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Dominant Role of the 5′ TAR Bulge in Dimerization of HIV-1 Genomic RNA, but No Evidence of TAR−TAR Kissing during in Vivo Virus Assembly

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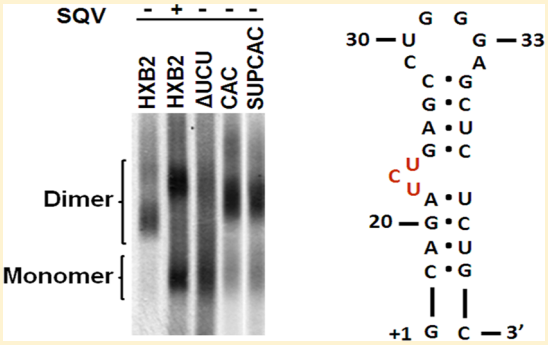
ABSTRACT: The 5′ untranslated region of HIV-1 genomic RNA (gRNA) contains two stem−loop structures that appear to be equally important for gRNA dimerization: the 57-nucleotide 5′ TAR, at the very 5′ end, and the 35-nucleotide SL1 (nucleotides 243−277). SL1 is well-known for containing the dimerization initiation site (DIS) in its apical loop. The DIS is a six-nucleotide palindrome. Here, we investigated the mechanism of TAR-directed gRNA dimerization. We found that the trinucleotide bulge (UCU24) of the 5′ TAR has dominant impacts on both formation of HIV-1 RNA dimers and maturation of the formed dimers. The ΔUCU trinucleotide deletion strongly inhibited the first process and blocked the other, thus impairing gRNA dimerization as severely as deletion of the entire 5′ TAR, and more severely than deletion of the DIS, inactivation of the viral protease, or most severe mutations in the nucleocapsid protein. The apical loop of TAR contains a 10-nucleotide palindrome that has been postulated to stimulate gRNA dimerization by a TAR−TAR kissing mechanism analogous to the one used by SL1 to stimulate dimerization. Using mutations that strongly destabilize formation of the TAR palindrome duplex, as well as compensatory mutations that restore duplex formation to a wild-type-like level, we found no evidence of TAR−TAR kissing, even though mutations nullifying the kissing potential of the TAR palindrome could impair dimerization by a mechanism other than hindering of SL1. However, nullifying the kissing potential of TAR had much less severe effects than ΔUCU. By not uncovering a dimerization mechanism intrinsic to TAR, our data suggest that TAR mutations exert their effect 3′ of TAR, yet not on SL1, because TAR and SL1 mutations have synergistic effects on gRNA dimerization.



Retroviruses package two identical copies of unspliced viral RNA that are noncovalently linked near their 5′ ends (ref 1 and references cited therein). This full-length viral RNA is called genomic RNA (gRNA). A dimeric genome appears to be essential for viral infectivity via, among others, facilitation of gRNA strand exchange during reverse transcription.[2−4] The reverse transcriptase typically switches four or five times from one gRNA strand to the other during provirus synthesis,[5] thus generating extensively recombinant DNA. Given the extreme dilution of gRNAs in infected cells and their minuscule diffusion coefficient (molecular mass of 3 million Da), crossovers to the other gRNA strand may be ineffective unless the two templates are proximal, i.e., dimeric. If at least one of the four or five crossovers is obligatory, because of an RNA nick or similar impediment, gRNA dimerization becomes essential for timely completion of reverse transcription, abundant viral progeny, and the high rate of evolution[6,7] because cells are not infrequently co-infected.[8,9] There is a direct correlation between gRNA dimerization level and gRNA recombination efficiency.[10]

HIV-1 genomic RNA dimerization appears to be largely controlled by the first 500 nucleotides (nt) from the 5′ end of gRNA[11] and by proteolytic processing of the Gag polyprotein Pr55gag.[12] Attempts to discover RNA sequences that control HIV-1 gRNA dimerization led to the identification of a dimerization site located in stem−loop 1 [SL1 (Figure 1)].[13−18] This dimerization site is a 6 nt palindrome, e.g., GCGCGC262, located in the apical loop of SL1 (Figure 1). It is named the dimerization initiation site (DIS), and indeed, it appears to initiate at least some level of gRNA dimerization by a "kissing" mechanism involving the formation of a duplex between two adjacent DISs.[1,19] However, destroying SL1 merely reduces by 50%[13,16,20−22] or <50%[1,23−26] the percentage of gRNA dimers extracted from native viruses.

