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Small finger protein of avian and murine retroviruses has nucleic acid annealing activity and positions the replication primer tRNA onto genomic RNA

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Retrovirus virions carry a diploid genome associated with a large number of small viral finger protein molecules which are required for encapsidation. Our present results show that finger protein p12 of Rous sarcoma virus (RSV) and p10 of murine leukaemia virus (MuLV) positions replication primer tRNA on the replication initiation site (PBS) at the 5′ end of the RNA genome. An RSV mutant with a Val–Pro insertion in the finger motif of p12 is able to partially encapsidate genomic RNA but is not infectious because mutated p12 is incapable of positioning the replication primer, $tRNA^{Trp}$. Since all known replication competent retroviruses, and the plant virus CaMV, code for finger proteins analogous to RSV p12 or MuLV p10, the initial stage of reverse transcription in avian, mammalian and human retroviruses and in CaMV is probably controlled in an analogous way.

Key words: retrovirus/primer tRNA/reverse transcription/finger protein

Introduction

Retroviruses have been shown to induce a variety of neoplastic and immunosuppressive diseases, including AIDS, and are widely distributed among animal species (reviewed by Lowy, 1985). Retrovirus virions are about 80–120 nm in diameter and consist of an inner core (nucleoid) surrounded by an outer envelope. The core corresponds to a ribonucleoprotein surrounded by the protein capsid. The viral RNA genome is in the ribonucleoprotein and it is composed of two identical positive sense RNAs with 5′ Cap and 3′ poly(A) structures (like most cellular mRNAs) (reviewed by Coffin, 1984). The two RNA subunits are annealed to each other at the dimer linkage structure (DLS) located at 300–400 nt from the 5′ end (Bender *et al.*, 1978; Murti *et al.*, 1981; Darlix *et al.*, 1982) and each subunit is 3.5–9 kb in length (reviewed by Van Beveren, 1984). A specific cell tRNA is hybridized to each subunit at the primer binding site (PBS; position 102–120 in RSV and 146–164 in Moloney murine leukaemia virus (MMuLV); Van Beveren *et al.*, 1984) where it is essential for priming reverse transcription of the RNA genome by reverse transcriptase (Varmus and Swanstrom, 1984). The diploid RNA genome within the ribonucleoprotein structure is associated with a few molecules of DNA polymerase (reverse transcriptase) and a large amount of a basic viral protein (nucleic acid binding protein; NBP) (Coffin, 1984) which belongs to the finger protein family (Berg, 1986; Covey, 1986; Darlix, 1986b; Vincent, 1986). In Rous sarcoma virus (RVS) virions ~1% of these protein molecules interact tightly with the viral RNA (Darlix and Spahr; 1982; Méric *et al.*, 1984).

In order to increase our understanding of the initiation of reverse transcription that is essential for virus replication we have examined how the replication primer tRNA was positioned onto the viral RNA.

Previous reports demonstrated that AMV reverse transcriptase forms a stable and specific complex with the replication primer $tRNA^{Trp}$ (Panet *et al.*, 1975; Araya and Litvak, 1979) and is able to partially unwind the acceptor stem of the tRNA favouring annealing of primer $tRNA^{Trp}$ to the RSV genome (Araya *et al.*, 1979). Here we provide biochemical and genetic data indicating that finger protein RSV p12 or MuLV p10 directs the annealing of the replication tRNA primer onto the primer binding site of the viral genome under physiological conditions. This hybridizing activity of RSV p12 or MuLV p10 also enhances the annealing of complementary 20–30mer oligonucleotides to viral RNA suggesting that the viral finger protein is required for the 5′→3′ transfer at the initial stage of minus strand viral DNA synthesis (Varmus and Swanstrom, 1984).

These experimental data indicate that interactions between the small viral finger protein, replication primer tRNA and retroviral genome control the initial stage of provirus synthesis in a manner reminiscent of the control of ColE1 plasmid replication by rom (Cesareni *et al.*, 1982; Tomizawa and Som, 1984) or by initiator protein (Patel and Bastia, 1987).

Results

Small finger protein of RSV and MuLV positions primer tRNA onto the PBS of viral RNA

The RSV-PrB *td* and MMuLV proviruses cloned in λ-EMBL3 and pBR322, respectively (Miller and Verma, 1984; Darlix, 1986a), were subcloned in pSP65 (Figure 1). For most of our studies recombinant template DNAs were opened within the 5′ *gag* coding sequence giving rise to viral RNA of 0.7 kb (Schinnick *et al.*, 1981; Schwartz *et al.*, 1983) upon transcription with SP6 RNA polymerase (see Figure 1).

RSV RNA (0.3–0.5 μg) and 5′-^{32}P-labelled $tRNA^{Trp}$ were mixed with p12 and the products were analysed by agarose gel electrophoresis following incubation and deproteinization. The results are shown in Figure 2A. The ethidium bromide stained gel reveals that RSV RNA with the 380 nt leader (lane 4) migrates as dimers and higher order multimers (lanes 5 and 6) after p12 treatment but not following treatment with reverse transcriptase (lane 7). The 5′ 255 nt of RSV leader RNA contain the repeat (R), the 5′ untranslated sequence (U5) and the primer binding site (PBS) (position 102–120) (Coffin, 1984) and the encapsidation sequence (E) (Katz *et al.*, 1986). No p12-induced dimeriza-

