Characterization of (+) Strand Initiation and Termination Sequences Located at the Center of the Equine Infectious Anemia Virus Genome†

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ABSTRACT: Permeabilized preparations of equine infectious anemia virus (EIAV) are shown here to support efficient and accurate synthesis of full-length double-stranded proviral DNA. When (−) and (+) strand products were analyzed by Southern blotting, a discontinuity, mapping approximately to the center of the EIAV genome, could be demonstrated for the (+) strand, predicting a second site for initiation of DNA synthesis and a specific mechanism of (+) strand termination. Precise localization of this (+) strand origin within the integrase (IN) coding region was achieved through its in vitro selection and extension into, and excision from, nascent DNA by purified recombinant p66/p51 EIAV reverse transcriptase (RT), suggesting that the EIAV genome harbors a central polypurine tract (cPPT). In addition, a model system was developed for evaluating whether sequences immediately downstream of the cPPT would terminate (+) strand synthesis in the context of strand displacement. Such a sequence was indeed discovered which functions in a manner analogous to that of the central termination sequence (CTS) of HIV, where A-tract-induced minor groove compression has been suggested to induce localized distortion of the nucleic acid duplex and termination of (+) strand synthesis. This interpretation is reinforced by experiments indicating that read-through of the CTS can be efficiently promoted by substituting 2,6-diaminopurine for adenine, thereby relieving minor groove compression. The nucleotide substitution can also shift the site of termination in strand displacement (+) strand synthesis. Collectively, our data support proposals that lentiviruses may have evolved specialized mechanisms for initiating and terminating (+) strand DNA synthesis at the center of their genomes.

Plus (+) strand synthesis in retroviruses requires selection of, and initiation from, the polypurine tract (PPT)[1] RNA primer, a short (13−16 nucleotides) purine-rich region located near the 3′ end of the (+) RNA genome generated through resistance of this RNA−DNA replication intermediate to ribonuclease H (RNase H)-mediated hydrolysis (*1*). After initiation, this primer must be accurately removed from the nascent (+) strand DNA to provide the appropriate 5′ long terminal repeat sequence for recognition by the retroviral integration machinery. A feature common to several lentiviruses, including visna virus (*2*), human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2, respectively) (*3*, *4*), simian foamy virus LK-3 (*5*), and human and simian spumaviruses (*6*), is the presence of a second copy of the PPT at the center of the genome (within the integrase coding region), utilization of which has been implicated from a specific (+) strand discontinuity. Although the replicative advantage derived from an additional (+) strand initiation site (designated the central or cPPT) is not immediately clear, altering this sequence in HIV-1 (while preserving the IN reading frame) has been shown to impair virus replication (*7*, *8*). In contrast, while the replication strategy of the yeast retrotransposon Ty1 also exploits a cPPT, its alteration does not alter transposition frequency (*9*). While attempts to define unifying structural features of the (+) strand PPT−(−) strand DNA hybrid have been made, the retroviral and retrotransposon PPTs vary in both size and sequence, indicating that further experimentation is necessary for fully understanding this highly specialized step in replication and, in the case of HIV, for exploiting its potential as a therapeutic target.

A unique (+) strand discontinuity at the center of HIV-1 proviral DNA also predicts the evolution of an additional mechanism for halting the polymerizing complex in its immediate vicinity as synthesis of the double-stranded proviral DNA nears completion. For HIV-1, Charneau et al.

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[1] Abbreviations: CTS, central termination sequence; EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; IN, integrase; kb, kilobases; *pol*, polymerase; PPT, polypurine tract; RNase H, ribonuclease H; RT, reverse transcriptase.

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(*10*) and Levigne et al. (*11*) have indeed demonstrated that termination is elicited through an element located downstream of the cPPT, which these authors designated the central termination sequence or CTS. Distinguishing features of the CTS are multiple A-tracts, which induce localized distortion of duplex DNA. The phenomenon of DNA bending and its role in mediating important biological events, including regulation of gene expression and chromatin structure, have been demonstrated (*12*−*15*). In HIV, the role of a CTS duplex would be realized when the polymerizing machinery, arriving at the cPPT following the second strand transfer event (*10*), encounters duplex DNA arising from cPPT-primed (+) strand synthesis and moves into the strand displacement mode. At the same time, the CTS is created during cPPT-primed (+) strand synthesis, a stage at which it must not present a barrier to the polymerizing enzyme. The CTS thus represents another unique structure encountered by RT, a multifunctional enzyme which accommodates several conformationally distinct nucleic acid duplexes during retroviral replication (*16*). However, our current understanding of CTS-mediated events is restricted to HIV-1, and its significance would benefit from detailed studies with additional lentiviruses.

Despite their structural similarity and amino acid sequence homology, the RTs of EIAV and HIV exhibit significant differences in their enzymatic properties. This was best demonstrated with p51 EIAV RT, which in contrast to its HIV-1 counterpart associates into a stable homodimer, but catalyzes distributive DNA synthesis (*17*, *18*). More recently, we showed that RNase H-mediated hydrolysis is more stringently controlled by EIAV RT; i.e., while the HIV enzyme cleaves an RNA−DNA hybrid some 17−24 nucleotides behind the DNA primer 3′ OH, the equine enzyme cleaves almost exclusively at position −17 (*19*). Finally, although HIV and EIAV share a common tRNA replication primer ($tRNA^{Lys,3}$), EIAV RT fails to initiate tRNA-primed (−) strand synthesis on the HIV-1 genome, while HIV-1 RT readily supports the equivalent event on the EIAV genome (*20*). On the basis of these findings, we elected to investigate whether (+) strand DNA synthesis in EIAV is mediated through a second cPPT whose sequence is similar to that of the 3′ PPT and, if so, whether cPPT-mediated (+) strand synthesis is subject to CTS-mediated termination. To answer the first of these questions, an endogenous EIAV reverse transcription system was developed which can be performed in the presence of culture medium and without prior virion concentration. Analysis of the products of such reactions with multiple (+) strand DNA probes suggests utilization of a cPPT. Surprisingly, EIAV (+) strand DNA synthesis was sensitive to dNTP concentrations, suggesting these might influence RNase H activity and "clearance" of (+) strand RNA from the RNA−DNA replication intermediate. Endogenous studies were complemented by a combination of in vitro assays with model RNA−DNA hybrids and purified recombinant enzymes, which allowed precise location of the origin of cPPT-primed (+) strand synthesis and evaluation of termination in its immediate vicinity at the EIAV CTS.

MATERIALS AND METHODS

Virus and Cell Culture. Equine dermal cells, chronically infected with the Malmquist strain of EIAV (ATCC VR-778; *21*), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Medium from culture flasks or roller-bottle cultures was harvested daily, clarified by centrifugation at 2600*g* for 15 min, and stored frozen at −70 °C with or without being concentrated by ultracentrifugation as previously described (*22*).

Endogenous RT Reaction. Cell culture medium containing EIAV was adjusted to 50 mM Tris-HCl (pH 8.1), 3 mM $MgCl_2$, 3 mM EGTA, 0.05% NP-40, and each dNTP at 100 μM, unless otherwise specified. The concentration of DMEM was 90%, which contributed 73 mM NaCl, 40 mM $NaHCO_3$, 22 mM HEPES, 1.6 mM $CaCl_2$, 4.9 mM $MgSO_4$, and 22 mM glucose, together with trace metals, amino acids, and vitamins. Permeabilized virions were incubated for 2 h at 39−41 °C, after which EDTA was added to a final concentration of 5 mM. Virions were precipitated by adding 0.5 volume of 30% polyethylene glycol (PEG) (8000 MW) and 0.4 M NaCl and incubating at 4 °C for 2 h. For direct labeling, [α-^{32}P]dCTP was included in the incubation cocktail. The precipitate was collected by centrifugation for 30 min and resuspended to 5−10% of the original medium volume in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.1% SDS. Proteinase K was added to a final concentration of 100 μg/mL, and the reaction mixture was incubated for 1 h at 56 °C. DNA samples were subjected to electrophoresis in alkaline agarose gels (1%), transferred to nitrocellulose membranes (*23*), and analyzed with the ^{32}P-labeled probes described below.

