Mutating a Conserved Motif of the HIV-1 Reverse Transcriptase Palm Subdomain Alters Primer Utilization†

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ABSTRACT: In order to investigate how primer grip residues of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) contribute toward the architecture of its palm subdomain and neighboring structural elements, the DNA polymerase and ribonuclease H (RNase H) activities of enzymes bearing aromatic substitutions at Trp^{229} and Tyr^{232} of the catalytically-competent p66 subunit were evaluated. Although all mutants retained RNase H function, the manner in which different RNA−DNA hybrids were hydrolyzed was affected. Depending on the nature of the substitution, DNA-dependent DNA synthesis was (i) unaffected, (ii) interrupted shortly after initiation, or (iii) stalled when the replication machinery encountered an intramolecular duplex on the single-stranded template. Evaluating (−) strand strong-stop DNA synthesis on an RNA template derived from the viral genome raises the additional possibility that DNA and RNA primers might be differentially recognized by the retroviral polymerase. In support of this, all mutants were unable to extend the HIV-1 polypurine tract (PPT) RNA primer into (+) strand DNA, despite supporting the equivalent event from an oligodeoxynucleotide primer. Collectively, our data illustrate that subtle alterations to primer grip architecture may manifest themselves in discrimination between oligoribo- and oligodeoxyribonucleic acid primers.

The availability of high-resolution crystal structures for human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT)[1] (Kohlstaedt *et al*., 1992; Jacobo-Molina *et al*., 1993; Unge *et al*., 1994; Stammers *et al*., 1994; Ren *et al*., 1995; Rodgers *et al*., 1995) has facilitated biochemical evaluation of the manner in which this highly versatile enzyme accommodates A- and B-form nucleic acid duplexes, and non-A, non-B RNA−DNA hybrids it encounters during retroviral replication. Based on the X-ray analysis of RT complexed with a short DNA duplex, Jacobo-Molina *et al*. (1993) designated the $\beta12-\beta13$ hairpin ($Phe^{227}-His^{235}$) of the catalytically-competent p66 subunit the *primer grip*, proposing that it maintains the primer terminus in the appropriate orientation for nucleophilic attack on an incoming dNTP (Figure 1[A]). At the same time, Smerdon *et al.* (1994) noted from the RT/Nevirapine cocrystal (Kohlstaedt *et al*., 1992) that p66 residues Phe^{227}, Trp^{229}, and Leu^{234} constitute immutable residues among those defining the non-nucleoside inhibitor binding pocket. Exploiting the architectural importance of these residues may therefore provide insights into novel approaches for inhibiting HIV replication via agents inducing alterations to subunit and/or subdomain geometry ("allosteric" inhibitors). Such efforts would benefit from a detailed analysis of primer grip function and the consequence of altering its architecture for accommodation and usage of nucleic acid duplexes.

The ability to reconstitute enzymatically-active HIV-1 RT from its p66 and p51 components (Le Grice *et al*., 1991; Howard *et al*., 1991; Lederer *et al*., 1992) has been exploited for preliminary evaluation of primer grip function via alanine scanning mutagenesis of "selectively-mutated" p66/p51 heterodimers. Notable among our findings was that a Leu^{234}Ala mutation in p66 inhibited dimerization (Ghosh *et al*., 1996), while Trp^{229}Ala substitution severely compromised DNA polymerase function and viral infectivity (Jacques *et al*., 1994). Although mutating Phe^{227} revealed little difference in enzyme activity in preliminary studies, a more stringent evaluation of RT-mediated events suggests that altering this residue perturbs events specifically dependent on RNA termini, namely, RNA 5′-directed RNase H activity (De Stefano, 1995; Palaniappan *et al*., 1996, 1997) and RNA-primed synthesis of (−) and (+) strand DNA (Powell *et al*., 1997). Finally, an unexpected result was that a Tyr^{232}Ala substitution had the consequence of directing RNase H cleavage exclusively toward template nucleotide −8 rather than −17 (Ghosh *et al*., 1996), the latter of which defines the spatial separation of the DNA polymerase and RNase H catalytic centers (Kohlstaedt *et al*., 1992; Jacobo-Molina *et al*., 1993). Collectively, these studies illustrate the importance of maintaining the structural integrity of the p66 $Trp^{229}-Tyr^{232}$ quartet comprising the $\beta12-13$ connecting loop (Met^{230} and Gly^{231}) and immediately adjacent residues (Trp^{229} and Tyr^{232}). Figure 1[A] illustrates that the primer grip lies within a four-stranded β-sheet at the base of the p66 thumb. Recent mutagenesis studies have indicated that alterations to α-helix H of the HIV-1 p66 thumb subdomain result in decreased processivity of DNA synthesis (Beard *et*

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[1] Abbreviations: HIV, human immunodeficiency virus; PPT, polypurine tract; RT, reverse transcriptase.

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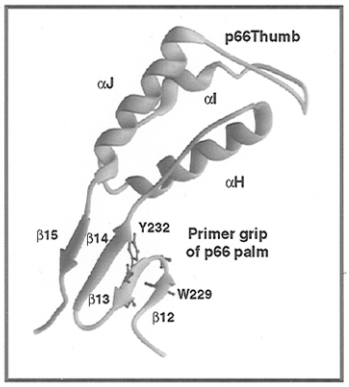
[A]



[B]

Virus	224	225	226	227	228	229	230	231	232	233	234
	Primer Grip Sequence										
HSRV	Q	K	T	V	E	F	L	G	F	N	I
HTLV-1	P	G	T	I	K	F	L	G	Q	I	I
IAP-H	A	D	T	G	L	F	L	G	S	K	I
BLV	P	S	P	V	P	F	L	G	Q	M	V
IAP-M	S	D	T	G	Q	F	L	G	S	V	V
MMTV	Y	D	N	L	K	Y	L	G	T	H	I
KERV-K	S	T	P	F	H	Y	L	G	M	Q	I
MuLV	Q	K	Q	V	K	Y	L	G	Y	L	L
FeLV	L	Q	E	V	T	Y	L	G	Y	S	L
GaLV	Q	R	E	V	T	Y	L	G	Y	L	L
HTLV-2	Q	T	K	V	T	Y	L	G	Y	L	L
BaERV	Q	T	K	V	T	Y	L	G	Y	I	L
MuRRS	Q	M	E	L	V	Y	L	R	Y	T	L
RSV	E	P	G	V	Q	Y	L	G	Y	K	L
MPMV	Q	D	P	Y	T	Y	L	G	F	E	L
HERV E	R	Q	Q	V	C	Y	L	G	F	T	I
SRV1	Q	D	P	Y	T	Y	L	G	F	E	L
SRV2	Q	D	P	Y	T	Y	L	G	F	Q	I
SMRV-H	S	E	P	F	S	Y	L	G	F	E	L
BIV	E	E	R	V	K	W	I	G	F	E	L
Visna	G	Y	P	A	K	W	L	G	F	E	L
CAEV	R	Y	P	A	K	W	L	G	Y	E	L
FIV	E	P	P	Y	T	W	M	G	Y	E	L
EIAV	V	P	P	Y	S	W	L	G	Y	Q	L
SIVmac	D	P	P	F	Q	W	M	G	Y	E	L
SIVmnd	E	P	P	F	H	W	M	G	Y	E	L
SIVagm	E	P	P	Y	E	W	M	G	Y	K	L
HIV-2	D	P	P	Y	H	W	M	G	Y	E	L
HIV-1	E	P	P	F	L	W	M	G	Y	E	L
		β11b-β12 loop		β12			β12-β13 loop		β13		

FIGURE 1: [A] Spatial relationship of the p66 primer grip motif of the p66 palm (the $\beta12-\beta13$ hairpin) of HIV-1 RT to its thumb subdomain. Subdomain designation is according to Jacobo-Molina *et al*. (1993). In addition to containing the primer grip, β-strands $11-15$ constitute a sheet at the base of the thumb. [B] Compilation of retroviral primer grip sequences. HIV-1 RT sequences between Glu^{224} and Leu^{234} serve as reference. Abbreviations: BaEV, baboon endogenous retrovirus; BIV, bovine immunodeficiency virus; BLV, bovine leukemia virus; CAEV, caprine arthritis−encephalitis virus; EIAV, equine infectious anemia virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; GaLV, gibbon ape leukemia virus; HERV, human endogenous retrovirus; HIV, human immunodeficiency virus; HSRV, human spumaretrovirus; HTLV, human T-cell leukemia virus; IAP-H, intracisternal A-particle (hamster); IAP-M, intracisternal A-particle (mouse); MMTV, mouse mammary tumor virus; MPMV, Mason−Pfizer monkey virus; MuLV, murine leukemia virus; MuRRS, murine retrovirus-related sequence; RSV, rous sarcoma virus (chicken); SIV, simian immunodeficiency virus; SMRV-H, squirrel monkey retrovirus; SRV, simian AIDS virus; Visna, visna virus (sheep).

al., 1994; Bebenek *et al*., 1995), presumably through altered contact to the nucleic acid duplex. A similar effect is realized when a segment at the tip of the thumb subdomain of the DNA polymerase Klenow fragment is deleted (Minnick *et al*., 1996). It is therefore not unreasonable to assume that altering the architecture at the base of the p66 thumb changes the geometry of this subdomain to impact on its ability to interact with the template-primer duplex.

