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Polypurine Tract Adjacent to the U_3 Region of the Rous Sarcoma Virus Genome Provides a *cis*-Acting Function

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The *v-src* coding region was deleted from cloned Rous sarcoma virus DNA, and the deleted clones were tested for infectivity by transfection. All of the coding region, as well as most of the sequences lying between *v-src* and the unique 3′ region (U_3), could be deleted without affecting viability. However, at least 9 and at most 29 of the nucleotides in the purine-rich tract adjacent to U_3 were necessary for growth, even in the presence of a helper virus. It is concluded that these nucleotides (the polypurine tract) provide a *cis*-acting function necessary for retrovirus replication.

Retrovirus mutants that may be complemented by a helper virus have been studied well (21). Little, however, is known of *cis*-acting mutations, primarily because of their lethality even in the presence of a helper virus. The mutant SE21Q1b, which is defective in packaging virion RNA, is the only *cis*-acting mutant that has been studied by classical techniques (12, 17).

DNA cloning and in vitro mutagenesis permit precise genomic sequence alterations, the products of which may be assayed by transfection of cloned DNA. With these techniques, mutants carrying a lethal *cis*-acting defect can be identified by their inability to replicate in the presence of a helper virus.

All retrovirus genomes sequenced to date contain an uninterrupted tract of purines immediately adjacent to the U_3 sequence in viral RNA (8, 18–20; see Fig. 2). There may be as few as 6 purines (spleen necrosis virus) or as many as 19 purines (mouse mammary tumor virus) in this tract. The immediate juxtaposition of this sequence to the point where plus-strand (coding-strand) synthesis is initiated suggests its involvement in plus-strand synthesis (1, 3, 4, 7, 9, 11, 14, 16, 20, 21). If this tract were involved in priming plus-strand DNA synthesis or defining the site of action of an RNase H-type enzyme, mutations in the region should be *cis* acting. This hypothesis was tested by deleting portions of the region adjacent to U_3 in vitro and transfecting the altered DNA, with or without cloned wild-type DNA, into permissive cells. Replication was assessed by measuring reverse transcriptase activity, and viral RNA was analyzed for the presence of the specific deletions.

MATERIALS AND METHODS

Cells. C/O chicken embryo fibroblasts (SPAFAS, Inc.) were grown in Dulbecco modified Eagle medium containing 5% calf serum, 5% fetal calf serum, and 0.3% (wt/vol) tryptose phosphate in the presence of penicillin and streptomycin.

Chemicals and enzymes. DNA polymerase and restriction enzymes were purchased from New England Biolabs; alkaline phosphatase was purchased from Bethesda Research Laboratories, Inc.; T4 polynucleotide kinase was purchased from P-L Biochemicals, Inc.; agarose and S1 nuclease were purchased from Sigma Chemical Co.; and acrylamide was purchased from Bio-Rad Laboratories.

Preparation of cloned retrovirus DNA. The large circular form of DNA from the Schmidt-Ruppin A strain (SR-A) of Rous sarcoma virus, cloned as a permuted linear into λgtWES, was a gift from W. DeLorbe (6). Subsequent manipulations were performed on fragments subcloned into pBR322 or related plasmids. Plasmids were grown in *Escherichia coli* HB101, amplified with chloramphenicol, and purified by RNase and pronase digestions of Triton X-100 lysates. Plasmid DNA to be 5′ end labeled or digested with *Bal* 31 exonuclease was passed over a Bio-Gel A-15M column to remove RNA.