Hybridization Experiments. Products of the endogenous reaction were evaluated with several oligonucleotides (Synthecell) spanning the complete (−) and (+) strands of the EIAV genome (Figure 2A). Oligonucleotides were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase according to standard protocols. Hybridization of these probes to immobilized DNA was performed in a hybridization buffer containing 5× SSC (1× SSC being 0.15 M NaCl and 0.015 M sodium citrate), 10× Denhardt's solution (0.2% ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin), 100 μg/mL herring sperm DNA, 50 mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 0.5% SDS, 5 mM EDTA, 1 mM EGTA, and 50% deionized formamide at 42 °C for several hours. This was followed by overnight hybridization at 42 °C in 6× SSC, 0.1% SDS, 2× Denhardt's solution, and 500 μL of tRNA. Thereafter, membranes were washed with 2× SSC for 30 min, 0.5× SSC, and 0.1% SDS for 20 min, and finally with 0.5× SSC, 0.1% SDS, and 0.1% sodium pyrophosphate for 20 min. All washes were conducted at the hybridization temperature (42 °C). In all cases, membranes were exposed to Kodak X-Omat AR film for autoradiography.

cPPT Selection and Extension. An RNA−DNA hybrid, within which the putative EIAV cPPT was embedded, was used to evaluate (+) strand synthesis at the center of the genome. Plasmid pECP, containing the appropriate sequence of the EIAV genome (J. W. Rausch, unpublished), was transcribed in vitro to generate an ∼120-nucleotide RNA, which was purified by denaturing polyacrylamide gel electrophoresis. A 20-nucleotide DNA primer was hybridized to the 3′ terminus of this RNA and extended with RNase H-deficient HIV-1 RT derivative (*24*) in a buffer of 10 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 80 mM NaCl, 5 mM DTT,

and 200 μM dNTPs for 60 min at 37 °C. DNA synthesis was terminated by extraction with an equal volume of 1:1 phenol/chloroform (v/v), after which nucleic acids in the aqueous phase were precipitated with 3 volumes of 100% ethanol and 0.1 volume of 3 M NaOAc (pH 5.0), and resuspended in nondenaturing gel-loading buffer (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol). The RNA−DNA hybrid was purified from a 10% polyacrylamide gel and quantified spectroscopically. To evaluate (+) strand synthesis, hybrid (50 nM) was incubated with recombinant wild-type EIAV RT (85 nM), 50 μM dNTPs, and [α-^{32}P]dATP in the reaction buffer described above for 60 min at 37 °C. Synthesis was terminated by thermal denaturation at 90 °C for 2 min, after which 50% of each mixture was supplemented with 0.3 volume of 1 N NaOH and incubated at 65 °C for 20 min to remove the (+) strand RNA primer. The remaining portion was stored at 4 °C. Products were precipitated, resuspended in a urea-based gel-loading buffer, and evaluated by high-resolution denaturing gel electrophoresis and autoradiography. For localization of the cPPT initiation site, the appropriate (−) strand DNA fragment of plasmid pEC (a gift from S. L. Payne) was sequenced using the dsDNA Cycle Sequencing System (Gibco BRL). For comparative purposes, equivalent experiments were conducted with the p66/p51 HIV-1 enzyme.

CTS-Mediated Termination of (+) Strand Synthesis. A 400 bp sequence of the EIAV genome, encompassing the cPPT and putative termination signal, was amplified by PCR and cloned into the pCR II vector using a TA cloning kit (Invitrogen). Single-stranded (−) strand DNA was prepared using a biotinylated (+) strand and nonbiotinylated (−) strand primer (*25*). The sequence was amplified by PCR and denatured, after which the biotinylated strand was bound to streptavidin by incubation at room temperature for 5 min. PCR products were fractionated by denaturing polyacrylamide gel electrophoresis and detected by UV shadowing. (+) strand DNA complexed with streptavidin is readily separated from the (−) strand via its altered electrophoretic mobility. This product was excised from the gel and eluted.

A 17-nucleotide DNA copy encompassing the EIAV cPPT (5′-AACAAAGGGAGGGAAAG-3′) was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase according to standard methodologies and annealed to the purified (−) strand template containing sequences complementary to the cPPT and surrounding regions. DNA synthesis was initiated from this primer by adding recombinant p66/p51 EIAV RT to a reaction mixture consisting of annealed template−primer, 200 μM dNTP mix, 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, and 10 mM DTT. The reaction mixture was incubated at 37 °C, and aliquots were removed at times indicated in the text and mixed with an equal volume of urea loading buffer [7 M urea, 0.5% (w/v) xylene cyanol, 0.5% (w/v) bromophenol blue, and 10 mM EDTA]. DNA synthesis products were fractionated by high-voltage gel electrophoresis through 8% (w/v) polyacrylamide/7 M urea gels in TBE buffer. After drying, gels were subjected to autoradiography using a Kodak "Biomax" system and quantified by phosphorimaging analysis (Molecular Dynamics) using Image Quant software provided by the supplier. To evaluate CTS-mediated termination in the context of strand displacement, a 51-nucleotide DNA displacement primer, containing the putative CTS and 14-nucleotide 5′ and 3′ flanking regions, was annealed to the (−) strand template together with the radiolabeled cPPT primer. For experiments incorporating 2,6-diaminopurine triphosphate in place of dATP during cPPT-primed (+) strand synthesis, the nucleotide analogue was substituted in the dNTP cocktail at a final concentration of 10 or 50 μM.

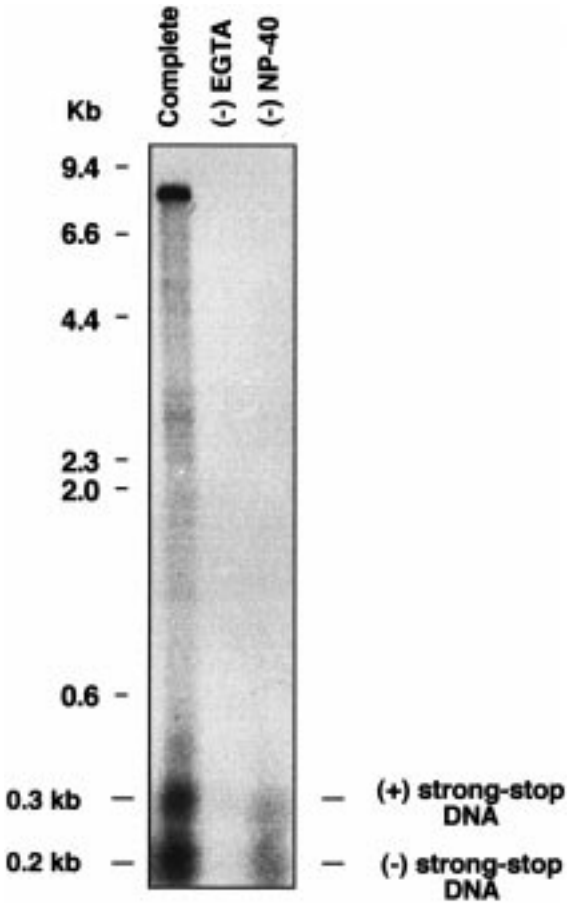


FIGURE 1: Direct labeling of endogenous reverse transcription DNA products. DNA products from endogenous reverse transcription were labeled directly by incorporation of radioactive dCTP. Although the concentration of the virions is not necessary for the endogenous reverse transcription reaction, both EGTA and NP-40 are required for full-length synthesis.

RESULTS

Endogenous EIAV Reaction. Retroviral reverse transcriptase activity (the exogeneous reaction) is most frequently evaluated under conditions where aliquots of cell culture medium are added to a cocktail containing template−primer and components essential for incorporation of labeled dNTP into polydeoxynucleotide. For the endogeneous reaction, however, virus is typically concentrated and resuspended in a cocktail optimized for production of high-molecular weight transcripts. A simplified endogeneous reaction protocol was developed for evaluating reverse transcription directly in cell culture medium to avoid concentration procedures which might potentially damage the synthetic capacity of virions. An analysis of the effect of cell culture medium on RT activity found that this inhibited synthesis of full-length DNA, but that the effect could be reversed by addition of EGTA (*22*).

