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Evidence for a gapped linear duplex DNA intermediate in the replicative cycle of human and simian spumaviruses

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Abstract

Two forms of linear DNAs have been found in simian (SFV1) and human (HSRV) spumaviruses: a linear duplex unsensitive to nuclease S_1 and a sensitive structure with a single-stranded gap. Two nuclease S_1 sensitive sites, mapping at the same position for both viruses, have been identified in the gapped structure. Using different molecular subgenomic clones of HSRV as probes in Southern blot analysis, one S_1 site was localized in the 3'LTR and the other near the middle of the molecule at about 6.5 kbp from the 5' end of the viral genome. The latter site was shown to correspond to a single stranded region within the linear duplex DNA. Nucleotide sequence analysis revealed that the polypurine tract (PPT) usually found at the 5' boundary of the 3'LTR of retroviruses, is duplicated in HSRV at the 3' end of the pol gene, near the gap. This suggests that the synthesis of plus strand DNA is discontinous, generating the gap.

INTRODUCTION

Besides Oncornavirinae and Lentivirinae, Spumavirinae constitute the third subfamily of Retroviridae. Within Spumavirinae, primate foamy viruses (monkeys, apes and human isolates) are of particular interest, because they cause latent infections in natural and experimental hosts (1). Moreover, these viruses are carried by lymphocytes and can suppress immune responses (2). Some primate foamy virus prototypes have been characterized biochemically and biologically (1, 3, 4, 5, 6, 7) but few data exist on the molecular replicative cycle of these viruses.

During our investigations on the molecular status of DNA replicative forms of simian foamy virus 1 (SFV1) (1) and the human spumaretrovirus (HSRV) (8) , careful preparations of native viral DNA revealed some fragility. The DNA was spontaneously broken in a non-random manner, always giving the same pattern in Southern blots when stored for more than 15 days at 4°C, in contrast to other DNA preparations. This suggests the possible presence of single stranded regions in foamy viral DNA, as previously reported for the replicative forms of other retroviruses (9, 10, 11).

In this study nuclease S_1 sensitive sites were identified and localized inside the SFV1 and HSRV DNA by using recently cloned specific probes of HSRV subgenomic fragments (12,13). One of these sites was shown to be due to a gap in the DNA molecule. In addition, the polypurine tract (PPT), localized at the 5' boundary of the 3'LTR used in retroviruses for initiation of plus strand DNA synthesis, was found to be duplicated in the gap region.

MATERIALS AND METHODS

Virus and cell cultures

SFV1, originally obtained from the National Institute of Health (Bethesda, Md, USA), was propagated on CF2Th dog cells as already described (4). HSRV, Epstein-Achong isolate (8) comes from the laboratory of M.A. Epstein (University of Bristol, United Kingdom) and was grown on human U373-MG neural cell line purchased from ATCC (Rockville, Md, USA). Dog and human cell lines were maintained, respectively, in Mc Coy and Eagle's Minimum Essential Media (MEM), supplemented with 10 % foetal calf serum. MEM was supplemented with non essential amino-acids and sodium pyruvate.

Preparation of viral DNA

Cultures were infected either with SFV1 or with HSRV at a multiplicity of infection of approximatively 0.5. SFV1 infected CF2Th cells were harvested at 24-48 hours post-infection. U373MG cultures inoculated with HSRV preparations were split 72 hours post-infection and harvested 72 hours later. Viral DNA was prepared by the Hirt method (14). Briefly : after washing with PBS, cells were resuspended in 10mM EDTA, pH 7.4 and lysed with 0.6% SDS. The cellular lysate was made 1M NaCl (3 ml/10^7 cells) and after 8 hours at 4°C, this suspension was spun for 30 minutes at 10^4 rpm in a SW27 rotor. The supernatant was deproteinized by proteinase K (Merck), digestion and phenol extraction.

Nuclease S_1 treatment

Viral DNA was incubated with nuclease S_1 (Boehringer Mannheim) in : 50 mM sodium acetate, pH 4.5, 200 mM NaCl, 1 mM $ZnSO_4$, for 15 minutes at 37°C with a concentration of 7 units/ug DNA.

Heat denaturation

HSRV DNA was heat denatured at 100°C for 5 minutes and quenched on ice.