The *v-src* coding region was removed from subclones of SR-A DNA as follows. The 5′-deletion endpoints were created by digesting an *Eco*RI 3.1-kilobase (kb) subclone (Fig. 1A) with either *Acc*I or *Bal*I, creating flush ends with the large fragment of *E. coli* DNA polymerase I, and ligating *Cla*I linkers to the modified restriction sites. The *Acc*I site is 884 base pairs downstream from the *Eco*RI site located in the envelope coding region; the *Bal*I site is 1,090 base pairs downstream from the same *Eco*RI site. All deleted variants are referred to as either 884 or 1090 followed by a second number that refers to the 3′ endpoint of the *v-src* deletion. The 3′ endpoints were generated by ligating a *Cla*I linker either to a *Pvu*II site (Fig. 1A) 273 base pairs upstream from the downstream long terminal repeat (LTR) or to endpoints created by *Bal* 31 exonuclease closer to the downstream LTR. The 3′-deletion endpoints were determined by DNA sequencing (13) and are located 273, 69, 29, or 8 base pairs upstream from the downstream LTR. By digesting the manipulated subclones with

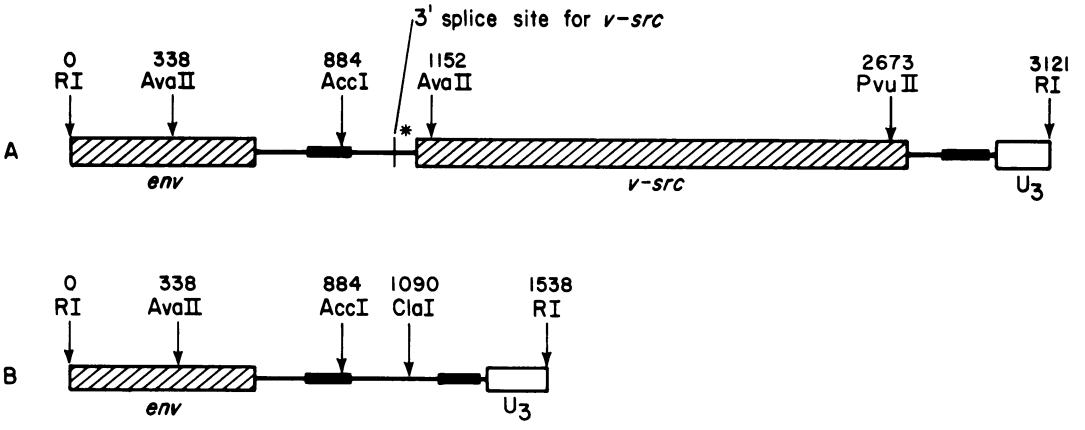

FIG. 1. (A) Schematic representation of the 3.1-kb *Eco*RI fragment containing the *v-src* coding region from an SR-A DNA clone. A terminal part of the envelope coding region is represented by the region labeled *env*. U_3 represents 177 base pairs of the unique 3' sequence in the downstream LTR. Restriction sites are marked above the horizontal; only two of the *Ava*II sites have been included for clarity. The *v-src* coding region was deleted from cloned DNA by using available restriction sites (from the asterisk representing a *Bal*I restriction site to a *Pvu*II restriction site shown); *Bal* 31 exonuclease was used to enlarge the deletion. All deleted clones are referred to by two numbers: the first is the distance from the upstream deletion endpoint to the *Eco*RI site in *env*, and the second is the distance from the downstream deletion endpoint to the beginning of the downstream LTR. The darkened boxes represent directly repeated sequences of 120 base pairs. (B) Analogous *Eco*RI fragment of the deletion 1090/−273. The *v-src* coding region has been replaced by a *Cla*I linker.

*Cla*I and ligating them to each other, the *v-src* coding region was removed and, in effect, replaced with a *Cla*I linker. Viral DNA clone 1090/−29, for example, refers to a *v-src* deletion that has lost the region from the *Bal*I site 1,090 base pairs downstream from the *Eco*RI site in *env* to a *Bal* 31-created site 29 base pairs upstream from the downstream LTR (Fig. 1A).

