HIV-1 reverse transcription is initiated from a primer binding site (PBS) located in the 5′-region of the genomic RNA. Additional interactions between the genomic RNA and the primer tRNA have been described in several retroviruses, including avian retroviruses, HIV-2 (6), and feline immunodeficiency virus (7), but not in murine leukemia virus (8).

In HIV-1, several alternative interactions between the viral RNA (vRNA) and tRNA^\text{Lys}_{\text{A}}^\text{Lys} have been proposed, but no consensus model has been reached. The most detailed in vitro structural studies of the initiation complex of reverse transcription were performed on the HIV-1 MAL isolate (9–11). They allowed us to build secondary (9) and tertiary (11) structure models of the MAL initiation complex. In these models, parts of the PBS and the downstream region (14) (Fig. 1) were required for efficient initiation of HIV-1 MAL reverse transcription in vitro (12, 13). However, no ex vivo data on the MAL reverse transcription is available. MAL is a complex recombinant of HIV-1 subtypes A, D, and I that contains a 23-nucleotide duplication, whereas other studies supported different intermolecular interactions (17) (analogous to helices 5D and 3E in Fig. 1b). Long-term replication experiments with mutant HXB2 viruses designed to use a non-cognate tRNA as primer (tRNA^\text{Lys}_{\text{A}}^\text{Lys}) indicated a functional role for the interaction between the anticodon and A-rich loops. Indeed, whereas mutant HXB2 viruses in which only the PBS was adapted were unstable and rapidly reverted, mutant HXB2 viruses in which the PBS and the viral A-rich loop were simultaneously adapted either to tRNA^\text{Lys}_{\text{A}}^\text{Lys} (18–20), tRNA^\text{Lys}_{\text{A}}^\text{Lys} (21), tRNA^\text{Lys}_{\text{A}}^\text{Lys} (22), or tRNA^\text{Lys}_{\text{A}}^\text{Lys} (23) could stably use these tRNA species as primer. Finally, in vitro and ex vivo analysis of HXB2 mutated in helix 1 (Fig. 1c) suggested another intermolecular interaction (between the 5′-strand of helix 1 and the T\text{V}C arm of tRNA^\text{Lys}_{\text{A}}^\text{Lys}), which has been named the PAS (primer activating signal)-anti-PAS interaction (24, 25).

Several factors complicate the study of the initiation of reverse transcription and may explain these discrepancies. First, several functional sites overlap in the PBS domain, and mutations may affect several steps in the HIV-1 life cycle (26, 27). Second, the HIV-1 RNA structure is versatile (14, 28), and

Reverse transcriptase (RT), an RNA- and DNA-dependent DNA polymerase that also harbors an RNase H domain, converts the single-stranded genomic RNA of retroviruses into a double-stranded DNA with duplicated long terminal repeats (1). It initiates DNA synthesis from a tRNA, tRNA^\text{Lys}_{\text{A}}^\text{Lys} in the case of the type 1 human immunodeficiency virus (HIV-1), that is selectively encapsidated into the viral particles (2, 3). During budding and maturation of the retroviral particles, the 18 3′-terminal nucleotides of the primer tRNA are annealed to the viral RNA prior to tRNA^\text{Lys}_{\text{A}}^\text{Lys} annealing, thus explaining that extensive interactions with the primer are not required. Interestingly, such interactions are required in HXB2 mutants designed to use a non-cognate tRNA as primer (tRNA^\text{Lys}_{\text{A}}^\text{Lys}) (21). In the latter case, the extended interactions are required to counteract a negative contribution associated with the alternate primer.

This work was supported by grants from the Agence Nationale de Recherches sur le SIDA (ANRS) (to C. E. and R. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Genetics and Microbiology, Faculty of Medicine, University of Geneva, Switzerland.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www.jbc.org

Received for publication, April 22, 2004, and in revised form, June 10, 2004
Published, JBC Papers in Press, June 11, 2004, DOI 10.1074/jbc.M404473200

Valérie Goldschmidt‡, Jean-Christophe Paillart‡, Mickaël Rigourd‡, Bernard Ehresmann‡, Anne-Marie Aubertin‡, Chantal Ehresmann‡, and Roland Marquet‡

From the Unité Propre de Recherche 9002 du CNRS conventionnée à l’Université Louis Pasteur, IBMC, 15 rue René DESCARTES, 67084 Strasbourg Cedex, France and the Institut de Virologie, UMR544 INSERM-Université Louis Pasteur, 67000 Strasbourg, France

Structural Variability of the Initiation Complex of HIV-1 Reverse Transcription*
mutations may induce aberrant structures, both in vitro and ex vivo, that obscure analysis (29). Third, in vitro systems used to analyze the initiation of reverse transcription might not adequately reflect the in vivo situation. Finally, the possibility remains that the structure of the initiation complex might not be conserved among HIV-1 isolates.

