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A defective human foamy provirus generated by pregenome splicing

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Foamy viruses are a group of retroviruses of complex structure which were thought to be non-pathogenic. The recent demonstration of neurological diseases in mice transgenic for human foamy virus (HFV) and the high prevalence of HFV sequences in Graves' disease question this idea. By PCR, we have detected HFV sequences with a non-random deletion in the *bel*1 transactivator gene in other autoimmune conditions. Sequence analysis revealed that this deleted area corresponds to the excision of a known intron in *bet*, one of HFV's regulatory genes. The same phenomenon was observed in both acute and chronic infections, *in vitro* or *in vivo*, although the deleted forms were distinctly more abundant in chronic states. The viral DNA containing the *bel*1 deletion is apparently part of an otherwise complete genome, strongly suggesting that this provirus derives from the reverse transcription of a spliced pregenomic RNA. *Bel*1-spliced provirus was shown to be defective when transfected into permissive cells. However, co-expression with the Bel1 transactivator led to functional *trans*-complementation and formation of viral particles. Splicing of the genome may be an important factor in HFV biology: genomes with the deletion may either interfere with wild-type virus expression or alter host cell functions through background expression of viral regulatory proteins.

Key words: alternative splicing/autoimmune disease/gene regulation/spumavirus/viral persistence

Introduction

Foamy viruses are a group of retroviruses which infect a wide variety of organisms ranging from cat to man (Flügel, 1991). In tissue culture, the distinct syncytial and foam-like cytopathic effects of these viruses led to the first isolation of a human retrovirus (Achong *et al.*, 1971). These viruses were, until recently, thought to be innocuous *in vivo*, and hence have not been studied in such great detail as the other human retroviruses. The recent description of progressive encephalopathy and myopathy in transgenic mice which harbour the complete human foamy virus (HFV) genome or only the viral regulatory genes has led to a renewed interest in this group of viruses (Bothe *et al.*, 1991; Aguzzi *et al.*, 1992). Moreover, our recent description of a high viral prevalence in Graves' disease, an autoimmune hyperthyroidism (Lagaye *et al.*, 1992), suggests the involvement of this virus in human disease.

HFV shares some structural homologies with the lentivirus and HTLV group, in particular by the presence of multiple complexly spliced regulatory genes in the 3′ portion of its genome, known as the *bel* region. The pattern of expression of the regulatory genes is highly complex due to the presence of several splice sites and multiply spliced transcripts (Figure 1) (Muranyi and Flügel, 1991). The *bel*1 gene encodes a regulatory protein which transactivates the long terminal repeats (LTRs) of both HFV and HIV-1 (Rethwilm *et al.*, 1990; Keller *et al.*, 1992; Lee *et al.*, 1992). Bel1 does not contain any regions of homology to either transactivation or DNA-binding motifs. Nevertheless, mutational analysis has identified such functional domains (He *et al.*, 1993; Venkatesh *et al.*, 1993). Other proteins, Bel2, Bel3 and Bet, whose contributions to viral biology have not yet been determined, can be expressed from this region. One of them, Bel3, shows partial homology with the HIV-2 Nef protein (Maurer and Flügel, 1987). Some splicing events lead to the formation of fusion proteins: in particular, Bet is generated by the fusion of the N-terminal end of Bel1 to the complete *bel*2 open reading frame. This fusion has no transactivating properties as Bet lacks the functional domains identified in Bel1 (He *et al.*, 1993).

Different host–virus states can be accounted for by a balance in viral gene expression, in particular early or immediate early genes. We report here that HFV proviral DNA exists in two distinct molecular forms, the variant one being derived from the wild-type virus by a single splicing event. This splice deletes a 301 bp segment from the *bel*1 gene, leading to inactivation of the gene and creating an intronless *bet* gene. Several chronically infected patients, animals and cell lines show a predominance of the spliced form by PCR or Southern blot analysis, suggesting that the switch of Bel1 to Bet may participate in the establishment or maintenance of latent infection. A *bel*1-deleted provirus,