Transfections. Before transfection, permuted viral DNA was separated from plasmid DNA by digestion with restriction endonuclease *Sal*I, the site into which the permuted viral DNA had been cloned, followed by ligation with T4 DNA ligase at a DNA concentration of 500 μg/ml. The ligation products were purified by phenol extraction and precipitated with ethanol. Approximately 5 μg of viral DNA was transfected into chicken embryo fibroblasts 30% confluent on a 10-cm plate by using modifications (24) of the technique of Graham and van der Eb (10). At 4 h after the transfection, culture medium was removed, and the cells were exposed to 2 ml of 15% glycerol (vol/vol) in the culture medium for 2 min and then placed in fresh culture medium. Cells were split as necessary, and 10 to 12 days later, 10 ml of culture medium was assayed for reverse transcriptase activity (15) with a polyribocytidylate template, an oligodeoxyguanidylate primer, and [α-^{32}P]dGTP at a concentration of 10 μM and a specific activity of 1 Ci/mmol.

Isolation of RNA. Total cellular RNA was prepared by washing confluent 10-cm plates twice with cold phosphate-buffered saline (150 mM NaCl, 4 mM KCl, 12 mM phosphate, pH 7.4) and adding 2 ml of 4 M guanidium thiocyanate in 50 mM Tris-hydrochloride (pH 7.6)–10 mM EDTA–2% Sarkosyl–2% 2-mercaptoethanol directly to the plates. Lysed cells were pooled, sheared through a 22-gauge needle, extracted twice with a 1:1 mixture of phenol-chloroform, and ethanol precipitated twice.

Labeling DNA fragments and DNA sequencing. The procedures of Maxam and Gilbert (13) were followed for end labeling and sequencing DNA fragments. Fragments were 5' end labeled with T4 polynucleotide kinase and 3' end labeled with the large fragment of *E. coli* DNA polymerase I.

S1 analysis. The Weaver and Weissmann (23) modifications of the Berk and Sharp (2) procedure were used for S1 analysis. Briefly, 30 μg of total cellular RNA was coprecipitated in ethanol with 0.25 pmol of end-labeled DNA. The pellet was dissolved in 40 μl of 80% formamide in standard hybridization buffer (2), heated to 68°C for 10 min, and then incubated at 48°C for 3 h. Next, 360 μl of cold S1 buffer (2) and 200 U of S1 nuclease were added, and the mixture was incubated at 28°C for 90 min. Then, 25 μl of 1 M Tris-chloride (pH 7.5) was added, the solution was extracted with a 1:1 phenol-chloroform mixture, and the aqueous phase was precipitated with ethanol. The pellet was suspended in 8 μl of 95% formamide loading buffer, heated to 90°C for 2 min, chilled, and then analyzed on a 0.35-mm-thick acrylamide-urea gel.

RESULTS

The *v-src* coding region was removed from cloned SR-A DNA and replaced by a *Cla*I site (Fig. 1A shows the *Eco*RI fragment of SR-A DNA that was manipulated). The prototype mutant (Fig. 1B) lost the *v-src* coding region but retained both copies of the 120-base-pair direct repeat flanking *v-src*, the *v-src* 3'-splice site, and all of the *gag*, *pol*, and *env* coding regions. The

TABLE 1. Results of reverse transcriptase assays of culture medium 2 weeks after transfection of viral DNA

Transfected DNA[a]	Reverse transcriptase[b]	Cell morphology
pBR322	−	Normal
SR-A	+	Transformed
1090/−273	+	Normal
884/−273	+	Normal
1090/−69	+	Normal
884/−69	+	Normal
1090/−29	+	Normal
884/−29	+	Normal
1090/−8	−	Normal
884/−8	−	Normal
SR-A + any variant	+	Transformed

[a] See text for description of deletions.
[b] +, $> 5 \times 10^5$ cpm; −, $<1 \times 10^4$ cpm.

mutant was cloned as a permuted linear, with the two LTRs in tandem, into the *Sal*I site of a variant of pBR322 missing the *Cla*I and *Hin*dIII recognition sites. Transfection of this DNA after digestion with *Sal*I and ligation produced a nontransforming, helper-independent virus similar to the avian leukosis viruses. Viral titers were equivalent to those of Rous sarcoma virus-infected cells.