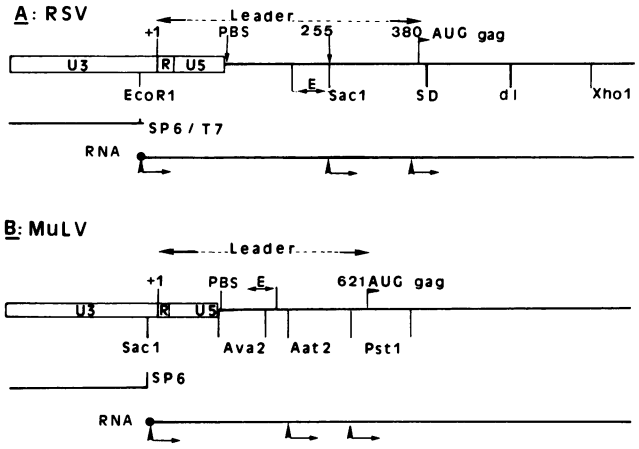

Fig. 1. Genetic manipulation of the 5′ leader of RSV and MuLV proviral genomes and synthesis of 5′ recombinant RSV and MuLV RNAs. (**A**) RSV DNA: 3′ untranslated region (U_3), repeat (R), 5′ untranslated region (U_5) (U_3-R-U_5 = LTR), primer binding site (PBS: position 102−120), encapsidation sequence (E), 3′ leader and 5′ *gag* coding sequence are shown together with restriction sites of interest. RSV DNA was manipulated at *Eco*RI, *Sac*I sites or using *Bal*31S and subcloned in the pSP65 DNA. pSP6-RSV DNA clones were linearized at the *Xho*I site (position 630) or at the *Eco*RI site (position 2310) for 5′ RNA at positions 255 or 360. The start of transcription *in vivo* is +1 (Cap) and is indicated by double arrows for the 5′ recombinant RNAs. Numbers refer to position in nucleotides (Darlix, 1986a). (**B**) MMuLV DNA: U_3-R-U_5 (LTR), PBS (146−164), E, 3′ leader and 5′ *gag* are shown. MMuLV DNA was manipulated at *Sac*I (position −31), *Aat*II (position 367) and *Pst*I (position 567) sites and subcloned in the pSP65 DNA. pSP65-MuLV DNA was linearized at the *Bst*EII site (position 725) or at the *Xho*I site (position 1530). The start of transcription *in vivo* is +1 (Cap) and is indicated by double arrows for the 5′ recombinant RNA (Schinnick *et al.*, 1981).

tion can be observed after removal of these 5′ 255 nt (Figure 2A, lane 2). A detailed study of RSV and MuLV RNA dimerization under the control of the E sequence and the small finger protein will be published elsewhere (see also Bender *et al.*, 1978; Murti *et al.*, 1981).

Figure 2B demonstrates that protein p12 also promotes efficient annealing of $tRNA^{Trp}$ to RSV RNA. RNA carrying the PBS site (lane 5) does not bind $tRNA^{Trp}$ (lane 6) unless sufficient levels of p12 are included in the reaction (lanes 7 and 8). Binding of $tRNA^{Trp}$ to RSV RNA is enhanced by AMV reverse transcriptase (lane 10) although the enzyme alone does not promote $tRNA^{Trp}$ binding (lane 9). Primer $tRNA^{Trp}$ very probably binds to the PBS since removal of the 5′ 255 nt of RSV RNA abolishes [^{32}P]-$tRNA^{Trp}$ annealing (lanes 1−4).

To confirm this, reverse transcription was carried out using virion 35S RNA and recombinant RNAs having 55 or 51 nt in addition to the 101 nt from the PBS to the 5′ Cap (Coffin, 1984; see legend to Figure 3). After preincubation with p12 and primer 5′-[^{32}P]$tRNA^{Trp}$, cDNA synthesis was performed using the three template RNAs. As expected for cDNA synthesis primed by $tRNA^{Trp}$ at the PBS, the length of the cDNA is ~101 nt using RSV 35S RNA and 156 or 152 nt using the respective recombinant RSV RNAs (Figure 3A, lanes 2−4). Moreover, cDNA is not synthesized in the absence of p12 (lanes 1 and 5) indicating that p12 is required to position the primer $tRNA^{Trp}$ on the PBS of RSV RNA. This RNA annealing activity appears to be a specific property of RSV-p12 (Figure 3B, lanes 2 and 3) since MuLV p30, RSV p27 and the nucleic acid binding protein T4Gp32 are