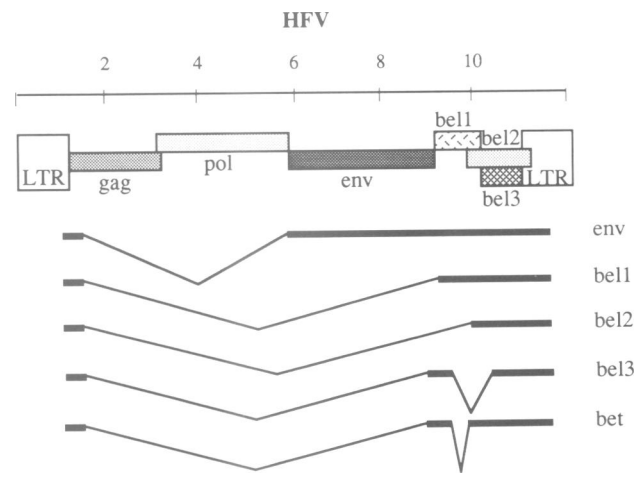

Fig. 1. Genetic organization and major mRNA species of HFV. The different known HFV genes are indicated. The complex splicing pattern of the virus was simplified by omitting micro-exons (after Muranyi and Flügel, 1991).

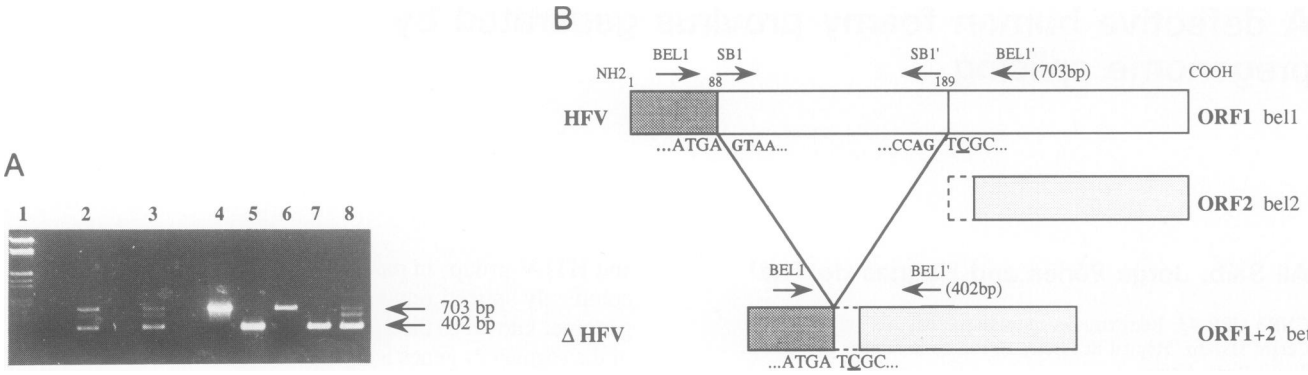

Fig. 2. (**A**) DNA from peripheral blood lymphocytes of autoimmune patients was analysed by PCR using the BEL1 primers. Lane 1, λ DNA digested with *Eco*RI and *Hin*dIII. Lanes 2, 3, 6 and 8, DNA from patients with Hashimoto's disease. Lane 7, DNA from a myasthaenic patient. Positive controls were pHFV13 (lane 4) and pΔHFV (lane 5). The 600 bp amplified fragment is due to a PCR artefact arising from the 703 bp fragment. (**B**) Schematic organization of the *bel*1, *bel*2 and *bet* genes. The primers used for PCR analysis are depicted by arrows and the size of the amplified fragments is shown in brackets. The 301 bp intron of the *bet* gene is indicated and the splice donor and acceptor sites are shown. The amino acid positions adjacent to the splice sites are indicated. The mutation near the acceptor site is underlined. The 5′ non-coding part of *bel*2 fused to *bel*1 is indicated by a dashed line, while the Bel2 protein is given as a solid line. ORF: open reading frame.

reconstructed from an infectious clone, was shown to be defective but could be rescued by *bel*1 expression. These observations highlight the importance of splicing in retroviruses and suggest that selected deletions of parts of the viral genome could be a strategy for changing the biology of the viruses.

Results

Detection in autoimmune patients of an HFV genome containing a deletion

In the course of characterizing the HFV sequences present in a patient suffering from myasthaenia gravis (MG) (A.Saïb, manuscript in preparation), we found that the amplified fragment in the *bel*1 region was ~300 bp shorter than normal (400 versus 700 bp) (Figure 2A, lane 7). However, four other PCR amplifications—from the LTR, *gag*, *env* and *bel*2 regions—were of the expected size and contained only a few point mutations (G_{3229} to A; G_{5087} to A; A_{5994} to G) relative to the prototype virus sequence (Flügel *et al.*, 1987). The nucleotide sequence of the shorter *bel*1 PCR fragment revealed a point mutation (T_{4665} to C) as well as a deletion (nucleotides 4362–4663), suggesting that, in this patient, the HFV provirus contains a 301 bp deletion in the *bel*1 gene. The borders of this deletion correspond exactly to the mapped splice donor and acceptor sites of the intron of the *bet* gene (Muranyi and Flügel, 1991) (Figure 2B). Interestingly, the point mutation maps near this splice acceptor site (Figure 2B) and may have altered the splicing pattern of the virus.