Thus, HIV-1 viral RNA contains one or several additional dimerization site(s) that seem to be as crucial as or more crucial than SL1. But where are these sites located? An exhaustive investigation of the 5′ untranslated region (Figure 1) found that the 5′ TAR (transactivation response element) stem−loop was at least as important as SL1 for HIV-1 gRNA dimerization and that its role was unrelated to the DIS.[26] The investigation revealed evidence of a role of U5-AUG duplex formation, but

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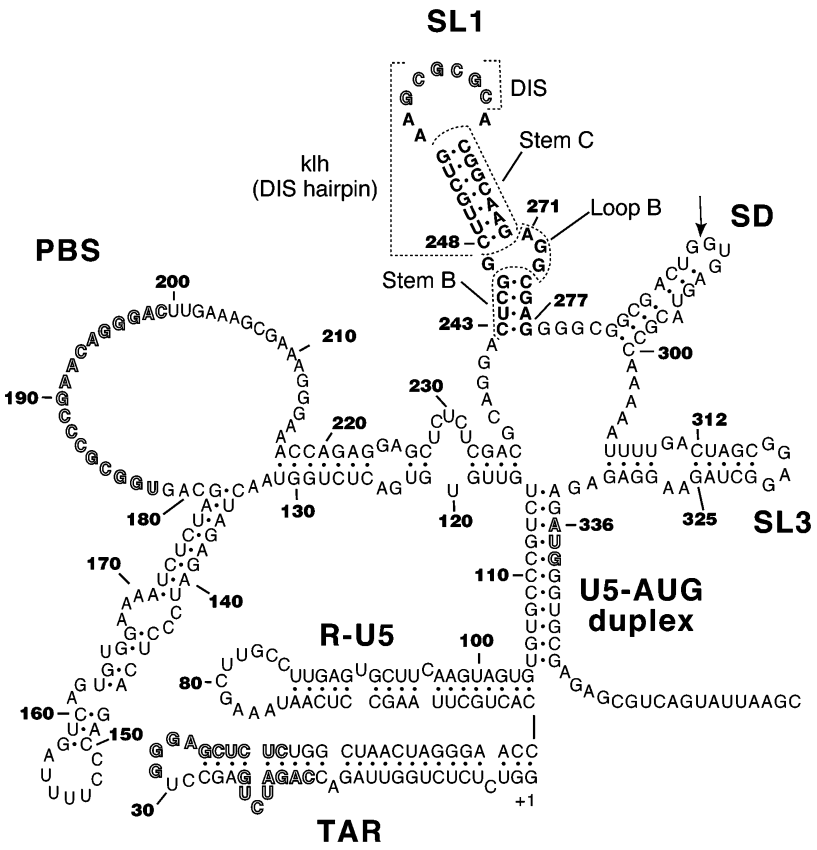

Figure 1. Postulated stem–loop diagram of the 5′ untranslated region (5′ UTR) of HIV-1_{HXB2} gRNA. An essentially similar alternative structure can be seen, for HIV-1_{NL4-3}, in ref 97. TAR palindromes 1 and 2, the TAR bulge (UCU24), the primer (tRNALys3) binding site (PBS), the dimerization initiation site (DIS), and the AUG initiation codon of the *gag* gene are highlighted. The cleavage site within the 5′ major splice donor (SD) is denoted with an arrow. The top part of SL1 is called the kissing-loop hairpin (klh), and the top part of the klh is the DIS. There is good evidence supporting the existence of the 5′ TAR, R-U5,[26] and stem–loops SL1[27] and SL3 at some point in the life cycle, and some evidence in favor of elements of the PBS and SD stem–loop structures.[97] Further evidence and attributions related to this model can be found in Figure 1 of ref 1.