A compilation of primer grip sequences from approximately 30 retroviral enzymes (Figure 1[B]; Xiong & Eickbush, 1990) shows a marked preference for Met or Leu at position 230, while Gly^{231} is invariant. The same compilation indicates a preference for aromatic residues with π-electron-rich side chains at positions 229 and 232. This opens the possibility that the aromatic residues might intercalate (via $\pi-\pi$ stacking interactions) with template-primer to aid in orienting the primer terminus, similar to a role proposed for Trp^{266} of the p66 thumb (Bebenek *et al*., 1995). While the majority of lentiviral enzymes share the Trp^{229}/Tyr^{232} combination at these positions, the combinations Trp/Phe, Tyr/Phe, Tyr/Tyr, and Phe/Phe appear equally acceptable by closely-related RTs. However, a striking feature of this compilation is the absence of Trp at position 232, suggesting that its bulkier indole side chain is less readily accommodated within the $\beta12-\beta15$ sheet. We therefore sought in this paper to define aromatic residues which could be tolerated at positions 229 and 232 of the HIV-1 enzyme.

Our studies have been extended to include events in the HIV replication cycle which impose a high degree of precision on the retroviral enzyme, namely, selection of the polypurine tract (PPT) primer from the RNA/DNA replicative intermediate and its extension as (+) strand DNA (Wöhrl & Moelling, 1990; Pullen *et al*., 1993; Powell & Levin, 1996). These assay systems were introduced based on recent observations from our laboratory that significant defects to RT function can only be revealed by developing screening methodologies closely mimicking *in vivo* events (Le Grice, 1995; Arts *et al*., 1996a,b). Using several such assays, data of this paper demonstrate that (a) DNA polymerase and RNase H functions of HIV-1 RT are dramatically altered by subtle alterations to the geometry of the p66 primer grip and (b) RNA and DNA primers may be differentially recognized by the replication machinery.

EXPERIMENTAL PROCEDURES

Construction and Purification of Selectively-Mutated p66/p51 HIV-1 RT. To alter Trp^{229} and Tyr^{232} of the p66 HIV-1 RT subunit, oligonucleotide-directed mutagenesis was performed using the unique site elimination (USE) mutagenesis kit of Pharmacia Biotechnology. The method is based on the procedure of Deng and Nickoloff (1992), and utilizes two primer systems to generate site-specific mutation. One primer, designated the target mutagenic primer, introduces the desired mutation, while the second primer converted a unique *Avi*II site of the p66 RT gene to a *Bfr*I site for selection of the desired mutation. Through this procedure, the single p66 mutations $W^{229}Y$, $W^{229}F$, $Y^{232}W$, and $Y^{232}F$ and the double mutations $W^{229}F/Y^{232}F$ and $W^{229}Y/Y^{232}W$ were created. The authenticity of the coding sequence of the mutated RT gene was confirmed by sequencing, using the cycle sequencing system of Life Technologies.

Mutant p66 polypeptides were reconstituted into heterodimer with wild-type p51 and purified to homogeneity by metal chelate and ion-exchange chromatography as described (Le Grice *et al*., 1995), yielding the selectively mutated heterodimers $p66^{W229Y}$/p51, $p66^{W229F}$/p51, $p66^{Y232W}$/

p51, $p66^{Y232F}$/p51, $p66^{W229Y,Y232W}$/p51, and $p66^{W229F,Y232F}$/p51. Purified proteins were stored at −20 °C in a buffer containing 50% glycerol (Le Grice *et al*., 1995) and remained stable for several months.

DNA-Dependent DNA Polymerase Activity. DNA-dependent DNA polymerase activity of each mutant was evaluated on a heteropolymeric 71-nt DNA template annealed to a 5′ end-labeled 36-nt DNA primer as described previously (Ghosh *et al*., 1996). Template and primer were hybridized at a 2:1 molar ratio in 10 mM Tris-HCl (pH 7.5), 25 mM NaCl, and 0.2 mM $MgCl_2$. Polymerase activity was monitored in a buffer of 50 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, and an equimolar ratio of RT and template-primer (0.1 pmol each). DNA synthesis was initiated by adding dNTPs to a final concentration of 50 μM. Samples were removed for analysis between 5 s and 5 min and mixed with a urea-based gel loading buffer, and the synthesis products were fractionated by high-resolution denaturing gel electrophoresis. Following autoradiography, reaction products were quantified by phosphorimaging (Molecular Dynamics). Data analysis was accomplished using Image Quant software provided by the supplier.

Oligonucleotide and $tRNA^{Lys,3}$-Primed (−) Strand Strong-Stop DNA Synthesis. RNA-dependent DNA synthesis activity was studied using an *in vitro* system resembling events during initiation of retroviral replication. This utilizes a primer binding site (PBS)-containing viral RNA template flanked by unique 5′ (U5) and repeat (R) sequences of the $HIV\text{-}1_{HXB2}$ genome as described elsewhere (Arts *et al*., 1996a,b). DNA synthesis was evaluated from 5′-^{32}P end-labeled oligodeoxy- and oligoribonucleotides hybridized to the PBS, in addition to an internally-labeled, synthetic copy of the HIV-1 replication primer, $tRNA^{Lys,3}$. RNA template and primer were annealed at a 1:1 molar ratio in buffer containing 10 mM Tris-HCl (pH 7.5) and 25 mM KCl by heating at 85 °C followed by gradual cooling to allow formation of secondary structures. Template/primer (0.3 pmol) was preincubated with RT (0.3 pmol) in 10 μL of buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, and 5 mM DTT. DNA synthesis was initiated by the addition of dNTPs to a final concentration of 200 μM and allowed to proceed for 60 min at 37 °C. Synthesis was quenched by the addition of a formamide-based loading buffer, after which the products were resolved by high-voltage denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

PPT-Primed (+) Strand DNA Synthesis. The ability to initiate DNA-dependent, (+) strand DNA synthesis from the 3′ PPT primer of HIV-1 was evaluated as described by Powell and Levin (1996). A 35-nt (−) strand DNA template containing the PPT complement and downstream 20 nt was annealed to one of the following four primers: (a) a 15-nt PPT-containing RNA, which mimics RNase H-mediated selection of the primer; this allows each mutant to extend the PPT without prior selection; (b) a 20-nt RNA primer which contains the PPT and an additional 5 nt at its 3′ terminus; this primer is used to study the ability of RT to process the PPT 3′ terminus prior to extension; (c) a 15-nt purine-rich RNA primer, containing the sequence immediately downstream of the 3′ PPT (5′-ACUGGAAGGGC-UAAU), hybridized to a 35-nt DNA primer (Powell & Levin, 1996); (d) a 15-nt DNA version of the PPT primer.

Template was annealed to each primer at a 1:10 molar ratio in 50 mM KCl by heating at 90 °C, followed by gradual cooling to room temperature. Wild-type or mutant RT was preincubated with these template/primer combinations at a 10:1 molar ratio in 10 μL of buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 6 mM DTT, and 10 units of RNasin. (+) strand synthesis was initiated by addition of dCTP, dGTP, and dTTP to a final concentration of 250 mM each, and 3 pmol of [α-^{32}P]dATP plus unlabeled dATP to a final concentration of 50 mM. Incubation was continued at 37 °C for 15 min. In this assay, DNA synthesis products were internally labeled by the addition of [α-^{32}P]dATP. The reactions were terminated by addition of formamide loading buffer. (+) strand products were fractionated by denaturing polyacrylamide gel electrophoresis (8% polyacrylamide/8 M urea) and processed as described above.