We wondered whether a similar situation would be encountered in other autoimmune patients. DNA from four patients with Hashimoto's disease was investigated and the shorter *bel*1 PCR fragment was found in three of them (Figure 2A, lanes 2, 3 and 8). One patient showed only the *bel*1 fragment without the deletion (Figure 2A, lane 6). The *bel*1 deletion is not generated by mutations near the splice acceptor site as the sequence of the 402 bp PCR fragment from one of these patients did not differ from the HFV prototype (the amplified fragments of the other patients were not sequenced). A pair of PCR primers (SB1), within the *bel*1 deletion, showed the presence of intact HFV sequences in all HFV positive patients. Thus, it cannot be concluded from the exclusive observation of a deleted *bel*1 gene with

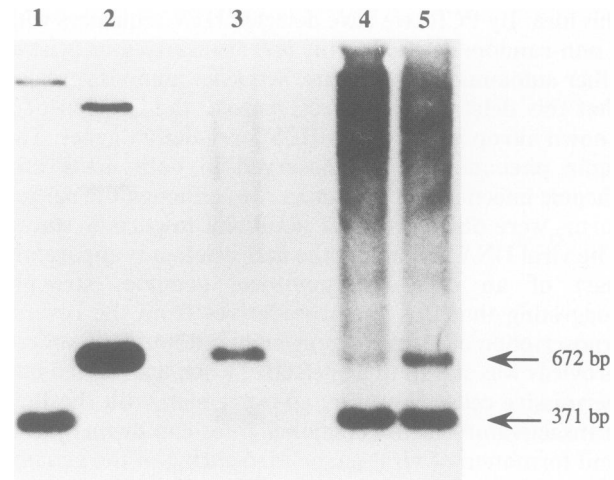

Fig. 3. Detection of the deletion in the *bel*1 region by Southern blot analysis using *Eco*RI and *Nco*I in acutely infected cells (lane 3), chronically infected HFV lysis-resistant DAMI cells (lane 4, a specific clone; lane 5, the parental pool). Positive controls were pΔHFV (lane 1) and pHFV13 (lane 2). The 475 bp *Pst*I–*Nco*I fragment, located in the *bel* region, was used as a probe. The ratio of deletion-bearing to intact *bel*1 genes was ~1:20 in the clone and 1:5 in the pool.

BEL1 primers, that the intact form is not present in trace amounts. Molecular cloning of this HFV variant was not attemped as only a minority of cells was virus-infected (Southern analysis was negative: data not shown). The functional consequence of such a deletion would be a switch from Bel1 expression to Bet expression (Figure 2B). The Bel1 protein was previously shown to be an essential transactivator of the HFV LTR (Löchelt *et al.*, 1991; He *et al.*, 1993; Venkatesh *et al.*, 1993). Therefore, deletion of a segment of the *bel*1 gene is likely to impair viral transcription and replication.

HFV in acute infection

A Hirt extract of viral DNA produced during acute HFV infection of permissive U373MG cells was examined by both PCR and Southern blot analysis. After amplification, both *bel*1 products were detected. However, the intact viral form

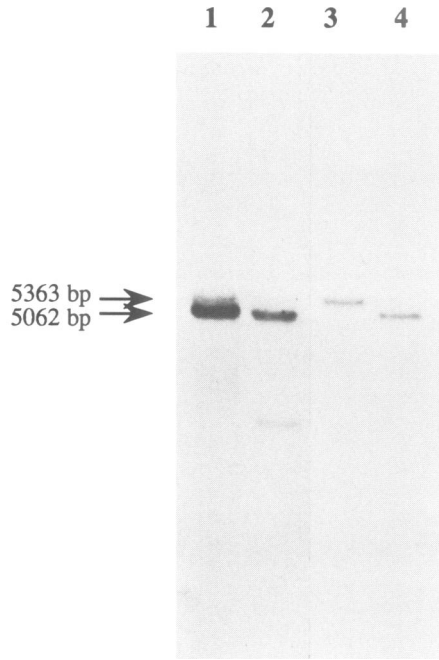

Fig. 4. Southern blot analysis of DNA extracted from chronically infected DAMI cells using *Hin*dIII [lanes 1 (the parental pool) and 2 (a specific clone)]. The pHFV13 (lane 3) and pΔHFV (lane 4) plasmids were used as positive controls. Blots were hybridized with the 5363 bp *Hin*dIII–*Hin*dIII fragment.