no evidence for the participation of the R-U5, PBS, SD (Figure 1),[26] and SL3 stemp–loop structures,[27] other than by preserving the conformation of 5′ or 3′ dimerization sites. The R-U5 stem–loop is crowned by an AAGCUU82 palindrome (Figure 1), but gRNA dimerization was not affected by deletion of this palindrome[20] or the entire stem–loop.[26] The stimulatory role of the U5-AUG duplex in gRNA dimerization can be direct or indirect depending on whether the duplex is formed inter- or intramolecularly;[26] intramolecular U5-AUG duplex formation may promote dimerization at least in part by displacing and exposing the DIS.[28] The role of TAR could be direct, via TAR–TAR kissing interaction, or indirect, via an effect on 3′ sequences excluding SL1.[26] Atomic force microscopic evidence suggests that the 5′ TAR may be directly involved in dimerization of 744 nt partial HIV-1 RNA transcripts under reasonably physiological conditions.[29]

On the basis of three previous studies,[26,29,30] it is tempting to consider the 5′ TAR as a second gRNA dimerization site. The TAR–TAR kissing hypothesis is supported by the presence of a phylogenetically conserved palindrome located in the apical loop of TAR.[30] This palindrome, termed palindrome 2 here, consists of nucleotides GGGAGCUCUC40 (Figure 1). Mild destabilization of palindrome 2 via the A34U mutation (it increases the predicted ΔG°_{37} of formation of the postulated TAR palindrome duplex from −14.2 to −10.0 kcal/mol) can reduce the level of dimerization of partial RNA transcripts that do not include the DIS.[30] [All reported ΔG°_{37} values are predicted values obtained from models using nearest-neighbor parameters (Materials and Methods).] However, no change in gRNA dimerization yield was seen when the same mutation was tested in the context of the whole HIV-1 particle with or without an inactivated DIS.[26] Song et al.[26] also studied the U37A mutation (it increases ΔG°_{37} to −11.4 kcal/mol), with no detectable change in dimerization level. This led to the suggestion that stronger destabilizations of palindrome 2 are needed to be effective in isolated virions.[26]

Here we tested the TAR–TAR kissing hypothesis by severely destabilizing the kissing potential of palindrome 2 via mutations G35A and G33U+G35A (Table 1) and studying the impact of these two mutations on gRNA dimerization in the context of WT, DIS-inactivated, and protease-inactive HIV-1_{HXB2}. Each of them reduced the level of gRNA dimerization, consistent with the kissing-loop hypothesis, and in contrast with mutations A34U and U37A.[26] However, when we introduced compensatory mutations to reconstitute an alternative palindrome with a duplex stability similar to that of the WT, the dimerization defect was not corrected. This is inconsistent with the kissing-loop hypothesis. We performed similar experiments with another TAR palindrome, termed palindrome 1 (nucleotides CAGAUCUG25), with a similar conclusion. From these and other experiments, notably an extraordinarily effective deletion of the TAR bulge (Figure 1), we conclude that TAR is very important for gRNA dimerization but plays its role not via

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Table 1. Impact of Mutations in TAR Palindromes 2 and 1 on the Predicted Gibbs Standard Free Energy of Duplex Formation at 37 °C and on the Percentage of gRNA Dimerization in HIV-1 Produced in the Absence or Presence of Saquinavir[c]

Mutant	Sequence	ΔG°_{37} (kcal/mol)	gRNA dimerization in: protease-active context (−SQV)	gRNA dimerization in: protease-inactive context (+SQV)
HXB2 (TAR palindrome 2)	5′– GGGAGCUCUC -40 CUCUGGAGGG – 5′	−14.2	100[a]	100[b]
G35A	5′– GGGA**AC**UCUC -40 CUCU**CA**AGGG – 5′	−4.9	82 ± 2	–
G33U+G35A	5′– GG**U**A**A**CU**C**UC -40 CU**C**U**CA**A**U**GG – 5′	+5.1	75 ± 1	63 ± 2.5
SUPC2	5′– GG**UGAUCAC**C -40 CC**ACUAGUG**G – 5′	−15.3	80 ± 1	74 ± 6
HXB2 (TAR palindrome 1)	5′– CAGAUCUG -25 GUCUAGAC – 5′	−8.7	100	100
ΔUCU	5′– CAGAG -25 GAGAC – 5′	–	52 ± 2.5	27 ± 1.5
CAC	5′– C**AGACAC**G -25 G**CAC**AGAC – 5′	–	92 ± 1.5	–
SUPCAC	5′– C**GUGCAC**G -25 G**CACGUG**C – 5′	−11.4	83 ± 1	–