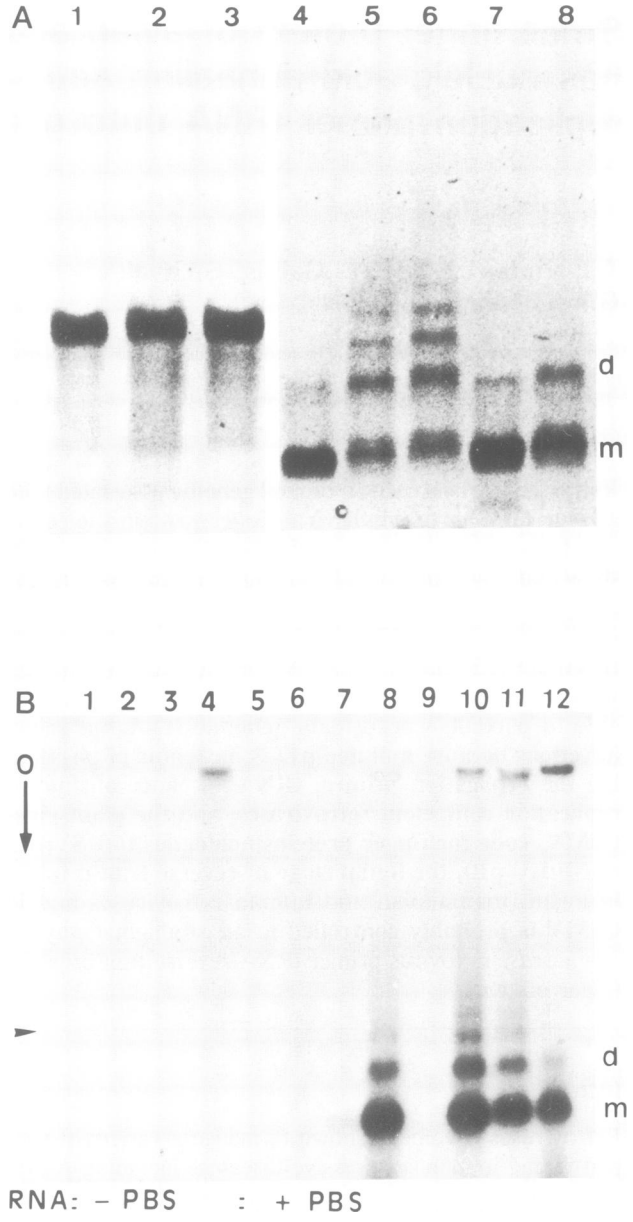

Fig. 2. Finger protein RSV p12 dimerizes viral RNA and anneals primer $tRNA^{Trp}$ to RSV RNA. RSV RNA, 5′-[^{32}P]$tRNA^{Trp}$ labelling and incubation conditions were done as described in Materials and methods. Incubations were at 40°C for 15 min in 10 μl containing 0.5 μg RNA 2.05 kb (positions 260−2310, see Schwartz *et al.*, 1983) without PBS (PBS$^-$) or 0.25 μg RNA 0.7 kb (leader to position 630) with PBS (PBS$^+$) and where indicated ~30 ng 5′-[^{32}P]$tRNA^{Trp}$ (2×10^6 c.p.m./μg). (**A**) Viral RNA dimerization. RSV RNAs were stained by ethidium bromide after agarose gel electrophoresis (see Materials and methods). m = monomer (0.7 kb) and d = dimer (1.4 kb). PBS$^-$ 2.05 kb RNA: with 30 ng $tRNA^{Trp}$ (**1**), plus 50 ng p12 (**2**) or 0.6 μg AMV DNA polymerase (**3**). PBS$^+$ 2.05 kb RNA: with 30 ng $tRNA^{Trp}$ (**4**), plus 25 ng p12 (**5**) or 50 ng p12 (**6**), 0.6 μg AMV DNA polymerase (**7**), or 25 ng p12 plus 0.6 μg AMV DNA polymerase (**8**). (**B**) Primer $tRNA^{Trp}$ annealing to RSV RNA. The gel was dried and autoradiographed (12 h). 0 is the origin of electrophoresis. PBS$^-$ RNA: alone (**1**), with 30 ng $tRNA^{Trp}$ (**2**), plus 0.6 μg AMV DNA polymerase (**3**), or 50 ng p12 (**4**). PBS$^+$ RNA: alone (**5**), with 30 ng $tRNA^{Trp}$ (**6**), plus 10 ng p12 (**7**), or 50 ng p12 (**8**), 0.6 μg AMV DNA polymerase (**9**), or AMV DNA polymerase plus 50 ng (**10**), 15 ng (**11**) or 10 ng (**12**) of p12.

unable to position $tRNA^{Trp}$ onto RSV RNA (Figure 3B, lanes 1,4 and 5, respectively).

Such experiments were repeated with MMuLV RNA,

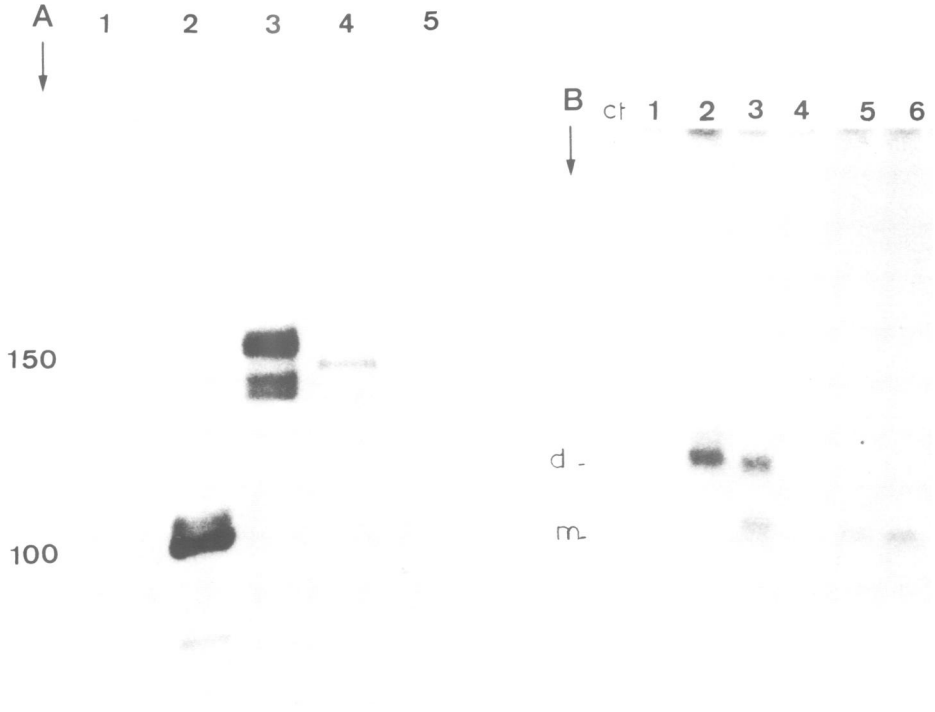

Fig. 3. RSV p12 positions $tRNA^{Trp}$ onto RSV RNA. (**A**) RSV RNA (0.7 kb) was prepared as described in Materials and methods except that the extra 55 or 51 nt at the 5′ end were obtained using proviral DNA template subcloned in pSP65 at the *Eco*RI sites in U_3 and 3′ *gag* (Schwartz *et al.*, 1983) (directly or upon treatment with mung bean nuclease). Binding of $tRNA^{Trp}$ was done in the presence of 50 ng p12. cDNA synthesis was performed at 43°C for 6 min in conditions previously described (Darlix *et al.*, 1977). ss cDNA labelled using [^{32}P]dCTP was analysed by 6% polyacrylamide–urea gel electrophoresis. Template virion 35S RNA minus p12 (**1**) or plus p12 (**2**), RNA 0.7 kb with 5′ 55 extra nt plus p12 (**3**) or minus p12 (**5**) and RSV RNA with 5′ 51 extra nt plus p12 (**4**). (**B**) RSV RNA (0.7 kb) with the 380 nt leader was incubated with protein p12, p27, AMV DNA polymerase or T4 gp32 (see also Figure 2). RNA (0.7 kb) with 0.6 μg AMV DNA polymerase (**1**) 50 ng p12 (**2**) or 15 ng p12 (**3**), 1 μg RSV p27 (**4**), T4Gp32 0.3 μg (**5**) or 0.5 μg (**6**). m = monomer (0.7 kb) and d = dimer (1.4 kb). The agarose gel was dried and autoradiographed (12 h).

primer $tRNA^{pro}$ and RSV p12. The results (not shown) indicate that p12 can also position primer $tRNA^{Pro}$ on the PBS of MMuLV RNA at a low efficiency. We therefore decided to investigate the behaviour of finger protein p10 of MuLV in these binding assays.

MMuLV RNA with the 621 nt leader (Figure 1 and Schinnick *et al.*, 1981) and 5′-[^{32}P]$tRNA^{Pro}$, were incubated with or without purified p10, deproteinized and analysed by agarose gel electrophoresis (Figure 4A). The 0.75 kb MMuLV RNA (lane 1) is dimerized by p10 (lanes 2, 4 and 5), but not by reverse transcriptase (lane 3) or *Escherichia coli* Rec A protein (lane 7). It is, however, dimerized by large amounts of MuLV p30 (lane 6). Autoradiography of the gel indicates that p10 is capable of annealing $tRNA^{Pro}$ to MMuLV RNA (lanes 11, 13 and 14) whereas reverse transcriptase (lane 12), MuLV p30 (lane 15) and *E.coli* Rec A protein (lane 16) were not.