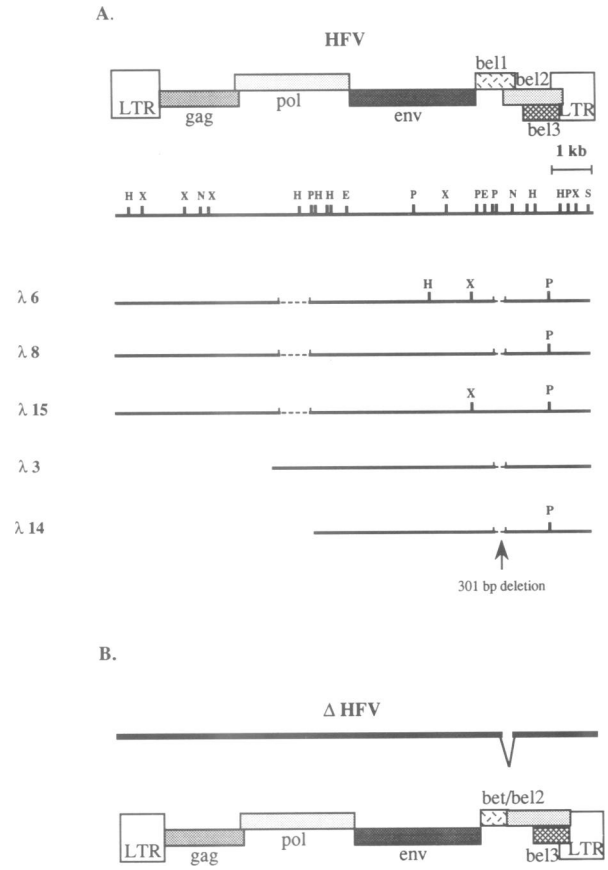

Fig. 5. (**A**) Maps of genomic λ clones isolated from DNA from the DAMI cell line. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; S, *Bsu*36I; X, *Xba*I. Only the viral portion of the clones is depicted. Polymorphic sites are indicated on the different clones. (**B**) Proposed genetic structure of ΔHFV which is derived from the reverse transcription of a spliced pregenomic RNA.

was much more abundant (data not shown). Importantly, in this model, we could also demonstrate the deletion by Southern blotting using restriction endonucleases *Eco*RI and *Nco*I, which have cleavage sites that flank the deletion (Figure 3, lane 3) (see Figure 5 for the localization of the *Eco*RI and *Nco*I sites). Using *Hin*dIII, which cuts both in the *env* gene [5′ to the most 5′ splice acceptor site known (Muranyi and Flügel, 1991)] and in the *bel*1 gene, we could again demonstrate, with an internal probe, the presence of a *bel*1-deleted form of the proviral DNA (not shown). Thus, it is most unlikely that our observations reflect the presence of a retrotranscribed putative subgenomic *env*–*bet* message (Figure 1) lacking the *gag* sequences, as has been shown for some subgenomic RNAs from animal retroviruses (Stacey, 1980; Bodor and Svoboda, 1989). Moreover, the *bel*1-deleted DNA sequences contain an LTR U5 motif, as demonstrated by the presence of the deletion using both *Eco*RI and *Bsu*36I restriction endonucleases (not shown). These data provide evidence that the HFV provirus exists in (at least) two forms, the shorter one being a spliced form of the wild-type one.

We subsequently wondered whether the deletion-containing HFV provirus could be derived from wild-type HFV. Thus, permissive AV3 cells were transfected with an infectious HFV clone (Löchelt *et al.*, 1991) and the presence of the *bel*1 deletion was investigated using PCR 1 week after transfection. Indeed, both the deletion-bearing (402 bp) fragment and the intact one were detected. Altogether, our data strongly suggest that the deletion in the provirus is generated by splicing out a fragment of the *bel*1 gene in the RNA pregenome.

