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Discontinuous Plus-Strand DNA Synthesis in Human Immunodeficiency Virus Type 1-Infected Cells and in a Partially Reconstituted Cell-Free System

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Human immunodeficiency virus type 1 (HIV-1) replication requires conversion of viral RNA to double-stranded DNA. To better understand the molecular mechanisms of this process, we examined viral DNA synthesis in a simple cell-free system that uses the activities of HIV-1 reverse transcriptase to convert regions of single-stranded HIV-1 RNA to double-stranded DNA in a single incubation. This system recapitulated several of the required intermediate steps of viral DNA synthesis: RNA-templated minus-strand polymerization, preferential plus-strand initiation at the central and 3′ HIV-1 polypurine tracts, and DNA-templated plus-strand polymerization. Secondary sites of plus-strand initiation were also observed at low frequency both in the cell-free system and in cultured virus. Direct comparison of viral and cell-free products revealed differences in the precision and selectivity of plus-strand initiation, suggesting that the cell-free system lacks one or more essential replication components. These studies provide clues about mechanisms of plus-strand initiation and serve as a starting point for the development of more complex multicomponent cell-free systems.

Human immunodeficiency type 1 (HIV-1), like other retroviruses, is a dimeric plus-stranded RNA virus that replicates through a DNA intermediate. After infection of a cell, the viral RNA is converted to double-stranded DNA by a complex process catalyzed by virus-encoded reverse transcriptase (RT), a unique DNA polymerase that utilizes both RNA and DNA templates. The resultant DNA is integrated into host cell chromosomes, and new genomic RNA and viral proteins are subsequently synthesized and packaged into progeny virions (reviewed in references 14 and 57).

Retroviral DNA synthesis occurs in multiple steps that can be grouped into events of minus-strand DNA synthesis (mostly RNA templated) and plus-strand DNA synthesis (mostly DNA templated). Minus-strand DNA synthesis is initiated from a cellular tRNA primer annealed to the primer binding site near the 5′ end of the genomic RNA. Polymerization from this primer first proceeds to the 5′ end of the RNA, generating minus-strand strong-stop DNA. This relatively short DNA strand (~170 nucleotides [nt] for HIV-1) then transfers inter- or intramolecularly to the 3′ end of the RNA (22) and serves as a primer for polymerization of the remaining ~9,000 nt of minus-strand DNA.

Plus-strand DNA synthesis is initiated from the resultant RNA-DNA duplex by RT and its intrinsic RNase H activity. RNase H selectively degrades the RNA strand of the RNA-DNA duplex, leaving short plus-strand RNA fragments annealed to the nascent minus-strand DNA (9). The polypurine tract fragment (PPT) near the 3′ end of the genome (3′ PPT) is used as an obligatory primer for plus-strand DNA synthesis in all retroviruses. A second copy of the PPT located near the center of the genome (cPPT) is also used to prime plus-strand DNA polymerization in HIV-1 and other lentiviruses, in spumaviruses, and in the retrotransposon Ty1 (10, 11, 18, 19, 25, 26, 33, 51). In addition, nonspecific plus-strand DNA synthesis initiation sites have been identified in regions upstream of the 3′ PPT in avian and murine retroviruses (references 5 and 9 and references therein). Plus-strand DNA initiated from the 3′ PPT forms the ~600-nt plus-strand strong-stop DNA which transfers intramolecularly to the 3′ end of the minus-strand DNA (22) to serve as primer for polymerization of the remaining plus-strand DNA.

Although the molecular biology of retroviral DNA synthesis is well characterized (14, 57), the multiple biochemical events required for DNA synthesis are only partially understood. Synthesis occurs within a poorly defined and structurally dynamic multicomponent complex (7, 8, 17, 49, 57). The HIV-1 complex is comprised of viral RNA, RT, nucleocapsid (NC), integrase (IN), matrix (MA), deoxyribonucleoside triphosphates (dNTPs), and possibly other viral and cellular factors (8, 17). In HIV-1, DNA synthesis is initiated in free virions, continues in the host cell cytoplasm, and is likely completed in the nucleus (36, 38, 55). Studies monitoring the time-dependent appearance of each nascent strand of an avian retrovirus show that plus-strand synthesis starts before completion of the minus-strand (58). Presumably HIV-1 minus- and plus-strand DNA synthesis also occurs simultaneously, and this synthesis must be coordinated to obtain the correct double-stranded DNA product for subsequent chromosomal integration.

Several of the biochemical events of retroviral DNA synthesis have been reconstituted in cell-free systems using purified viral components. Early (tRNA priming, strong-stop DNA synthesis, first-strand transfer), intermediate (elongation, plus-strand initiation), and late (strand displacement, integration) steps of viral DNA synthesis have all been examined in vitro by using purified retroviral enzymes (reviewed in references 2, 9, 32, 43, and 54). These systems have yielded valuable information about the biochemical mechanisms driving individual steps of retro-

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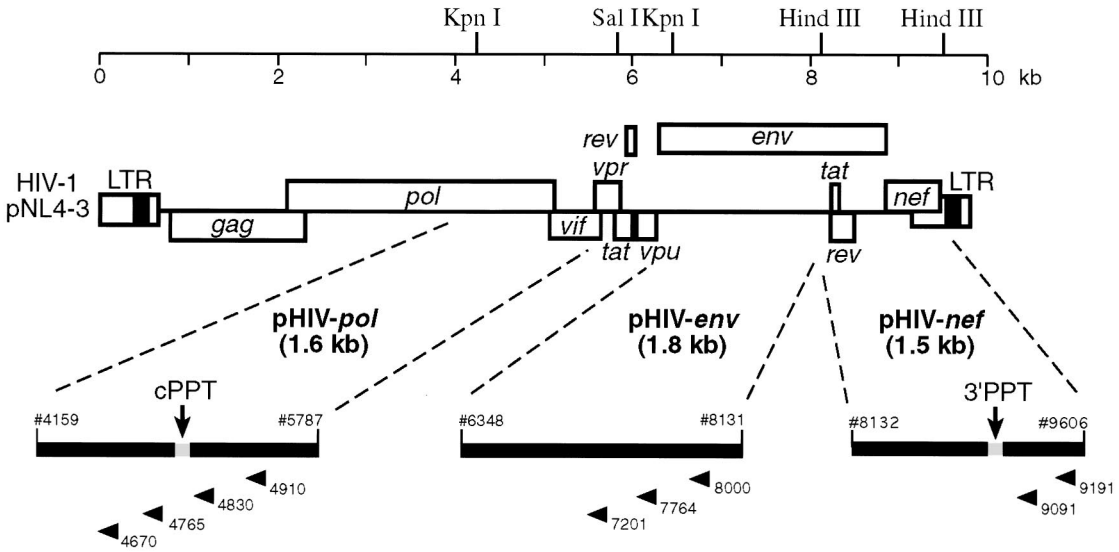

FIG. 1. Schematic of the HIV-1_{NL4-3} proviral genome. Locations of the *Kpn*I/*Sal*I (nt 4159 to 5787), *Kpn*I/*Hin*dIII (nt 6348 to 8131), and *Hin*dIII/*Hin*dIII (nt 8132 to 9606) fragments used to create pHIV-*pol*, pHIV-*env*, and pHIV-*nef* are indicated. Plus-strand RNAs were generated from these subclones as described previously (31). Oligonucleotide primers used in the cell-free system (indicated by arrowheads; not to scale) are positioned at their approximate locations along the RNA templates (black horizontal bars at the bottom). Primer numbers correspond to the position of the primer's 3′-terminal nucleotide in the HIV-1_{NL4-3} proviral sequence (31). Locations of the cPPT and 3′ PPT in the *pol* and *nef* RNAs are indicated. LTR, long terminal repeat.

viral DNA synthesis and have provided tools for the discovery of antiviral drugs.

Despite these successes, it has not been possible to obtain the complete conversion of genomic retroviral RNA to full-length double-stranded DNA in a reconstituted cell-free system. Experiments directly comparing events of DNA synthesis in replicating viruses with those catalyzed by purified retroviral enzymes in vitro indicate that simple cell-free reaction systems are incomplete. For example, both the efficiency and fidelity of DNA synthesis catalyzed by purified RTs are significantly lower than achieved by the virus during replication in a cell (references 27, 31, 37 and 56 and references therein). Discrepancies between viral and cell-free DNA synthesis are also observed in response to antiviral drugs (34). These findings suggest that viral and/or cellular factors affect retroviral DNA synthesis in a manner that is poorly understood. The relative low processivity of purified RTs is particularly perplexing and is a major barrier to the synthesis of long stretches of retroviral DNA in cell-free systems (1, 31, 39, 59). Both viral and cellular proteins have been shown to stimulate the activities of purified RTs in vitro (3, 24, 27, 35, 53, 60). However, none of these factors increase activity to the level required for polymerization of full-length viral DNA.