Analysis of products synthesized under these conditions is presented in Figure 1, and indicates small amounts of

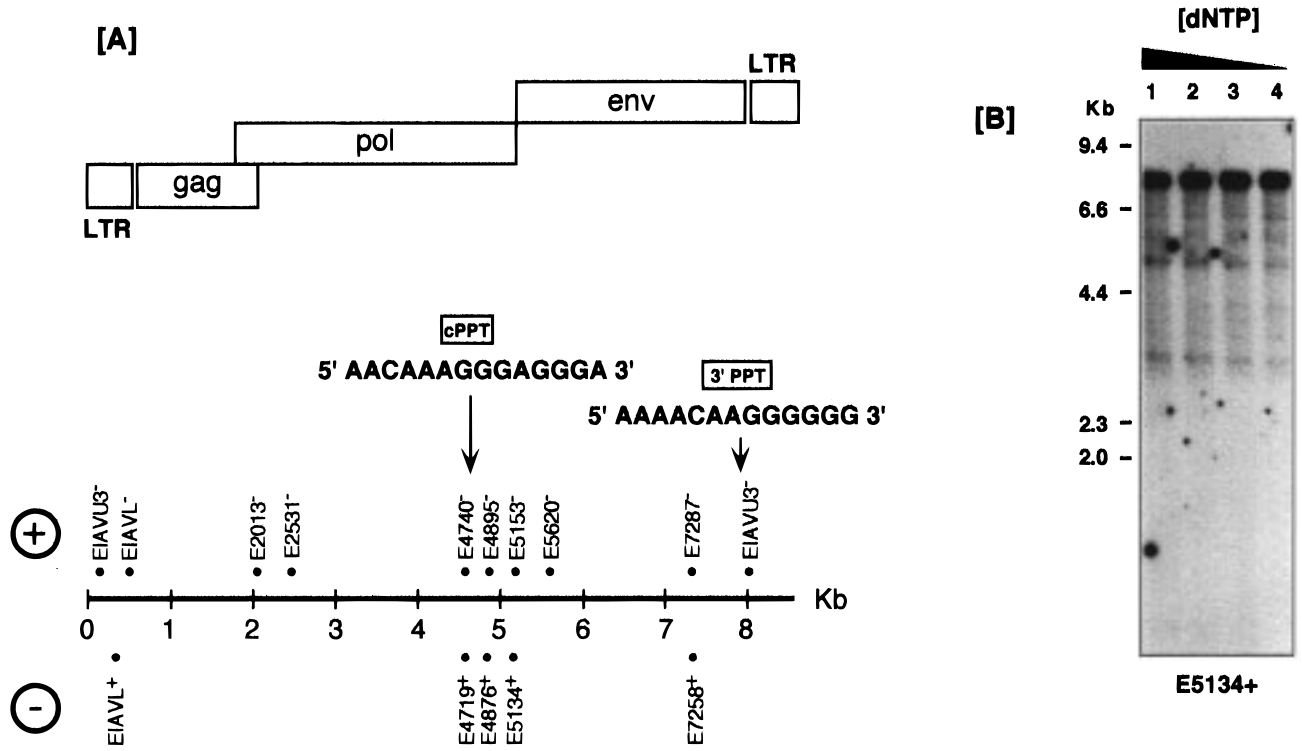


FIGURE 2: Map of oligonucleotide probes for Southern hybridization. DNA products of the endogenous RT reaction were analyzed by Southern hybridization using oligonucleotide probes. (A) Schematic outline of the EIAV genome and location of the probes (22-nucleotide) used to evaluate (−) and (+) strand DNA products relative to EIAV proviral DNA. Notations refer to the position of the 5′ nucleotide of each probe. (B) Endogenous reverse transcription assays as a function of dNTP concentration: lane 1, 100 μM; lane 2, 50 μM; lane 3, 25 μM; and lane 4, 12.5 μM. (−) strand DNA synthesis products were analyzed by Southern hybridization using oligonucleotide probe E5134$^+$, which hybridizes to the 3′ end of the *pol* gene.

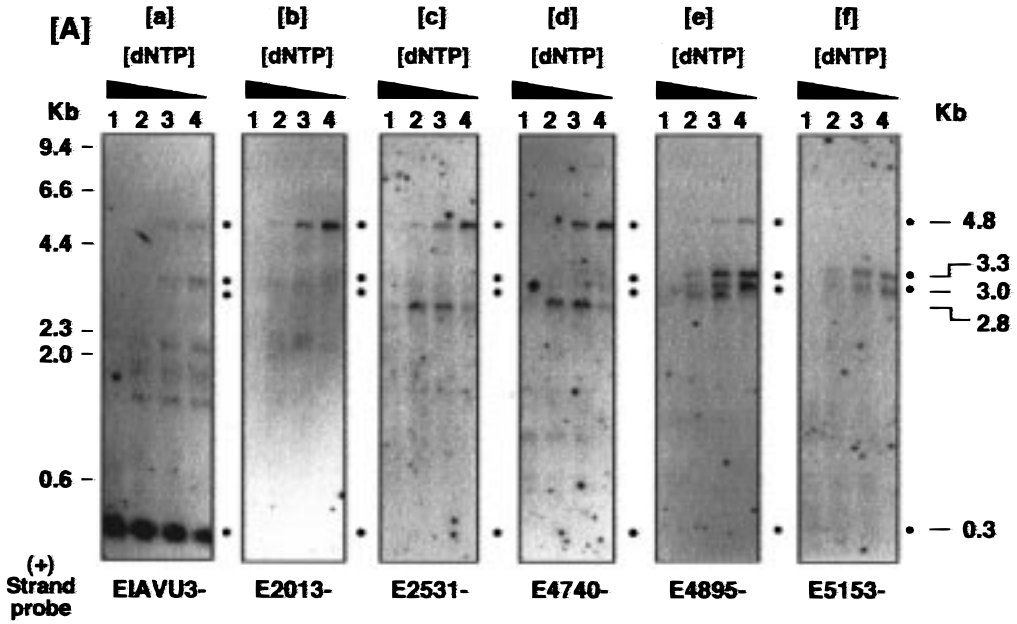
several DNA species between 2.0 and 5.0 kb, in addition to three major products. The latter comprised full-length genomic DNA (~8.0 kb) and two short products whose sizes (0.3 and 0.2 kb) correspond to (+) and (−) strand strong stop DNAs, respectively. Under the conditions used, DNA synthesis was evaluated in a reaction volume of 120 μL, of which the EIAV culture supernatant contributed 100 μL. Synthesis was completely abolished in the absence of EGTA, illustrating its necessity for removing trace elements exerting an inhibitory influence, while small amounts of (+) and (−) strand strong stop DNA were evident when NP-40 was withheld. This latter observation may represent small amounts of disrupted virions in the preparation, since it was somewhat variable. Despite this, the data of Figure 1 indicate the efficiency and ease with which endogenous RT activity can be evaluated with EIAV culture supernatants.

Comparison of (+) and (−) Strand DNA Synthesis. In previous work, it was demonstrated that synthesis of full-length EIAV (−) strand DNA is not absolutely dependent on elevated dNTP concentrations in the assay mixture (*14*, *22*). To further characterize (−) and (+) strand DNA products as a function of dNTP concentration, these were varied from 100 to 12.5 μM, and nascent transcripts identified as (−) or (+) strand DNA via Southern hybridization using oligonucleotide probes complementary to both strands of the DNA provirus (Figure 2A).

Analysis of (−) strand DNA synthesis products indicated no dependency of product formation on dNTP concentration (Figure 2B); i.e., DNA products were made in approximately equivalent proportions at dNTP concentrations ranging from 100 to 12.5 μM. The major product was genome-length (−) strand DNA, and minor species were not characterized further. At 3 μM dNTP, equivalently sized DNA products were synthesized, but the total amount of product decreased slightly (data not shown). In contrast, the dNTP concentration exerted a strong influence on the products of (+) strand DNA synthesis (Figure 3A). At 100 μM, virtually no (+) strand DNA product could be identified, with the exception of the 0.3 kb strong stop product. However, as the dNTP concentration was decreased, accumulation of the largest (+) strand transcripts was observed. Decreasing this to 6.2 μM reduced the total amount of (+) strand DNA that was synthesized (data not shown).

EIAV (+) Strand Synthesis Is Discontinuous. As indicated in Figure 2A, the oligonucleotide probes complementary to the EIAV (+) strand encompass the entire proviral DNA sequence. An analysis of endogenous DNA products using these multiple probes allowed mapping of their origins and an assessment of the DNA products most significantly affected by dNTP concentration (Figure 3A).