The 3′ boundary of the *v-src* deletion was extended using *Bal* 31 exonuclease, yielding mutants that had lost portions of the downstream 120-nucleotide direct repeat. These mutants produced virus in as high a titer as the wild type (Table 1). However, when the 3′ deletion was extended to the polypurine tract adjacent to the downstream LTR, leaving only eight nucleotides of the tract, no detectable virus was produced after transfection (Table 1). Figure 2 shows both the nucleotide sequence adjacent to the downstream LTR and the endpoints of three

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ATCGAT(-69)
  ↓
  GGGCTTCGGT TGTACGCGGT TAGGAGTCCC CTCAGGATAT
  -70        -60        -50        -40

ATCGAT(-29)              ATCGAT(-8)  ┌──────┐
  ↓                        ↓         │ U3   │
  AGTAGTTTCG CTTTTGCATA GGGAGGGGGA  │AATGTAGTCT
  -30        -20        -10         └──────┘
```

FIG. 2. Nucleotide sequence adjacent to the downstream LTR (marked U_3). Arrows represent the downstream limits of several deletions involving the *v-src* coding region and most of the downstream 120-base-pair repeat. The sequence ATCGAT, the *Cla*I recognition sequence, joins these downstream deletion endpoints to endpoints upstream of the *v-src* coding region (Fig. 1). The numbers in parentheses above the ATCGAT sequences refer to nucleotide positions from the beginning of the downstream LTR. The downstream 120-base-pair direct repeat ends nine nucleotides upstream from the beginning of U_3 (position −9).

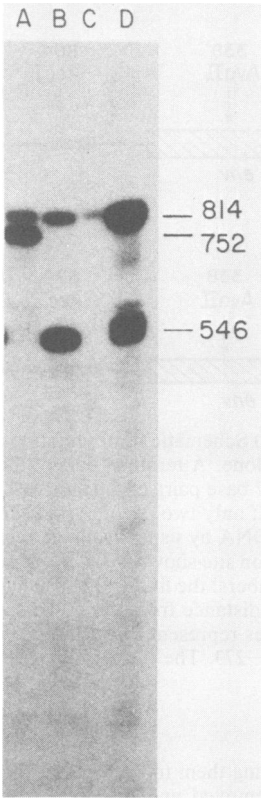

FIG. 3. Acrylamide (7.2%)–urea gel of S1 digestion products after hybridization of an end-labeled double-stranded DNA probe to total RNA from infected cells. The DNA probe was made by 3′ end labeling the 814-base-pair *Ava*II fragment shown in Fig. 1A. Bands represent the distance from the *Ava*II site in *env* to downstream discrepancies in homology between RNA and DNA. S1 digestion products are shown after hybridization with: (A) RNA from cells cotransfected with SR-A and 1090/−69 DNAs; (B) RNA from cells cotransfected with SR-A and 884/−69 DNAs; (C) uninfected chicken fibroblast RNA; (D) RNA from cells infected with SR-A that had been passaged several times after transfection.

deletion mutants in that region. Only the mutant at −8 was not viable.

To verify that the viral RNA produced from transfected cells was the complement of the DNA transfected into the cells, viral RNA was analyzed using S1 nuclease and cloned DNA probes. The 814-base-pair-long *Ava*II fragment from SR-A DNA was 3′ end labeled and purified (Fig. 1A). The DNA strand labeled at position 338 can hybridize to viral RNA and be protected from S1 nuclease to the point where the RNA and DNA sequences diverge. Wild-type RNA should protect the entire fragment, whereas the deleted viral RNAs should afford protection only to the 5′ extent of the deletions (i.e.,

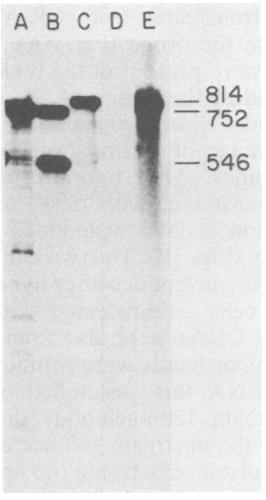