To obtain a more detailed understanding of biochemical mechanisms involved in the multistep synthesis of retroviral DNA, we describe a partially reconstituted cell-free HIV-1 DNA synthesis system that uses purified HIV-1 RT and its associated RNase H to convert regions of plus-strand HIV-1 RNA to double-stranded DNA in a single incubation. This system was used to characterize the specificity and efficiency of initiation of plus-strand DNA synthesis over approximately 850 nt of HIV-1 RNA. A direct comparison with viral DNA synthesis in HIV-1-infected cells showed that the cell-free system recapitulates the major events of plus-strand initiation with preferential synthesis starting from both the 3′ and central PPTs. However, the precision of initiation at the PPTs and at other ancillary initiation sites in the HIV-1 genome is lower in the cell-free system compared with replicating virus. These experiments provide clues about mechanisms of initiation of plus-strand synthesis and serve as a starting point for the development of more complex, multicomponent cell-free systems that reconstitute all steps of retroviral DNA synthesis.

MATERIALS AND METHODS

Materials. Virion and recombinant HIV-1 RT (p66-p51 heterodimer) and the HIV-1_{NL4-3} subclones pHIV-*pol* and pHIV-*env* were prepared as described previously (31). pHIV-*nef* was prepared from pNL4-3 by subcloning a 1.5 kb *Hin*dIII fragment (nt 8132 to 9606 [Fig. 1]) into pGEM-7Zf(−) by using standard methods (50). pNL4-3 was a kind gift of Arnold Rabson (New Jersey Center for Advanced Biotechnology and Medicine, Piscataway). pGEM-7Zf(−), rNTPS, RNasin, T7 RNA polymerase, and *Taq* DNA polymerase were from Promega. T4 polynucleotide kinase and Sequenase kits were purchased from U.S. Biochemical Corp. [γ-^{32}P]ATP (3,000 Ci/mmol), [α-^{32}P]dCTP (6,000 Ci/mmol), and Hybond N+ nylon blotting membranes were from Amersham. Ultrapure dNTPs and Sephadex G-50 were from Pharmacia LKB Biotechnology, Inc. Oligonucleotides were synthesized by Operon Technologies, Inc. All other reagents were of the highest available grade from Fischer Scientific or Gibco BRL.

Plus-stranded RNA was generated by in vitro transcription from pHIV-*nef*, pHIV-*pol*, and pHIV-*env* as described previously (31). RNAs were gel purified through 2% low-melting-temperature agarose and isolated by using GELase (Epicentre Technologies) according to the manufacturer's instructions. RNAs were quantitated either by UV absorbance at 260 nm or by including a trace amount of [α-^{32}P]UTP (400 Ci/mmol) in the RNA synthesis reaction followed by scintillation counting. Similar values were obtained by each method.

RNase H-minus HIV-1 RT. A 1.7-kb *Eco*RI/*Hin*dIII HIV-1 RT fragment encoding full-length HIV-1_{HXB2} RT was subcloned from pRT4 (31) into M13mp19 by using standard methods (50). The resulting phage, mpRT4, was used to engineer an HIV-1 RT point mutant (D443N) as detailed by Mizrahi et al. (39). HIV-1 RT containing this mutation lacks detectable RNase H activity while retaining full polymerase activity (39). A 328-bp *Kpn*I fragment containing the mutation was subcloned back into *Kpn*I-cut pRT4, replacing the wild-type sequence. The presence of the mutation was confirmed by DNA sequencing.

Recombinant D443N HIV-1 RT was expressed and purified from *Escherichia coli* DH5αI^{Q} as described previously for the wild-type HIV-1 RT (31), with the following modifications. Twelve grams of cell paste was used for the purification, and the desalted RT-containing fractions from the phosphocellulose column were pooled and loaded onto a 1-ml MonoS column (5 by 5 cm; Pharmacia LKB Biotechnology) at a flow rate of 0.5 ml/min. The column was washed with 5 ml of buffer M, and the RT was eluted with a 30-ml linear gradient of 0 to 0.5 M NaCl in buffer M. RT-containing fractions eluting between 0.2 and 0.3 M NaCl (approximate concentrations) were pooled and concentrated in a Centriprep-10 ultrafiltration device (Amicon). The purified D443N HIV-1 RT (>95% homogeneous; 99% p66 as determined from Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)) was stored at −80°C in multiple aliquots until needed.

Cell culture and HIV-1 infection. HeLaT4 cells were a gift of Michael Emmerman (Fred Hutchinson Cancer Research Center, Seattle, Wash.) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and hygromycin (100 μg/ml). HeLa-CD4-LTR-β-gal cells were from the AIDS Repository (National Institutes of Health, Bethesda, Md.) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, G418 (200 μg/ml), and hygromycin (100 μg/ml) (30). HIV-1_{IIIB} virus was a gift of Arnold Rabson and was amplified by inoculating HeLaT4 cells. Five days after infection, supernatant was collected and virus titer was determined on HeLa-CD4-LTR-β-gal cells as described previously (30).

To obtain low-molecular-weight DNA from HIV-1-infected cells, 2.5×10^6 HeLaT4 cells were first treated with Polybrene (2 μg/ml) for 30 min and then incubated for 2 h with 2×10^6 infectious units of HIV-1_{IIIB}. Low-molecular-weight DNA was extracted from cells 0, 4, 8, 12, 16, 20, and 24 h after infection, using the Hirt procedure (20).

Cell-free DNA polymerization. Primers 20 nt in length (20-mers) were hybridized to RNA at a molar ratio of 0.9:1 (38 nM primer/43 nM template) in reaction buffer C (25 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 30 mM KCl, 2 mM dithiothreitol) as described previously (31). Primers were either unlabeled or 5′ end labeled with [γ-^{32}P]ATP for analysis of plus-strand or minus-strand DNA synthesis, respectively. Polymerization reactions (15-μl total volume) were conducted as described previously (27, 31) except with either 2 or 30 nM primer-template and 2 or 30 nM HIV-1 RT active sites (27) in reaction buffer C. Polymerizations were conducted at 37°C for 0 to 120 min. Minus-strand polymerization was stopped by the addition of EDTA to 50 mM final concentration and 5′-^{32}P-labeled minus-strand DNA products were analyzed by electrophoresis on a 7 M urea–8% polyacrylamide gel and visualized by autoradiography (27, 31); plus-strand products were analyzed as described below.

Analysis of nascent plus-strand DNAs. Products from cell-free reactions were incubated with 0.3 N NaOH for 1 h at 65°C, neutralized by the addition of 0.3 M sodium acetate (pH 5.0)–0.1 M Tris-HCl (pH 8.0), and passed through 0.5-ml Sephadex G-50 spin columns (31). Plus-strand DNAs were detected, and 5′ ends were mapped by Southern blotting or linear PCR.

For blotting, combined DNA products from two reactions (starting with 30 nM primer-template) were resolved by electrophoresis on a 7 M urea–8% polyacrylamide gel. The gel was overlaid with Hybond N+ membrane wetted with 10× Tris-borate-EDTA (50), and one piece of dry gel blot paper (Schleicher & Schuell) also wetted with 10× Tris-borate-EDTA. This in turn was covered with three pieces of gel blot paper and a glass plate, and the DNA products were transferred to the membrane through capillary action for approximately 12 h. Essentially 100% of DNAs from 10 to >250 nt in length transferred to the membrane in this procedure. The membrane was removed, incubated with 0.4 N NaOH for 15 min, and washed with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (50) for 5 min at 25°C. The blot was incubated with 200 ng of oligonucleotide probe (labeled with terminal transferase and [α-^{32}P]dCTP as described by Gibco BRL) in a 30-ml solution containing 7% SDS, 10% polyethylene glycol 8000, 0.25 M NaCl, and 0.15 M sodium phosphate (pH 7.0) for 3.5 h at 42°C. The membrane was washed two times with 50 ml of 2× SSC–0.1% SDS for 10 min at 25°C, dried, and exposed either to film or a phosphorimaging screen (Molecular Dynamics).