In total, five major EIAV (+) strand products were identified using the nine oligonucleotide probes indicated in Figure 2A, with lengths of 4.8, 3.3, 3.0, 2.8, and 0.3 kb. The 0.3 kb product was the major species detected by the EIAVU3- probe, representing (+) strand strong stop DNA, while the origin of minor species around 2.0 kb is uncertain. However, no probe detected a full-length, 8 kb (+) strand product; i.e., the 4.8 kb DNA was the largest (+) strand product observed. This transcript was detected not only by the EIAVU3- probe but also with EIAVL-, E2013-, E2531-, E4740-, and E4895-. Collectively, the hybridization data position the 4.8 kb product at the 5′ end of the EIAV genome,



[B]

Probe / Frag.	EIAV L-	E2013-	E2531-	E4740-	E4895-	E5153-	E5620-	E7287-	EIAVU3-
4.8 Kb	+	+	+	+	+	-	-	-	+
3.3 Kb	-	-	-	-	+	+	+	+	+
3.0 Kb	-	-	-	-	+	+	+	+	-
0.3 Kb	-	-	-	-	-	-	-	-	+

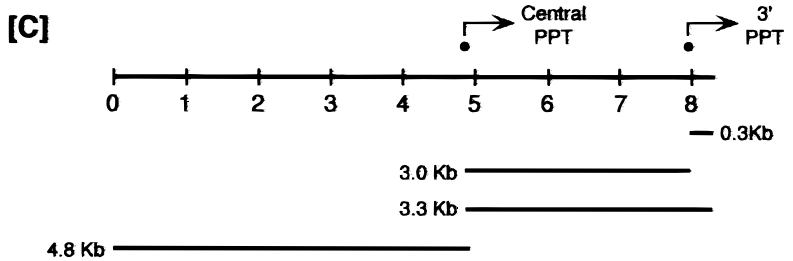


FIGURE 3: Accumulation of EIAV (+) strand products as a function of dNTP concentration. (A) (+) strand DNA products were synthesized by endogenous reverse transcription at dNTP concentrations of 100 (lanes 1), 50 (lanes 2), 25 (lanes 3), and 12.5 μM (lanes 4) and examined by Southern hybridization with the (+) strand oligonucleotide probes indicated in Figure 2B. (B) Hybridization pattern of the major bands shown in panel A. The notation + indicates the presence of a band when products are hybridized with the given probe. In addition to the probes indicated in panel A, data are summarized for oligonucleotides E5620$^-$ and E7287$^-$, which localize to the *env* gene, and EIAVL$^-$, which localizes to the 5′ leader region (see Figure 2B). (C) Four major EIAV (+) strand products (4.8, 3.3, 3.0, and 0.3 kb) and their origins on the EIAV genome. The 0.3 kb fragment represents (+) strand strong stop DNA initiating from the 3′ PPT, while the 3.0 and 3.3 kb products represent products initiated from a presumptive cPPT. Finally, the 4.8 kb DNA represents a strand transfer product whose synthesis initiates at the 3′ PPT and terminates near the center of the EIAV genome.

synthesis of which is initiated at the 3′ PPT. Subsequent relocation of the (+) strand strong stop product to the 5′ end of the EIAV genome following strand transfer allows synthesis of the 4.8 kb DNA to approximately the center of the genome. The 3.3 and 3.0 kb products were detected by E4895-, E5153-, and E7287- probes, while the former was also detected by EIAVU3-. Such results indicate that both products represent the same general region of the 3′ end of the viral genome. However, the 3.0 kb fragment did not include the 3′ PPT since its product was not detected by

EIAVU3-. Taken together with the absence of an intact (+) strand (~8.0 kb) product, the most likely explanation for the probing data of Figure 3A would be a second site for initiation of EIAV (+) strand DNA synthesis at the center of the genome and in keeping with related lentiviruses (*2*−*6*) and the yeast retrotransposon Ty1 (*9*). As indicated in Figure 3C, the 3.0 and 3.3 kb products appear to have initiated from the same site (possibly a central PPT), while the latter represents DNA synthesis through the U3 region by displacement of the (+) strand strong stop product. A 2.8 kb (+) strand product was identified by probes E2531-, E4740-, and E4895-. Unlike other DNA products, this did not initiate from the defined 3′ or the putative central PPT. Initiation occurred within a 500 bp region located between the sequences that hybridized to probes E2013- and E2531-, i.e., at the beginning of the *pol* open reading frame. The 2.8 kb product accumulated at intermediate dNTP concentrations, but was severely reduced as these were lowered, under which conditions the 4.8 kb transcript was most abundant. Since the 2.8 and 4.8 kb species are transcribed from the same region of the genome, the presence of one might be expected to preclude the other.

In Vitro Mapping of the Central PPT. The data of Figure 3 tentatively located a second (+) strand origin near the center of the EIAV genome, which would be consistent with findings from related lentiviruses, including HIV and visna. On the basis of the hybridization patterns of oligonucleotides E4740$^-$ and E4895$^-$ (Figure 3B), this site was located between nucleotides 4700 and 4900 of the EIAV genome. Within this region, a candidate EIAV cPPT sequence 5′-A-A-C-A-A-G-G-G-A-G-G-G-A-A-A-G-3′ was identified in the IN coding region of the *pol* gene [nucleotides 4856−4872 of the proviral DNA sequence of Kawakami et al. (*26*)]. Although the all-purine nature of this putative PPT is interrupted by a single pyrimidine near its 5′ end, Lauermann et al. (*27*) have demonstrated that a pyrimidine-containing PPT of the HIV-2_{ROD} isolate is stably maintained in culture. Punctuation of the PPT by a pyrimidine residue is also evident in the EIAV 3′ PPT (*19*), as well as in caprine arthritis/encephalitis, visna, bovine leukemia, and human T-cell leukemia virus (*28*). To evaluate the central (+) strand initiation site experimentally, an in vitro system was developed which, in a single reaction, examines the ability of recombinant p66/p51 EIAV RT to (i) select the putative cPPT from a hybrid of (+) strand RNA and (−) strand DNA encompassing this region, (ii) extend the cPPT into (+) strand DNA, and (iii) eliminate the RNA primer, as would likely occur during replication. High-resolution denaturing polyacrylamide gel electrophoresis, together with DNA sequencing reactions on the same template, allowed precise location of any (+) strand initiation site. This methodology (*19*) is presented diagrammatically in Figure 4A, involving the initial preparation of the cPPT-containing RNA−DNA hybrid with RNase H-deficient RT (*24*) and subsequently offering the purified hybrid to wild-type p66/p51 EIAV RT in the presence of dNTPs, one of which is radiolabeled. For comparison, a similar PPT selection and/or extension experiment was performed with recombinant p66/p51 HIV-1 RT.

Figure 4B indicates, in the absence of alkaline hydrolysis, two major (+) strand DNAs differing in size by approximately 12 nucleotides as the products of reactions catalyzed by p66/p51 HIV-1 RT and three products with the EIAV enzyme. The larger of these was eliminated following NaOH treatment, mapping the 5′ end of the EIAV (+) strand product immediately downstream of the G-G-G-A-G-G-G-A sequence of the putative cPPT. The HIV-1 enzyme, when required to select a heterologous PPT, initiates DNA synthesis two bases 5′ to that used by EIAV RT. Taken together, these data are in keeping with our recent observations that the RNase H activities of these enzymes have distinguishing characteristics (*19*). For both enzymes, correctly sized (+) strand DNA is also evident in the absence of alkali treatment, indicating they have the capacity to select, extend, and remove the PPT primer in a single reaction. In combination with the Southern analysis summarized in Figure 3, our in vitro data imply a second, central site for initiation of (+) strand synthesis in EIAV. Minor amounts of (+) strand DNA four nucleotides larger than that originating at the 3′ end of the proposed cPPT are evident in the absence and presence of alkali treatment, suggesting either minor heterogeneity in selection of the (+) strand initiation site or incomplete alkali treatment. However, the termination model proposed by Levigne et al. (*11*) indicates that cPPT-initiated (+) strand DNA would be subject to displacement up to the CTS and removed by host enzymes prior to integration. Under such conditions, and if it is assumed that an equivalent termination mechanism exists for EIAV, such heterogeneity may be tolerated without impairing viral replication kinetics.

CTS-Mediated Termination of EIAV (+) Strand Synthesis. The existence of a second site for initiation of (+) strand synthesis near the center of the EIAV genome suggests a need for an efficient termination mechanism for halting the replication machinery which initiated (+) DNA synthesis at the 3′ PPT and arrives at the cPPT following second strand transfer. For HIV-1, a sequence ~100 bp downstream of the cPPT containing multiple A-tracts efficiently halts the replicating enzyme, and was designated the central termination sequence or CTS (*10*, *11*). First reported by Klarmann et al. (*29*) as a strong pausing site, a distinguishing feature of the CTS is its ability, in the context of duplex DNA [which at late stages in replication would reflect displacement synthesis of (+) DNA initiated from the cPPT], to induce minor groove compression and halt progression of the retroviral polymerase. Using this precedent, and on the basis of the hybridization data obtained with oligonucleotides E4895$^-$ and E5153$^-$ (Figure 3B), a related sequence was identified ~100 nucleotides downstream of the EIAV cPPT between nucleotides 4964 and 4985 (Figure 5A). We therefore investigated whether this sequence could induce termination of the EIAV replication machinery in vitro.