Evaluation of RNase H Activity. RNase H activities of wild-type and mutant RT were initially determined in the absence of DNA synthesis with a heteropolymeric 90-nt 5′ end-labeled RNA template-annealed to a 36-nt DNA primer, as described previously (Ghosh *et al*., 1995, 1996; Rausch *et al.,* 1996). Radiolabeled hybrid was preincubated with enzyme at a 1:2 molar ratio in 80 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 80 mM NaCl, and 5 mM DTT. Hydrolysis was initiated by the addition of $MgCl_2$ to a final concentration of 10 mM and continued incubation at 37 °C for 10 min. Samples were removed at the times indicated in the text and quenched with urea-based gel loading buffer. Products were resolved by denaturing polyacrylamide gel electrophoresis, visualized by autoradiography, and quantified by phosphorimaging (Molecular Dynamics).

In a second analysis, mutants were evaluated for their ability to cleave the PPT RNA/(+) strand DNA junction *in trans*. The 35-nt DNA template described above was hybridized to the 15-nt PPT RNA primer at a 1:10 molar ratio according to Powell and Levin (1996). The primer was fully extended by incubating with T4 DNA polymerase in the presence of [α-^{32}P]dATP in 15 μL of RT buffer for 15 min at 37 °C. This hybrid containing an RNA−DNA chimera was then incubated with wild-type or mutant RT at a 1:10 molar ratio in the same reaction buffer for 15 min at 37 °C. Reactions products were processed as described above. In this assay, correct cleavage at the PPT/(+) strand DNA junction releases a 20-nt (+) strand DNA fragment.

Gel-Mobility Shift Analysis of Replication Complexes. Binding to duplex DNA following primer extension by a single nucleotide was performed according to Guo *et al.* (1995). Template and primer were hybridized at a 1:1 molar ratio in 50 mM KCl at 90 °C with gradual cooling to room temperature. RT was preincubated with this substrate at a 10:1 molar ratio in assay buffer containing [α-^{32}P]dATP to allow a one-nucleotide extension of the primer. Glycerol was then added to a final concentration of 20% (v/v) and the complex loaded onto a native polyacrylamide gel (6%). Electrophoresis was performed in a buffer of 25 mM Tris-HCl (pH 8.0) and 62 mM glycine for 60 min at room temperature at a constant voltage of 200 V. Following electrophoresis, gels were dried and subjected to autoradiography.

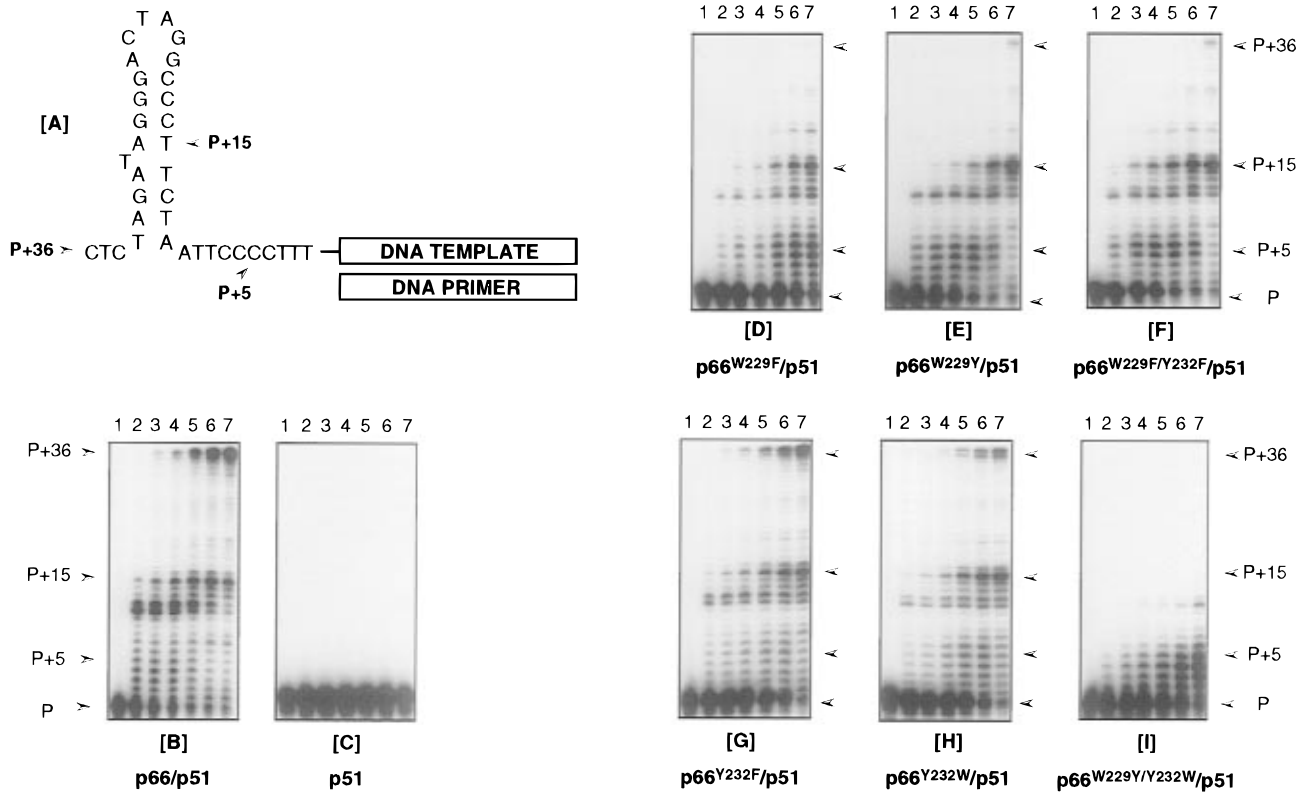


FIGURE 2: DNA-dependent DNA polymerase activity of HIV-1 RT primer grip mutants on a heterologous 71-nt/36-nt template-primer. The template-primer combination is outlined schematically in panel [A], and has been shown to contain a short hairpin on the template due to intramolecular base pairing (Wöhrl *et al*., 1995). Panels [B]−[I] illustrate a time course of DNA synthesis for each mutant, wherein lanes 2−7 represent product evaluation after 5, 15, 30, 60, 120, and 300 s, respectively. Unextended primer is represented by lane 1. The definitions P+5 etc. refer to the 3′ terminus of the extended primer. In the experiments of panels [B]−[I], the 36-nt DNA primer was ^{32}P-labeled at its 5′ terminus.

RESULTS

Primer Grip Mutations Induce Stalling during DNA Synthesis. The DNA-dependent DNA polymerase activity of each mutant was initially evaluated by determining the time-course of synthesis on a heteropolymeric template-primer whose single-stranded template contains a short region of intramolecular base pairing (Figure 2[A]). This hairpin (Wöhrl *et al*., 1994, 1995a,b) induces transient pausing of the replication machinery (possibly reflecting its gradual disruption by the fingers subdomain, thereby allowing the single-stranded template access to the catalytic center), but is eventually resolved by wild-type RT, leading to synthesis of a full-length, 72-nt product (P+36, Figure 2[B]). Under the same conditions, substitution of Trp^{229} with either Phe (Figure 2[D]) or Tyr (Figure 2[E]) enhances stalling at this hairpin, yielding minimal full-length product. A similar phenotype was noted for enzyme harboring the double mutation Trp^{229}Phe/Tyr^{232}Phe (Figure 2[F]). Visual inspection of panels 2[E] and 2[F], in addition to phosphorimaging (not shown), indicates that the original 36-nt primer was virtually exhausted after 5 min, suggesting the deficiency lay not at initiation but rather in an ability to disrupt a stretch of three consecutive G-C base pairs of the template hairpin. In addition to this pause, mutant p66^{W229F}/p51 experiences difficulty in iterative insertion of dG, indicated by accumulation of P+4−P+7 products. Collectively data of Figure 2[D]−[F] indicate that altering Trp^{229} of the primer grip may enhance stalling at regions of intramolecular base pairing.