Southern Blots

DNAs were resolved by electrophoresis in 0.7% agarose gels and

transferred to nitrocellulose filters. Prehybridization and hybridization were carried out in 50% formamide at 42°C (15). Filters were washed under stringent conditions : 5 x 5 minutes in 2 x SSC, 0.1% SDS at 25°C and 2 x 15 min. in 0.1 x SSC, 0.1% SDS at 50°C.

Molecular probes

Five HSRV specific probes were prepared from recombinant clones pHSRV-H-C55, pHSRV-B52 and pHSRV-E-D2 (12,13):

- C55 : the Hind III insert covering the 3' part of pol, env and bel genes.

- B52 : the BamHI-EcoRI insert covering the bel genes and the 3'LTR.

- D2-HH : the 3.65 kbp Hind III fragment covering a part of the 5'LTR, gag and the 5' part of pol excised from the D2 clone.

D2-HH and C55 do not overlap ; B52 and D2-HH share a part of the LTR (see the map in figure 3).

- gag : the 1.8 kbp HinPI fragment excised from the D2 clone.

- bel : the 1.2 Kbp EcoRI-Hind III fragment excised from the D2 clone.

DNA probes were labelled with ^{32}P using random priming (16).

Nucleotide sequence analysis

The search for the PPT duplication in HSRV and related viruses was performed using CITI 2 (Paris, France) software.

RESULTS

Nuclease S_1 sensitivity

This study was performed with Hirt supernatant DNAs extracted from SFV1 and HSRV infected cells. To determine whether viral DNAs contain nuclease S_1 sensitive sites, DNAs digested or undigested with nuclease S_1, which is specific for single-stranded DNA, were run on agarose gels, transferred to nitrocellulose by the Southern procedure, and hybridized with ^{32}P labelled specific probes under stringent conditions. The specific recombinant probes used in these experiments, C55, B52, and D2-HH, correspond to well-defined regions of the HSRV genome as described in Materials and Methods. The three probes together represent the quasi-totality of the genome.

The autoradiograms of the same preparations hybridized and rehybridized with the three probes are shown in figure 1. The results of Southern blot analysis, clearly indicated a comparable molecular structure for SFV1 and HSRV, since similar patterns were obtained for the DNAs of both viruses, independent of the hybridizing probes, sizing the uncleaved viral

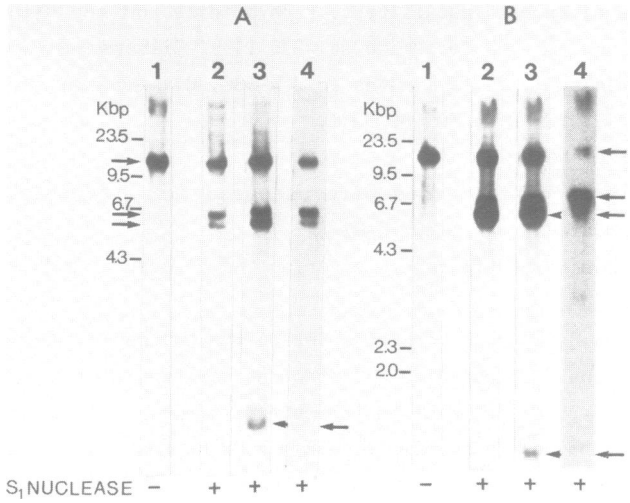

Figure 1 : Southern-blot analysis of viral DNAs digested by nuclease S_1 : SFV1 DNA (panel A) and HSRV DNA (panel B). Undigested DNAs (lanes 1) hybridized with the C55 probe. The same pattern was obtained with the three probes (data not shown). Nuclease S_1 digested DNAs hybrized with C55 (lanes 2), B52 (lanes 3) and D2-HH (lanes 4). Bacteriophage λ DNA digested by Hind III was used as a molecular-weight marker.

DNA at 13 kbp (1A and 1B). It is remarkable that the sizes of both viral DNAs are identical and appear greater than the size (12.2 kbp) determined recently for HSRV by nucleotide sequencing (12, 13). The apparent discrepancy between these values may be explained by the relatively low accuracy of agarose gel electrophoresis as compared with nucleotide sequencing. It is also relevant to note that only the linear form of the viral DNA was detected. Under the experimental conditions used, we were not able to observe circular or supercoiled forms. Comparisons between undigested and nuclease S_1 digested DNAs hybridizing with the three probes, C55 (2A and 2B), B52 (3A and 3B) or D2-HH (4A and 4B) revealed that in addition to the 13 kbp band, two bands of 6.5 kbp and 5.5 kbp were generated. However, D2-HH probe, representing the 5' part of the viral genome, preferentially detected the 6.5 kbp band. Moreover, when the blot was probed with either B52 or D2-HH, a minor band of about 0.8 kbp was also detectable.