The (+) strand DNA synthesis profile from an oligodeoxynucleotide hybridized to the cPPT of a (−) strand DNA template is shown in panel i of Figure 5B. In the absence of strand displacement synthesis, full-length (+) strand product is evident with prolonged incubation. However, even under these conditions, we observed accumulation of nascent DNA approximately 100 nucleotides downstream of the cPPT, i.e., within the putative termination sequence. When the nomenclature of Charneau et al. (*10*) was adopted, the strong and weak termination sites were designated Ter 1 and Ter 2, and their exact locations, extrapolated from a DNA sequencing ladder, are indicated in Figure 5C. It is interesting to note that termination of DNA synthesis at Ter 1 occurs *after* the $(A)_6$ tract has been synthesized, i.e., once the DNA poly-

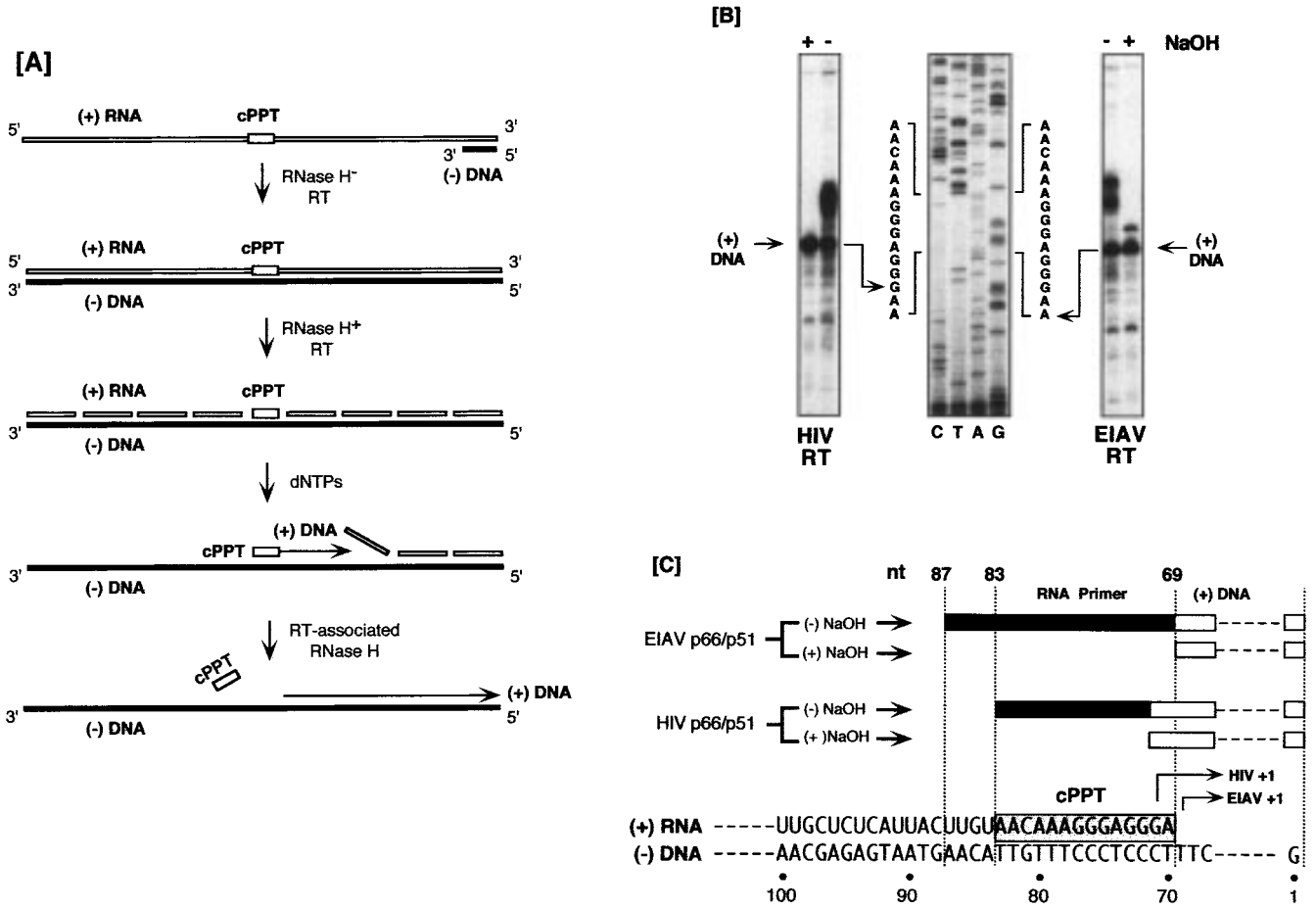


FIGURE 4: In vitro localization of the EIAV cPPT. (A) Schematic representation of the model cPPT localization system. A (−) strand DNA primer is hybridized at the 3′ terminus EIAV (+) strand RNA and extended in the presence of an RNase H-deficient RT (*24*). The resulting (+) strand RNA−(−) strand DNA hybrid is purified by nondenaturing polyacrylamide gel electrophoresis and incubated with wild-type p66/p51 EIAV or HIV-1 RT in the presence of dNTPs, one of which is radiolabeled. If the PPT is selected from the RNA−DNA hybrid, this will be extended into (+) strand DNA. As the replication machinery leaves the PPT, the RNA primer will be removed by RT-associated RNase H activity. Using this system, a 69-nucleotide product represents (+) strand DNA devoid of the PPT. (B) Labeled (+) strand products resulting from the combined selection and extension assay. For comparative studies, the p66/p51 heterodimers of HIV-1 and EIAV RT were evaluated. Following (+) strand synthesis, the reaction mixture was evaluated directly or treated with NaOH to eliminate the RNA primer. A DNA sequencing ladder was used to precisely locate the site of (+) strand initiation. The proposed cPPT and site of (+) strand initiation have been indicated. (C) Summary of the cPPT selection and extension experiment. HIV-1 and EIAV RT cleave the PPT primers differently at both 5′ and 3′ termini as indicated. The putative EIAV cPPT, as selected by the native enzyme, is enclosed within the gray box.

merase catalytic center contains a 6 bp A•T DNA duplex. Although weak, Ter 2 defines a second position within the CTS at which the replication machinery transiently pauses, but is bypassed with prolonged incubation. However, in contrast to the data of Levigne et al. (*11*), Ter 2 is localized to the 3′ end of a $(T)_5$ tract of nascent (+) strand DNA.

In an attempt to duplicate events occurring in vivo as EIAV (+) strand synthesis nears completion, cPPT-primed DNA synthesis was evaluated in the presence of a second, (+) strand oligodeoxynucleotide complementary to (−) strand CTS and flanking sequences. Under these conditions, panel ii of Figure 5B indicates that the CTS-containing duplex induces virtually complete arrest of DNA synthesis at the position defined as Ter 1. P2, depicted in panel ii of Figure 5A, was designed to include 14 nucleotides both 5′ and 3′ to the putative CTS (enclosed within the bars of Figure 5B), which simultaneously evaluates the capacity of EIAV RT for strand displacement synthesis prior to CTS-mediated termination. Since (a) P2 sequences up to and including the A-A-A-A-A-A sequence of the CTS must be displaced prior to arrest of (+) strand synthesis and (b) analysis of DNA synthesis profiles does not reveal enhanced termination as the replication machinery encounters the 5′ terminus of P2, the data of Figure 5B do not reflect an inability to catalyze strand displacement synthesis. Moreover, a second oligonucleotide hybridized immediately after the CTS failed to interrupt DNA synthesis from the cPPT (data not shown). We therefore speculate that, like HIV, EIAV has evolved a mechanism to specifically arrest the replication machinery as the last step of (+) strand synthesis.

Alterations in the Nucleic Acid Geometry Relieve CTS-Mediated Termination. Replacement of adenine with the nucleotide analogue 2,6-diaminopurine (DAP) in duplex DNA has the consequence of abolishing minor groove compression and A-tract-induced curvature, reflecting the increased stability of the double helix through additional Watson−Crick base pairing (*30*−*32*). On the basis of these findings, we elected to investigate whether termination at the CTS might be relieved by substituting the triphosphate of DAP for dATP in our model (+) strand synthesis system, the results of which are presented in Figure 6.