In order to circumvent these problems and to compare the in situ structure of the MAL and NL4.3 initiation complexes of reverse transcription, we performed the first structural probing of the HIV-1 genomic RNA in infected cells (i.e., prior to tRNA\textsuperscript{A\textsubscript{L}} annealing) and in viral particles (i.e., after tRNA\textsuperscript{A\textsubscript{L}} annealing and virus maturation). These structural data were consistent with the in vitro probing data we collected on the MAL, NL4.3, and HXB2 complexes, and validated the in vitro functional studies we performed on the wild-type (WT) MAL and HXB2 isolates and on HXB2 mutants utilizing tRNA\textsubscript{His} as primer. Taken together, our results show that in HXB2/NL4.3 and, according to extensive sequence analysis, in 86% of HIV-1 isolates, there is no stable interaction between the anticodon loop of tRNA\textsuperscript{A\textsubscript{L}} and the A-rich loop. This loop-loop interaction clusters to the A and G HIV-1 subgroups and recombinants of the former subgroup, including MAL. In HIV-1 MAL, this interaction counteracts a negative contribution of the vRNA. This interaction also exists in the mutant HIV-1 HXB 2 viruses using tRNA\textsubscript{His} as primer, where it neutralizes a negative contribution of the tRNA\textsubscript{His} primer. This last result explains why these mutant HXB2 viruses do not behave as the WT HXB2 isolate.

**EXPERIMENTAL PROCEDURES**

**Primers, Templates, and RT Used for in Vitro Studies—**Native tRNA\textsuperscript{A\textsubscript{L}} was purified from beef liver as described (30). Its sequence and post-transcriptional modifications are identical to those of human tRNA\textsuperscript{A\textsubscript{L}}. Synthesis and purification of tRNA\textsubscript{His} were previously described (31). ORN\textsubscript{His} and ORN\textsubscript{His} were obtained from Dharmaco Research Inc. Primers were \textsuperscript{32}P-labeled at their 5'-end according to published procedures (11).

WT MAL, WT HXB2, HXB2 His, and HXB2 His-AC-GAC RNAs were transcribed in vitro and purified as described (31). The MAL and HXB2-derived RNAs correspond to nucleotides 1–311 and 1–295 of the genomic RNA, respectively. A plasmid expressing HXB2 HIV-1 RT was kindly provided to us by Dr. Torsten Unger (Upsala, Sweden), together with the protocols for protein overexpression and purification.

**Annealing of the Primers to the Templates—**Viral RNA and tRNA\textsuperscript{A\textsubscript{L}}, tRNA\textsubscript{His}, ORN\textsubscript{His}, or ORN\textsubscript{His} were denatured in water for 2 min at 90 °C and chilled on ice. Annealing was performed at 70 °C for 20 min in sodium cacodylate (pH 7.5) 50 mM, KCl 300 mM. Annealing efficiency was routinely checked by native polyacrylamide gel electrophoresis and was found to be higher than 95% in all experiments.

**Minus Strand Strong Stop DNA Synthesis—**In a standard experiment, vRNA was annealed with \textsuperscript{32}P-labeled primer (10 nm final concentration) and preincubated for 4 min at 37 °C with 25 nm RT in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 6 mM MgCl\textsubscript{2}, 1 mM DTE. Reverse transcription was initiated by adding dNTP (50 \muM each). At times ranging from 15 to 60 min, DNA synthesis was stopped by an equal volume of formamide containing 50 mM EDTA, and the products were analyzed on 8 or 14% denaturing polyacrylamide gels and quantified with a Bioimager BAS 2000 (Fuji).

**Data Analysis—**Curve fitting was performed with version 4.08 carbon of the Igor Pro Software (WaveMetrics Inc.). Global curve fitting was performed as described in the software manual. In this procedure, data sets obtained with different primers were simultaneously fit with a bi-exponential function. The amplitude parameters were considered as "local" parameters that could adopt different values for each primer, whereas the fast and slow rate constants were considered as "global" parameters with identical values for the two primers. The global fitting approach was validated by the limited increase of the \textit{k} value divided by the number of data points, as compared with "simple" fitting, despite the fact that the number of adjustable parameters was reduced. In all cases, this value was < 6 \times 10^{-4}.

**Chemical Probing of tRNA in Vitro—**After hybridization, the vRNA/tRNA complexes were incubated at 20 °C for 15 min in the annealing buffer supplemented with 5 mM MgCl\textsubscript{2} before probing with dimethyl sulfate (DMS). After addition of 1 \muM of yeast total tRNA, RNA was modified by addition of 1 \muM of 10-fold diluted DMS for 5 or 10 min. RNA modification was stopped with 200 \muM of ethanol and 50 \muM of sodium acetate (0.3 M, pH 5.3) containing 1 \muM of glycogen. Modified bases were detected by primer extension with reverse transcriptase as previously described (14).

**Infectious Molecular Clones—**The HIV-1 NL4.3 molecular clone was used to generate a chimerical construct, pNL4.3/MAL, containing nucleotides 61–274 from the MAL isolate substituted for the homologous NL4.3 region. To obtain this construct, the AflII-BssHII fragment of pJCB (32) was PCRamplified using primers p8-I5/MAL (5'-GTC TCT GTT AGA CCA G-3') and pA5S-BssHII (5'-CCG CCTT GGC TCT TGC C GTT CCC CGC TTC AAC AGG CGG CC-3'). The annealing of nucleotides correspond to changes introduced to restore a subtype B dimerization initiation site (33) and allow subsequent cloning into the pNL4.3 molecular clone. The PCR product was digested with AflII and BssHII and cloned between the same restriction sites of pNL4.3 (34), generating pLTR5-MAL. Finally, the AattI-SphI fragment of pLTR5-MAL was substituted for the homologous region of pNL4.3.