In an other nuclease S_1 digestion experiment (figure 2, lanes c and c'), DNAs samples were run for a longer period of time and hybridized with the two probes corresponding to the gag and bel regions (see Materials and methods). Both probes revealed a doublet for the 13 kbp band. The 6.5 kbp

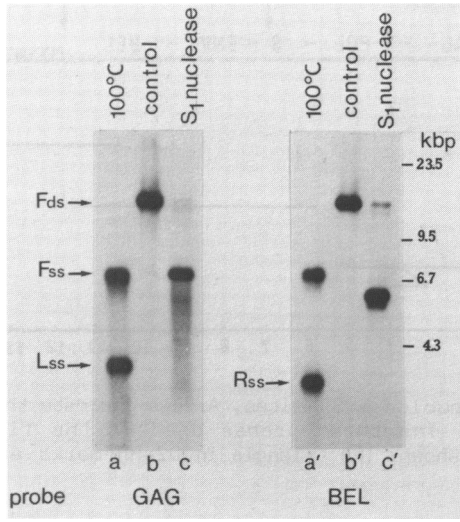

Figure 2 : Southern-blot analysis of denaturated HSRV DNA. Duplicate samples of HSRV DNA were electrophoresed in the same gel at 4°C transferred to nitrocellulose and hybridized either to gag or bel specific probes. Heat-denatured DNA (lanes a and a'); control DNA (lanes b and b'); nuclease S_1 digested DNA (lanes c and c'). Bacteriophage λ DNA digested by Hind III was used as a molecular-weight marker.

fragment was detected with the gag probe and the 5.5 kbp fragment with the bel probe. It is relevant that only for the 5.5 kbp a doublet was detected indicating an other S_1 site near one of its extremities.

All these results show that SFV1 and HSRV DNAs contain two populations of linear duplex DNA molecules since a minority was always resistant to nuclease S_1 digestion. Concerning the nuclease sensitive fraction, one S_1 site could be unambiguously localised at 6.5 kbp from the 5' end of the viral genome. Based on the doublets at 13 and 5.5 kbp and the 0.8 kbp fragment a second S_1 site was localized in the 3' LTR. This is summarized in a tentative nuclease S_1 map of viral DNA (figure 3).

The gap

The presence of nuclease S_1 sensitive sites can be due either to an actual single stranded gap (11), or to an unpaired DNA conformation, as is the case in the promoter region of some eukaryotic genes (17,18). In order to discriminate between these two possibilities, HSRV DNA was heat denatured and analyzed by Southern blotting hybridization with the gag and bel specific probes, described in Materials and Methods (figure 2). Control and

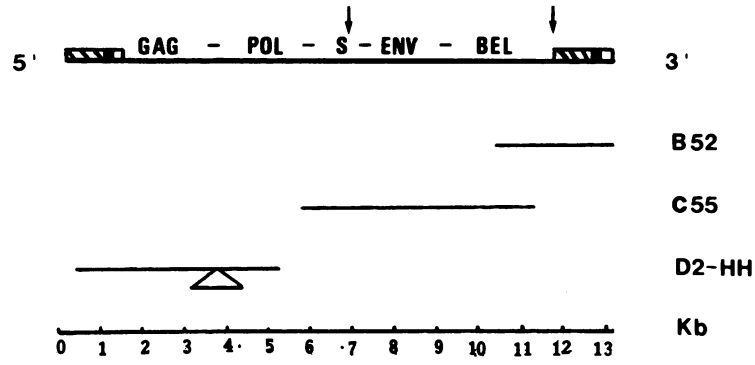

Figure 3 : Map of nuclease S_1 sites. Arrows indicate the localization of nuclease S_1 sites. The three probes used in the first nuclease S_1 experiment are also shown. The triangle in D2-HH marks a deletion in the pol gene.

nuclease S_1 digested DNAs were run in parallel. When HSRV DNA was denatured three single-stranded fragments were revealed (fig. 2 lanes a and a'). This indicates the presence of a gap in the double-stranded DNA molecule since in the case of an unpaired DNA structure one would expect to detect only one band. The fragments Fss, detected by both probes can be identified as a full length single-strand. Two others, named Lss and Rss were detected respectively by specific gag and bel probes. Therefore Lss and Rss fragments can be identified as the single-stranded molecules corresponding to the 6.5 Kbp and 5.5 Kbp double-stranded fragments, generated by nuclease S_1 digestion.