The probe used in each analysis was the same oligonucleotide used as primer for the initial minus-strand DNA synthesis (Fig. 1 and 2). Thus, the probes hybridize only to nascent plus-strand DNAs that have been extended by HIV-1 RT back to the 5′ end of the nascent minus-strand DNA initiated from the same primer-probe oligonucleotide sequence. Since all plus-strand products detected by these probes must have a common 3′ end, bands migrating through the gel at different rates have unique 5′ ends that can be identified by their electrophoretic mobility relative to a sequencing ladder created with the same primer.

To detect plus-strand DNA products by linear PCR, 30 to 60 μg of Hirt DNA (determined by absorbance of Hirt extracts at 260 nm) or 0.1 volumes of cell-free reaction products (starting with 2 nM primer-template and 2 nM HIV-1 RT) were incubated in reaction mixtures (50-μl total volume) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM each dNTP, 0.6 pmol of 5′-^{32}P-labeled oligonucleotide primer, and 6 U of *Taq* DNA polymerase. Cycling was done as described previously (11) except that after 30 cycles, 6 more U of *Taq* was added and PCR was resumed for 30 additional cycles. Reactions were brought to 100 μl with H_2O and passed through 0.5-ml Sephadex G-50 spin columns (27, 31). Products were analyzed by urea PAGE and visualized with a Molecular Dynamics PhosphorImager.

RESULTS

Cell-free HIV-1 DNA synthesis. To gain a better understanding of the biochemical mechanisms of double-stranded HIV-1 DNA synthesis and to study individual steps of this process, we developed a cell-free system in which regions of single-stranded HIV-1 RNA are converted to double-strand DNA in a concerted reaction catalyzed by purified HIV-1 RT (Fig. 2). Starting with plus-stranded HIV-1 RNA annealed to an oligodeoxyribonucleotide primer, double-stranded HIV-1 DNA is synthesized by using the three catalytic activities of RT: (i) RNA-templated DNA polymerase, (ii) RNase H, and (iii) DNA-templated DNA polymerase. Thus, this cell-free system reconstitutes several intermediate steps of HIV-1 DNA synthesis in a single incubation. The efficiencies and specificities of both minus- and plus-strand DNA polymerization can be examined in parallel and compared directly with events occurring in HIV-infected cells.

(i) Minus-strand DNA synthesis. To examine minus-strand DNA polymerization, a 1.5-kb RNA derived from the 3′ end of HIV-1 (pHIV-*nef* [Fig. 1]) and a 1.7-kb RNA derived from the central *pol* region of the HIV-1 genome (pHIV-*pol* [Fig. 1 and reference 31]) were hybridized to 5′-^{32}P-labeled oligodeoxyribonucleotide primers and incubated with purified, recombinant HIV-1 RT in the presence of dNTPs. The reaction products were analyzed by urea PAGE. For each primer-template (30 nM hybrid), the concentration of RT remained fixed (30 nM active sites) and the incubation time was varied between 0 and 120 min (Fig. 3). At this concentration of RT, the majority of hybridized primers were extended within 5 to 10 min.

Examination of the reaction products from the *nef* template revealed numerous sites of paused or interrupted DNA synthesis (indicated as R10 to R17), including a relatively strong site at the start of the G run at the 3′ end of the 3′ PPT (R11 [Fig. 3A]). This is similar in location and sequence to a pause site at the 3′ end of the cPPT (R3 [Fig. 3B and reference 31]). Most of the pause sites on the *nef* template occurred in homopolymeric runs of like nucleotides with the exception of R13 and R15. On average approximately 40 nt were polymerized after 2.5 min of incubation. This increased to 95 nt after 90 min of incubation, when 60% of the primers were extended beyond the 3′ PPT and therefore available as substrates for RNase H cleavage and subsequent initiation of plus-strand DNA synthesis.

As observed previously (31), distinct polymerization pause sites also occurred on the *pol* template (R1 to R4 [Fig. 3B]). Sequences R1 and R2 are particularly strong barriers to DNA polymerization, and only a fraction of primers (≤10%) were extended beyond the cPPT after 120 min of incubation. This finding reflects the inherent low processivity of HIV-1 RT (1, 31, 39, 59) which severely limits the amount of RNA-DNA duplex formed at and beyond the cPPT relative to the 3′ PPT (compare the intensities of bands longer than 50 nt for each set of reactions, Fig. 3). A mutant HIV-1 RT (D443N) lacking RNase H activity (39) had essentially wild-type DNA polymerase activity and yielded minus-strand DNA products very similar to those of wild-type RT on both the *pol* and *nef* RNA templates (Fig. 3B, lane 11, and data not shown).

(ii) Plus-strand DNA synthesis from the 3′ and central PPTs. A blotting assay was used to identify nascent plus-strand DNA products formed after initial synthesis of minus strands (Fig. 2, step 3, left). DNA synthesis reactions were carried out as in Fig. 3 except the oligonucleotide primers were not radioactively labeled. After alkaline hydrolysis of the RNA strand and its fragments, the DNA reaction products were resolved by urea PAGE and transferred to a nylon membrane. Plus-strand DNAs were detected by probing with ^{32}P-labeled strand-specific oligonucleotides, and the 5′ ends of the nascent DNAs were mapped against DNA sequencing ladders.

Both the 3′ and central PPTs strongly directed the initiation of plus-strand DNA synthesis in this cell-free system (Fig. 4). Reactions starting with either *nef* (Fig. 4A) or *pol* (Fig. 4B) RNA yielded plus-strand DNA products with 5′ ends mapping predominantly in the PPTs. After prolonged incubation and overexposure to film, additional weak products were also detected at surrounding non-PPT sites (see below). No plus-strand products were detected when the D443N RNase H-

minus HIV-1 RT was substituted for wild-type RT (Fig. 4B, lane 8, and data not shown), even though minus strands were synthesized almost equally well by both RTs (Fig. 3B). Similarly, no plus-strand products were formed in reactions lacking minus-strand oligonucleotide primers (data not shown).

Plus-strand product formation was linear up to 20 and 40 min at the 3′ and central PPTs, respectively. After an initial 3- to 5-min lag time, the rates of nascent plus-strand product formation (Fig. 4) were approximately equal to the rates of minus-strand product extension beyond each PPT (Fig. 3). This finding indicates that once RNA-DNA duplex is formed at each PPT, RNase H cleavage and subsequent extension of the RNA primer by RT occur rapidly.

The blot assay revealed four prominent bands with 5′ termini that mapped to the last three G residues and adjacent A residue in each PPT (Fig. 4, center, indicated with arrows). The amounts of each of the four products were similar, and the relative ratios of these products remained constant during the course of incubation. The patterns of products at the 3′ and central PPTs were essentially the same. Identical results were obtained in assays using recombinant HIV-1 RT purified in a different laboratory as well as with multiple independent RNA preparations (data not shown). These data, together with the requirement for active RNase H, show that the plus-strand initiation observed does not result from contaminating RNases or priming by non-HIV-1 nucleic acid fragments that may be present at low levels in the RNA preparations.

(iii) Plus-strand synthesis from non-PPT sites. Low levels of plus-strand DNAs were detected with 5′ ends at non-PPT sites on both the *nef* and *pol* RNAs. This finding indicates that RNA sequences lacking canonical PPTs can also serve as primers in the cell-free system. To evaluate the utilization of non-PPT sites as primers of plus-strand synthesis, primer-template combinations on the *pol*, *env*, and *nef* RNAs were incubated with either wild-type or RNase H-minus HIV-1 RT for 40 min under standard polymerization conditions, and the resulting plus-strand DNA products were analyzed by blotting. Two representative blots are shown in Fig. 5. Initiation of plus-strand DNA synthesis occurred from multiple sites in the *pol* template upstream from the cPPT as well as in *env* (Fig. 5A and B, respectively, lanes 1). No plus-strand initiation products were detected on either template in reactions containing RNase H-minus HIV-1 RT (lanes 2), demonstrating that active RNase H is required for the reaction and that the initiation sites are specific for HIV-1 RT and not due to nonspecific RNase or oligonucleotide contaminants. Reactions using other primer-templates (4670 · *pol*, 4910 · *pol*, 7201 · *env*, 7764 · *env*, and 9191 · *nef* [Fig. 1]) also demonstrated RNase H-dependent initiation of plus-strand DNA polymerization at multiple sites (data not shown). Reactions performed with wild-type HIV-1 RT purified in a different laboratory yielded essentially identical results. Unprimed RNA incubated with wild-type HIV-1 RT failed to synthesize plus-strand DNA, ruling out any contribution of hairpin self-priming to plus-strand product formation (48).