Defects in DNA polymerase function appeared less severe; when Tyr^{232} was substituted with either Phe (Figure 2[G]) or Trp (Figure 2[H]); i.e., mutants p66^{Y232F}/p51 and p66^{Y232W}/p51 RT display slightly less activity than the wild-type enzyme, but a qualitatively similar stalling pattern. In contrast, interchanging residues 229 and 232 has severe consequences for DNA-dependent DNA polymerase function (Figure 2[I]). Ninety percent of DNA synthesis catalyzed by p66^{W229Y,Y232W}/p51 RT terminates following addition of 6 nt of the primer. Under identical conditions, p51 HIV-1 RT yields no product, ruling out the possibility that the data of Figure 2[I] reflect disruption of the mutant and residual p51 activity (in these mutants, the p51 subunit is unaltered). Furthermore, phosphorimaging analysis (not shown) indicates that the 36-nt primer is virtually exhausted over the 5-min incubation period, suggesting again that the defect does not lie at initiation. Interestingly, our template sequence demands iterative insertion of dA and dG immediately after initiation, raising the possibility that such events are compromised by altering primer grip architecture.

Stability of DNA-Dependent DNA Synthesis Complexes. To determine the relative stability of replication complexes formed by each of the primer grip mutants and a DNA primer-template duplex after single-nucleotide incorporation, we performed a gel-mobility shift assay (Figure 3). This procedure (Guo *et al*., 1995) involves extension of a DNA primer by a single, radiolabeled nucleotide, followed by fractionation of the nucleoprotein complex through a non-denaturing polyacrylamide gel. Thus, in this assay, the amount of label incorporated is a measure of polymerase activity, while the fraction of label which is shifted is a measure of RT·primer-template stability. Phosphorimaging

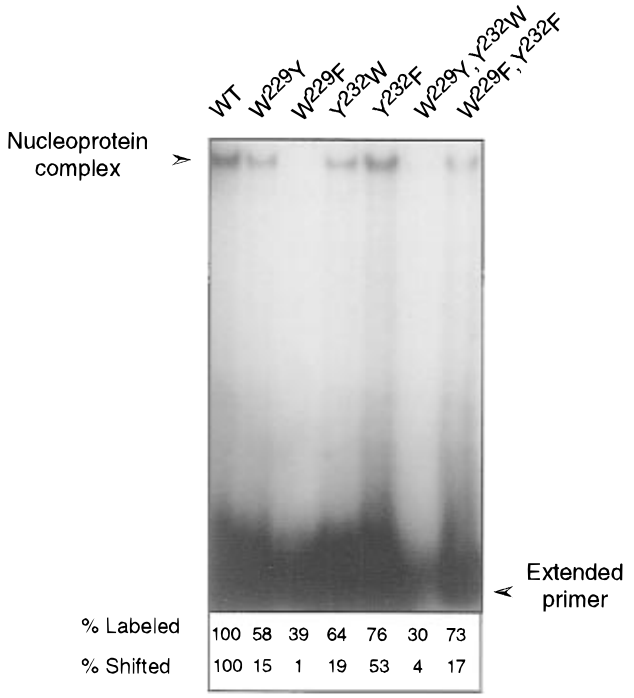


FIGURE 3: Stability of (+) strand initiation complexes. A gel-mobility shift analysis was performed immediately after a single, radiolabeled nucleotide was added to the (+) strand PPT RNA primer; i.e., the assay *simultaneously* assesses synthetic capacity and stability of the ternary complex. Values for the extent of labeling, as well as the amount of shifted complex (in each case relative to the parental enzyme), are presented at the bottom of the panel, and were determined by phosphorimaging. The nature of the p66 primer grip mutation is indicated above each lane.

can then be applied to quantify the extent of synthesis and amount of shifted complex.

As indicated in Figure 3, all mutants were slightly less active than wild-type RT in single-nucleotide addition. However, considerably greater differences were noted for the stability of the replication complexes. Although mutants p66^{W229F}/p51 and p66^{W229Y,Y232W}/p51 retained 30−40% of wild-type primer extension activity, there was a 100-fold and a 25-fold reduction in the stability of their replication complexes, respectively. Likewise, the synthetic capacity of mutants p66^{W229Y}/p51, p66^{Y232W}/p51, and p66W^{229F,Y232F}/p51 was 58−73% of the parental enzyme, while the stability of the replication complexes was reduced by 5−7-fold. In general, only mutant p66^{Y232F}/p51 provided data comparable to wild-type RT. Although essentially qualitative, the data of Figure 3 provide preliminary evidence that subtle alterations to primer grip architecture can reduce the stability of HIV-1 RT·primer-template complexes formed after addition of a single nucleotide.

Evaluation of (−) Strand Strong-Stop DNA Synthesis. In the experiments of Figures 2 and 3, the ability of primer grip mutants to accommodate and productively use duplex DNA was assessed. However, we have recently demonstrated that the nature of the primer [i.e., as the DNA strand of B-form duplex DNA *vs* an RNA strand in a non-A, non-B RNA−DNA hybrid (Federoff *et al*., 1993)] can influence its usage (Powell *et al*., 1997) Furthermore, data from our laboratory have also highlighted specific defects to tRNA-primed (−) strand synthesis (i.e., from an A-form duplex) which go otherwise unnoticed during the equivalent oligonucleotide-primed event (Arts *et al*., 1996a,b). We therefore evaluated RNA-dependent DNA synthesis using oligonucleotide and tRNA primers, the results of which are presented in Figure 4.

Data from DNA-primed (−) strand strong-stop synthesis (Figure 4, panel [B]) parallel those obtained with a heteropolymeric template-primer (Figure 2). Substituting Trp^{229} with Tyr or Phe (lanes [B]2 and [B]3, respectively) reduces the efficiency of strong-stop DNA synthesis more severely than when Trp or Phe replace Tyr^{232} (lanes [B]4 and [B]5, respectively). Likewise, the double substitution Trp^{229}Phe/Tyr^{232}Phe yields a considerably less active enzyme (lane [B]7), while interchanging Trp^{229} and Tyr^{232} results in prematurely-terminated cDNAs (lane [B]6). Surprisingly, mutant p66^{W229F}/p51 has more difficulty when synthesizing from an RNA primer than from its DNA counterpart (lane [C]3 *vs* lane [B]3), an observation which also holds true for the double mutant p66^{W229F,Y232F}/p51 (lane [C]7 *vs* lane [B]7). The opposite holds for mutant p66^{W229Y}/p51, namely, RNA-primed (−) strand synthesis (lane [C]2) is more efficient that the DNA-primed event (lane [B]2). Once again, substituting Tyr^{232} with Trp or Phe appears acceptable (lanes [C]4 and [C]5, respectively), while the Trp^{229}−Tyr^{232} interchange yields primarily stalled cDNAs (lane [C]6). Although a complex pattern emerges from these oligonucleotide-primed experiments, they are in keeping with recent data that alterations to residues immediately flanking the primer grip (Pro^{226} and Phe^{227}) result in differential primer usage (Powell *et al*., 1997).

Finally, all mutants show a dramatic reduction in synthetic capacity when challenged to catalyze the equivalent event from an intact (76-nt) tRNA primer (Figure 4, panel [D]). Only mutants p66^{Y232W}/p51 and p66^{Y232F}/p51 support this event, and even then at substantially reduced levels (lanes [D]4 and [D]5, respectively). Interestingly, we recently documented that initiation of HIV-1 (−) strand synthesis requires the replicating enzyme to disrupt critical intermolecular tRNA−viral RNA interactions immediately 5′ to the PBS (Isel *et al*., 1995; Arts *et al*., 1996a,b; Lanchy *et al*., 1996). The paucity of prematurely-terminated products in Figure 4[D] thus suggests that altering the primer grip may weaken contacts with the tRNA replication primer, or positioning of its 3′ terminus at the polymerase catalytic center, with the consequence that these loop−loop interactions cannot be disrupted. These defects can be likened to increased pausing at intramolecular structures in Figure 2.

Primer Grip Mutants Cannot Support RNA-Primed, DNA-Dependent DNA Synthesis. During (−) strand synthesis in retroviruses, the tRNA primer provides the appropriate 3′-OH for initiation (Marquet *et al*., 1995). In contrast, RNase H activity is required for selection of the appropriate primer terminus in order to initiate second or (+) strand synthesis from the PPT (Champoux, 1993). The PPT RNA/(−) strand DNA duplex may also adopt a configuration favored by the replication machinery, since this appears to be the major initiation site among potential (+) strand RNA primers, with the exception of the central PPT (Powell & Levin, 1996). The ability of primer grip mutants to support this specialized event was therefore evaluated, the results of which are shown in Figure 5.