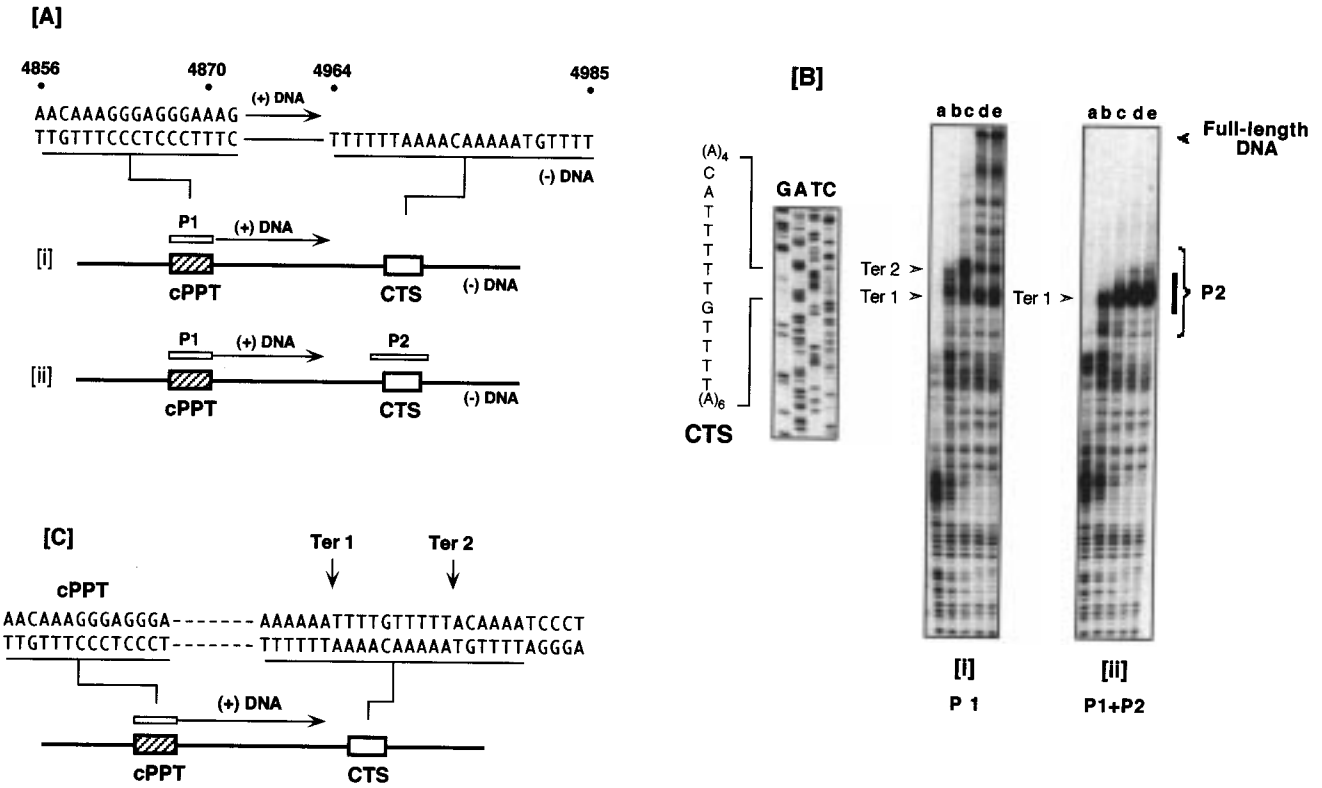


FIGURE 5: Termination of EIAV (+) strand synthesis in the vicinity of the cPPT. (A) Schematic diagram for determining (+) strand sequences in the vicinity of the cPPT inducing pausing and termination of (+) strand synthesis. A labeled (+) strand cPPT-containing DNA primer annealed to EIAV (−) strand DNA (P1) is extended by EIAV RT. Downstream of this, the putative CTS is indicated, to which a second oligonucleotide (P2) was hybridized to evaluate termination of (+) strand synthesis in the context of strand displacement synthesis. Nucleotide numbering is based on the EIAV proviral DNA sequence of Kawakami et al. (*26*). (B) Time course of cPPT-primed strand DNA synthesis. In part i of panel B, (+) strand DNA was synthesized in the absence of displacement synthesis, while in part ii of panel B, the replication machinery that was initiated from the cPPT was evaluated in the presence of a second oligonucleotide hybridized to the proposed CTS. The location of the second oligonucleotide is shown in brackets, within which the position of CTS sequences is indicated. In both cases, (+) strand products were evaluated after 10 s (lanes a), 30 s (lanes b), 1 min (lanes c), 5 min (lanes d), and 10 min (lanes e). Major and secondary termination sites have been designated Ter 1 and Ter 2, respectively. (C) Localization of termination sites within the putative EIAV CTS.

Initially, cPPT-primed (+) strand synthesis with each adenine derivative was evaluated in the absence of strand displacement synthesis (Figure 6A). Visual inspection of each time course indicated equivalent levels of (+) strand product between the cPPT and CTS, indicating to a first approximation that DAPTP and dATP are incorporated with equal efficiency by EIAV RT. As shown earlier, although considerable pausing at the CTS is evident in dATP-supported reactions, product does accumulate. However, the level of pausing is substantially reduced in the presence of DAPTP, with a concomitant increased yield of full-length (+) strand DNA. Surprisingly, when pausing at Ter 1 is relieved, this is accompanied by an increased level of pausing at Ter 2, although this is likewise alleviated with prolonged incubation, presumably made possible through multiple binding events. In Figure 6B, cPPT-primed (+) strand synthesis was evaluated in the presence of a second oligonucleotide complementary to the CTS and flanking sequences to mimic strand displacement synthesis. In the presence of dATP, complete cessation of (+) strand synthesis immediately following completion of the $(A)_6$ tract of the CTS is again evident. In the presence of DAPTP, Ter 1-mediated termination is indeed abolished and full-length DNA is synthesized, although at levels considerably lower than had been anticipated. The reason for this is evident in the shorter autoradiographic exposure of the same experiment, indicating that once Ter 1-mediated termination is relieved, Ter 2 sequences have a dominant influence on pausing. What is at first glance an apparently contradictory situation can be explained by the presence of A-tracts on both the nascent (+) strand DNA and (−) strand template. Initially, the replication machinery synthesizes a (+) strand $(A)_6$ tract complementary to a template $(T)_6$ tract of the CTS, inducing minor groove compression. As predicted, this can be relieved by substituting DAPTP for dATP. However, the situation at Ter 2 is reversed; i.e., the (−) strand template contains an $(A)_5$ tract whose $(T)_5$ complement will be synthesized on the (+) strand. Thus, once Ter 2 is copied, the resulting 5 bp A·T duplex retains the potential to induce minor groove compression and halt the replicating enzyme, but in this case, it is insensitive to DAPTP incorporation. Ter 2 might therefore be considered a cryptic termination sequence whose effect is only realized once Ter 1-mediated termination is eliminated or bypassed. The combined effect of Ter 1 and Ter 2 would therefore ensure complete cessation of 3′ PPT-primed (+) strand synthesis at late stages of EIAV replication following second strand transfer.

DISCUSSION

A recurring feature with retroviruses and retrotransposons is a second site for initiation of (+) strand synthesis near the center of the genome (*2*−*6*). Furthermore, rather than