The position of each initiation site in the HIV-1 sequence was identified by comparing band locations to sequencing markers, and the molar yields of plus-strand DNAs from all reactions were calculated from standards coelectrophoresed and blotted along with the reaction products. Although some initiation sites will be underrepresented in the blot assay due to pauses in plus-strand synthesis, this analysis permits an estimate of the relative usage of each plus-strand initiation site during conversion of single-stranded RNA to double-stranded DNA in the cell-free system (Fig. 5C).

Synthesis of HIV-1 plus-strand DNA occurred most efficiently from the 3′ and central PPTs. The percentages of nascent RNA-DNA duplexes cleaved and converted to detectable plus-strand DNAs at the 3′ and central PPTs were approximately 40 and 30, respectively (Fig. 5C). At non-PPT sites, plus-strand initiation occurred on average every 20 nt along the

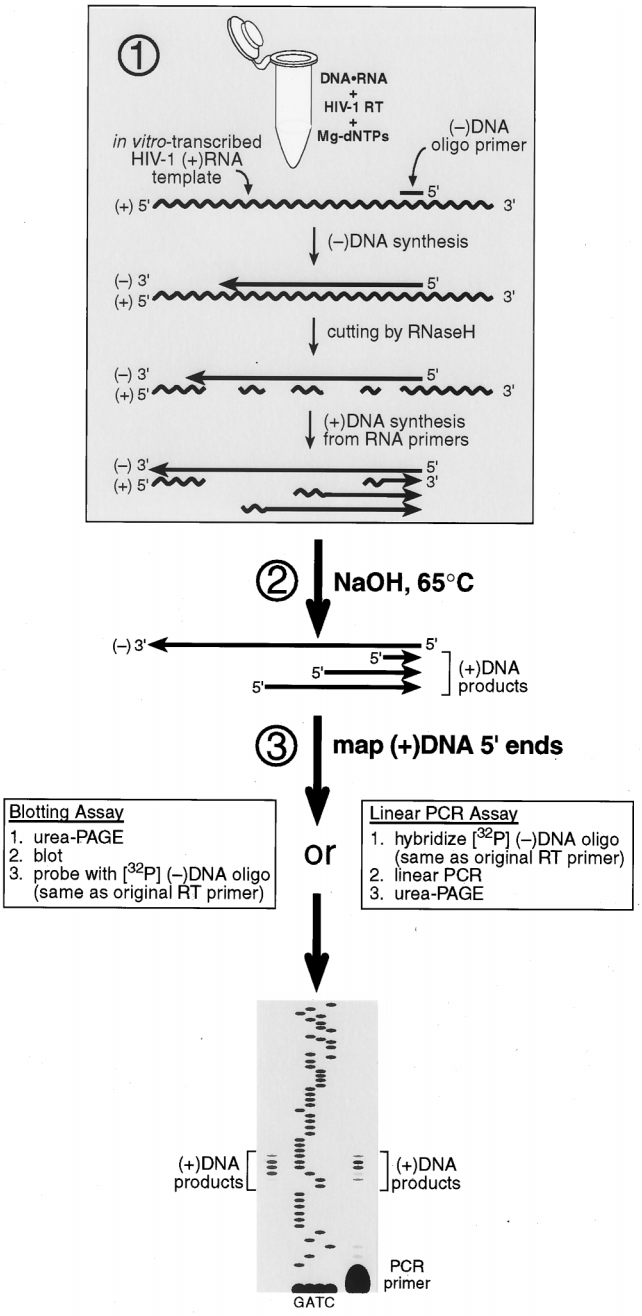

FIG. 2. Cell-free system to study HIV-1 DNA synthesis. A synthetic minus-strand [(−)DNA] oligonucleotide primer 20 nt in length (straight horizontal line) was annealed to in vitro-transcribed HIV-1 RNA (wavy line) and incubated with purified HIV-1 RT in the presence of Mg^{2+} and dNTPs. In a single incubation (step 1), RT catalyzes minus-strand DNA polymerization, RNase H cleavage, and subsequent plus-strand polymerization primed from RNase H-generated RNA fragments and templated by the nascent minus-strand DNA. After treatment with alkali at 65°C to remove RNA (step 2), nascent plus-strand DNAs were identified and 5′ ends were mapped (step 3) by two methods: a blotting assay and a linear PCR assay. Both assays use a strand-specific ^{32}P-oligonucleotide probe identical to the original primer used for minus-strand polymerization (step 1). See Materials and Methods for details.

pol and *env* sequences studied. The percent conversion of RNA-DNA duplex to plus-strand DNA at any single non-PPT site was ≤5, demonstrating that the PPTs were utilized preferentially but not exclusively by HIV-1 RT in the cell-free system.

Plus-strand initiation in HIV-1-infected cells. (i) Initiation at the cPPT. To directly compare plus-strand initiation sites generated in the cell-free system with those used by replicating virus, HeLaT4 cells (2.5×10^6) were infected with HIV-1 (2×10^6 infectious units), and low-molecular-weight DNAs were extracted at several time points after infection to map 5′ ends of HIV-1 plus-strand DNAs, using a strand-specific, linear PCR assay (Fig. 2, step 3, right). The first site mapped was the cPPT, because it is a known requisite plus-strand DNA synthesis initiation site for HIV-1 (10, 11, 25, 26). DNA extracted from cells 8 and 16 h after HIV-1 infection contained plus-strand 5′ ends mapping to the 3′ margin of the central PPT (Fig. 6A, lanes 2 and 3), while mock-infected cells lacked any bands in this region (Fig. 6A, lane 1). Plus-strand 5′ ends at the cPPT were detected at 4, 8, 12, 16, and 20 h after infection in three independent experiments (data not shown). A control for pausing by *Taq* DNA polymerase revealed no pause sites at the cPPT. These data confirm that there is a DNA plus-strand discontinuity at the cPPT during HIV-1 replication in culture (10, 11, 25, 26).

The PCR product at the cPPT was either a single or double band, depending on the amount of Hirt DNA extract used in the amplification reaction (Fig. 6B). The upper band, obtained primarily from PCRs using low amounts of Hirt extract, maps to the last G residue in the PPT (Fig. 6A, lanes 2 and 3; Fig. 6B, lane 1). As the amount of Hirt extract was increased, a band one nucleotide shorter was generated that maps to the A residue immediately adjacent to the PPT (Fig. 6B, lanes 2 and 3). *Taq* DNA polymerase as well as other polymerases add a single nontemplated nucleotide when polymerizing to the 5′ ends of templates (12). The efficiency of nontemplated nucleotide addition is dependent on reaction conditions (in particular the salt concentration) and the polymerase/DNA ratio (41). It is likely that the doublet generated in the linear PCR mapping reactions results from both precise 5′-end mapping (lower band) and nontemplated +1 addition (upper band). The single upper band observed in assays using low amounts of Hirt extract (Fig. 6A, lanes 2 and 3; Fig. 6B, lane 1) presumably results from preferential formation of +1 addition products under these assay conditions. Thus, initiation of plus-strand DNA synthesis during HIV-1 replication in culture occurs with relative high precision, generating a plus-strand DNA discontinuity with the 5′-terminal nucleotide being the A residue immediately adjacent to the cPPT (Fig. 6C).

The linear PCR assay was also used to map plus-strand DNAs generated in the cell-free system, thereby permitting a direct comparison of plus-strand initiation sites in the two systems. As observed with the blotting assay (Fig. 4B), the linear PCR analysis confirmed that plus strands are preferentially initiated from the cPPT (Fig. 6A, lanes 4 and 5) and that initiation requires active RNase H (lane 6). In contrast to the precise plus-strand 5′ ends generated in replicating virus, both recombinant HIV-1 RT and RT purified directly from HIV-1 virions initiated plus-strand synthesis from multiple sites within the cPPT (Fig. 6A, lanes 4 and 5). The same band patterns were observed at different concentrations of input PCR template (data not shown). After accounting for +1 addition by *Taq* DNA polymerase, the positions of the plus-strand 5′ ends mapped by linear PCR were essentially the same as those mapped in the blotting assay (Fig. 4B; summarized in Fig. 6C). These positions were also confirmed by mapping with Klenow DNA polymerase, which yields minimal +1 addition (data not shown) (13).

