybridization with Bel-specific oligonucleotide probes Belp (panel b) and Belm (panel c) revealed Fss and Rss respectively. Control and nuclease S1-digested DNAs were run in parallel and, as expected, both oligonucleotide probes gave a positive signal with double-stranded fragments containing the Bel gene. In similar experiments with gag-specific oligonucleotide probes, Fss and Lss were detected (data not shown).

Based on the reactivity of heat-denatured HSRV DNA with strand-specific oligonucleotide probes, we conclude that the gap is located on the plus strand of the DNA.

**Mapping the gap region**

To estimate the size of the gap and to verify that the gap and the PPT map at the same position (Kupiec et al., 1988), we performed Southern blot hybridizations with two specific probes: C55-hh, the 680 bp HinpI fragment encompassing the gap region, and Sm, the 20 base oligonucleotide probe consisting of nucleotides 1050 to 1069. Nucleotide numbering is according to Flügel et al. (1987).

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Fig. 1. Reactivity of heat-denatured HSRV with strand-specific oligonucleotide probes. DNAs were electrophoresed in a 0.7% agarose gel at 4 °C, transferred to nitrocellulose and hybridized to probe C55 (a) or to the oligonucleotide probes Belp (b) or Belm (c). Bacteriophage λ DNA digested with HindIII was used as size markers (kbp). Lanes 1, HSRV DNA heat-denatured at 100 °C; lanes 2, control; lanes 3, S1 nuclease-digested DNA.

Fig. 2. Schematic representation of the central region of HSRV DNA. The positions of the PPT, the probes used and the gap (GAP) are indicated. The hatched boxes represent the HinpI fragment and Sm, the oligonucleotide probe consisting of nucleotides 1050 to 1069. Nucleotide numbering is according to Flügel et al. (1987).
Human spumavirus proviral DNA synthesis

Fig. 3. Extent and mapping of the gap. Viral DNA was restricted with HincI (lanes 1) and an aliquot was digested further with nuclease S1 (lanes 2). Southern blots were performed in 1.5% agarose gels and hybridized with probe C55-hh (a) or the oligonucleotide probe Sm (b). Numbers indicate the size of DNA digestion products (bp) calculated by comparison with known fragments of HaeIII-cleaved φX174 DNA.

and 190 bp (Fig. 3a); only the smaller one is visualized using the Sm probe (Fig. 3b). These data allowed us to estimate the extent of the gap to be 120 ± 10 bases and to map its 3' end to the PPT (Fig. 2). It is interesting to note that after nuclease S1 digestion (Fig 3a), the 680 bp band, which migrates slightly slower than the HincI broad band, is probably derived from ungapped DNA.

Kinetics of viral DNA synthesis

We previously hypothesized that, in foamy viruses, plus-strand DNA synthesis is initiated at both PPTs, thus generating the gap. Alternatively, the two major plus-strand molecules identified above might result from processing of intact full-length, linear duplex DNA. To examine these possibilities, Hirt supernatant DNAs were extracted from cells at various times p.i. and denatured with glyoxal. Analysis of blotted DNAs hybridized with the LTR probe (Fig. 4) showed that the three major species (Fss, Lss and Rss) appeared simultaneously and were detectable 15 min p.i. As the viral infection progressed, the amount of these molecules increased. Moreover, a doublet of about 1-2 kb was also seen, the lower band being predominant. These short fragments, which hybridized to the LTR probe exclusively, could represent the entire HSRV LTR.

Since no full-length DNA strands accumulated before the two plus-strand fragments appeared, our data suggest that the gap is generated by dual initiation of plus-strand DNA rather than by processing from a full-length plus strand.

Discussion

In this study we have demonstrated that the gap in linear duplex HSRV DNA is localized in plus-strand DNA. This gap was estimated to be 120 bases long and maps at the 3' end of the internal PPT site. Kinetic analysis of proviral DNA synthesis showed that a full-length minus strand and two subgenomic plus strands appear together even at early times after infection. Based on the size of the R fragment and its positive hybridization with the LTR probe, the 1.2 kb LTR-specific fragment may represent a strong-stop plus-strand DNA intermediate (Varmus & Swanstrom, 1982). These results support our previous hypothesis on the dual initiation of plus-strand DNA synthesis, being primed at the conventional 3' LTR PPT and also at the internal PPT site at the 3' end of the gap.

The gap in HSRV DNA is delimited at the 3' end by the functional PPT. Furthermore, analysis of the HSRV sequence (Flügel et al., 1987) revealed the presence of another PPT, GAGAAGAAGAA, at the 5' end of the gap, extending from nucleotides 886 to 906. This is also observed in the nucleotide sequence of the gap region of the SFV LK3 isolate (Schweizer et al., 1989). Based on
these observations and the identification of such tracts at the 3' end of the \textit{pol} gene of the simian virus prototype, SFV\textsubscript{1} (Mergia \textit{et al.}, 1990; our unpublished results), we speculate that the gap in SFV\textsubscript{1} is similarly delimited. Thus, the gap appears to be determined by these PPTs rather than randomly generated.

The theory of dual initiation of plus-strand synthesis to generate a gap in proviral DNA may be extended to the entire spumavirus family. Thus, the process first described in visna virus (Blum \textit{et al.}, 1985; Sonigo \textit{et al.}, 1985), the prototype of the lentivirus family, has to be considered as a more general feature of retrovirus DNA replication.

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\textbf{References}


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