**Cell Culture, Transfection, and Infection—**HeLa cells were grown in Dulbecco's modified Eagles's medium. CEMx174 cells were grown in RPMI 1640 medium. All cells were supplemented with 10% heat-inactivated fetal calf serum. HeLa cells were transfected with 10 \muM of WT and mutant plasmids using the calcium phosphate coprecipitation method. Progeny viruses were quantified by a micro RT assay of the cell culture supernatants (35). Five million CEMx174 cells were infected with equivalent amounts of virus as determined by RT activity. One hour after infection, the cells were diluted in 20 ml of RPMI 1640.

**In Vivo RNA Modification—**At 72 h after infection, CEMx174 cells were washed twice with phosphate-buffered saline and suspended in 30 \muL of this buffer. Progeny viruses were clarified by centrifugation and passed through a 0.22-\mum cellulose acetate filter (Millipore). The culture supernatant was then pelleted through a 20% sucrose cushion by ultracentrifugation with a SW28 rotor at 27,000 rpm for 2 h at 4 °C. Virus pellets were suspended in 90 \muL of phosphate-buffered saline. Cells and viruses were treated with 3 \muM of DMS for 4 and 8 min at 37 °C. Reaction was stopped by adding 1 ml of Tris(1 mM), and RNA was extracted as described by the supplier. Modified bases were detected by primer extension as described (14).

**Sequence Alignment—**An alignment of the 538 HIV-1 sequences encompassing nucleotides 550–700 was recovered from the HIV sequence data base (www.hiv.lanl.gov) (36). (Numbering is according to the U3 start of HXB2, it corresponds to nucleotides 95–245 of the HXB2 genomic RNA). This alignment was checked with ClustalX (37) and improved manually in a text editor.

**RESULTS**

In order to gain further insight into the initiation complex of HIV-1 reverse transcription and to conciliate divergent results, we studied the structure of this complex ex vivo, thus preventing any potential artifact due to in vitro primer annealing and RNA renaturation, and to the absence of viral and/or cellular cofactors. In addition, we compared the initiation complexes formed by the MAL and NL4.3/HXB2 isolates. These isolates significantly differ in their sequence and RNA secondary structure of the PBS domain before tRNA\textsuperscript{A\textsubscript{L}} annealing (Fig. 1, a and c).

**In Situ and in Vitro Structural Probing of NL4.3 and HXB2 Initiation Complexes—**DMS, which modifies adenines at position N1 and cytosines at N3 when the Watson-Crick side of these nucleotides is not base-paired (38), has been extensively used to probe the structure of retroviral initiation complexes in vitro (8–11, 13, 24, 29, 31, 39). In order to extend these results to more biologically relevant contexts, we probed the structure of the vRNA directly in infected cells (i.e., prior to tRNA\textsuperscript{A\textsubscript{L}} annealing) and in viral particles (i.e., after tRNA\textsuperscript{A\textsubscript{L}} annealing and virus maturation). Infected cells and purified virions were treated with DMS, and the RNA was extracted after chemical modification. Modified nucleotides in the genomic RNA were specifically detected by extension of primers complementary to sequences located 3' of the major splice donor site, which could not anneal to spliced HIV RNA. The results described below
represent the first successful in situ probing of HIV RNA.

We first studied CEMx174 cells acutely infected with NL4.3, as well as their progeny virions (Fig. 2a). In the absence of DMS treatment, a strong pause was observed in the PBS of NL4.3 RNA extracted from cells, but not in the RNA extracted from virions. This specific pausing might be due to RNA degradation by cellular RNases during RNA extraction, or to selective exclusion of the degraded RNA from the virions. This pause decreased further extension of the primer, producing a slightly weaker signal in the cells, as compared with virions. The reactivity profile of NL4.3 RNA in cells is fully compatible with the secondary structure model presented in Fig. 1c: most reactive nucleotides are located in bulges (i.e. A-132, A-133, A-159), internal and apical loops (i.e. C-151, C-152, A-157, A-170, A-171, A-172) and in the single-stranded stretch 3' to the PBS; a few are located in unstable helical regions.

When compared with cells, nucleotides A-191, A-192, C-193, A-194, and A-198 are protected in virions, as expected from tRNA\textsubscript{3\text{\Lys}} annealing to the PBS. However, this protection was incomplete, in keeping with previous studies revealing suboptimal PBS occupancy in virions (40). Beside these changes, the DMS modification pattern of NL4.3 RNA was remarkably similar in cells and in virions, with only minor differences in the band intensities. The most pronounced differences were located at A-216, A-147, and C-151, which were reactive in the cells, and became protected in the virions (Fig. 2a). Remarkably, adenes 168–170 were not protected in virions, indicating that the tRNA\textsubscript{3\text{\Lys}} anticodon loop did not interact with the NL4.3 A-rich loop (Fig. 2a). Taken together, our in situ probing experiments on NL4.3 RNA indicated that this RNA underwent little, if any, structural rearrangement during formation of the initiation complex of reverse transcription. In addition, they did not reveal any interaction between tRNA\textsubscript{3\text{\Lys}} and the vRNA outside the PBS.