(ii) Initiation at non-PPT sites. The use of ancillary initiation sites in the cell-free system (Fig. 5) suggested that replicating virus might also initiate plus-strand DNA synthesis from non-PPT sites at low frequency. Several mapping primers were used to search for non-PPT plus-strand discontinuities in Hirt extracts of HIV-1-infected cells. These primers were designed to examine regions of the HIV-1 genome that (i) were identified as secondary initiation sites in the cell-free system (primers 4910 and 8000), (ii) contain PPT homology (primers 893, 2772, 4394, and 7201), and/or (iii) were recently implicated (38) as sites of plus-strand discontinuity (primers 5532 and 7201; primer names refer to the genomic position of each primer's 3′ terminus [Fig. 1]).

The analyses using mapping primers 893, 4394, 4910, 5532, and 8000 yielded no clearly discernible plus-strand discontinuities at all time points assayed (4, 8, 12, and 16 h postinfection). We estimate that in these experiments, the linear PCR assay will detect discontinuities present at levels ≥10% of the cPPT discontinuity (Fig. 6). Thus, the absence of detectable ancillary plus-strand discontinuities in replicating virus is consistent with the low-frequency usage of most secondary initiation sites in the cell-free system (<10% of the PPTs [Fig. 5C]). An assay with higher sensitivity will be required to evaluate the usage of many of these sites. One exception is initiation at position 7930. In the cell-free system, this is a preferred secondary initiation site used ~20% as frequently as the cPPT (Fig. 5B and C). No plus-strand discontinuity corresponding to this initiation site was detected in extracts of HIV-1-infected cells.

When Hirt extracts were assayed by using the 7201 mapping primer, a new plus-strand discontinuity was detected approximately 65 nt from the primer (Fig. 7, lanes 2 to 5; band marked with an arrow). This discontinuity was detected as early as 4 h and persisted until at least 16 h postinfection. The band corresponding to the discontinuity was absent in mock-infected cells (Fig. 7, lane 1) and was observed in three independent experiments. Control experiments on recombinant templates confirmed that the band did not result from pausing by *Taq* DNA polymerase during the linear PCR assay (data not shown). The 5′ end of the discontinuity mapped to position 7137 in HIV-1_{NL4-3} (corresponding to position 7145 in HIV-1_{HXB2}) within the HIV-1 *env* gene. The sequence immediately upstream of 7137 is purine rich but is not a canonical PPT.

The cell-free reactions catalyzed by purified HIV-1 RT were also analyzed by linear PCR for plus-strand DNA initiation sites in this region of *env* (Fig. 7, lanes 6 to 8). A region of RNase H-dependent plus-strand initiation sites was identified at approximately 50 nt from primer 7201 (initiation sites marked with arrows). However, site 7137 was not a significant initiation site in the cell-free system. Nearly identical band patterns were observed for plus-strand 5′ ends generated by both virion and recombinant HIV-1 RTs (Fig. 7, lanes 6 and 7).

DISCUSSION

To better characterize the molecular and biochemical events of HIV-1 DNA synthesis, we developed a cell-free system that converts regions of single-stranded HIV-1 RNA to double-stranded DNA by using the activities of HIV-1 RT (Fig. 2). This system recapitulated several intermediate steps of viral DNA synthesis, including preferential initiation of plus-strand DNAs from the central and 3′ PPTs (Fig. 4). Secondary sites of plus-strand initiation were also observed at low frequency (Fig. 5), suggesting that HIV-1, like other retroviruses, may generate plus-strand discontinuities during normal virus replication.

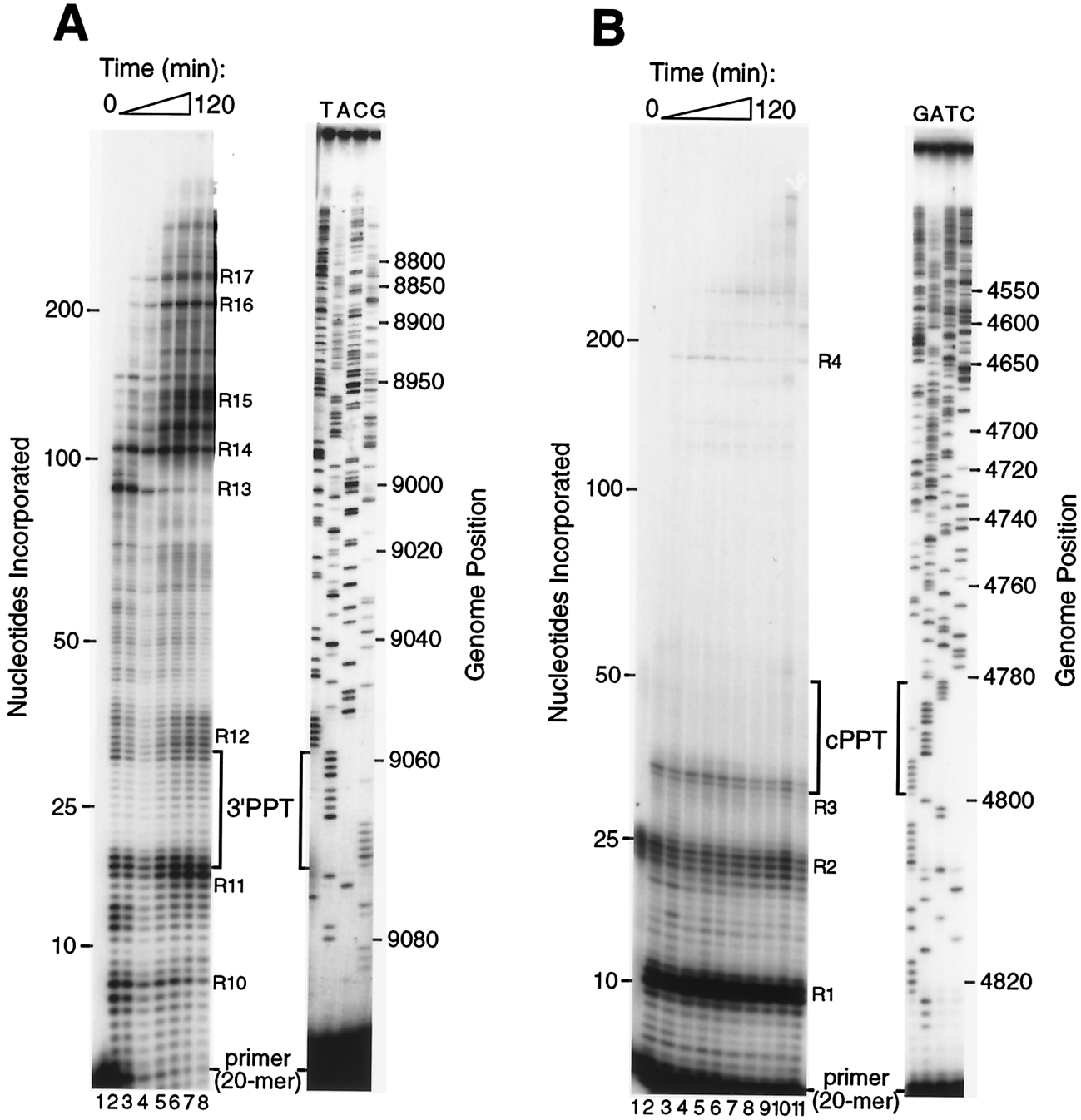

FIG. 3. Cell-free minus-strand DNA synthesis. Single-stranded RNAs from pHIV-*nef* (A) and pHIV-*pol* (B) were hybridized individually to 20-mer primers 9091 and 4830, respectively (Fig. 1). 5′-^{32}P-labeled primer-template (30 nM) was incubated with HIV-1 RT (30 nM active sites) as described in Materials and Methods. Reaction times were varied from 0 to 120 min, products were resolved by urea PAGE, and the resulting autoradiographs are shown. The number of nucleotides incorporated, template pause sites (R1, R2, etc.), and the HIV-1_{NL4-3} sequence positions are indicated in the margins. (A) Primer 9091 · *nef* RNA. Incubation times were 0, 2.5, 5, 10, 30, 60, 90, and 120 min (lanes 1 to 8, respectively). The location of the 3′ PPT is indicated for both the reaction lanes and the plus-strand template sequence lanes (TACG). (B) Primer 4830 · *pol* RNA. Incubation times were 0, 1, 2.5, 5, 7.5, 10, 20, 40, 60, and 120 min (lanes 1 to 10, respectively). Lane 11 is a 120-min incubation with the D443N HIV-1 RT mutant lacking RNase H activity. The residual unextended primer in lanes 1 to 11 is due to the molar excess of primer 4830 relative to *pol* RNA template used in this reaction set. The location of the cPPT is indicated for both the reaction lanes and the plus-strand sequence lanes (GATC).