Deletion of the 5′ 367 nt, R U5, PBS and E (see Figure 1B) prevents both RNA dimerization (Figure 4A, lanes 8 and 9) and annealing of primer $tRNA^{Pro}$ (lanes 17 and 18). MuLV cDNA made in the presence or absence of p10 with 5′-[^{32}P]$tRNA^{Pro}$ primer for 6 min was analysed by PAGE. As shown in Figure 4B, MMuLV cDNA having the expected length of 255 nt (75 nt of $tRNA^{Pro}$ plus 180 nt of DNA, see Figure 1B) is made only in the presence of p10 (lane 2).

Annealing activity of the viral finger protein

In order to determine whether the nucleic acid annealing activity of the viral finger protein is restricted to viral RNA and primer tRNA, we synthesized oligonucleotides complementary to RSV or MuLV RNA and used them in hybridization assays.

A 5′-^{32}P-labelled 19mer complementary to positions 545–564 of RSV RNA (Schwartz *et al.*, 1983) was incubated with RSV RNA at an oligomer/RNA ratio of 3. It efficiently hybridizes to RSV RNA in the presence of p12 (Figure 5, lanes 5 and 6) but only poorly using reverse transcriptase alone (lane 7) or T4Gp32 protein (lanes 8 and 9). Deletion of positions 545–564 abolishes hybridization (lanes 1–3) as expected. Similar experiments were carried out using a 36mer complementary to positions 350–386 of RSV leader or a 28mer complementary to positions 280–307 of MuLV leader. Results of these annealing assays confirm that RSV p12 and MuLV p10 enhance hybridization of a 5′-[^{32}P]oligonucleotide to its complementary sequence on the viral RNA (Figure 5, lanes 10 and 11).

Thus, infectivity of virions appears to be dependent on the annealing activity of the small viral finger protein responsible for viral RNA dimerization and encapsidation (results to be published elsewhere and Méric and Spahr, 1986), and for primer tRNA hybridization at the initiation site for proviral DNA synthesis by reverse transcriptase.

A Val–Pro insertion in the finger motif of p12 impairs initiation of proviral DNA synthesis

Formation of the diploid viral genome is a process closely linked to RNA encapsidation, thus RSV mutants with deletions in the p12 coding sequence are unable to package