A 20-nt (+) strand DNA is derived from reactions containing wild-type HIV-1 RT and either a 15-nt PPT

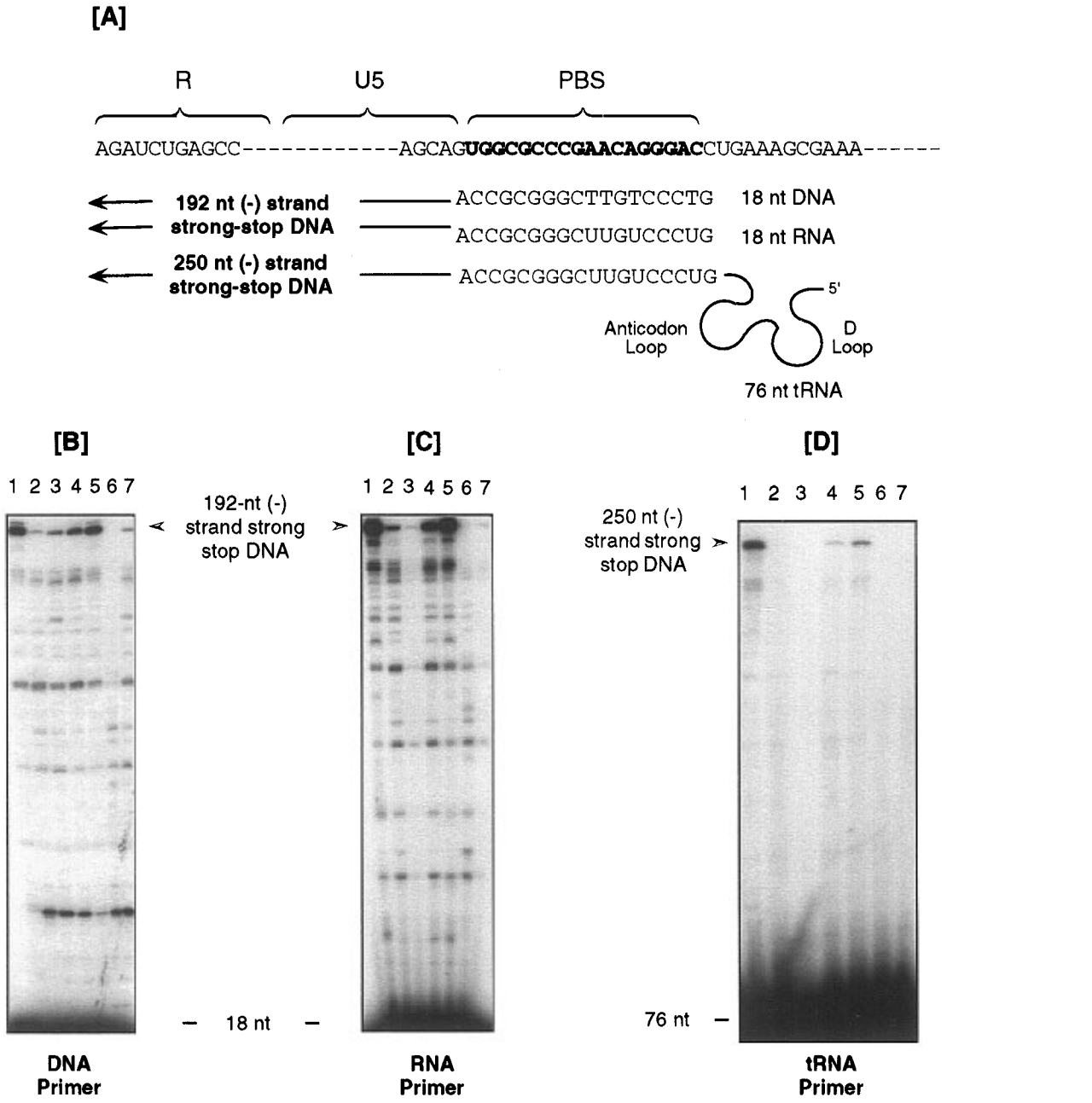


FIGURE 4: Oligonucleotide and tRNA-primed (−) RNA-dependent DNA polymerase activity of primer grip mutants. Panel [A] indicates the template-primer combinations and expected lengths of (−) strand strong-stop DNA. For these experiments, oligonucleotide primers were uniquely 5′-^{32}P end-labeled, while synthetic tRNA^{Lys,3} was internally-labeled during *in vitro* transcription. Notations R, U5, and PBS refer to repeat, unique 5′, and primer binding sites of the HIV-1 genome, respectively Panels [B]−[D] illustrate the products of DNA-, RNA-, and tRNA-primed events. For each panel, lanes 1−7 represent wild-type, p66^{W229Y}/p51, p66^{W229F}/p51, p66^{Y232W}/p51, p66^{Y232F}/p51, p66^{W229Y,Y232W}/p51, and p66$^{W229F,\ Y232F}$/p51 RT, respectively.

primer or a 20-nt derivative extended at its 3′ end by 5 nt (Figure 5[B], [i], lanes 1 and 2, respectively). These results illustrate (a) that the 15-nt PPT primer is removed from (+) strand DNA via RNase H activity and (b) that the 20-nt primer is first processed at the appropriate PPT 3′ terminus before being utilized as primer. The specificity of this event is indicated by the fact that a purine-rich RNA primer, having the sequence of the first 15 bases downstream of the 3′ PPT (Powell & Levin, 1996), when hybridized to an equivalently-sized DNA template failed to support DNA synthesis (Figure 5[B], [i], lane 3). Despite retaining DNA polymerase function, primer grip mutants were inactive in PPT-primed (+) strand synthesis (Figure 5[B], [ii]−[vii], lanes 1 and 2). In one case, i.e., mutant p66^{Y232F}/p51, no activity was seen with the 20-nt RNA primer (Figure 5[B], [v], lane 2), but a small amount of activity could be detected with the 15-nt RNA PPT primer (Figure 5[B], [v], lane 1). Interestingly, this mutant was the only one with the ability to form a nucleoprotein complex having a stability comparable to that of wild-type RT (Figure 3). A similar correlation between ability to extend an RNA PPT primer and formation of a stable complex following addition of one nucleotide has also been observed with several alanine-scanning primer grip mutants (Powell *et al*., 1997). All mutants were, however, almost as active as the parental enzyme in catalyzing extension from a PPT-bound DNA primer (Figure 5[B],