In order to check if the same complex was formed in vitro in the absence of cofactors, we probed NL4.3 and HXB2 RNA before and after tRNA\textsubscript{3\text{\Lys}} annealing (Fig. 2, b and c). For these experiments, the potassium chloride concentration was increased to 300 mM, in order to stabilize and more easily detect weak tertiary interactions (11). In vitro, a strong pause was
observed in the PBS of NL4.3 and HXB2 RNA when tRNA$^{\text{lys}}$ was annealed at this site (Fig. 2, b and c). Otherwise, in vitro DMS modification patterns were remarkably similar to those obtained in situ (compare Fig. 2a to Fig. 2, b and c). In vitro, tRNA$^{\text{lys}}$ annealing protected the PBS nucleotides and A-147 in both templates. In addition, the reactivity of C-179 increased upon primer annealing to HXB2 RNA. However, formation of the initiation complex did not induce extensive rearrangements of NL4.3 and HXB2 RNA (Fig. 2, b and c). In addition, the A-rich loop of both RNAs (nucleotides 165–170) remained accessible to DMS after tRNA$^{\text{lys}}$ annealing, indicating that it did not stably interact with the anticodon loop. The one-nucleotide shift observed around position 215 in the modification patterns of NL4.3 and HXB2 RNA (Fig. 2, b and c) was due to sequence differences between these HIV-1 isolates (Fig. 1c).

In Situ and In Vitro Structural Probing of MAL PBS Domain—Next, we performed in situ structural probing of the MAL initiation complex. However, MAL replicates poorly in most cell types (41), and we were unable to obtain enough material for in situ DMS probing by using the MAL molecular clone. Therefore, we substituted nucleotides 61–274 (including the complete PBS domain) of MAL for the homologous region of the NL4.3 molecular clone. The resulting chimerical NL4.3-MAL clone was infectious and replication kinetics of NL4.3 and MAL were similar (data not shown). This clone thus allowed us to probe the MAL PBS domain in cells and in virions.

A very strong RT stop was observed in the PBS of NL4.3-MAL RNA extracted from cells, but not from virions. As a result, the intensity of the bands located 5’ of this stop strongly decreased (Fig. 3a). In order to correct this bias, we overexposed this part of the gels to equalize the band intensities (Fig. 3b). In sharp contrast with NL4.3 (Fig. 2a), the DMS modification patterns of NL4.3-MAL RNA were very different in infected cells and in virions (Fig. 3, a and b). Remarkably, these patterns were very similar to those obtained prior and after in vitro tRNA$^{\text{lys}}$ annealing (Fig. 3c). The nucleotides in the PBS, as well as A-122, A-134, A-138, A-147, C-158, A-165, A-166, A-211, A-212, and C-213 were more reactive in cells and prior to in vitro tRNA$^{\text{lys}}$ annealing, than in virions and after in vitro tRNA$^{\text{lys}}$ annealing (Fig. 3). Conversely, the reactivity of nucleotides A-130, A-131, A-150, C-151, A-157, A-206, A-207, and A-218 increased in viral particles and after tRNA$^{\text{lys}}$ annealing in vitro (Fig. 3). Hence, our probing data indicated that extensive structural rearrangements took place during formation of the MAL initiation complex of reverse transcription in situ and in vitro. Indeed, as previously shown (9) for the in vitro data, the modification patterns of NL4.3-MAL in cells and virions are compatible with the secondary structure models depicted in Fig. 1, a and b, respectively.

The only significant difference between in vitro and in situ data concerned protection of adenines 164–167, which resulted from the interaction between the viral A-rich loop and the anticodon loop of tRNA$^{\text{lys}}$ (Fig. 1b). This protection was less pronounced in situ than in vitro (Fig. 3, b and c). Several factors might explain this difference. First, the higher potassium chloride concentration and the lower temperature used for in vitro probing experiments are expected to stabilize this interaction (11). Second, nucleocapsid protein, which covers the initiation complex of reverse transcription in virions, might destabilize this labile interaction (42). Quantification indicated that the reactivity of adenines 164 and 167 was 25 and 20% lower in virions than in cells, respectively, while the reactivity of adenines 165 and 166 was 35% lower. Since, adenines 165 and 166 were more strongly protected than A-164 and A-167, both ex vivo and in vitro, the same loop-loop interaction most likely took place in the MAL initiation complex in vitro and inside the viral particles.

Initiation of Reverse Transcription Using tRNA$^{\text{lys}}$ or ORN$^{\text{lys}}$ as Primers—In order to correlate our structural data with the efficiency of reverse transcription of the MAL and HXB2 isolates, we analyzed the rate of primer extension during (−) strand strong stop DNA synthesis. With both templates, we compared the extension rate of tRNA$^{\text{lys}}$ and ORN$^{\text{lys}}$, an oligoribonucleotide corresponding to the 18 3’-terminal nucleotides of tRNA$^{\text{lys}}$ that does not make any intermolecular interaction outside the PBS.

Little difference was observed between the tRNA$^{\text{lys}}$-primed
Initiation of HIV-1 Reverse Transcription

reverse transcription of MAL and HXB2 RNA. However, extension of ORN₃ exchange was slower on MAL than on HXB2 RNA (Fig. 4, a and b). With the MAL template, 50% of ORN₃ₙₙₙ were extended in −2.5 min, whereas >50% of tRNA₃ₙₙₙ were extended in 5 min (Fig. 4b). With the HXB2 RNA, half of the tRNA₃ₙₙₙ and ORN₃ₙₙₙ primers were extended in −60 and −80 s, respectively.