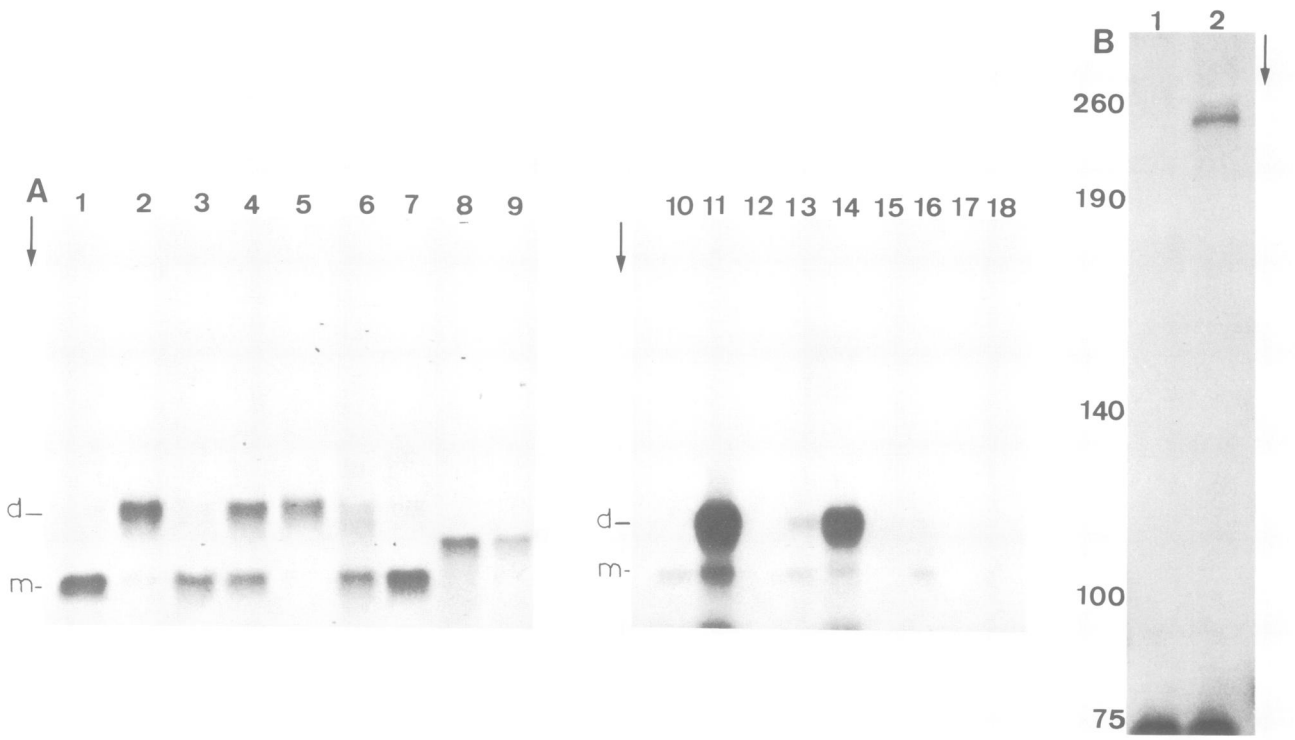

Fig. 4. Finger protein p10 of MuLV dimerizes viral RNA and positions primer $tRNA^{Pro}$ onto MuLV RNA. (**A**) MuLV RNA synthesis, 5′-[^{32}P]$tRNA^{Pro}$ labelling and incubation conditions are described in Materials and methods and Figures 1–3. All incubations were done in 10 μl and contained 20 ng $tRNA^{Pro}$ (2×10^6 c.p.m./μg). **Lanes 1–7**: 0.75 kb MuLV RNA with complete 621 nt leader (PBS). (1) control, (2) plus 50 ng p10, (3) plus 1 μg reverse transcriptase, (4) plus 20 ng p10, (5) plus 80 ng p10, (6) plus 0.5 μg p30, (7) plus 1 μg *E.coli* Rec A. **Lanes 8** and **9**: 1 kb MuLV RNA lacking the 5′ first 367 nt of the leader (PBS$^-$). (8) plus 20 ng p10, (9) plus 0.1 μg p10. **Lanes 10–18**: as above except that the gel was fixed, dried and autoradiographed (12 h). (**b**) MuLV RNA (0.75 kb) was incubated with 5′-[^{32}P]$tRNA^{Pro}$ with or without 50 ng p10 in 10 μl (see above). This incubation (2 μl) was taken and MuLV cDNA synthesis was performed at 43°C for 6 min (Darlix *et al.*, 1977). The reaction was stopped by addition of 0.1% SDS and heated at 100°C for 1 min. MuLV cDNA labelled at its 5′ end by [^{32}P]$tRNA^{Pro}$ was analysed by 6% PAGE in 7 M urea. (**1**) Without p10, (**2**) with p10. Markers (nt) are indicated on the left.

their RNA (Méric and Spahr, 1986). However, one mutant named PrC-1 (Méric and Spahr, 1986) with a Val–Pro insertion in the first finger motif of p12 is partially able to dimerize and encapsidate viral RNA but virions are not infectious. Proviral DNAs of the wild-type and of PrC-1 were transfected into avian fibroblasts (CEF) and 24 h later medium was collected every 12 h for 2 days. Wild-type and PrC-1 virions were collected, genomic RNAs purified and assayed for their capability to promote cDNA synthesis *in vitro*. As shown in Figure 6A, cDNA and high mol. wt DNA are made with wild-type RNA but not with PrC-1 RNA because the mutated p12 of PrC-1 is probably unable to position primer $tRNA^{Trp}$ on the PBS.

To demonstrate further the absence of replication primer $tRNA^{Trp}$ on PrC-1 genomic RNA we used 5′-[^{32}P]oligomer complementary to the 3′ 25 nt of $tRNA^{Trp}$. Genomic RNA from wild-type and PrC-1 virions was purified by sucrose gradient centrifugation (see Materials and methods), RNA from the 30–70S and 4–10S fractions was denatured, spotted on a nylon membrane and probed with 5′-[^{32}P]oligomer complementary to $tRNA^{Trp}$. As shown in Figure 6B, $tRNA^{Trp}$ is present in wild-type and PrC-1 viruses (spots 2 and 4) as well as on the wild-type genomic RNA (spot 3) but absent from the PrC-1 genome (spot 1). Thus mutated finger protein p12 is very probably unable to position replication primer $tRNA^{Pro}$ on the PBS of genomic RNA, and PrC-1 is not infectious because it cannot direct initiation of proviral DNA synthesis.

Discussion

Finger proteins p12 of RSV and p10 of MuLV are small proteins that have a nucleic acid binding and unwinding activity but no apparent sequence specificity *in vitro* (Schulein *et al.*, 1978; Smith and Bailey, 1979; Sykora and Moelling, 1981). Like all retroviral NBPs, RSV p12 and MuLV p10 are very basic and can be subdivided into three domains: a basic amino terminus domain followed by two (RSV p12, HIV p15, MMTV p14) (Schwartz *et al.*, 1983; Moore *et al.*, 1987; Covey, 1986; Darlix, 1986), or one (MuLV p10) (Schinnick, 1981) finger motif (Cys-X_2-Cys-X_3-His-X_4-Cys), and a COOH domain rich in proline and basic residues. In addition, the finger motif called the Cys–His box is shared by all members of the finger protein family (Covey, 1986; Darlix, 1986; Vincent, 1986) and is a potential metal binding domain in nucleic acid binding proteins (Berg, 1986); these proteins can bind to DNA and RNA and are of fundamental importance for the regulation of gene expression as demonstrated by the recent work on transcription factors IIIA (Miller *et al.*, 1985) and Sp1 (Kadonaga *et al.*, 1987) and on proteins regulating the development of *Drosophila* (Vincent *et al.*, 1985; Boulay *et al.*, 1987).

Nucleic acid annealing activity of finger proteins RSV p12 and MuLV p10 and reverse transcription

The nucleic acid annealing activity of these small finger proteins (Figures 2–5) takes place under physiological condi-

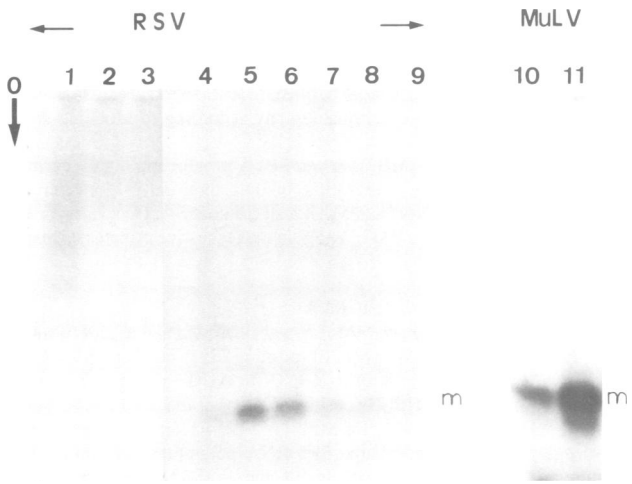

Fig. 5. Annealing activity of finger proteins RSV p12 and MuLV p10. Assays and analyses are described in Materials and methods. A 19mer complementary to proteins 545–556 (Schwartz *et al.*, 1983; Pugatsch and Stacey, 1983) was chemically synthesized, purified and 5′-^{32}P-labelled. RNA (0.3 μg) was used with a 3-fold molar excess over the oligonucleotide. RSV RNA (2 kb) with a deletion of positions 400–600 with 19mer (**1**), plus p12 50 ng (**2**) or AMV DNA polymerase 0.6 μg (**3**); RSV RNA (0.7 kb) (see Figure 1) with 19mer (**4**), plus p12 25 ng (**5**) or 50 ng (**6**), AMV DNA polymerase 0.3 μg (**7**) or 0.6 μg (**8**), or T4 gene 32 protein 0.5 μg (**9**). A 28mer complementary to positions 280–307 of MMuLV (Schinnick *et al.*, 1981) was chemically synthesized, purified and 5′-^{32}P-labelled. MMuLV RNA (0.3 μg) with the 621 nt leader was incubated with 30 ng oligomer (**10**), plus 10 ng MuLV p10 (**11**). The agarose gel was dried and autoradiographed (1 day). 0 = the origin of electrophoresis and m = 0.7 kb.