FIG. 4. Acrylamide (7.2%) gel of S1 digestion products after hybridization of the same 3'-end-labeled *Ava*II DNA probe used in experiments described in the legend to Fig. 3 to: (A) RNA from cells transfected with 1090/−29 DNA; (B) RNA from cells cotransfected with 1090/−29 and 884/−29 DNAs; (C) RNA from cells cotransfected with SR-A and 1090/−8 DNAs; (D) RNA from cells transfected with 1090/−8 DNA. (E) Undigested DNA probe.

position 884 or 1090 in Fig. 1A). The S1 fragments, resulting from hybridization to RNA from cells cotransfected 3 weeks earlier with both SR-A (wild-type) and 1090/−69 DNAs, are shown in Fig. 3A. The 814-nucleotide (full-length) band was the result of protection by SR-A RNA, and the 752-nucleotide band was the result of protection by 1090/−69 RNA (1,090 − 338 = 752). Since the DNA probe is in molar excess during hybridization, the band intensities roughly correlate with the relative concentrations of RNA species. There was a similar result after cotransfection of SR-A and 884/−69 DNAs (Fig. 3B). The full-length band resulted from SR-A RNA, and the 546-nucleotide band resulted from 884/−69 RNA. After hybridization of probe to SR-A RNA, known to contain a high percentage of naturally occurring transformation-defective variants, S1 digestion yielded a new band at 546 nucleotides (Fig. 3D) mapping within the 120-base-pair direct repeat upstream of *v-src* (Fig. 1A and 3D).

The same 3'-end-labeled *Ava*II fragment was hybridized to RNA from cells transfected with 1090/−29 DNA alone (Fig. 4A). As expected, the full-length fragment was not protected, but the 752-nucleotide fragment was present. When 1090/−29 and 884/−29 DNAs were cotransfected, the resulting viral RNA protected the DNA probe to positions 752 and 546 in roughly equal proportions (Fig. 4B). When 1090/−8 was cotransfected with SR-A DNA, the cells became morphologically transformed and the viral RNA protected the full-length fragment, but no band was seen at 752 nucleotides, suggesting that the 1090/−8 virus did not grow in the presence of a helper virus (Fig. 4C). The faint bands at position 546 in Fig. 4A and C probably represent deleted genomes similar to those detected in Fig. 3D. RNA from cells transfected with 1090/−8 DNA alone gave no protection (Fig. 4D).

Similar mapping data were collected using a 5'-end-labeled *Eco*RI 3.1-kb fragment shown in Fig. 1A. The DNA strand labeled at position 3121 could hybridize to viral RNA and be pro-

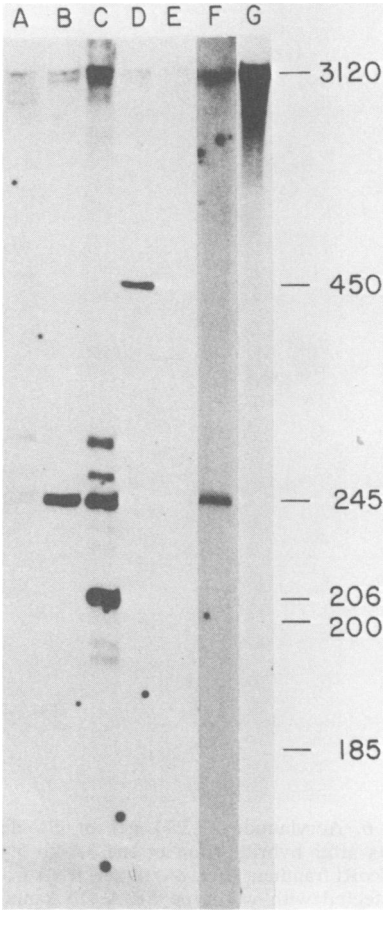

FIG. 5. Acrylamide (7.2%) gel of S1 digestion products after hybridization of the 3.1-kb 5'-end-labeled *Eco*RI fragment (Fig. 1A) to: (A) RNA from cells transfected with SR-A DNA; (B) RNA from cells transfected with 1090/−69 DNA; (C) RNA from cells cotransfected with 1090/−69 and 884/−29 DNAs; (D) RNA from cells transfected with 884/−273 DNA; (E) RNA from cells cotransfected with SR-A and 1090/−8 DNAs; (F) RNA from cells cotransfected with SR-A and 1090/−69 DNAs. (G) Undigested DNA probe.

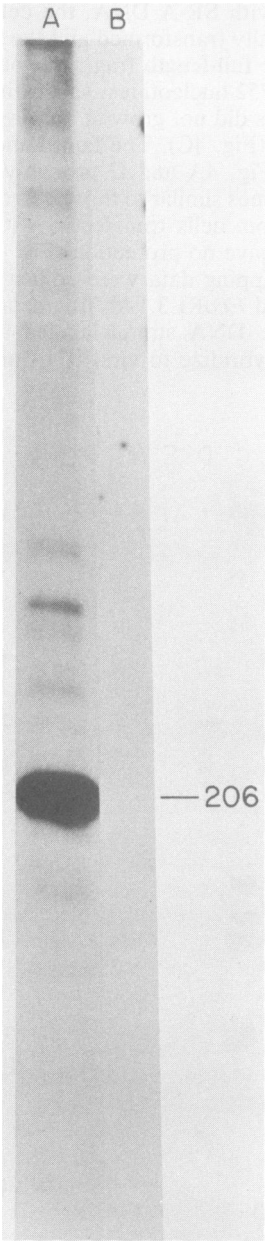