Initiation of reverse transcription of HXB2 and MAL RNA followed bi-exponential kinetics, as previously observed for MAL (15, 43). Previous work showed that the fast reaction corresponds to primer extension inside a productive primer-template:RT complex, whereas the slow reaction is limited by a conformational change of a non-productive primer-template conformation (43). For each template, kinetics of primer extension was analyzed by global fitting (see “Experimental Procedures”). These fits indicated that, for each template, a mixture of “fast” and “slow” initiation complexes coexisted (Fig. 4, b and c).

Substituting ORN₃ₙₙₙ for tRNA₃ₙₙₙ did not change the extension rate of these particular complexes but affected the ratio of fast and slow initiation complexes, as reflected by changes in the amplitudes of the fast and slow reactions (Aₙₙₙₙₙ and Aₙₙₙₙₙₙ) (Fig. 4c). With the MAL RNA as template, tRNA₃ₙₙₙₙₙ formed 67% of fast initiation complex, but only 23% of fast complex was formed when the primer was ORN₃ₙₙₙ. The situation was dramatically different when the template was HXB2 RNA, since the fraction of fast complex was only slightly reduced (from 62 to 52%) when ORN₃ₙₙₙ was substituted for tRNA₃ₙₙₙ.

Interestingly, tRNA₃ₙₙₙₙₙ extension was faster when this primer was annealed to HIV-1 MAL RNA, as compared with HXB2 RNA (Fig. 4b), as reflected by the 5-fold difference in kₙₙₙₙₙ (Fig. 4c). On the other hand, only a 2-fold difference in kₙₙₙₙₙ was measured between MAL and HXB2 templates.

The kinetics of the initiation of reverse transcription of MAL and HXB2 RNA were fully consistent with our structural study in showing major differences between these isolates. In HIV-1 MAL, replacing tRNA₃ₙₙₙ with ORN₃ₙₙₙ had a strong negative effect on the initiation of reverse transcription. At the opposite, substituting ORN₃ₙₙₙ for tRNA₃ₙₙₙ had only minor consequences on HXB2 reverse transcription, indicating that intermolecular interactions between tRNA₃ₙₙₙ and this template were not required outside of the PBS to promote efficient initiation. In addition, the 5-fold difference in kₙₙₙₙₙ for the initiation complexes of these two isolates suggested that they adopt different structures.

Formation of the fast initiation complex was favored by interactions between the primer and MAL RNA outside the PBS, but such interactions were not required with HXB2 RNA. These results suggest that these “additional” interactions were required to counteract a negative effect (such as steric hindrance) that originated from the MAL RNA, rather than from the primer.

Initiation of Reverse Transcription of the HXB2 Mutants Using tRNA₃ₙₙₙ as Primer—The absence of interaction between the A-rich loop of the WT HXB2 RNA and the anticodon loop of tRNA₃ₙₙₙ is due to the replacement of ex vivo and in vitro studies on HXB2 mutants that use tRNA₃ₙₙₙ as their reverse transcription primer. In these mutants, an interaction between a mutated A-rich loop and the anticodon loop of tRNA₃ₙₙₙ was required for efficient replication (18–20) and initiation of reverse transcription (31). Indeed, the results we obtained with the WT HXB2 RNA suggest that the mutant HXB2 virions designed to utilize tRNA₃ₙₙₙ as primer do not reflect the WT situation.

To solve this contradiction, we studied the initiation of reverse transcription of RNAs derived from the HXB2 His and HXB2 His-AC-GAC molecular clones of HIV-1. HXB2 His is identical to WT HXB2, except that the PBS is complementary to the 18 nucleotides at the 3'-end of tRNA₃ₙₙₙ. HXB2 His-AC-GAC contains four additional substitutions introduced by site-directed mutagenesis into the A-rich loop, and three point mutations selected during replication in tissue culture. The selected mutations are crucial for efficient viral replication (18–20) and for stable interaction between the mutated A-rich loop and the tRNA₃ₙₙₙ anticodon loop (31).

We followed the kinetics of the initiation of reverse transcription of HXB2 His and HXB2 His-AC-GAC RNA using either...
With tRNA\textsuperscript{His} as primer, HXB2 His-AC-GAC RNA, which interacts with the tRNA\textsuperscript{His} anticodon loop (31), was reverse-transcribed efficiently, while HXB2 His RNA was not (Fig. 5a). The tRNA\textsuperscript{His} primer formed 82% of fast complex with HXB2 His-AC-GAC RNA, compared with 8% with HXB2 His RNA (Fig. 5b). However, HXB2 His RNA reverse transcription was efficiently primed by ORN\textsuperscript{His}. Indeed, ORN\textsuperscript{His} initiated reverse transcription of HXB2 His and HXB2 His-AC-GAC RNAs at very similar rates (Fig. 5c), and both templates formed > 80% of fast complex with this primer (Fig. 5b).

Formation of the fast complex required an interaction between the anticodon loop of tRNA\textsuperscript{His} and the vRNA, but no interaction outside of the PBS was required when reverse transcription was initiated by ORN\textsuperscript{His}. Thus, the interaction between the anticodon loop of tRNA\textsuperscript{His} and the vRNA most likely counteracts a negative contribution originating from tRNA\textsuperscript{His}, rather than from the template.

Sequence Alignments—Our experimental data showed that the MAL and NL4.3/HXB2 initiation complexes of reverse transcription are structurally and functionally different. However, MAL is a particular isolate because it is a complex recombinant of HIV-1 subtypes A, D, and I, and it contains a 23-nucleotide duplication, including part of the PBS and the downstream region (14). To check whether the MAL isolate is an oddity, we aligned the 538 sequences from the HIV-1 database (www.hiv.lanl.gov) that contain the region corresponding to nucleotides 95–245 of the HXB2 genomic RNA (Fig. 6 and data not shown). We found 74 sequences (14%) containing an insertion identical to or very similar to the MAL insertion.