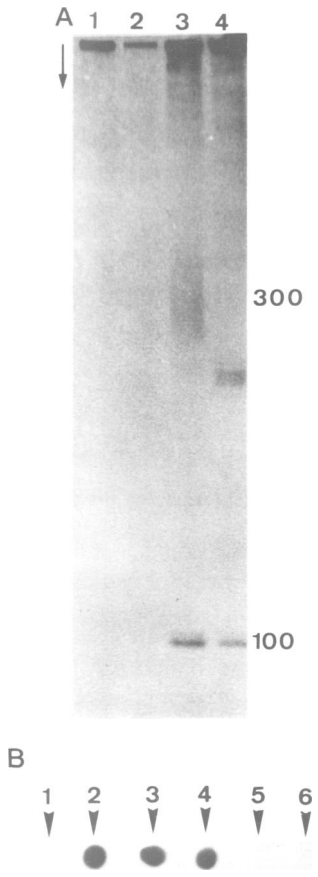

Fig. 6. A Val–Pro insertion in the Cys–His domain of p12 impairs initiation of viral cDNA synthesis. pAPrC (PrC wild-type) and PrC-1 proviral clones were constructed by C.Méric (Méric and Spahr, 1986). PrC-1 has a Val–Pro insertion in the first Cys–His box of p12. Three 10 cm dishes of CEFs (2×10^7 cells) were transfected (control, pAPrC and PrC-1) and 1 day later medium was collected for 2 days. Virions and RSV RNA 30–70S were purified according to published procedures (Darlix *et al.*, 1977). A third of the purified virions was used in order to estimate the amount of virus produced by means of Western analysis (SDS–PAGE followed by immuno-detection with anti-RSV p27 serum). (**A**) 101 nt cDNA synthesis was in 15 μl using RSV RNA PrC or PrC-1 in conditions described in Figure 3. After cDNA synthesis and alkali treatment, ^{32}P-101 cDNA was analysed by 6% PAGE. RNA template 45–70S PrC-1 (**1**) and PrC (**3**) RNA template 30–40S, PrC-1 (**2**) and PrC (**4**) RNA. (**B**) 30–70S and 4–10S RNAs from wild-type and PrC-1 virions were denatured and spotted on nylon membranes (Maniatis *et al.*, 1981). An oligomer complementary to the 3′ 25 nt of tRNA^{Trp} was 5′-^{32}P-labelled with [γ-^{32}P]ATP and T4 kinase (10^8 c.p.m./μg) and was used at 2×10^6 c.p.m./ml in the hybridization reaction [5 × SSC, 0.5% SDS, 50 μg/ml poly(A)] for 12 h at 58°C. 30–70S RNA PrC-1 (**1**) or PrC (**3**), 4–10S RNA PrC-1 (**2**) or PrC (**4**). With control cells no RSV RNA nor tRNA^{Trp} is detected in pellets (**5** and **6**).

tions (see Materials and methods) and it is inhibited at 0.3 M NaCl (not shown). Moreover the RNA concentration used (20–50 ng/μl) is at least 10-fold less than in the virions assuming a radius of 30 nm for the core and a diploid genome of 6000 kd.

The function of the small finger proteins can be understood if both the nucleic acid unwinding property and the *cis*-acting sequences of the target RNAs are taken into account. RSV leader (or MuLV leader) and replication primer tRNA are both highly structured at 37°C (Panet *et al.*, 1975; Araya and Litvak, 1979; Fournier *et al.*, 1976; Darlix *et al.*, 1986) and hybridization seems unlikely without destruction of the secondary structures. Unwinding of these RNA structures, which promotes the base pairing interactions between complementary sequences (e.g. 3′ primer tRNA and PBS of genomic RNA), is performed by p12 (or p10) (Figures 2 and 4). In the presence of reverse transcriptase in addition to p12, tRNA^{Trp} binds more efficiently to PBS. This is to be expected since reverse transcriptase is able to partially unwind the acceptor stem of tRNA^{Trp} (Panet *et al.*, 1975; Araya and Litvak, 1979). Cooperation between reverse transcriptase and p12 is probably not restricted to interactions with primer tRNA and viral RNA but may also involve protein–protein interactions since they associate tightly *in vitro* and during reverse transcriptase purification (Moelling *et al.*, 1979). Consequently RSV p12 or MuLV p10 can be viewed as a necessary co-factor of reverse transcriptase in primer tRNA binding to the initiation site for reverse transcription. In addition this nucleic acid annealing activity of RSV p12 and MuLV p10 directs the formation of the diploid genome (RNA dimerization) in the presence of the E sequence. Deletion of E, which contains inverted repeats (Murti *et al.*, 1981; Schinnick *et al.*, 1981; Pugatsch and Stacey, 1983) results in the abolition of RNA dimerization (see Figures 2–4; to be published in detail elsewhere). The viral finger protein may also participate in the 5′→3′ cDNA transfer at the early stage of provirus synthesis. This transfer is achieved by RNase H digestion of the reverse transcribed 5′ RNA template (Darlix *et al.*, 1977) followed by base pairing between the R sequence at the 3′ end of cDNA and its complement at the 3′ end of viral RNA (Varmus and Swanstrom, 1984). RSV p12 and MuLV p10 might accelerate this necessary 5′→3′ transfer because of their annealing

activity (Figure 5). In agreement with this conclusion, full length or high mol. wt viral DNA is synthesized in virions only in the presence of p12 (Boone and Skalka, 1981) whereas RSV cDNA 101 nt accumulates in the absence of p12 (Darlix *et al.*, 1977). Furthermore, recent data show that MuLV proviral DNA synthesis occurs *in vivo* in core particles (Brown *et al.*, 1987).

The active site for annealing activity of RSV p12 and MuLV p10 probably involves the Cys−His finger motif, as deletion of this domain in RSV p12, or a Val−Pro insertion, impairs viral RNA packaging and dimerization as well as initiation of proviral DNA synthesis (Méric and Spahr, 1986; and Figure 6).