This possibility was confirmed by mapping a new plus-strand discontinuity at position 7137 in nascent HIV-1 DNA isolated from cultured virus (Fig. 7). Direct comparison of the viral and cell-free products revealed differences both in the location (Fig. 5 and 7) and precision (Fig. 6) of plus-strand initiation, suggesting that the cell-free system lacks one or more essential replication components.

The conversion of HIV-1 RNA to double-stranded DNA involves initial minus-strand DNA synthesis using RNA as template. In accord with previous observations (27, 31, 59), multiple polymerization pause sites occurred during DNA synthesis in our cell-free synthesis system, particularly at the start of homopolymeric ribonucleotide runs (Fig. 3). The multiple pause sites prevented the polymerization of significant quan-

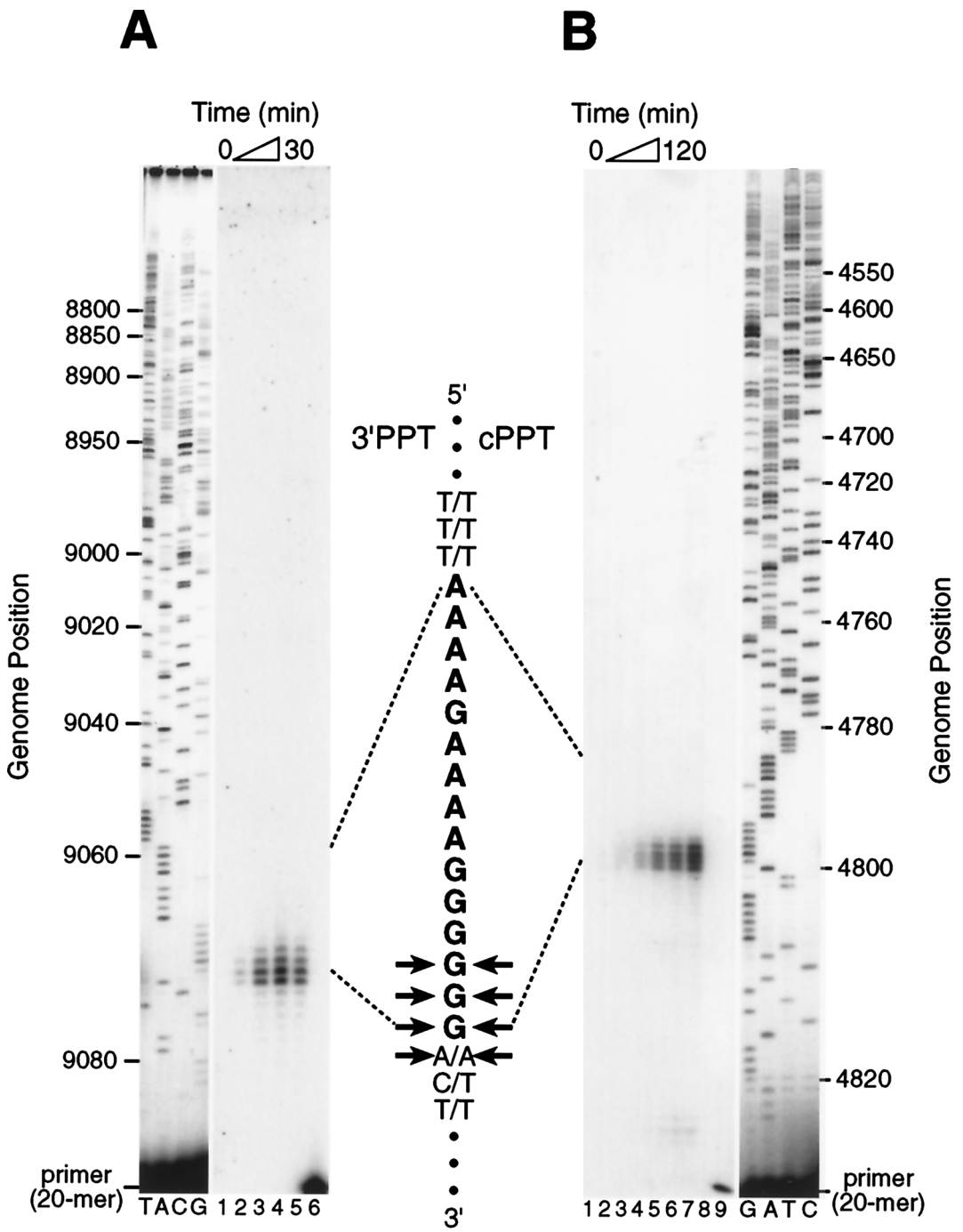

FIG. 4. Cell-free plus-strand DNA synthesis from the 3′ and central PPTs. DNA synthesis reactions were carried out as described in the legend to Fig. 3 except that the 20-mer primers were not radioactively labeled. Reaction products were separated by urea PAGE, blotted, and probed with ^{32}P-labeled 9091 or 4830 minus-strand DNA oligonucleotides (for panels A and B, respectively) as described in Materials and Methods. HIV-1_{NL4-3} sequence positions are indicated in the margins alongside the plus-strand sequence lanes (TACG [A] and GATC [B]). The sequence of the PPT (bold) and flanking nucleotides are indicated in the center. The flanking sequences are shown as pairs where the left side refers to the 3′ PPT and the right side refers to the cPPT sequence. The arrows point to the 5′-terminal nucleotides of the major plus-strand DNA products. (A) Plus-strand DNA synthesis initiation from the 3′ PPT. Reaction times were 0, 5, 10, 20, and 30 min (lanes 1 to 5, respectively). Lane 6, 20-mer marker used for alignment with sequencing lanes. (B) Initiation from the cPPT. Reaction times were 0, 5, 10, 20, 30, 40, 60, and 120 min (lanes 1 to 7, respectively). Lane 8, cell-free reaction (120 min) using HIV-1 RNase H-minus RT (D443N); lane 9, 20-mer marker.

tities of products longer than 200 to 300 nt. This finding indicates that purified HIV-1 RT is incapable of complete polymerization of minus-strand DNA under the conditions of our cell-free system. These data also illustrate one of the challenges of reconstituting the multistep process of retroviral DNA synthesis in a cell-free system. It is similarly difficult to synthesize full-length DNA in endogenous HIV-1 RT reactions in permeabilized virions; the yields are low and very sensitive to changes in detergent and ionic strength (6).