In addition, we found that the insertion 3′ to the PBS strongly correlates with substitutions 5′ to the PBS (Fig. 6). In the isolates with an insertion, substitutions 5′ to the PBS stabilize the hairpin structure with the A-rich sequence in the
Fig. 6. Alignment of representative HIV-1 sequences of the M group with and without insertion 3’ to the PBS. The subgroup or CRF is indicated in the second column. The asterisks indicate nucleotides that co-vary with the presence of an insertion. Numbers above and below the alignment are according to the MAL and HXB2 RNA sequences, respectively.

TABLE I

| Position (according to MAL) | 150 | 151 | 153 | 157 | 159 | 163–164 | 164 |
|-----------------------------|-----|-----|-----|-----|-----|--------|-----|
| Isolates with an insertion  |     |     |     |     |     |        |     |
| A: 100                      | C: 100 | C: 98.3 | A: 91.5 | U: 79.7 | Δ: 96.6 | A: 91.5 |
| G: 1.7                      | G: 8.5 | G: 15.3 | A: 3.4 | Δ: 8.5 |
| HXB2                        |     |     |     |     |     |        |     |
| C                           | U    | U    | U    | U    | A    | G    | G    |
| Isolates without insertion  |     |     |     |     |     |        |     |
| C: 67.1                     | U: 59.9 | U: 86.2 | U: 83.5 | A: 83.5 | G: 82.5 | G: 87.0 |
| A: 17.7                     | C: 29.4 | C: 7.6 | A: 7.6 | U: 7.9 | Δ: 8.9 | A: 12.8 |
| U: 14.3                     | A: 2.7 | G: 3.7 | G: 7.1 | C: 4.9 | Δ: 0.6 | A: 8.6 | Δ: 0.2 |
| G: 0.3                      | Δ: 1.5 | A: 2.5 | C: 1.7 | G: 3.7 |
| Δ: 0.5                      | G: 0.5 | G: 0.5 | G: 0.5 | G: 0.5 |

Initiation of HIV-1 Reverse Transcription

apical loop, as observed in MAL (Fig. 1a). In the isolates without insertion, the hairpin structure with the A-rich sequence in an internal loop is favored, as observed in HXB2 and NL4.3 (Fig. 1e). Interestingly, stabilizing the “MAL-like structure” (with the A-stretch in the apical loop) in HXB2 has been shown to decrease tRNA\textsubscript{3Lys} annealing and the initiation of reverse transcription (44, 45). The MAL and HXB2 sequences to the PBS were representative of the isolates with and without an insertion 3’ to the PBS, respectively (Fig. 6 and Table I). The only divergences were found at nucleotides 159 and 161 of the isolates G-160 and A-161 base pair with C-170 and U-169, and the five adenes of the A-tract are located in the apical loop (Fig. 1a). Thus, it is likely that the MAL and HXB2/NL4.3 isolates are structurally and functionally representative of the HIV-1 isolates with and without an insertion downstream of the PBS, respectively.

Interestingly, the insertion 3’ to the PBS is not evenly distributed among the HIV-1 groups, subgroups and circulating recombinant forms (CRFs) (36). The insertion is only found in subgroups G (100%) and A (22%), especially in sub-subgroup A2 (100%), of the major (M) group of HIV-1, and in CRFs (Fig. 6). Indeed, the insertion is found in 100% of the CRFs 01_AE, 02_AG, 04_cpx, and 06_cpx that are actively spreading in Africa and Asia.

DISCUSSION

Previous studies of the HIV-1 initiation of reverse transcription provided divergent results regarding possible interactions between tRNA\textsubscript{3Lys} and the vRNA outside the PBS and their functional roles (9–13, 15–20, 24, 25). These divergences may originate from different in vitro experimental conditions, the lack of direct ex vivo structural information, and the use of different HIV-1 isolates. To solve these discrepancies, we developed in situ probing of the HIV-1 genomic RNA and performed a detailed comparison of the MAL and NL4.3 isolates.

The experiments we reported here represent the first ex vivo probing of HIV-1 RNA. The DMS modification profiles of the genomic RNA in cells and in virions appeared remarkably similar to those obtained prior and after in vitro tRNA\textsubscript{3Lys} annealing, respectively (Figs. 2 and 3). This result is surprising given that in vitro probing was performed in the absence of any protein. In addition, less than 1% of viruses are able to complete their life cycle. However, the majority of virions have been shown to be able to initiate reverse transcription (47). The only significant difference between in vitro and ex vivo DMS probing concerns weak tertiary interactions, which appeared easier to detect in vitro when using a higher ionic strength.

Our in vitro and in situ probing experiments revealed marked differences between the MAL and NL4.3/HXB2 PBS domains. While the MAL PBS domain undergoes very extensive structural rearrangements upon tRNA\textsubscript{3Lys} annealing, in agreement with previous in vitro studies (9–11,13), the structure of the NL4.3/HXB2 PBS domain is only marginally affected. In addition, the A-rich loop of the MAL RNA is protected in the initiation complex, most likely by the anticodon loop of tRNA\textsubscript{3Lys} (9, 10), but the NL4.3/HXB2 A-rich loop is not.