FIG. 6. Acrylamide (7.2%) gel of S1 digestion products after hybridization of the 3.1-kb 5'-end-labeled *Eco*RI fragment (Fig. 1A) to: (A) RNA from cells cotransfected with wild-type SR-A DNA plus 1090/−29 DNA that had been deleted of most of *gag*, *pol*, and *env*; (B) RNA from uninfected cells.

tected from S1 nuclease to the position where the RNA and DNA sequences diverged. The *Eco*RI site in U_3 lies 177 base pairs downstream from the start of the LTR. Therefore, S1-digested hybrids from the deleted variants will generate fragments larger than 177 nucleotides. RNA from cells transfected with SR-A DNA was hybridized to the probe (Fig. 5A). A few very faint bands were present in the region of 200 to 300 nucleotides, the region of the downstream 120-nucleotide direct repeat, representing a small percentage of natural transformation-defective genomes. After hybridization to RNA from cells transfected with 1090/−69 DNA, the expected band at 246 nucleotides (69 + 177) could be seen (Fig. 5B). The two expected bands at 206 and 246 nucleotides after hybridization to RNA from cells cotransfected with 1090/−69 and 884/−29 DNAs were also found (Fig. 5C). The other minor bands were artifacts produced by 884/−29 RNA; this variant had lost *v-src* and the downstream 120-nucleotide direct repeat, but retained the upstream 120-nucleotide direct repeat. The distance between the upstream 120-nucleotide direct repeat and the downstream LTR in 884/−29 was almost the same as the distance between the downstream 120 nucleotide direct repeat and the downstream LTR in the wild-type genome. Presumably, the 120-nucleotide direct repeat in 884/−29 RNA partially protects the region of the downstream 120-nucleotide direct repeat in the DNA probe, thus generating the artifactual bands at approximately 260 and 280 nucleotides (Fig. 5C). These extra bands represent regions where the upstream and downstream direct repeats diverged in sequence (they are only 80% homologous). Cotransfection of SR-A DNA with 1090/−69 DNA yielded the expected band at 246 nucleotides, but cotransfection of SR-A DNA with 1090/−8 DNA did not yield any bands in this region, particularly around 185 nucleotides (Fig. 5E and F). This again shows that deleting all but eight nucleotides adjacent to the downstream LTR produced an inviable genome, even if it was transfected in the presence of a helper genome.

Although the inability to detect bands representing the −8 mutant suggests that this genome is *cis* defective, the assay must be shown to detect *trans*-defective deletions. Therefore, the 1090/−29 variant was further deleted for most of its *gag*, *pol*, and *env* coding regions. The DNA was cotransfected with wild-type DNA, and viral RNA was analyzed using the *Eco*RI 5'-end-labeled probe (same as Fig. 5). An intense band at 206 nucleotides was observed as expected (Fig. 6). Wild-type RNA alone did not produce this band (Fig. 5A). This verified that a *trans*-defective virus genome, or recombinants thereof, could be detected easily with this technique, thus further strengthening the proposal that the −8 deletion is a lethal *cis*-acting mutation.