Although the yields of longer minus-strand DNA in our

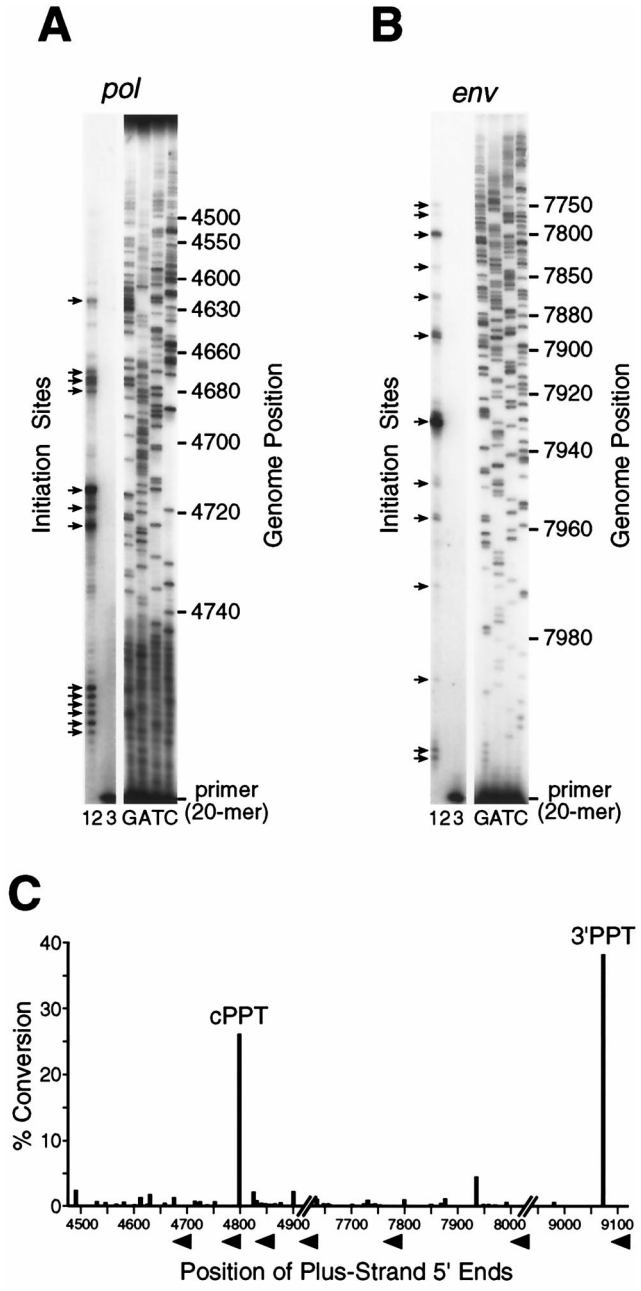

FIG. 5. Cell-free plus-strand synthesis from non-PPT sites. DNA synthesis reactions were carried out for 40 min as described in Materials and Methods and the legends to Fig. 3 and 4. Plus-strand initiation sites are indicated with arrows to the left of each panel, and HIV-1 sequence positions are marked alongside the plus-strand sequence lanes (GATC) in the right margins. (A) Plus-strand products generated from primer 4765 · *pol* RNA (Fig. 1). (B) Plus-strand products generated from primer 8000 · *env* RNA (Fig. 1). Lanes: 1, reactions with wild-type HIV-1 RT; 2, reactions with RNase H-minus HIV-1 RT (D443N); 3, 20-mer markers. (C) Conversion efficiencies for each initiation site. Percent conversion for each site was calculated from the molar quantity of an individual plus-strand product (determined from standards used in each blot [data not shown]) relative to the molar quantity of minus-strand DNA extended ≥18 nt beyond the initiation site giving rise to that product (Fig. 3 and data not shown). Calculations were made separately for each initiation site, thus normalizing for differences in the efficiencies of minus-strand DNA synthesis across different regions of the RNA. Each bar in the graph represents a unique initiation region spanning one to five contiguous nucleotides. The percent conversion of some initiation sites may be underestimated due to polymerization pause sites during plus-strand DNA synthesis (31); incompletely extended plus strands will not be detected by the probe. The numbers on the *x* axis correspond to positions in HIV-1_{NL4-3} proviral DNA. The approximate positions of the different 20-mer primers-probes used to generate these data are shown as arrowheads (not to scale). The data represent average values obtained in two or three independent experiments. Standard errors ranged from ±12% conversion at the cPPT to ±1 to 3% at the non-PPT sites. The location of each initiation site was highly reproducible in independent experiments.

simple cell-free system were low, sufficient amounts of RNA-DNA duplex were generated to allow detection of products resulting from the subsequent steps of initiation and polymerization of plus-strand DNA (Fig. 4 and 5). The cell-free system preferentially directed plus-strand DNA polymerization from the 3′ and central PPTs in a manner dependent on minus-strand synthesis and active RNase H. Initiation from both PPTs occurred with nearly equal efficiencies (Fig. 5C) despite the different sequences flanking each PPT (Fig. 4). This finding suggests that the PPT sequence alone contains sufficient information to direct effective plus-strand initiation. These data confirm and extend previous findings that the PPT sequence in preformed short RNA-DNA duplexes directs the initiation of plus-strand synthesis by purified RTs (16, 23, 42, 44, 45).

A key difference between our system and other assays of RT/RNase H activity is that we start with single-stranded HIV-1 RNA and incubations proceed until the formation of double-stranded DNA. Thus, our system recapitulates three major steps required for viral DNA synthesis: (i) minus-strand synthesis by RT, (ii) cleavage of RNA-DNA intermediates by RNase H, and (iii) plus-strand primer recognition and extension by RT. These steps take place in a single incubation and must occur in a coordinated fashion, presumably mimicking viral events required for the synthesis of both DNA strands (58). A somewhat similar system that relies on RNA hairpin self-priming for minus-strand DNA synthesis and subsequent plus-strand synthesis by the Moloney murine leukemia virus RT has been described previously (48). Our system makes no a priori assumptions about plus-strand initiation sites, and the use of synthetic minus-strand oligonucleotide primers permits a screen of initiation site usage across relatively large regions of the HIV-1 genome. Although the 3′ and central PPTs were used preferentially as initiation sites, plus-strand polymerization also occurred at low frequencies from numerous other sites over the ~850 nt of HIV-1 RNA examined (Fig. 5C). The putative primers of the non-PPT initiation sites do not exhibit any particular sequence motif (data not shown). Approximately 50% of the sequences are purine rich (≥70% purine), though there is no correlation between purine content and conversion efficiency. This finding shows that purified HIV-1 RT is able to create and extend RNA primers of various sequence albeit with reduced efficiency relative to the PPTs. RTs from murine leukemia virus and Rous sarcoma virus have also been reported to extend non-PPT primer sequences in simple cell-free systems (15, 45, 47, 58).

Preferential utilization of the canonical PPTs in our system is consistent with that observed in less complex cell-free reactions (16, 23) and with the established role of PPTs as requisite plus-strand initiation sites in all retroviruses. Short purine-rich RNA-DNA duplexes adopt a relatively stable and unique helical conformation in solution (46). Mutations disrupting the PPT helical conformation abolish priming, while nondisruptive sequence changes only slightly reduce priming ability (42), demonstrating that substrate structure is a critical factor for recognition by HIV-1 RT. The lack of proper helical conformation may be responsible for the low efficiency of initiation at non-PPT sites in our system (Fig. 5C) and for the failure to detect multiple plus-strand initiation sites in other HIV-1 cell-

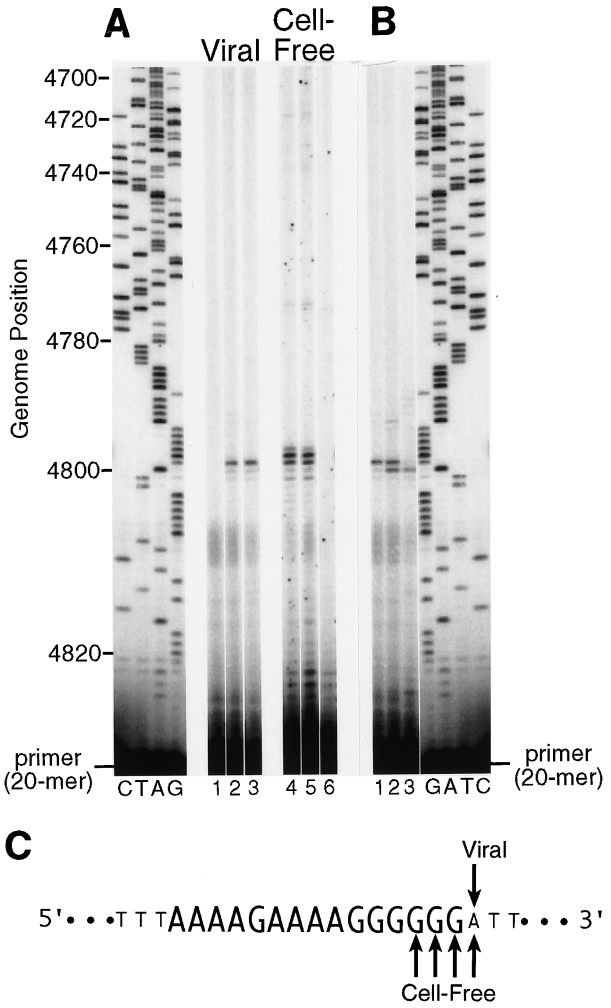

FIG. 6. Comparison of plus-strand initiation from the cPPT in replicating virus and the cell-free system. DNA extracted from HIV-1-infected cells or cell-free reaction products generated with primer 4830 · *pol* RNA (40-min reactions) were subjected to linear PCR with 5′-^{32}P-labeled oligonucleotide 4830 as described in Materials and Methods. The data were obtained with a PhosphorImager after PCR product separation by urea PAGE. (A) Products of linear PCR on Hirt extracts (30 μg) from cells 0, 8, and 16 h after infection with HIV-1 (lanes 1 to 3, respectively) and on cell-free DNA products from incubations with recombinant HIV-1 RT (lane 4), RT purified from HIV-1 virions (lane 5), and RNase H-minus HIV-1 RT (lane 6). The plus-strand sequence is shown (lanes CTAG) with HIV-1 NL4-3 sequence positions indicated in the left margin. (B) Products of linear PCR on increasing quantities of Hirt DNA (30, 45, and 60 μg in lanes 1 to 3, respectively) extracted 8 h after HIV-1 infection. The plus-strand sequence is shown (lanes GATC). (C) Summary of cPPT initiation sites used in the virus (arrow above the sequence) and in the cell-free system (arrows below the sequence). The arrows point to the 5′-terminal nucleotides of the major plus-strand DNA products after correction for +1 nucleotide addition catalyzed by *Taq* DNA polymerase. The positions indicated for the cell-free system were determined by both linear PCR (A) and blotting assays (Fig. 4B). The PPT sequence is indicated in large bold letters.

free systems that use substrates with relatively short RNA strands (23, 42).