HFV genome status in chronic experimental infections

To examine whether we could detect a *bel*1 deletion in other models of HFV infection, peripheral blood lymphocytes of chronically infected rabbits (M.Canivet and M.C.Guillemin, unpublished data) were analysed with BEL1 primers. While in three of the five HFV infected rabbits, both deletion-containing and intact proviruses were present, the other two contained only the deleted form (not shown). Again, Southern blot analysis was negative. The molecular findings in these experimentally infected animals, which are otherwise healthy, closely resemble those found in autoimmune patients.

A cellular system of chronic HFV infection was recently established in a megakaryocytic cell line, DAMI (C.Robert, F.Rozain, J.Wybier-Franqui, J.Lasneret, M.Canivet and J.Périès, manuscript in preparation). These cells, which were isolated as HFV lysis-resistant lines, no longer produce infectious viral particles, although the HFV genome is present. We tested the HFV genome in DAMI cells for the presence of the *bel*1 deletion. By PCR, almost exclusively this form was detected (not shown). The availability of cloned cell lines which contain HFV genomes offered an opportunity to characterize further the DNA sequences which harbour the *bel*1 deletion. On Southern blots, the deletion-bearing restriction fragment was found in a 20-fold excess over the intact *bel*1 gene (Figure 3, lane 4). Using other restriction endonucleases which flank the deletion, we show again that the deleted *bel*1 sequence belongs to a provirus as it extends 5′ to the most 5′ splice acceptor site (Figure 4) and contains a U5 motif (not shown). By genomic cloning, we isolated two proviruses which have a 301 bp deletion

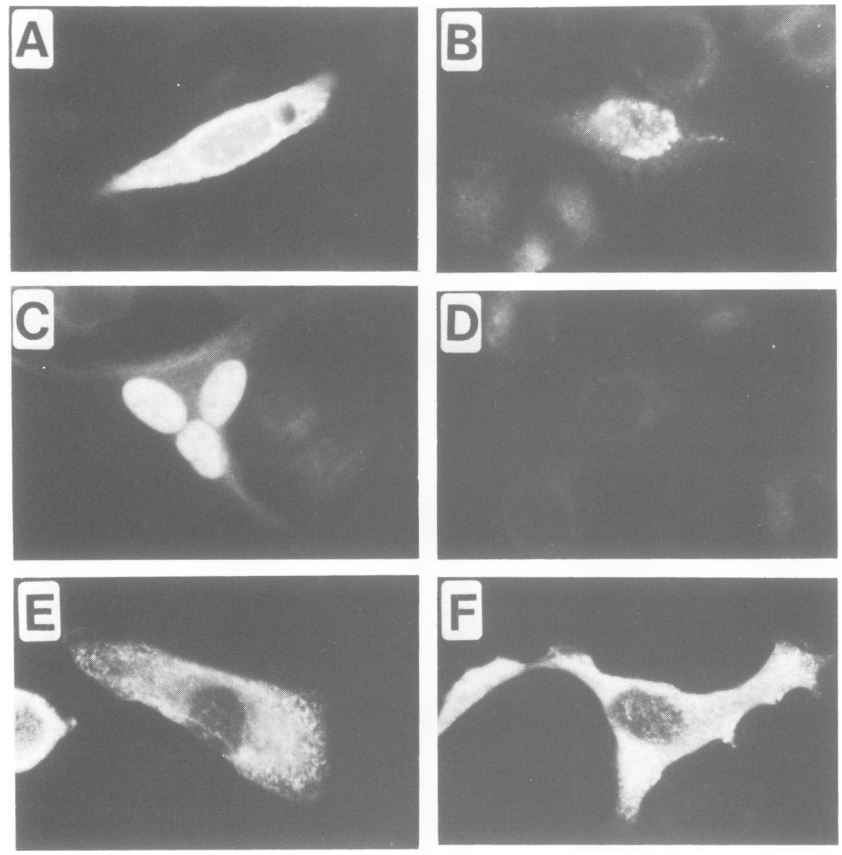

Fig. 6. Immunofluorescence analysis of transfected AV3 cells. (**A**) Cells transfected with pHFV13 stained with the rabbit polyclonal anti-HFV serum. (**B** and **C**) Cells transfected with pBCbel1 and stained with a mouse polyclonal anti-Bel1 serum 24 (B) or 48 h (C) post-transfection. (**D**) Cells transfected with pΔHFV and stained with the rabbit polyclonal anti-HFV serum. (**E**) Cells co-transfected with pΔHFV and pBCbel1 revealed with a monoclonal anti-Bet antibody, D11. (**F**) Acellular supernatants from cells co-transfected with pΔHFV and pBCbel1 were used to infect pBCbel1-transfected AV3 cells. Three days post-infection cells were stained with the monoclonal anti-Bet antiserum.