[a]Dimerization level in mutant viruses relative to that of HIV-1_{HXB2}, multiplied by 100. [b]Dimerization level in protease-inactive mutant HIV-1 relative to that of control, multiplied by 100. Controls are HIV-1_{HXB2} produced in the presence of 0.6 μM saquinavir. [c]Standard free energies of duplex formation of the indicated palindromes were predicted from nearest-neighbor parameters, as described in Materials and Methods, i.e., without considering flanking nucleotides and loop constraints. These ΔG values may be poor approximations of experimental numbers, but the $\Delta\Delta G$ values ($\Delta G_{wt} - \Delta G_{mut}$) should approximate rather well the degree of kissing impairment[119] because our mutations do not modify the flanking nucleotides.[120] When nucleotides involved in a kissing interaction are appropriately constrained by a stem–loop, the observed ΔG°_{37} of duplex formation can be more negative than the value predicted by simple duplex models, as in complexes of transfer RNAs with complementary anticodons[121] or in kissing interactions between two GACG loops from the gRNA dimerization site of Moloney murine leukemia virus (MMLV).[122,123] However, experimental studies of the effect of DIS mutations on ΔG values of SL1–SL1 kissing interactions indicate that the experimental ΔG values are more negative than the predicted ΔG values by a rather constant and sequence-independent amount when the flanking nucleotides are unchanged.[119,120] This suggests that the experimental difference in ΔG°_{37} ($\Delta\Delta G^{\circ}_{37}$) between two different TAR–TAR kissing interactions is approximated well by the predicted thermodynamic parameters for short oligoribonucleotide duplexes, because the amount in question cancels out in $\Delta\Delta G^{\circ}_{37}$ calculations. Furthermore, the apical loop of TAR is structurally heterogeneous and malleable,[63] and the whole upper TAR stem–loop must unfold to productively expose palindrome 2 or 1. This triples the size of the apical loop. There might be far fewer loop constraints in that context (a ≥17 nt apical loop) than in the context of the 4 nt GACG loop of MMLV gRNA, the 7 nt anticodon loop of tRNA, and the 9 nt apical loop of SL1.

TAR–TAR kissing but in an indirect manner possibly involving viral or cellular proteins.

MATERIALS AND METHODS

Plasmid Construction. Proviral vector pVC21.BH10, derived from the IIIB strain of HIV-1,[16] encodes an infectious HIV-1_{HXB2} clone, representing the wild type. Mutant proviral vectors were constructed from pSVC21.BH10 by polymerase chain reaction (PCR) mutagenesis, using primers listed in Table 2. To prepare those mutants, we synthesized a PCR-produced DNA fragment extending from *Hpa*I to *Spe*I restriction sites and ligated the fragment into the restriction-digested pVC21.BH10 vector. To prepare mutant G33U +G35A+ΔDIS, G33U+G35A primers were used to amplify a

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Table 2. Primers Used To Construct Most of the Studied Mutants

mutation	forward primer[a]
G35A	ctggttagaccagatctgagcctgggaActctctggctaactagggaaccc
G33U+G35A	ctggttagaccagatctgagcctggTaActctctggctaactagggaaccc
SUPC2	ctggttagaccagatctgagcctggTGATCACctggctaactagggaaccc
CAC	ggtctctctggttagaccagaCACgagcctgggagctctctg
SUPCAC	ggtctctctggttagaccGTGCACgagcctgggagctctctg
ΔUCU	tggtctctctggttagaccaga gagcctgggagctctctg

[a]Substitutions are shown as uppercase letters, and the UCU deletion is shown by a space in the primer sequence.

segment from the ΔDIS mutant vector.[26] After PCR mutagenesis and ligation, the inserted DNA fragments were sequenced (ACGT Inc., Toronto, ON) to verify that the desired mutation, and no other mutation, was introduced.