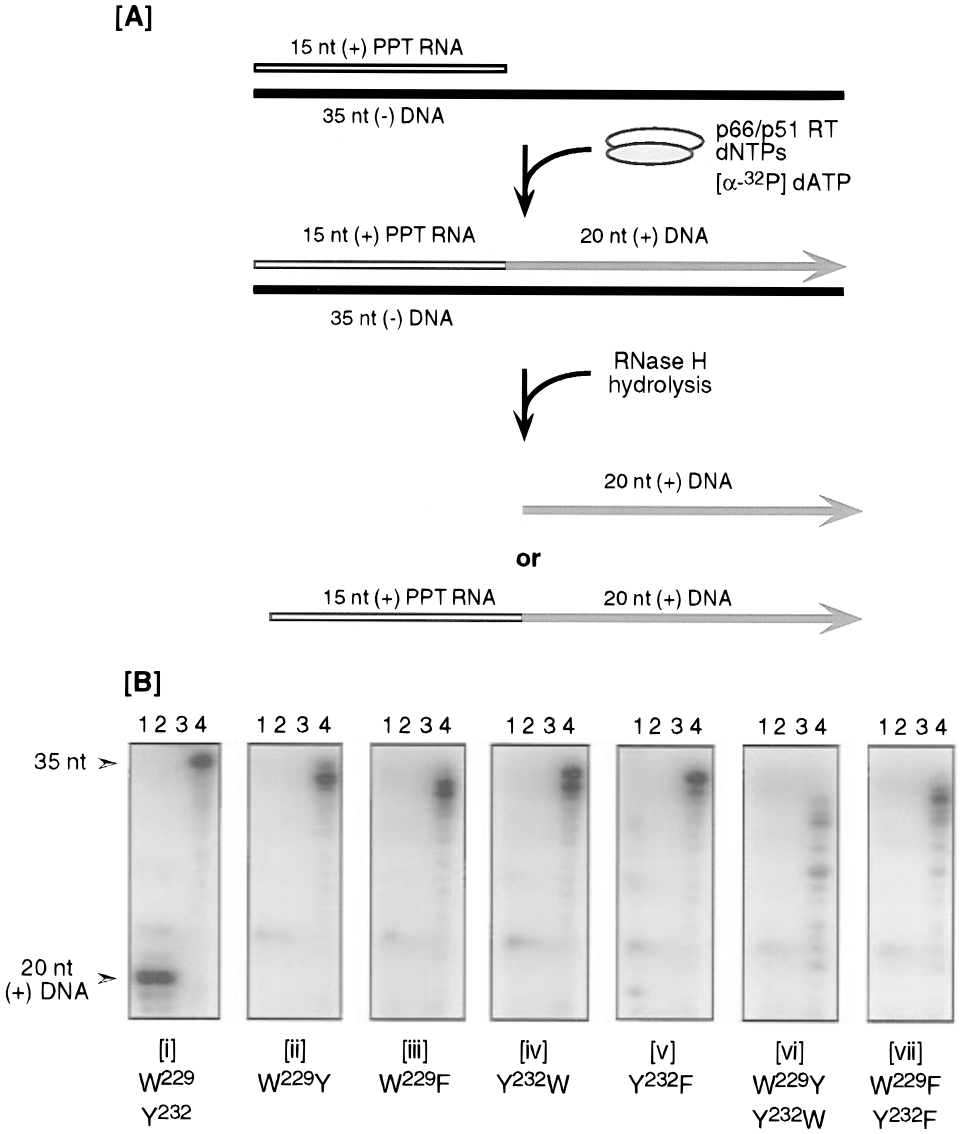


FIGURE 5: Ability of primer grip mutants to select and extend the (+) strand HIV-1 PPT primer. The assay system (Powell & Levin, 1996), outlined schematically in panel [A], comprises a 35-nt (−) strand DNA to which a PPT-containing RNA primer is hybridized. In the presence of RT and dNTPs, primer is extended into (+) strand DNA, which is freed from the RNA−DNA chimera through RT-associated RNase H activity. Inclusion of a [^{32}P]dNTP in the reaction identifies nascent, (+) strand DNA as a 20-nt product when released from the PPT, or a 35-nt fragment when present as an RNA−DNA chimera. In addition to the 15-nt RNA PPT primer, (+) strand synthesis is evaluated with (i) a 20-nt PPT RNA, extended at its 3′ terminus by 5-nt, thereby assessing the ability of the RNase H domain to select the appropriate PPT 3′-OH prior to (+) strand synthesis; (ii) a 15-nt RNA primer hybridized downstream of the PPT, which evaluates the efficiency of nonspecific RNA-primed (+) strand synthesis; and (iii) a 15-nt DNA primer hybridized to the PPT. [B] Ability of HIV-1 enzymes to use each primer for (+) strand synthesis. For each panel: lanes 1, 15-nt PPT RNA primer; lanes 2, 20-nt PPT RNA primer; lanes 3, downstream RNA primer; lanes 4, 15-nt PPT DNA primer. The migration of free (+) strand DNA (20 nt) and the predicted PPT-associated form (35-nt) is indicated. The p66 primer grip mutation is indicated below each panel.

[i]−[vii], lanes 4). As might have been predicted, mutant p66^{W229Y,Y232W}/p51 stalled at several positions of the template (Figure 5[B], [vi], lane 4).

Thus, despite their ability to initiate DNA-dependent DNA synthesis on a heteropolymeric template from a DNA primer (Figure 2), (+) strand DNA-dependent DNA synthesis from an RNA primer imposes severe constraints on mutants with an altered primer grip. The data of Figure 5 have particular importance for mutants p66^{Y232W}/p51 and p66^{Y232F}/p51, which were earlier demonstrated to be efficient DNA polymerases on a wide variety of substrates (see Figures 2 and 4).

Alterations to RNase H Functions. The inability of primer grip mutants to process and extend the PPT primer (Figure 5[B]) precluded evaluation of RNase H-mediated PPT selection. However, this could be addressed by supplying enzyme *in trans* to a preformed substrate containing the PPT RNA/(+) strand DNA chimera (Powell & Levin, 1996). The ability of T4 DNA polymerase to efficiently extend the PPT was exploited to prepare such a substrate (Figure 6[A]), and the RNase H function of each enzyme is presented in Figure 6[B]. As expected, wild-type p66/p51 RT cleaved precisely at the RNA/DNA junction to liberate a 20-nt (+) strand DNA. All primer grip mutants were capable of catalyzing the same reaction, indicating that the specificity of cleavage was not compromised. However, the efficiency of PPT cleavage was reduced with each mutant, assessed at 33−44% of wild-type by phosphorimaging.

Release of (+) strand DNA from the PPT requires single and precise endonucleolytic cleavage at the RNA/DNA

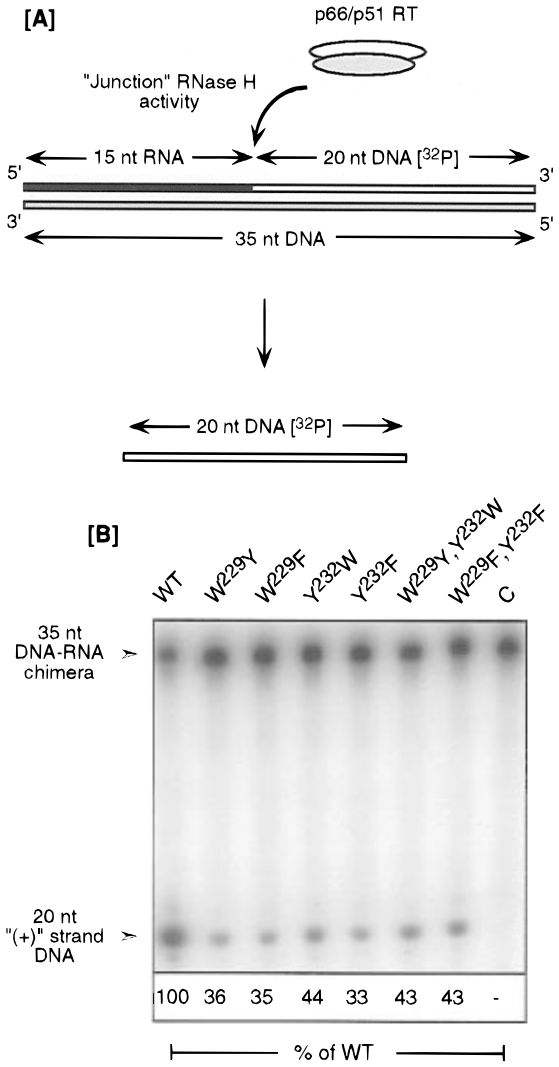


FIGURE 6: Ability of primer grip mutants to process a PPT RNA/(+) strand DNA chimera. [A] Schematic representation of the assay system. Extending the 15-nt PPT RNA primer by bacteriophage T4 DNA polymerase in the presence of a ^{32}P-labeled dNTP generates a radiolabeled 35-nt RNA−DNA chimera representative of events following initiation of (+) strand synthesis. Correct cleavage of this chimera by RT-associated RNase H at the PPT/(+) strand DNA junction releases a 20-nt DNA fragment. [B] "Junction" RNase H activity of primer grip mutants. The p66 mutation is indicated above each lane. WT, wild-type p66/p51 HIV-1 RT; C, control reaction lacking RT. The migration positions of the 35-nt RNA−DNA chimera and 20-nt (+) strand DNA are indicated at the side of the panel. Values below the panel represent the extent of RNase H-mediated cleavage, determined by phosphorimaging. Values are presented as a percentage of the wild-type enzyme activity.