in the *bel*1 gene as well as *gag* sequences (Figure 5). Both also have another 800 bp internal deletion within the reverse transcriptase and RNase H genes. This additional deletion may have occurred either during cloning or during the establishment of the chronically infected DAMI cell line. Two partial clones containing the deleted *bel*1 gene and extending into the *pol* gene 5′ of the *env* gene splice acceptor sites were also characterized. Finally, a clone was found containing the same 800 bp *pol* gene deletion but an intact *bel* region. Thus, the DNA which contains the *bel*1 deletion cannot be a putative reverse transcribed doubly spliced *env*−*bet* RNA. The most likely mechanism for generating these two proviruses is splicing in the pregenomic RNA.

Functional analysis of a bel1-deleted HFV provirus

A plasmid, pΔHFV, containing the *bel*1 deletion was derived from the infectious HFV clone, pHFV13 (see Materials and methods). To test the functional properties of this construct, pΔHFV was transfected into three cell lines, COS-7, U373MG and AV3, which are all permissive for HFV replication. Viral expression was assessed 2−4 days post-transfection by immunofluorescence using a rabbit polyclonal anti-HFV serum. While pHFV13-transfected cells were strongly positive (Figure 6A), the results were negative with the pΔHFV construct (Figure 6D). Similar results were obtained using other antigen-specific monoclonal antibodies, in particular the mouse monoclonal anti-Bet, D11 (not shown). Then we tried to establish whether this apparently defective *bel*1-deleted HFV genome could be rescued by Bel1. For that purpose, AV3 cells were co-transfected with both a Bel1 expression vector (pBCbel1) and pΔHFV, and analysed by immunofluorescence using D11 and a rabbit polyclonal anti-gag serum which will only reveal the provirus-derived proteins. While *bel*1-transfected cells did not stain with D11, strong staining was detected in the nuclei with a mouse polyclonal anti-Bel1 serum (Figure 6B and C). Cells transfected with both plasmids presented a strong positive signal with the Bel1 antiserum (not shown) as well as with monoclonal antibody D11 (Figure 6E) and the anti-gag serum (not shown). This establishes that Bel1 is essential for viral replication and protein expression, as previously shown using crude deletion analysis (Löchelt *et al.*, 1991; He *et al.*, 1993; Venkatesh *et al.*, 1993). These data suggest that functional *trans*-complementation between *bel*1 and pΔHFV could lead to formation of viral particles. Therefore, acellular supernatants from AV3 cells (4 days after transfection), co-transfected with the two plasmids were transferred on to transiently *bel*1-transfected AV3 cells. Two days later, cells were analysed by immunofluorescence using the monoclonal antibody, D11 (Figure 6F) and an anti-gag serum (not shown). A few strongly positive cells were found, establishing that the deletion-bearing pregenome can be packaged into viral particles.

Discussion

Our data show that the HFV provirus exists in two distinct molecular forms, which differ by the splicing out of a fragment in the *bel*1 transactivator gene. The deletion-bearing provirus (subsequently referred to as ΔHFV) is apparently associated with chronic forms of HFV infections, functionally defective, but can be packaged to form viral particles in the presence of exogenous Bel1. To our knowledge, this observation constitutes the first example of splicing-induced alterations in both genome structure and biology of a human retrovirus.

The splicing pattern of human retroviruses can be highly complex and some spliced regulatory genes were discovered long after the molecular characterization of the viral genome (Berneman *et al.*, 1992; Ciminale *et al.*, 1992). However, all spliced viral RNAs described to date used the 5′ donor site located at th LTR−*gag* junction as the first donor site (Coffin, 1991). Thus, singly or multiply spliced RNAs never contained *gag* sequences (note that the reverse transcription and integration of such subgenomic viral mRNAs lacking *gag* sequences is well known (Stacey, 1980; Bodor and Svoboda, 1989). Our observation of a pregenome in which only a regulatory gene is spliced out is important in that respect, as it establishes the existence of a new type of mRNA. Previous molecular analysis of unintegrated HFV proviral DNA using S1 nuclease analysis had already shown the presence of a shorter fragment in the *bel* region (Kupiec *et al.*, 1988; Tobaly-Tapiero *et al.*, 1991). This result, which was not understood at that time, can now be attributed to a deleted *bel*1 region in some proviral DNA.