Cell Culture, Transfection, and Collection of Virus-Containing Cell Supernatants. HeLa cells were cultured at 37 °C in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (Invitrogen), ampicillin, and streptomycin (Invitrogen). Cells were seeded in 150 mm × 25 mm Petri dishes containing 25 mL of culture medium, and 40−80% confluent cells were collected 24 h later. Cells were washed twice with preheated phosphate-buffered saline and transfected with 18 μg of proviral DNA, using the polyfect transfection reagent (Qiagen). Supernatants (25 mL) were harvested 48 h post-transfection, centrifuged, and filtered through a 0.2 μm pore size filtropur S filter (Sarstedt) with a 20 mL syringe to remove cell debris. To prepare supernatants containing protease-inactive HIV-1, 0.6 μM saquinavir (Hoffmann-La Roche) was added shortly after transfection.[12]

Virus Purification and Isolation of HIV-1 Viral RNA. Filtered supernatants were centrifuged at 35000 rpm (SW41 rotor, 4 °C, 1 h) through a 2 mL, 20% (w/v) sucrose cushion in phosphate-buffered saline. The virus pellet was dissolved in 400 μL of sterile lysis buffer [50 mM Tris (pH 7.4), 50 mM NaCl, 10 mM EDTA, 1% (w/v) SDS, 50 μg/mL tRNA, and 100 μg/mL proteinase K]. The solution was incubated at 37 °C for 30 min and extracted twice at 4 °C with an equal amount of a buffer-saturated phenol/chloroform/isoamyl alcohol mixture (25:24:1) (Invitrogen), as described previously.[20] The aqueous phase was precipitated overnight at −80 °C with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol and centrifuged at 14000 rpm in an Eppendorf 5145 microcentrifuge at 4 °C for 30 min. The gRNA pellet was rinsed with 70% ethanol (v/v) and dissolved in 10 μL of buffer S [10 mM Tris (pH 7.5), 100 mM NaCl, 10 mM EDTA, and 1% SDS].[1,31]

Electrophoretic Analysis of HIV-1 gRNA. To assess the dimerization of viral gRNA, nondenaturing Northern (RNA) blot analysis was used.[1] Electrophoretic conditions were 4 V/cm at 4 °C for 4 h in a 1% (w/v) agarose (Bioshop Canada) gel in TBE_2 [89 mM Tris-borate and 2 mM EDTA (pH 8.3)]. Typically, viral RNA loaded on a gel lane was isolated from 12 mL of filtered supernatant for mutants and 4 mL of filtered supernatant for WT. After electrophoresis, the gel was heated at 65 °C for 30 min in 10% (v/v) formaldehyde, and the embedded RNAs were diffusion transferred to a Hybond N+ nylon membrane (Amersham). The membrane was dried at 37 °C for 1.5−2 h, cross-linked (3000 J in a UV Stratalinker), and prehybridized at 42 °C for 2 h in 10 mL of 6× SSPE [1× SSPE consists of 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.4)], 50% (v/v) deionized formamide, 10% (w/v)