In line with these results, our in vitro functional studies indicate that interactions between tRNA\textsubscript{3Lys} and the vRNA are required outside the PBS for efficient initiation of reverse tran-
The interaction between the vRNA and the tRNAHis anticodon reverse transcription of these mutants (Fig. 5) suggests that indeed, our study reveals that these mutants do not be-

rangement of the MAL RNA during tRNA3
action between the anticodon and A-rich loops drives the rear-

single stranded (Fig. 1

RNA, and the junction between helices 2 and 7F is already

the opposite, helices 1 and 2 pre-exist in HXB2 (and NL4.3)

However, neither our DMS probing data, nor the sim-

antiPAS interaction

take place in the HXB2 reverse transcription initiation com-

different problems highlights the adaptability of the HIV-1

vRNA, rather than of the primer. This convergent solution to

by the deletion, and it would be interesting to test nucleotide

However, this effect might be due to RNA misfolding induced

The interaction between the anticodon and A-rich loops is

between helices 2 and 7F are directly recognized by RT, but

helix 6C is not (11). Noteworthy, none of these elements exits in

The interaction between the vRNA and the tRNAHis anticodon loop fulfills different functions in WT MAL and in the HXB2 mutants using tRNAHis. In the former case, it counteracts a negative effect of the vRNA, rather than of the primer. This convergent solution to different problems highlights the adaptability of the HIV-1 initiation complex of reverse transcription.

The interaction involving the tRNA3Lys anticodon loop is not the only intermolecular interaction that has been proposed to take place in the HXB2 reverse transcription initiation complex. Berkhour and co-workers (24, 25) identified the PAS-antiPAS interaction in vitro, in the absence of nucleocapsid protein. However, neither our DMS probing data, nor the similar kinetics of reverse transcription of HXB2 RNA observed with tRNA3Lys and ORN3Lys are in keeping with this proposal. One cannot exclude that the mutants designed to test this interaction do not reflect the WT situation (24, 25, 29), as reported above for the HXB2 mutants utilizing tRNAHis as primer.

Alternatively, Iwatani et al. (17) recently proposed that parts of the anticodon stem and variable loop of tRNA3Lys interact with nucleotides 143–149 of NL4.3 RNA. Our in vitro data would suggest that this interaction does not take place in NL4.3. However Iwatani et al. (17) only observed this interaction in the presence of nucleocapsid protein. In addition, mutation of the proposed interaction had pronounced effects in a short template that could not form helix 1, but limited effects in a template forming this helix (17). Our in situ probing data only partially support the existence of this interaction. Therefore, additional experiments will be required to conclude about the existence of this interaction in the context of the full-length genomic RNA in vivo.

The present study revealed an unexpected variability of the HIV-1 initiation complex of reverse transcription in vitro and in vivo. We cannot exclude that detailed studies of other HIV-1 isolates will expand the repertoire of structures allowing efficient initiation of reverse transcription.

Acknowledgments—We thank G. Bec for tRNA3Lys purification, P. Walter for the gift of HIV-1 RT, and C. Beyer for technical assistance with full culture. We thank C. Isel for the critical reading of the manuscript.

REFERENCES

1. Gilboa, E., Mitra, S. W., Goff, S., and Baltimore, D. (1979) Cell 18, 93–100

2. Marquet, R., Isel, C., Ehresmann, C., and Ehresmann, B. (1995) Biochimie 77, 113–124

3. Le Grice, S. F. (2003) Biochimie 85, 14349–14355

4. Aiyar, A., Cobrinik, D., Ge, Z., Kung, H. J., and Leis, J. (1992) J. Virol. 66, 2464–2472

5. Morris, S., Johnson, M., Stavnezer, E., and Leis, J. (2002) J. Virol. 76, 7571–7577

6. Freund, F., Boulme, F., Litvak, S., and Tarrago-Litvak, L. (2001) Nucleic Acids Res. 29, 2757–2765

7. Miller, T. J., Ehresmann, B., Hubsher, U., and Le Grice, S. F. (1991) J. Biol. Chem. 267, 27721–27730

8. Fosse, P., Mogul, M., Keith, G., Westhof, E., Ehresmann, C., and Ehresmann, B. (1998) J. Mol. Biol. 281, 736–746

9. Isel, C., Ehresmann, C., Keith, G., Ehresmann, B., and Marquet, R. (1995) J. Mol. Biol. 247, 236–250

10. Isel, C., Marquet, R., Keith, G., Ehresmann, C., and Ehresmann, B. (1993) J. Biol. Chem. 268, 25269–25272

11. Isel, C., Westhof, E., Massire, C., Le Grice, S. F. J., Ehresmann, C., and Marquet, R. (1999) EMBO J. 18, 1038–1048

12. Isel, C., Lanchay, J. M., Le Grice, S. F. J., Ehresmann, C., Ehresmann, B., and Marquet, R. (1996) EMBO J. 15, 917–924

13. Goldschmidt, V., Rigourd, M., Ehresmann, C., Le Grice, S. F. J., Ehresmann, B., and Marquet, R. (2003) J. Biol. Chem. 278, 42525–42534