This phenomenon appears to be common among foamy viruses. Southern blot analysis of simian foamy virus 6-infected cells using a human *bel*1 probe reveals two distinct DNA fragments (J.Tobaly, personal communication). A similar result was obtained with the bovine foamy virus (Renshaw *et al.*, 1991). In addition, molecular cloning of bovine foamy virus proviral DNA showed non-random deletions in the 3′ regulatory region (Renshaw *et al.*, 1991).

In cauliflower mosaic virus and figwort mosaic virus (two caulimoviruses), a similar situation is encountered (Hirochika *et al.*, 1985; Scholthof *et al.*, 1991). These viruses share significant biological homologies with the retroviruses, including for example, a reverse transcribed RNA pregenome. Defective HBV viruses containing spliced genomes (lacking the envelope S and *pol* genes) are also found in the serum and liver of some chronically HBV-infected patients (Terré *et al.*, 1991).

The defectivity of ΔHFV was shown using a poorly sensitive assay, which only reveals abundant viral proteins. We cannot exclude the possibility that small amounts of viral proteins are synthesized. Indeed, it has been noted that the HFV LTR can exert a weak promoter activity in the absence of Bel1 (Rethwilm *et al.*, 1990). The recent demonstration of an internal promoter in the *env* gene could account for a basal level of expression of the *bel* genes (Löchelt *et al.*, 1993). *Bel*-encoded regulatory proteins might play a role in the control of gene expression by interfering with either transcriptional or post-transcriptional controls. In that sense, the functional consequence of the deletion could be a shift from *bel*1 to *bel*2, *bet*, *bel*3 expression with impairment of LTR transcription and a block in viral replication. We are currently addressing this question by establishing stable transformants of the deletion-containing HFV provirus in permissive cells and analysing ΔHFV protein expression.

How does the presence of deleted forms of HFV relate to the biology of the virus? The presence of ΔHFV DNA could be a mere scar of a past HFV infection. On the other hand, ΔHFV may both significantly modify the course of HFV infection and/or play a role by itself. In cells which harbour the two genomes, the ΔHFV LTR may titrate out the Bel1 protein produced by the HFV, cutting the autoregulatory loop of Bel1 on its own gene. Alternatively, ΔHFV-produced regulatory proteins (possibly Bel2/Bet) might antagonize Bel1 activity. Defective interfering viral particles have been reported for many different types of viruses and impede wild-type virus replication or expression by various molecular mechanisms (Huang and Baltimore, 1970). Should ΔHFV interfere with HFV functions, the data reported here would constitute the first example of defective interfering particles generated by splicing.

ΔHFV alone may exert some biological functions through low levels of early viral gene expression. In cells where the viral replicative cycle cannot be achieved, continuous exposure to early viral proteins of several DNA viruses often induces transformation [reviewed in Benjamin and Vogt (1991)]. Less dramatic effects were associated with viral gene expression in the absence of viral replication, leading to the loss of cell differentiation [reviewed in Oldstone (1989)]. Such pertubations may be relevant to our recent proposal of an association between HFV infection and autoimmune diseases (Lagaye *et al.*, 1992), as lymphocyte (de)differentiation could lead to these disorders. Finally, ΔHFV may exert an effect on non-infected cells; like HTLVI tax or HIV tat (Marriott *et al.*, 1991), the Bet protein can be excreted (M.L.Giron, J.Wybier-Franqui, F.Rozain, M.C.Guilleman, J.Périès and R.Emanoil-Ravier, manuscript in preparation) and function as a 'virokine'. The switch from *bel*1 to *bet* expression, induced by the deletion, may be important in that respect.

Materials and methods

Animals, cell cultures and virus

Rabbits were infected with a single intravenous dose of HFV particles (1.5×10^6 p.f.u.). Peripheral blood lymphocytes were separated and DNA analysed 2 years after infection.

Mycoplasma-free HFV stocks were grown on U373MG cells, a human neural cell line, maintained in Eagle's minimum essential medium supplemented with non-essential amino acids, sodium pyruvate and 10% fetal calf serum (FCS). AV3, a human amniotic cell line, was maintained in McCoy's modified 5A medium and 10% FCS. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. DAMI, a human megakaryocytic cell line, was propagated in RPMI medium/10% horse serum.