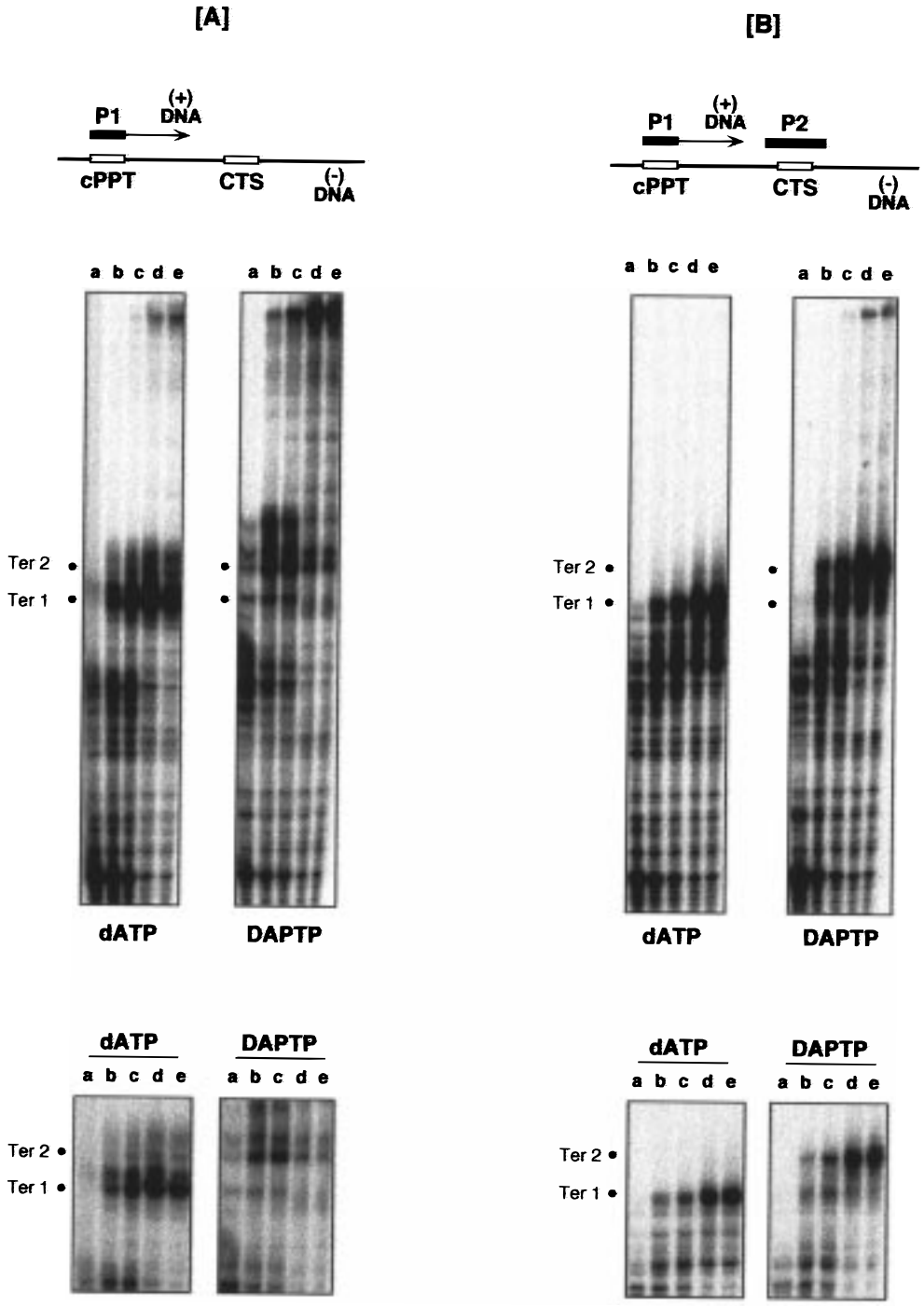


FIGURE 6: Effect of substituting DAPTP for dATP in (+) strand DNA on CTS-mediated termination. cPPT-primed (+) strand synthesis was evaluated from primer P1 in the absence of strand displacement synthesis (A) or in the presence of a second oligonucleotide hybridized to the CTS and flanking sequences (P2) (B). (Top panels) Schematic representation of (+) strand synthesis systems. (Middle panels) Time course of (+) strand synthesis mediated by dNTP cocktails containing either dATP or 2,6-diaminopurine triphosphate (DAPTP). For each time course, DNA synthesis was evaluated after 10 s (lanes a), 30 s (lanes b), 1 min (lanes c), 5 min (lanes d), and 10 min (lanes e). The major termination sites of the CTS have been indicated. (Bottom panels) Limited autoradiographic exposure results from the same experiments illustrating termination events around Ter 1 and Ter 2.

relying on strand displacement synthesis to halt the polymerizing complex arriving upstream of this element, designated the cPPT, HIV-1 appears to have evolved a specific mechanism for efficiently terminating DNA synthesis downstream of the cPPT at late stages of (+) strand synthesis (*10*, *11*). The study described here presents evidence for a (+) strand discontinuity within the EIAV proviral genome, consistent with the notion of a second origin of (+) strand synthesis. To augment endogenous studies, a simple in vitro system utilizing recombinant p66/p51 EIAV RT was developed to localize the second site of (+) strand initiation and follow release of the PPT primer from nascent (+) strand DNA. The EIAV cPPT lies within the IN coding region of the *pol* gene, although the sequence deviates from its 3′ counterpart (+). We also demonstrate in vitro that an A-tract-containing duplex downstream of the cPPT efficiently halts the replication machinery in the context of strand displacement synthesis. Although not shown here, this sequence also

terminates HIV-1 RT-mediated (+) strand synthesis on the EIAV genome with equal efficiency. Our data thus support proposals that structural features of the duplex proviral genome can function as efficient terminators of polymerization (*10*, *11*), and might be considered formally analogous to prokaryotic sequence-dependent transcriptional terminators (*33*).

An unexpected feature of our studies was the sensitivity of (+) strand synthesis products, other than (+) strand strong stop DNA, to elevated dNTP concentrations, while (−) strand synthesis was unaffected. One interpretation for these findings is that, although the 3′ PPT is efficiently used to initiate (+) strand synthesis, the second strand switch [relocating (+) strand strong stop DNA to the 5′ end of the recently synthesized (−) strand] is sensitive to elevated dNTP concentrations. This event of retroviral replication (*34*) requires that the tRNA primer, once partly used as the template for (+) strand synthesis, is removed by RT-associated RNase H activity (*35*−*37*), making (+) and (−) strand PBS sequences available for hybridization. Recently, it was reported that the RNase H activity of HIV-1 RT could be inhibited by nucleotide monomers and dimers (*38*) which might impair PPT selection, strand transfer efficiency, and subsequent (+) strand synthesis. The 2.8 kb (+) strand DNA was mapped to the 5′ end, but the initiation and termination sites were not precisely identified. This species lies within the region that constitutes the 4.8 kb (+) strand product and is a major product at intermediate dNTP concentrations, where the level of the 4.8 kb transcript is low. As the 4.8 kb species accumulated at lower dNTP concentrations, the yield of the 2.8 kb transcript decreased. Conceivably, the specificity of (+) strand primer production may be regulated by the dNTP concentration in the endogenous reaction, allowing cryptic initiation sites to be utilized at intermediate concentrations. An alternative scenario is that dNTP-mediated inhibition of the RNase H activity manifests itself in inefficient removal of genomic RNA from the RNA−DNA replication intermediate. This would place a considerable burden on the replication machinery, mandating extensive strand displacement synthesis for removing excessively long RNA remnants. Under such circumstances, reduced RNase H activity would be predicted to affect (−) strand DNA synthesis on the (+) strand RNA genome less severely than (+) strand synthesis on an "RNA-saturated" (−) strand DNA genome, as was observed experimentally.

In vitro data indicate that EIAV RT efficiently selects the cPPT from an RNA−DNA hybrid and removes it from nascent (+) strand DNA. High-resolution mapping predicts the cPPT sequence 5′-AACAAAGGGAGGGA-3′, a distinguishing feature of which is its punctuation by a pyrimidine residue. However, the same holds for the EIAV 3′ PPT proposed by Kawakami et al. (*26*) (5′-AAAAACAAGGGGGG-3′) and was demonstrated in our laboratory to be accurately selected from a (+) RNA−(−) DNA hybrid by homologous and heterologous RTs in vitro (*19*). Furthermore, Lauermann et al. (*27*) have documented that a pyrimidine-containing PPT of the HIV-2_{ROD} isolate is stably maintained in culture, suggesting adherence to an all-purine PPT is not absolute. The contiguous stretch of G residues at the 3′ end of the EIAV cPPT, proposed as a critical element for initiation (*28*), is also punctuated by an A residue. Despite this, HIV-1 and EIAV RT are shown here to select the cPPT (albeit with subtle differences) for initiation of (+) strand synthesis. It therefore appears that sequence identity constitutes only part of the mechanism of PPT selection, in favor of which are spectropolarimetric studies (*39*) indicating that the HIV-1 (+) strand RNA−(−) strand DNA PPT is structurally different from a random RNA−DNA hybrid.