The viral finger protein, retrovirus and retroid elements

In conclusion, the specific annealing property of finger protein RSV p12 is essential in the life cycle of the virus for export of a replication competent diploid RSV genome. This conclusion is also valid for MuLV p10 as indicated by our data and independent findings: (i) genetic evidence shows that MuLV reverse transcriptase is not involved in the annealing of $tRNA^{Pro}$ to genomic RNA (Levin and Seidman, 1981), (ii) packaging of primer tRNA is independent of genomic RNA encapsidation (Levin and Seidman, 1979), and (iii) the drug actinomycin D, which intercalates at GC pairs and results in helix unwinding, blocks genomic RNA encapsidation *in vivo* (Levin and Rosenak, 1976) and retroviral RNA dimerization *in vitro* (J.L.Darlix, unpublished results).

More generally the central role of the small viral finger protein in the life cycle of RSV or MuLV may well apply to all retroviruses, retroid elements and the plant virus CaMV, since all code for a finger protein analogous to RSV p12 or MuLV p10 and have elements similar to the RSV PBS, R and replication primer tRNA (Covey, 1986; Darlix, 1986b; Grimsley *et al.*, 1986; Mietz *et al.*, 1987). Finally, it is remarkable that initiation and control of DNA replication appear to be well conserved from plasmids to retroviruses with respect to the origin of replication, primer RNA and a protein having RNA annealing activity (Cesareni *et al.*, 1982).

Materials and methods

Plasmid constructions

Standard procedures were used for restriction digestion and plasmid construction (Maniatis *et al.*, 1982). *E.coli* 1035 (rec A^-) was used for plasmid manipulation and preparation. RSV-PrB *td* provirus cloned in λ-EMBL3 was partially digested with *Eco*RI and subcloned in pSP64. The 5′ leader was manipulated using *Sac*I and *Bal*31S (slow) in order to remove the 5′ 255 nt (*Sac*I) or 360 nt of the viral genome (Darlix, 1986a). cDNA v-*src* (Chang and Stoltzfus, 1985) was digested with *Eco*RI and subcloned in pSP65 DNA.

MMuLV cloned in pBR322 (pMLV-k) (Miller and Verma, 1984) was digested with *Sac*I, subcloned in pSP65 and further manipulated at the *Aat*II site (position 367) and the *Pst*I site (position 567).

Sequences were verified by means of dideoxynucleotide sequencing of plasmid DNA using AMV DNA polymerase or sequenase and oligonucleotide primers synthesisized using an Applied Biosystems machine. Plasmid DNAs were isolated from *E.coli* 1035 by the alkaline lysis method followed by RNase treatment and two phenol−chloroform extractions (Maniatis *et al.*, 1982).

Proteins and enzymes

RSV p12 and p27 were purified by acid−urea gel electrophoresis and recovered by electro-elution in 20 mM sodium acetate, precipitated and stored in 10 mM Tris−HCl pH 7, 10 mM DTT, 100 mM NaCl and 40% glycerol. RSV p12 was also purified by HPLC according to Henderson *et al.* (1981) and kindly provided by P.F.Spahr (Geneva).

MMuLV p12, p15 and p30 were purified by acid−urea gel electrophoresis and p10 by DNA cellulose chromatography according to Moelling *et al.* (1979).

T4 gene 32 protein (T4Gp32), *E.coli* Rec A protein and highly purified AMV DNA polymerase were gifts of D.Caput (ELF-Biorecherche, Toulouse), R.Devoret (Paris) and M.Gazeau (Genofit SA, Geneva), respectively. According to SDS−PAGE analysis the respective proteins appeared to be pure.

SP6 RNAs, tRNAs and RSV RNA

pSP65 carrying RSV, v-*src* or MMuLV was linearized at the *Xho*I (position 640), *Eco*RI (position 2316) or *Hin*dIII (position 2737) sites for RSV RNAs, at the *Pvu*II site (position 7800), 671 nt 3′ to AUG−v-*src*) (Schwartz *et al.*, 1983) for v-*src* and *Bst*EII (position 725) or at the *Xho*I site (position 1530) (Schinnick *et al.*, 1981) for MMuLV. The linearized plasmid DNA (2−5 μg) was transcribed by the SP6 RNA polymerase in 100 μl final volume and, following DNase treatment, the viral RNA preparation was extracted twice with phenol−chloroform, purified by Sephadex G75 chromatography, precipitated with ethanol and dissolved in sterile double distilled H_2O.

Beef liver $tRNA^{Trp}$ and $tRNA^{Pro}$ were purified as described (Fournier *et al.*, 1976), 5′-^{32}P-labelled using T4 kinase and [^{32}P]ATP, and repurified by 6% polyacrylamide−urea electrophoresis. Polynucleotides of 19, 28 and 36 nt (1 μg) were 5′-^{32}P-labelled according to this protocol.

Genomic 60S and 30S RNA was prepared from RSV or MuLV virions as already described (Darlix *et al.*, 1977).

Cell culture

NIH-3T3 and chicken embryo fibroblasts (CEF) prepared from Valo eggs were grown in Dulbecco's modified Eagle's medium supplemented with 2−5% fetal calf serum (Boehringer) at 37°C in the presence of 5% CO_2.

Transfection of chicken embryo fibroblasts

Cells, either freshly prepared or frozen in the presence of glycerol, were used for transfection. DEAE−dextran mediated transfection was done using the infectious PrC proviral clone, the non-infectious PrC-1 proviral clone (Méric and Spahr, 1986) and an infectious PrB *td* proviral clone (E.Bieth and J.L.Darlix, unpublished results). For transient assays, medium was collected every 12 h for 2 days (the day after transfection) and clarified by centrifugation (15 000 *g*). Virions were purified by centrifugation (30 000 r.p.m. for 1 h) and the amount of viral proteins p27 and p12 quantitated by immunoblotting following SDS−PAGE (Burnette, 1981) using rabbit anti-p27 and anti-p12 sera (Méric *et al.*, 1984).

p10/p12 assay

This was done using 10 μl of 50 mM Tris−HCl pH 7.1, 1 mM $MnCl_2$, 0.01 mM $ZnCl_2$, 5 mM DTT, 50 mM NaCl or in conditions of reverse transcription (40 mM Tris−HCl pH 8.3, 60 mM NaCl, 6 mM $MgCl_2$, 5 mM DTT) at 37−40°C for 15−30 min. The reaction was stopped by addition of 10% phenol, 1% SDS, 10 mM EDTA and 1% β-mercaptoethanol (final concentrations). RNA was analysed by 0.8% agarose gel electrophoresis in 50 mM Tris−borate, pH 8.3, 1 mM EDTA at 7 V/cm. After electrophoresis the agarose gel was washed in H_2O (2 × 20 min) and RNA was visualized by ethidium bromide staining (1 μg/ml). In order to detect [^{32}P]tRNA the agarose gel was fixed in 5% TCA, dried and autoradiographed.