Immunofluorescence and DNA transfections

AV3 and COS-7 cells were transfected with Lipofectin reagent (Gibco, BRL), according to the manufacturer's instructions. Transfected cells were fixed 24, 48 or 60 h post-transfection with 4% paraformaldehyde at 4°C for 20 min and permeabilized with methanol at 4°C for 5 min. The antibodies used were either serum from HFV-infected rabbits, a mouse polyclonal antiserum against a glutathione S-transferase(GST)−Bel1 fusion protein made in *Escherichia coli* (A.Saïb, manuscript in preparation), a rabbit polyclonal antiserum against a GST−gag fusion protein (provided by N.Morin, Montpellier), or a mouse monoclonal antibody against the Bet protein, D11, all at a 1/100 dilution. An anti-IgG FITC-coupled antibody (Biosys, France) was used as second fluorescent conjugate.

DNA analysis

Peripheral blood lymphocytes from patients or infected rabbits were isolated from blood samples by centrifugation over Ficoll/Hypaque. DNA was

extracted as described by Gross-Bellard *et al.* (1973). Viral DNA was extracted according to Hirt (1967). Southern blots, prepared by standard procedures (Sambrook *et al.*, 1989), were hybridized overnight with an [α-^{32}P]dCTP-labelled probe at 42°C in 5 × SSC, 0.1% SDS, 5 × Denhardt's, 50% formamide and 100 μg of denatured salmon sperm DNA per ml. Washings were performed by 0.1 × SSC, 0.1% SDS buffer at 60°C for 30 min. The plasmid pHFV13 was used as a probe for PCR hybridization. A 5363 bp *Hin*dIII–*Hin*dIII fragment of this plasmid containing the 3′ part of the *pol* gene and the entire *env* and *bel* genes [nt 1–5363, from the nucleotide sequence of Flügel *et al.* (1987)] was used for Southern blot hybridization. The 475 bp *Pst*I–*Nco*I DNA fragment (nt 4329–4805, in the *bel*1 gene) was used as a probe in some experiments.

PCR

Enzymatic amplifications of the *bel*1 region were performed with the following primer couples: BEL1: 5′-GCT GAC TAT TGC TGA GGA AC-3′ (nt 4202–4221) and 5′-AAG TTT GGT AGG TTG CTG GA-3′ (nt 4904–4885); SB1 (internal to the deletion): 5′-GTC ACT CTG TAA AAG ACT TA-3′ (nt 4368–4387) and 5′-TGG TAG AAG GTT TAA CAA GA-3′ (nt 4645–4662). The PCR program consists of 35 cycles of denaturation at 94°C for 1 min, followed by annealing at 54°C (BEL1) or 48°C (SB1) for 1 min and extension at 72°C for 1 min.

DNA cloning and sequencing

PCR products were subcloned in the pCR vector (TA Cloning Kit, InVitrogene), and sequenced using Sequenase (United States Biochemical) according to the manufacturers' instructions. To eliminate *Taq*-generated mutations, each PCR amplification was performed in duplicate and cloned; for each reaction two clones were sequenced. A genomic DNA library (8×10^5 primary clones) was constructed from the chronically infected DAMI cell line by standard procedures in a λEMBL3 vector using a partial *Sau*3A digest (Sambrook *et al.*, 1989).

Plasmid constructions

Plasmid pC2 was constructed by inserting the *Xba*I–*Bgl*II DNA fragment of the C55 plasmid (Flügel *et al.*, 1987) into the *Xba*I and *Bam*HI sites of pUC18. The pCRBEL1 plasmid (which contains the *bel*1 deletion) was obtained by subcloning the *bel*1 PCR product of the myasthaenic patient in the pCR vector (TA Cloning Kit, InVitrogene). To construct the pC2PCR plasmid, the *Bam*HI–*Cla*I DNA fragment of the pC2 plasmid was replaced with the corresponding fragment of the pCRBEL1 plasmid. The *Sal*I–*Ecl*XI fragment of the infectious pHFV13 plasmid (Löchelt *et al.*, 1991) was transferred to the corresponding sites of pBluescript SK+ creating the plasmid pHFVN. pΔHFVN was generated by replacing the *Nhe*I–*Cla*I DNA fragment of pHFVN with the corresponding fragment of pC2PCR. Finally, the pΔHFV plasmid was made by introducing the *Sal*I–*Ecl*XI DNA insert into pAT153 (Sambrook *et al.*, 1989).

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