Abrupt cessation of (+) strand synthesis in vivo near the center of the EIAV genome argues against a fortuitous termination event. Different scenarios might be invoked to account for this. For example, once copied into (+) strand DNA, the cPPT-containing duplex adopts a structure different from the (+) RNA−(−) DNA hybrid representing the initiation complex, invoking dissociation of RT. Alternatively, once the cPPT is copied into (+) strand DNA, EIAV RT is incapable of strand displacement synthesis, with the consequence that the replication machinery arriving from upstream is halted by duplex DNA originating from the cPPT. Since the experiments whose results are depicted in Figures 4 and 5 evaluate (+) strand synthesis from RNA (Figure 4B) and DNA primers hybridized to the cPPT (Figure 5B), it is unlikely that a cPPT-containing DNA duplex induces dissociation. Moreover, since retroviral replication requires strand displacement synthesis through at least one long terminal repeat, it is unlikely that the latter mechanism alone accounts for specific termination. The more likely scenario is that proposed for HIV-1, namely, that structural features invoked when the CTS is copied, in concert with strand displacement synthesis, inhibits translocation of RT which has polymerized through the cPPT during late (+) strand synthesis. This is supported by the observation that (a) EIAV RT can partially bypass this element in the absence of displacement synthesis [a necessary requirement for permitting elongation of cPPT-primed (+) DNA] and (b) oligonucleotide probe $E4895^{-}$, located between the cPPT and CTS, hybridized to the 4.8, 3.3, and 3.0 kb (+) strand fragments (Figure 3), indicating that they share a common sequence. The EIAV CTS differs from its HIV counterpart (*10*, *11*) in that its $(A)_n$ tracts are not in phase. However, since we observe almost complete cessation of (+) strand synthesis at Ter 1, phasing does not appear to be critical to (+) strand termination. This is strengthened by substituting DAP for A in nascent (+) strand DNA, which eliminates Ter 1-mediated termination and potentiates a second termination event at Ter 2. Thus, both HIV and EIAV can be considered as having two terminators within the CTS, but their influence on the replication machinery is mediated simply by A-tract-induced minor groove compression and contacts with structural elements of the retroviral polymerase.

Somewhat fortuitously, we demonstrate here that occupying the DNA polymerase catalytic center with a 4 bp A·T duplex (between Ter 1 and Ter 2) has little consequence for DNA synthesis, while flanking 5 bp (Ter 2) and 6 bp duplexes (Ter 1) efficiently stall RT in the context of strand displacement synthesis. The crystal structure of p66/p51 HIV-1 RT containing duplex DNA (*40*) indicates multiple contacts between amino acids of α-helices H and I of the p66 thumb subdomain and the nucleic acid. Gln^{258}, Lys^{259}, Gly^{262}, Lys^{263}, and Trp^{266} of α-helix H contact the sugar−phosphate backbone of primer nucleotides 3−6; Asn^{265} contacts template nucleotides 6 and 7, while α-helix I residues Ser^{280}, Arg^{284}, Gly^{285}, and Thr^{286} interact with the sugar−phosphate backbone of template nucleotides 7−9.

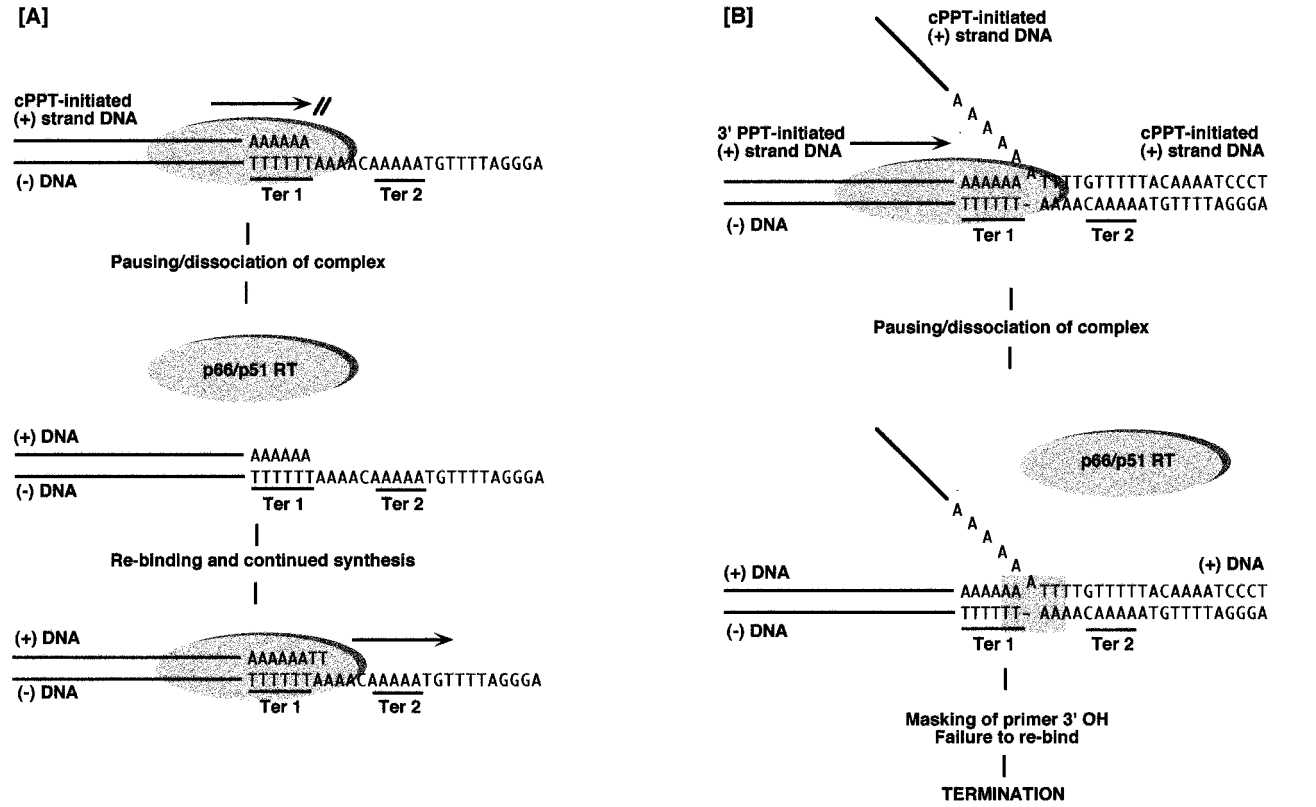


FIGURE 7: Proposed model for the EIAV CTS function. In the absence of strand displacement synthesis, i.e., cPPT-primed (+) strand synthesis (A), copying a (−) strand $(T)_6$ tract of the CTS generates a 6 bp A·T duplex. While pausing and dissociation most likely occur at this sequence (mediated by minor groove compression), a free 3′ OH is available for rebinding and continued (+) strand synthesis. At a later stage, 3′ PPT-initiated (+) strand DNA which completes the second strand jump is extended over the cPPT, after which strand displacement synthesis continues up to and including the $(A)_6$ tract of the CTS (B). The same 6 bp A·T duplex induces dissociation, but in this, the 3′ terminus of the nascent (+) strand is masked from RT by the displaced strand, leading to cessation of (+) strand synthesis. RT, which can bypass termination at Ter 1, will be subject to the same negative control at Ter 2.

Cumulatively, these observations (*41*) demonstrate important contacts with the sixth and seventh base pairs of duplex DNA occupying the polymerase catalytic center. Ter 1-mediated termination of EIAV RT occurs after addition of the last A residue of an $(A)_6$ tract, which locates the first A·T base pair of this duplex in the immediate vicinity of the α-helix H−α-helix I hairpin. Since minor groove compression will be most pronounced at this position, this may induce "dislocation" of nucleic acid from the p66 thumb, impaired translocation, and arrest of DNA synthesis. Reducing the length of the A·T duplex occupying the catalytic center by a single base pair still places DNA with altered minor groove geometry sufficiently close to the p66 thumb to alter translocation, while a 4 bp A·T duplex originating at the primer terminus appears to have lost this feature; i.e., template and primer nucleotides 6 and 7 recover the appropriate spatial relationship with respect to the α-helix H−α-helix I hairpin. Although these features could account for pausing and termination, the nucleic acid substrate must afford the retroviral polymerase some measure of flexibility, since "early" (+) strand synthesis from the cPPT must by definition continue through the CTS, while "late" (+) strand synthesis, following second strand transfer, is terminated. The model of Figure 7, invoking dissociation of RT following pausing at this element, could account for both scenarios. In the absence of strand displacement synthesis, the 3′ OH of the prematurely terminated, cPPT-primed (+) strand remains accessible for rebinding of RT and continued synthesis. In contrast, following dissolution of the replication complex at the CTS in the context of strand displacement synthesis, the nascent primer terminus is essentially masked from RT by the displaced (+) strand.

Regardless of the termination mechanism, a consequence would be duplex proviral DNA with a redundant, single-stranded sequence of ∼100 nucleotides (*11*). How might this be removed and the resulting gap repaired? One candidate for the former event is the host-encoded flap endonuclease (FEN1), which has been implicated in removal of Okazaki fragments (*42*). Rumbaugh and co-workers have recently demonstrated that purified FEN1 will accurately remove the single-stranded overlap in a single step, after which human DNA polymerase and ligase could repair the ensuing gap (*43*). The requirement for an equivalent virus-encoded function would thus be obviated. This would also be consistent with the data of Miller et al. (*44*) that indicate that (+) strand discontinuities in HIV proviral DNA need not be repaired until after integration.

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