junction. However, we (Schatz *et al*., 1990; Ghosh *et al*., 1995; Rausch *et al*., 1996) and others (Furfine & Reardon, 1991; Gopalakrishnan *et al*., 1992; Peliska & Benkovic, 1992; DeStefano *et al*., 1993; Post *et* al., 1993; Palaniappan *et al*., 1996) have shown that RNase H activity of HIV-1 and related retroviral RTs is a combination of endonuclease (or synthesis-dependent) and directional processing (or synthesis-independent) activities, the latter of which contributes toward efficient DNA strand transfer (Cirino *et al*., 1995; Ghosh *et al*., 1995; C. E. Cameron and S. F. J. Le Grice *et al*., unpublished observations). RNase H activity was therefore determined on a second substrate, comprising a 90-nt ^{32}P end-labeled RNA template hybridized to a 36-nt DNA primer (Figure 7[A]). A typical hydrolysis profile for wild-type RT is given in Figure 7[B], indicating the major 71- and 62-nt hydrolysis products and the temporal sequence with which they arise. A qualitatively similar profile was obtained for mutants p66^{W229Y}/p51 (Figure 7[C]) and p66^{W229Y,Y232W}/p51 (Figure 7[G]). Although the rate of hydrolysis was altered, the profiles of mutants p66^{Y232W}/p51 (Figure 7[E]) and p66^{Y232F}/p51 (Figure 7[F]) were qualitatively similar to those of the wild-type enzyme; i.e., the −17 hydrolysis product precedes the −8 product. However, an altered RNase H hydrolysis profile was highlighted with two mutants. The most prominent of these was p66^{W229F,Y232F}/p51 RT (Figure 7[H]), where the −8 hydrolysis product accumulates faster than the −17 intermediate. A similar phenotype, but with slightly reduced hydrolysis kinetics, was also evident for p66^{W229F}/p51 RT (Figure 7[D]). These enzymes differ from wild-type RT, which generates the −17 cleavage intermediate almost quantitatively from the intact substrate before hydrolyzing this as far as position −8. Phosphorimaging was used to quantify differences in the rate of appearance of the −17 and −8 intermediates for three mutants (p66^{Y232W}/p51, p66^{W229F}/p51, and p66$^{W229F/Y232F}$/p51), the results of which are shown in Figure 8. Although unusual, the properties of p66^{W229F,Y232F}/p51 and p66^{W229F}/p51 RT can be likened to those of a recently-documented mutant (p66^{Y232A}/p51; Ghosh *et al*., 1996), which we proposed might result from slippage of the nucleic acid duplex and repositioning of template nucleotide −8 at the RNase H catalytic center.

DISCUSSION

Given the importance of the primer grip motif of HIV-1 RT (Jacques *et al*., 1994; Ghosh *et al*., 1996), the present study was undertaken with two goals. First, we were prompted to evaluate the consequences of aromatic substitutions at positions 229 (Trp) and 232 (Tyr) of the p66 β12−β13 hairpin, since sequence compilations (Xiong & Eickbush, 1990; Figure 1[B]) indicate that alternative residues can be accommodated at either position by related retroviral enzymes. Second, we wished to assess enzymatic functions with experimental systems reflecting specific steps in retroviral replication, using the precedent that increased sophistication of the screening methodology aids detection of defects in RT function which would otherwise go unnoticed (Arts *et al*., 1996a,b). In following this strategy, we demonstrate here that Trp^{229} and Tyr^{232} of the primer grip are sensitive to aromatic substitutions, and that enzymes otherwise retaining polymerase and RNase H activity fail to support steps requiring a specialized form of that particular function.

Notable among our observations was the inability of primer grip mutants to initiate (+) strand DNA synthesis from the PPT RNA primer (Figure 5), despite (a) retaining RNase H (Figures 6 and 7) and DNA polymerase activities (Figure 2) mandated by this event, and (b) catalyzing (+) strand synthesis from a related DNA version of the PPT primer. The hybrid of (+) strand PPT RNA and (−) strand DNA specifying initiation of (+) strand synthesis thus appears to represent a structure uniquely sensitive to primer grip alterations, which otherwise permit DNA synthesis. In support of this contention, it has recently been demonstrated by spectropolarimetry that the PPT-containing hybrid differs significantly from other RNA−DNA hybrids (Powell & Levin, 1996). Conceivably, the (+) strand initiation complex

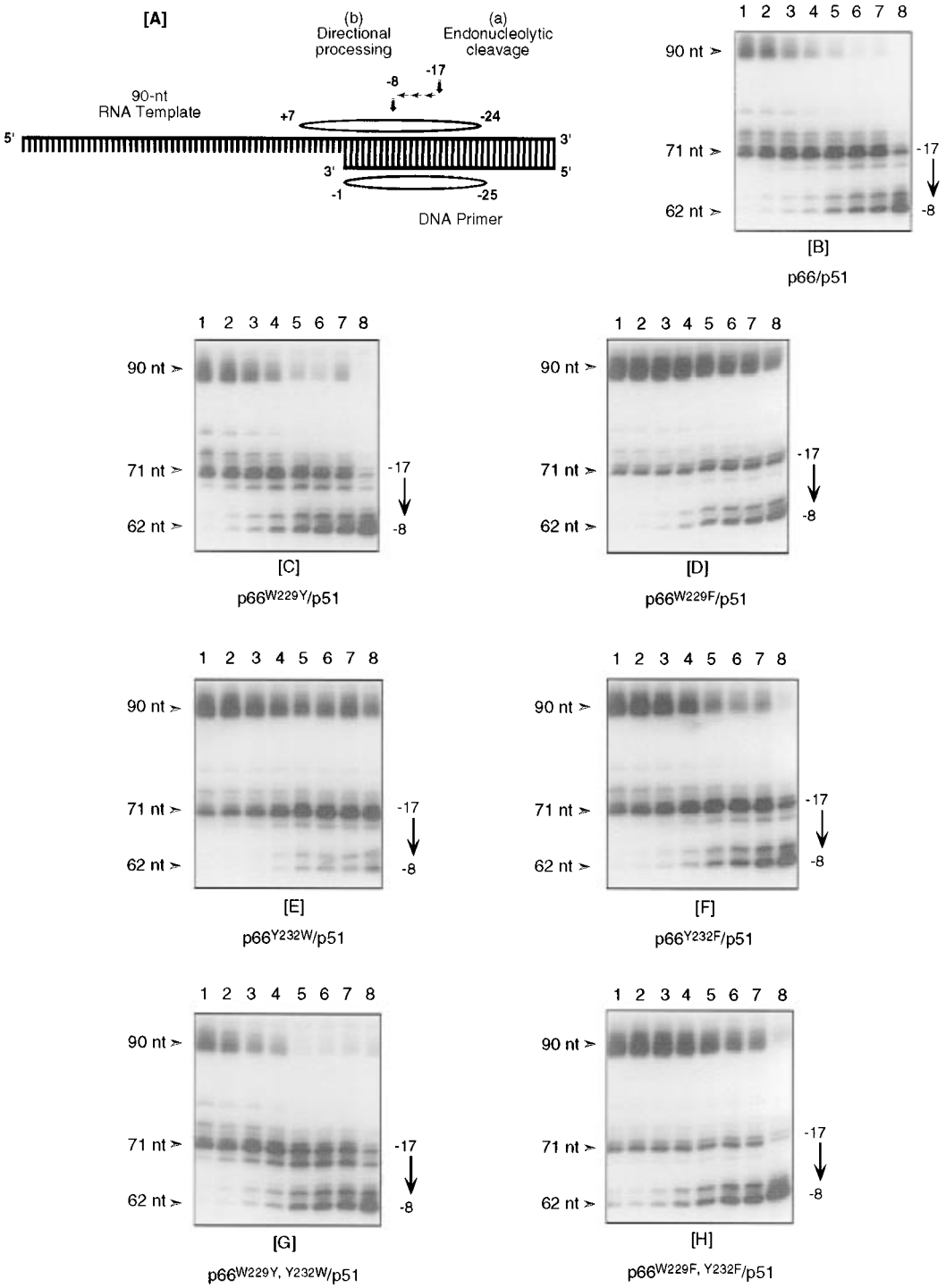


FIGURE 7: RNase H hydrolysis profiles of primer grip mutants. The substrate of panel [A] comprises a 5′ end-labeled 90-nt RNA template with a 36-nt DNA primer hybridized to the 3′ terminus. Template nucleotides +7 and −24 and primer nucleotides −1 and −24 indicate the extent to which the template-primer duplex is occupied by HIV-1 RT (Wöhrl *et al*., 1995b). Endonucleolytic and directional processing RNase H activities, giving rise to −17 (71-nt) and −8 (62-nt) hydrolysis products, respectively, have been illustrated. A typical RNase H profile from wild-type HIV-1 RT is presented in panel [B], and those of the mutants in panels [C]−[H]. Numbers on the left of each panel refer to the size of the template hydrolysis product, while those on the right indicate its position of hydrolysis. For each panel, lane designations 1−8 represent product analysis after 5 s, 15 s, 30 s, 1 min, 2 min, 3 min, 4 min, and 10 min, respectively.

may be "strained" when required to accommodate this structurally-unique hybrid, and altering primer grip geometry would have the consequence of positioning the primer terminus unfavorably for attacking an incoming dNTP. Alternatively, general contacts between the p66 thumb subdomain and the nucleic acid hybrid may be weakened when the primer grip architecture is compromised, thereby resulting in template slippage.