The initiation sites utilized in our cell-free system were compared directly to those in replicating HIV-1 in culture (Fig. 6 and 7). We found that the virus also preferentially generates a plus-strand discontinuity at the cPPT. This observation is consistent with reports of others (11, 25) and presumably reflects an intermediate product after initiation of plus-strand synthesis from the cPPT. The 5′ end of plus-strand DNA initiated from the cPPT was first detectable 4 h postinfection; the amount increased at 8 h and remained constant up to 20 h after infection (Fig. 6 and data not shown). These data closely agree with previous studies of the kinetics of linear HIV-1 DNA synthesis (4, 29).

We also detected a second plus-strand discontinuity in HIV-1 DNA in extracts of replicating virus in culture (Fig. 7). The 5′ end of this plus-strand discontinuity was mapped to HIV-1_{NL4-3} sequence position 7137 (corresponding to position 7145 in HIV-1_{HXB2}). The discontinuity was detected in extracts from three independent experiments and was not an artifact generated by *Taq* DNA polymerase pausing in the mapping assay. We estimate that this discontinuity was present in 20 to 30% of replicating viruses (assuming 100% initiation at the cPPT [10, 26]). Recently Miller et al. reported multiple plus-strand discontinuities in the 3′ half of HIV-1, including one roughly mapped to a 100-nt region encompassing position 7137

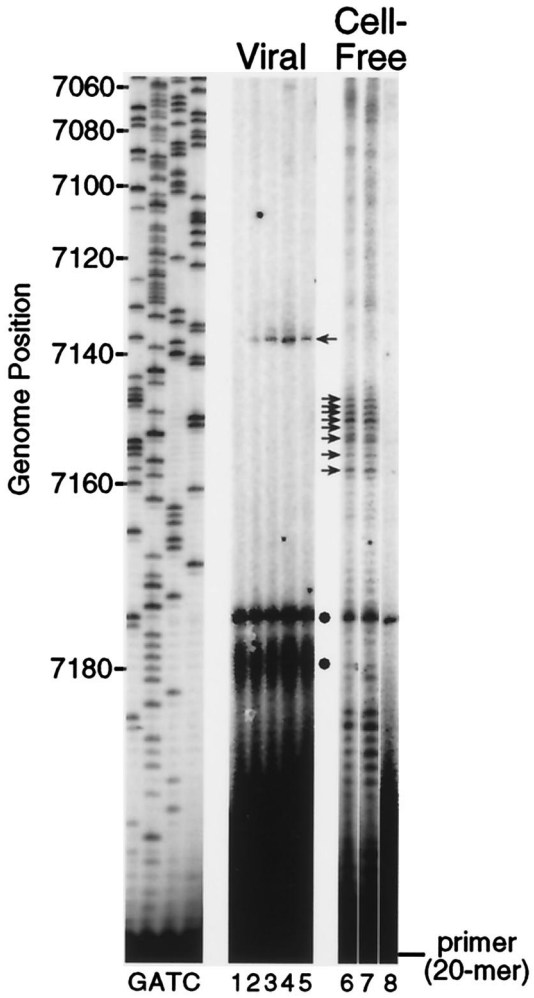

FIG. 7. Identification of a non-PPT plus-strand discontinuity in replicating HIV-1. DNA extracted from cells 0, 4, 8, 12, and 16 h after HIV-1 infection (30 μg of DNA; lanes 1 to 5, respectively) were subjected to linear PCR using 5′-^{32}P-labeled primer 7201, and the resultant PCR products were analyzed by urea PAGE as in Fig. 6. Lanes 6 to 8, products of linear PCR on cell-free reaction products from primer 7201 · *env* RNA (Fig. 1) generated by recombinant HIV-1 RT (lane 6), virion HIV-1 RT (lane 7), or RNase H-minus HIV-1 RT (lane 8). The 5′ ends of plus-strand discontinuities are indicated by arrows. Black dots indicate background bands observed in control PCRs lacking HIV-1 DNA. HIV-1 NL4-3 sequence positions are indicated alongside the plus-strand sequence lanes (GATC) in the left margin.

(38). Together these data show that HIV-1, like other retroviruses (reviewed in references 5 and 9), synthesizes viral DNA with discontinuous plus strands.

The putative primer sequence for site 7137 is purine rich and shares sequence identity with a portion of the HIV-1 PPTs. We have recently identified another HIV-1 plus-strand discontinuity in extracts of infected cells at HIV-1_{NL4-3} position 1136 (data not shown). This new plus-strand initiation site in the HIV-1 *gag* gene shows strong sequence similarity to 7137. Experiments are in progress to further characterize this and other ancillary initiation sites.

Both the frequency and specificity of plus-strand initiation in our cell-free system differed from that in replicating virus (Fig. 5C, 6, and 7). We observed notable differences in plus-strand initiation between the two systems at site 7137 as well as other sites (Fig. 5 to 7 and data not shown). Similarly, the precision of initiation within the PPTs themselves was lower in the cell-free system compared to replicating virus (Fig. 4 and 6). The plus-strand DNAs generated in the cell-free system were distributed among four major products with 5′ ends mapping at and around the 3′ margin of the cPPT. In contrast, replicating virus generated plus-strand DNAs with 5′ ends mapping almost exclusively to the A residue immediately adjacent to the cPPT. The heterogeneity of plus-strand 5′ ends in cell-free systems (Fig. 4 and 6; references 23 and 44) might be attributed to imprecise RNase H cleavage at the PPT (16). It is unclear why, in the context of replicating virus, RT/RNase H initiates plus-strand synthesis precisely from the 3′ end of the PPT (Fig. 6 and reference 11), while both recombinant HIV-1 RT and RT purified directly from HIV-1 virions are much less precise (Fig. 6). Accessory factors and/or the unique environment of the multiprotein preintegration complex (7, 8, 17, 49) may be required for efficient and precise viral DNA synthesis (27, 31).

There are several potential consequences of discontinuous plus-strand DNA synthesis during HIV-1 replication. Based on the distribution of PPT-like sequences in the HIV-1 genome and on the frequency of ancillary initiation sites detected in replicating virus (two ancillary sites detected out of ~1,800 nt analyzed [Fig. 7 and data not shown]), we estimate that as many as 10 non-PPT initiation sites could be used during HIV-1 plus-strand DNA synthesis. If initiation from each non-PPT site occurs with an efficiency comparable to that of site 7137 (20 to 30% of the cPPT [Fig. 7]), then two to three non-PPT initiation sites are likely to be used during the synthesis of any single provirus in a cell. Previously we suggested that the low processivity of HIV-1 RT might be overcome, in part, by the use of multiple plus-strand initiation sites (31). The resultant strand discontinuities would also facilitate recombination by a strand displacement/assimilation mechanism (28; reviewed in references 5 and 21). Regardless of purpose, plus-strand discontinuities must eventually be resolved in order to generate a contiguous strand. Recent studies show that this resolution occurs predominantly after viral DNA integration (38). Initiation events occurring downstream of the 3′ PPT would produce long terminal repeats defective for integration and would result in aborted replication (40, 52).

The cell-free system described in this report was designed specifically to examine the multiple molecular events surrounding initiation of plus-strand HIV-1 DNA synthesis. Cell-free systems have also been developed to study multistep mechanisms occurring early in HIV-1 DNA synthesis (tRNA priming, minus-strand strong stop DNA synthesis, and subsequent minus-strand transfers [reviewed in references 2 and 54]). When combined, these systems should provide valuable information about the complex biochemical and molecular processes required for coordinated conversion of single-stranded viral RNA to double-stranded DNA. Several lines of evidence suggest that simple cell-free systems lack one or more essential replication components (27, 31, 34, 37, 56). It will be informative to determine how other retroviral proteins found in preintegration complexes (NC, CA, MA, IN, Vpr, and p6 [7, 8, 17, 49]) affect retroviral DNA synthesis in these concerted cell-free reactions.

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