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References

Araya,A. and Litvak,S. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 631−637.

Araya,A., Sarih,L. and Litvak,S. (1979) *Nucleic Acids Res.*, **6**, 3831–3843.
Bender,W., Chien,H., Chattopadhyay,S., Vogt,P.K., Gardner,M.B. and Davison,N. (1978) *J. Virol.*, **35**, 888–896.
Berg,J.M. (1986) *Science*, **232**, 485–487.
Boone,L.R. and Skalka,A.M. (1981) *J. Virol.*, **37**, 109–116.
Boulay,J.L., Dennefeld,C. and Alberga,A. (1987) *Nature*, **330**, 395–398.
Brown,P.O., Bowerman,B., Varmus,H.E. and Bishop,J.M. (1987) *Cell*, **49**, 347–356.
Burnette,W.J. (1981) *Anal. Biochem.*, **112**, 195–203.
Cesareni,G., Muesing,M.A. and Polisky,B. (1982) *Proc. Natl. Acad. Sci. USA.*, **79**, 6313–6317.
Chang,L.J. and Stoltzfus,M. (1985) *J. Virol.*, **53**, 969–972.
Coffin,J. (1984) In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), *RNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 1, pp. 281–368.
Covey,S.N. (1986) *Nucleic Acids Res.*, **14**, 623–633.
Darlix,J.L. (1986a) *J. Mol. Biol.*, **189**, 421–434.
Darlix,J.L. (1986b) *Biochimie*, **68**, 941–949.
Darlix,J.L. and Spahr,P.F. (1982) *J. Mol. Biol.*, **160**, 147–161.
Darlix,J.L., Bromley,P.A. and Spahr,P.F. (1977) *J. Virol.*, **23**, 659–668.
Darlix,J.L., Spahr,P.F., Bromley,P.A. and Jaton,J.C. (1979) *J. Virol.*, **29**, 597–611.
Darlix,J.L., Zuker,M. and Spahr,P.F. (1982) *Nucleic Acids Res.*, **10**, 5183–5196.
Davis,N.L. and Rueckert,R.R. (1972) *J. Virol.*, **10**, 1010–1020.
Fournier,M., Dorizzi,M., Sarger,C. and Labouesse,J. (1976) *Biochimie*, **58**, 1159–1165.
Grimsley,N., Hohn,T. and Hohn,B. (1986) *EMBO J.*, **5**, 641–646.
Henderson,L.E., Copeland,T., Sowder,R.C., Smythers,G.W. and Oroszlan, S. (1981) *J. Biol. Chem.*, **256**, 8400–8406.
Kadonaga,J., Carner,K.R., Masiarz,F.R. and Tjian,R. (1987) *Cell*, **51**, 1079–1080.
Katz,R.A., Terry,R.W. and Skalka,A.M. (1986) *J. Virol.*, **59**, 163–167.
Levin,J.G. and Rosenak,M.J. (1976) *Proc. Natl. Acad. Sci. USA.*, **73**, 1154–1158.
Levin,J.G. and Seidman,J.G. (1978) *J. Virol.*, **29**, 328–335.
Levin,J.G. and Seidman,J.G. (1981) *J. Virol.*, **38**, 403–408.
Lowy,D.R. (1985) In Fields *et al.* (eds), *Virology*. Raven Press, NY, pp. 235–263.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1983) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Méric,C. and Spahr,P.F. (1986) *J. Virol.*, **60**, 450–459.
Méric,C., Darlix,J.L. and Spahr,P.F. (1984) *J. Mol. Biol.*, **173**, 531–538.
Mietz,J.A., Grossman,Z., Meders,K.K. and Kuff,E.L. (1987) *J. Virol.*, **61**, 3020–3029.
Miller,A.D. and Verma,I.M. (1984) *J. Virol.*, **49**, 214–222.
Miller,J., McLachlan,A.D. and Klug,A. (1985) *EMBO J.*, **4**, 1609–1614.
Moelling,K., Sykora,K.W., Dittmar,K.E., Scott,A. and Watson,K.F. (1979) *J. Biol. Chem.*, **254**, 3738–3742.
Moore,R., Dixon,M., Smith,R., Peters,G. and Dickson,C. (1987) *J. Virol.*, **61**, 480–490.
Murti,K.G., Bondurant,M. and Tereba,A. (1981) *J. Virol.*, **37**, 411–419.
Panet,A., Haseltine,W., Baltimore,D., Peters,G., Harada,F. and Dahlberg,J. (1975) *Proc. Natl. Acad. Sci. USA.*, **72**, 2535–2539.
Patel,I. and Bastia,D. (1987) *Cell*, **51**, 455–462.
Pugatsch,T. and Stacey,D.W. (1983) *Virology*, **128**, 505–511.
Schinnick,T.M., Lerner,R.A. and Sutcliffe,J.G. (1981) *Nature*, **293**, 543–548.
Schulein,M., Burnette,W.N. and August,J.T. (1978) *J. Virol.*, **26**, 54–60.
Schwartz,D.E., Tizard,R. and Gilbert,W. (1983) *Cell*, **32**, 853–859.
Smith,B.J. and Bailey,J.M. (1979) *Nucleic Acids Res.*, **7**, 2055–2072.
Sykora,K.W. and Moelling,K. (1981) *J. Gen. Virol.*, **55**, 379–391.
Tomizawa,J.I. and Som,T. (1984) *Cell*, **38**, 871–878.
Van Beveren,C., Coffin,J. and Hughes,S. (1984) In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), *RNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, pp. 559–122.
Varmus,H. and Swanstrom,R. (1984) In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), RNA Tumor Viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 1, pp. 369–512.
Vincent,A. (1986) *Nucleic Acids Res.*, **14**, 4385–4391.
Vincent,A., Colot,H.V. and Rosbash,M. (1984) *J. Mol. Biol.*, **186**, 149–166.

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