These results demonstrate that the linear spumavirus DNA actually contains a gap, localized at about 6.5 kbp from the 5' of the molecule.

Correlation with PPT duplication

It has been previously suggested that the plus strand DNA synthesis of another retroviral agent, Visna virus, is initiated both at the conventional PPT site in the 3'LTR and at a second PPT site near the 3' end of the pol gene, generating a gap in this region (19). Thus, we searched for the presence of PPTs in the gap region of HSRV. A careful analysis of the recently published sequence (12) revealed four polypurine tracts near the 3' end of the pol gene. One of these, localized 15 bp from the 3' end of pol gene at position 6337-6348 is a perfect repeat of an eleven nucleotide long sequence found in the 3' LTR PPT.

Duplications of the 3'LTR PPT at the 3' end of the pol gene have

Table 1 : Sequence homology between the PPTs of HSRV and three lentiviruses.

POSITIONS	SEQUENCES (5'-3')	VIRUSES	REFERENCES
10806-10831 (LTR)	AGAGAGGAAGTAAGGAGGAGAGGGTG	HSRV	12,23
6337-6348 (pol)	AGGAGAGGGTGG	HSRV	12,23
8837-8851 (LTR)	AAAAAGAAAGGGTGG	VIV	20
and	* ** ******		
4723-4737 (pol)			
14-30 (LTR)	AAAAAAAGAAAGGGTGG	CAEV	21
	* ** ******		
8626-8640 (LTR)	AAAAGAAAAGGGGGG	HIV-1	22
and	** * **** *		
4331-4346 (pol)			
	$A^{AA}_{GG}A^{A}_{G}AGGG^{T}_{G}G$	Consensus	

PPT sequence duplicated in HSRV and its homologous sequence in VIV, CAEV and HIV-I are underlined. Each of these viruses was compared with HSRV; asterisks indicate conserved nucleotides. Positions are given or calculated according to the references indicated.

already been reported for other retroviruses belonging to the Lentivirinae subfamily (19,20). A comparative analysis between the PPTs of HSRV and of three lentiviruses : VIV (Visna virus), HIV-1 (human immunodeficience virus) and CAEV (caprine arthritis-encephalitis), is shown in table 1. It is relevant to note that the eleven nucleotide long PPT of HSRV presents only two mismatches with VIV and CAEV PPTs, and three mismatches with HIV-1 PPTs.

DISCUSSION

Our data show that viral DNAs of HSRV and SFV1 contain two populations of linear DNA molecules : those that are nuclease S_1 resistant and those that are nuclease S_1 sensitive. One nuclease S_1 site was localized near the middle of the molecule and an other site close to or in the 3'LTR. Denatu-

ration experiments provide evidence that the sensitivity to nuclease S_1 is due to a gap in one of the DNA strands close to which a duplication of the conventional PPT site can be identified. Since this sequence is the primer binding site for retroviral plus strand DNA synthesis, we hypothesized that in foamy viruses plus strand DNA synthesis is initiated at the two PPT sites generating a gap, as has been reported for VIV (11, 19, 22).

Our findings are in agreement with those reported by Neumann-Haefelin et al. (9) concerning the presence of nuclease S_1 sites in the unclassified simian foamy isolate, LK3, but differ as regards the localization and the biological significance of these sites. In contrast with these authors, we have shown here by using cloned probes covering practically the entire genome, that nuclease S_1 cleaves foamy virus DNAs assymetrically.

In addition, with other recently reported observations (12,23) our present data establish an extensive similarity between foamy virus and Visna virus DNA structure, concerning : i) the predominance of linear DNA molecules, ii) the presence of a gap near the middle of the molecule, iii) the duplication of the PPT at the 3' end of the pol gene, iiii) the sequence homology between HSRV and VIV PPTs.

At least two non-exclusive interpretations can be proposed to account for the gapped viral DNA structure in the replicative cycle. In the first one, gaps in the linear DNA molecules are never filled in, and must be considered as failures in the viral DNA synthesis, thus preventing these sequences from growing into complete functional genomes. The second involves a maturation of linear DNA processing from a gapped immature form to a full-length mature DNA molecule. In either case, such DNA structure may correspond to a critical point in the replication pathway of these viruses.

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