A clue to understanding the consequences of primer grip mutations might lie in the DNA synthesis pattern of Figure 2, where the replication machinery must disrupt a small region of intramolecular base pairing in order to synthesize as far as the template 5′ terminus. Replacing Trp^{229} with Tyr or Phe, or the double $Trp^{229}Phe/Tyr^{232}Phe$ alteration, has the consequence of halting RT primarily within the hairpin, and at its most stable position, namely, a stem comprising

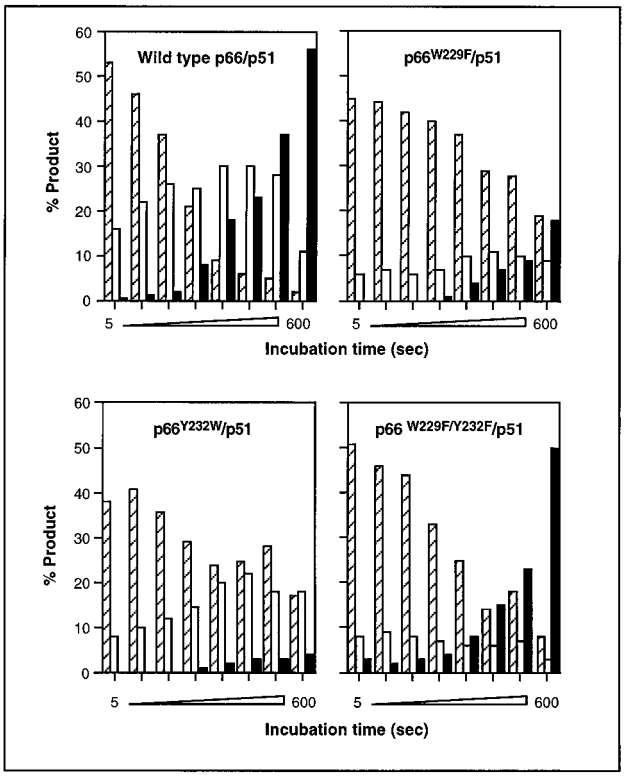


FIGURE 8: Quantitative evaluation of the RNase H hydrolysis profiles of wild-type p66/p51 RT and primer grip mutants p66^{W229F}/p51 and p66^{Y232W}/p51 and the double mutant p66$^{W229F/Y232F}$/p51 via phosphorimaging. For each enzyme, loss of the initial 90-nt substrate (dashed boxes) is presented together with accumulation of the 71- (−17) and 62-nt (−8) products (open and filled boxes, respectively).

three consecutive G-C base pairs (P+15). Thus, DNA synthesis *per se* appears less affected than the ability of the N-terminal p66 fingers subdomain to unwind this structure before the template is introduced at the catalytic center. As outlined above, minor alterations to the architecture of the β-sheet at the base of the p66 thumb (Figure 1[A]) may introduce flexibility into this subdomain, with the consequence that contact of α-helix H with the DNA template is reduced, resulting in DNA slippage. A similar interpretation has been proposed for thumb mutants of HIV-1 RT (Bebenek *et al*., 1995) and the DNA polymerase Klenow fragment (Minnick *et al*., 1996). It is not unreasonable to consider that the ability of the fingers subdomain to interact with, and displace, intra- or intermolecular template duplexes would likewise be controlled by the rigidity with which the template-primer duplex is accommodated in the nucleic acid binding cleft. In favor of this postulate, mutants p66^{Y232W}/p51 and p66^{Y232F}/p51 are active in oligoribonucleotide-primed (−) strand synthesis, yet inefficient when catalyzing the same event from the intact HIV-1 replication primer tRNA^{Lys,3} (Figure 4). This is a step we and others have demonstrated is critically-dependent on disruption of intermolecular tRNA−viral RNA loop−loop interactions (Isel *et al*., 1995; Arts *et al*., 1996a,b; Lanchy *et al*., 1996). The same proposal may also hold true for mutant p66^{W229Y,Y232W}/p51, whose DNA-dependent DNA synthesis activity is >90% inhibited after extending a DNA primer by 5−7 nucleotides. Based on enzymatic footprinting experiments of HIV-1 replication complexes (Wöhrl *et al*., 1995a,b) and the sensitivity of RT to ddNTP inhibition as a function of the single-stranded template overhang (Boyer *et al*., 1994), the fingers subdomain would be predicted to contact the single-stranded template ~6−7 nt ahead of the DNA polymerase catalytic center. The fingers subdomain of an enzyme stalled at position +4, as noted for p66^{W229Y,Y232W}/p51 RT, would be positioned directly at the base of the template hairpin. A complete inability of the fingers subdomain to resolve this structure would be reflected in cessation of DNA synthesis shortly after initiation, as we have observed experimentally in Figure 2[I]. Although further experimentation will be required to provide more accurate mechanistic information, our data strengthen the need to establish more sophisticated screening methods for evaluating *modes* of polymerase and RNase H activity rather than *overall levels* of activity (Le Grice, 1995).

While a complex pattern arises from our study, a consistent feature appears to be the differential response to the nature of the primer during DNA synthesis. Figure 2 indicates alterations to processivity of DNA-dependent DNA synthesis, although the rate at which the 36-nt primer is exhausted does not markedly differ between mutants (M. Ghosh, unpublished results). In contrast, Figure 5 indicates that these mutants are deficient in RNA-primed, DNA-dependent DNA synthesis from the PPT, while DNA-primed, DNA-dependent DNA synthesis is achieved on the same template, differing only in alterations to processivity of mutant p66^{W229Y,Y232W}/p51. Events at the DNA polymerase active site thus appear sensitive to the nature of the template-primer duplex, which in the case of Figure 5 compares a non-A/non-B RNA−DNA hybrid with B-form duplex DNA. To a lesser extent, the notion of differential primer usage holds true during (−) strand strong-stop DNA synthesis (Figure 4), where p66^{W229Y}/p51 RT catalyzes RNA-primed events more efficiently than mutant p66^{W229F}/p51, but the opposite is evident during DNA-primed events on the same template. Furthermore, we recently demonstrated that the RNA-primed (−) strand DNA synthesis capacity of HIV-1 RT mutants p66^{P226A}/p51 and p66^{F227A}/p51 is affected to a significantly greater extent than DNA-primed events on the same template. Furthermore, these mutants are unable to initiate (+) strand synthesis from the PPT RNA primer, while doing so from its DNA counterpart (Powell *et al*., 1997).

Finally, it is important to stress the value of assessing alterations to the polymerase domain in the context of RNase H function and *vice versa*, both qualitatively and quantitatively. An example of this is p66^{W229Y,Y232W}/p51 RT which, despite severely altered DNA polymerase function, retains full RNase H activity, eliminating the possibility of gross structural changes in the heterodimer. Second, we have highlighted additional RT mutants, p66^{W229F}/p51 and p66^{W229F,Y232F}/p51, which favor cleavage of an RNA−DNA hybrid at template nucleotide −8 over the position conventionally determined by the spacial separation of the catalytic centers (−17). Although an explanation for preferential −8 cleavage remains to be established, agents altering RNase H specificity through perturbation of primer grip architecture could render the second DNA strand transfer event of HIV replication (Telesnitsky & Goff, 1993) amenable to therapeutic attack. In this case, pausing of the replication machinery after copying 18 nt of tRNA^{Lys,3} allows RNase H-mediated cleavage at the penultimate ribonucleotide of the (−) DNA/tRNA junction and intramolecular DNA strand transfer (Telesnitsky & Goff, 1993; Ben Artzi *et al*., 1996). However, should RNase H cleavage occur 8 nt behind the

replication machinery, this might alter the efficiency of strand transfer by reducing homology between the donor and acceptor templates from 17 to 8 nt. In addition, should strand transfer be permitted, allowing continued (+) strand synthesis, incomplete tRNA removal may have the consequence that additional sequences would be copied at the terminus of the 3′ LTR, which may alter recognition by HIV integrase. While perhaps a long-term goal, the success of such novel strategies will depend on the ability to highlight the qualitative differences in RT function outlined here.

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