DISCUSSION

As shown here, the entire *v-src* coding region can be removed from Rous sarcoma virus DNA

without affecting virus production after transfection. Moreover, most of the region between *v-src* and U_3 is dispensable, the only requirement being retention of at most 29 of the nucleotides adjacent to U_3 (the polypurine tract). The function of these nucleotides is unknown, but it is shown here that they are *cis* acting.

During retrovirus replication, there are many steps that may rely at least partially on *cis*-acting sequences, such as transcriptional promotion, transcriptional termination, RNA splicing, RNA encapsidation, initiation of minus-strand DNA synthesis, the two 5′-to-3′ "leaps," initiation of plus-strand DNA synthesis, and provirus integration. Although involvement of the polypurine tract in any one of these steps is theoretically possible, in all likelihood the polypurine tract is involved in the initiation of plus-strand DNA synthesis. The plus-strand fragment observed earliest during DNA synthesis in vivo and in vitro is the size of an LTR, and it hybridizes to LTR-encoded fragments (1, 4, 5, 7, 9, 11, 14, 16, 20, 21). It has been referred to as plus-strand strong-stop DNA (14). The 5′ end of plus-strand strong-stop DNA lies in the region where the polypurine tract is joined to U_3.

Although plus-strand strong-stop DNA is the most prominent plus-strand fragment observed during retrovirus DNA synthesis, in the avian system a heterogeneous collection of fragments also appears (4, 5, 11, 21). Therefore, the polypurine-U_3 junction is not the only site of plus-strand initiation. If other sites for plus-strand initiation are available, then why should deletion of the polypurine tract be lethal? According to some models for retrovirus DNA synthesis, the 5′ end of plus-strand strong-stop DNA ultimately becomes the 5′ end of the full-length DNA plus strand (1, 7, 9, 16). This probably results from displacement of the 5′ portion of plus-strand strong-stop DNA followed by extension of the minus strand (5, 7, 16), thereby creating the upstream LTR. Therefore, the 5′ terminus of plus-strand strong-stop DNA apparently defines the upstream limit of both LTRs. Incorrect initiation of plus-strand strong-stop DNA could create aberrant LTRs, a lethal defect that a helper virus would not complement.

The −8 derivatives contain 10 of the 13 purines of the polypurine tract (Fig. 2). Spleen necrosis virus has a tract of only six purines adjacent to U_3, yet it grows very well (18). Since the −8 derivatives were lethal, it appears that merely a stretch of purines is not sufficient in this region. Although the functions of the polypurine tract and its associated upstream sequences are unknown, it seems likely that this region "signals" an enzyme to cleave the viral RNA precisely at this point. Perhaps the RNase H activity of reverse transcriptase could create such a break. The free 3′ end of the RNA could then act as a primer for DNA synthesis.

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