14. Baudin, F., Marquet, R., Isel, C., Darlix, J. L., Ehresmann, B., and Ehresmann, C. (1995) J. Mol. Biol. 229, 382–397

15. Arts, E. J., Ghosh, M., Jacobs, J. M., Ehresmann, B., and Le Grice, S. F. J. (1996) J. Biol. Chem. 271, 9054–9061

16. Arts, E. J., Steier, S. R., Li, X. G., Rausch, J. W., Howard, K. J., Ehresmann, B., North, T. W., Wohler, H., Goody, R. S., Wainberg, M. A., and Le Grice, S. F. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10063–10068

17. Iwatani, Y., Rosen, A. E., Guo, J., Musier-Forsyth, K., and Levin, J. G. (2003) J. Biol. Chem. 278, 14191–14195

18. Wakefield, J. K., Kang, S. M., and Morrow, C. D. (1996) J. Biol. Chem. 271, 9666–975

19. Zhang, Z., Kang, S. M., LeBlanc, A., Hajduk, S. L., and Morrow, C. D. (1996) Virology 226, 306–317

20. Zhang, Z. J., Kang, S. M., Li, Y., and Morrow, C. D. (1998) C. R. Acad. Sci. Paris 7, 394–406

21. Kang, S. M., Zhang, Z. J., and Morrow, C. D. (1997) J. Virol. 71, 207–217

22. Kang, S. M., Zhang, Z., and Morrow, C. D. (1999) Virology 257, 95–105

23. Dupuy, L. C., Kelly, N. J., Elgavish, T. E., Harvey, S. C., and Morrow, C. D. (2003) J. Virol. 77, 8756–8764

24. Beersens, N., Groot, F., and Berkhour, B. (2001) J. Biol. Chem. 276, 31247–31256

25. Beersens, N., and Berkhour, B. (2002) J. Virol. 76, 2329–2339

26. Esposito, D., and Craige, R. (1986) EMBO J. 17, 5832–5843

27. Vicenzi, E., Dimitrov, D. S., Engelman, A., Mgone, T. S., Purcell, D. F., Leonard, J., Englund, G., and Martin, M. A. (1994) J. Biol. Chem. 269, 7879–7890

28. Huthoff, H., and Berkhour, B. (2001) RNA 7, 143–157

29. Goldschmidt, V., Ehresmann, C., Ehresmann, B., and Marquet, R. (2003) Nucleic Acids Res. 31, 850–858

30. Bénas, P., Bec, G., Keith, G., Marquet, R., Ehresmann, C., Ehresmann, B., and Dumas, P. (2000) RNA 6, 1347–1355

31. Rigourd, M., Goldschmidt, V., Brule, P., Morrow, C. D., Ehresmann, B., Ehresmann, C., and Marquet, R. (2003) Nucleic Acids Res. 31, 5764–5775

32. Paillart, J. C., Marquet, R., Skripkin, E., Ehresmann, B., and Ehresmann, C. (1994) J. Biol. Chem. 269, 37486–37493

33. Brunel, C., Marquet, R., Rompy, M., and Ehresmann, C. (2002) Biochimie (Paris) 84, 925–944

34. Paillart, J. C., Berthoux, L., Ottmann, M., Darlix, J.-L., Marquet, R., Ehresmann, B., and Ehresmann, C. (1996) J. Virol. 70, 8348–8354

35. Moog, C., Wick, A., Le Ber, P., Kirn, A., and Aubertin, A. M. (1994) Antiviral Res 24, 275–288
Initiation of HIV-1 Reverse Transcription

35931

36. Kuiken, C. L., Foley, B., Hahn, B., Korber, B., McCutchan, F., Marx, P. A., Mellers, J. W., Mullins, J. I., Sodroski, J., and Wolinsky, S. (eds) (2000) Human Retroviruses and AIDS 2000: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences, Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM

37. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003) Nucleic Acids Res. 31, 3497–3500

38. Brunel, C., and Romby, P. (2000) Methods Enzymol. 318, 3–21

39. Isel, C., Keith, G., Ehresmann, B., Ehresmann, C., and Marquet, R. (1998) Nucleic Acids Res. 26, 1198–1204

40. Gabor, J., Cen, S., Javanbakht, H., Niu, M., and Kleiman, L. (2002) J. Virol. 76, 9096–9102

41. Alizon, M., Wain-Hobson, S., Montagnier, L., and Sonigo, P. (1986) Cell 46, 63–74

42. Darlix, J. L., Lapadat-Tapolski, M., de Rocquigny, H., and Roques, B. P. (1995) J. Mol. Biol. 254, 523–537

43. Lanchy, J. M., Ehresmann, C., Le Grice, S. F. J., Ehresmann, B., and Marquet, R. (1996) EMBO J. 15, 7178–7187

44. Beerens, N., Klaver, B., and Berkhout, B. (2000) J. Virol. 74, 2227–2238

45. Beerens, N., and Berkhout, B. (2000) J. Biol. Chem. 275, 15474–15481

46. Zuker, M. (2003) Nucleic Acids Res. 31, 3406–3415

47. Huang, Y., Wang, J., Shalom, A., Li, Z., Kharchid, A., Wainberg, M. A., and Kleiman, L. (1997) J. Virol. 71, 726–728

48. Liang, C., Li, X., Rong, L., Insu, Y., Quan, Y., Kleiman, L., and Wainberg, M. A. (1997) J. Virol. 71, 5750–5757

49. Li, Y., Zhang, Z., Wakefield, J. K., Kang, S. M., and Morrow, C. D. (1997) J. Virol. 71, 6315–6322