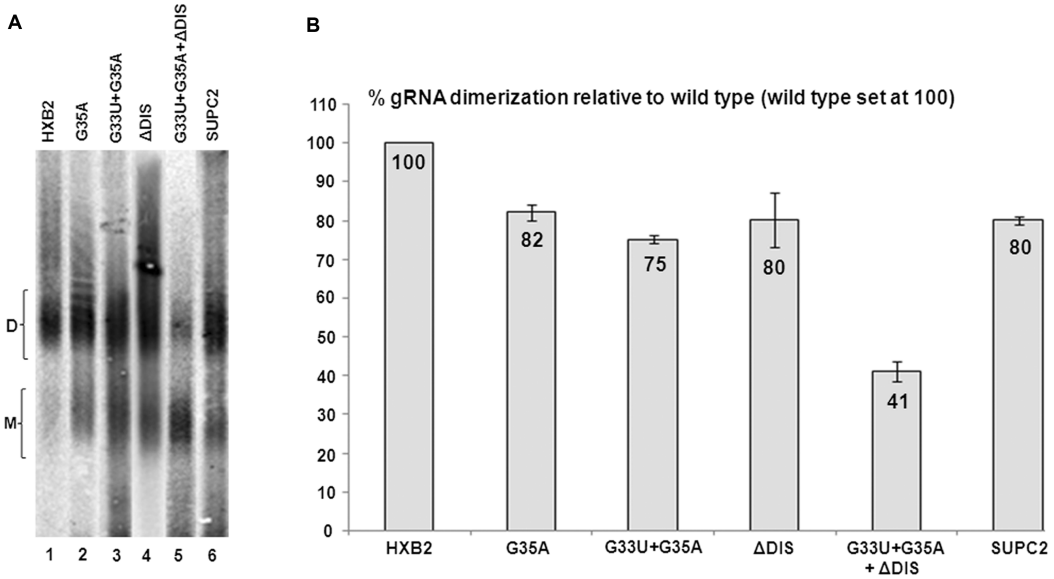

Figure 2. TAR palindrome 2 stimulates gRNA dimerization by controlling a site other than SL1 but does not seem to be involved in TAR−TAR kissing. Dimerization level and electrophoretic migration of gRNAs extracted from HIV-1_{HXB2} virions mutated in palindrome 2 and/or the DIS. Viral gRNAs were electrophoresed on a nondenaturing agarose gel (1%, w/v) and analyzed by Northern blotting (D, dimer; M, monomer). Autoradiographic exposure times varied from 1 to 8 h. The bar graph quantifies the result of densitometric analyses from at least four independent transfections for each mutant. Margins of errors, here and elsewhere in the paper, are standard errors of the mean. The dimerization level is independent of the amount of gRNA electrophoresed or the concentration of vector DNA used in transfection (40-fold range tested with WT and ΔDIS HIV-1) (not shown and ref 26). This is why highly reduced gRNA packaging or gRNA production need not impair gRNA dimerization. For example, deletion of SL3 (Figure 1),[27] mutations N+, L+, and C36S in the nucleocapsid protein (NC) of HIV-1_{HXB2},[12,43] and mutations R32G and S3 in NC of HIV-1_{NL43}[20] severely impair gRNA packaging without affecting gRNA dimerization. Note that highly weakened gRNA dimerization need not impair gRNA packaging either.[22,124]

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dextran sulfate, 1.5% SDS, 5× Denhardt's reagent, and 100 μg/mL salmon sperm DNA. Then, the nylon membrane was hybridized overnight in 10 mL of prehybridization buffer devoid of Denhardt's reagent in a rotating hybridization oven at 42 °C to approximately 25 μCi of ^{35}S-labeled antisense RNA 636−296 [a 356 nt RNA that is the antisense version of the region of residues 296−636 HIV-1 gRNA prepared with the SP6Megascript kit (Ambion)].[15] This was followed by two 30 min washes in 1× SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at room temperature and 35 °C, respectively, and one 30 min wash in 0.2× SSC and 0.1% SDS at 45 °C, followed by drying at room temperature and exposure to Kodak BioMax MR X-ray film.[16]

Densitometric Analysis. The autoradiographs were scanned and analyzed using NIH version 1.6.3. Care was taken to scan variously exposed films to guard against overexposed or underexposed bands. To evaluate the proportion of dimers, we typically considered the monomer and dimer bands to be equal in width. Material located elsewhere in the gels (e.g., extraneous or very slow migrating material in some lanes) was not taken into account in the calculation of the percentage of dimers.[12] All results regarding gRNA dimer migration, RNA monomer migration, and the percentage of gRNA dimers are reported as the average ± standard error of the mean.

ΔG°_{37} Values and RNA Folding. Predicted ΔG°_{37} values for duplex formation between two separate oligonucleotides (Table 1) were obtained as described by Turner et al.[32] and Serra and Turner[33] using the thermodynamic parameters described by Matthews et al.[34] Folding of TAR stem−loop structures (Figure 5) and longer HIV-1 RNAs, as well as their predicted ΔG values, was generated using the algorithm MFold (version 3.5).[34,35]

RESULTS

HeLa cells were transfected in parallel with equal amounts of pSVC21.BH10 or mutant proviral vectors. Proviral vector pSVC21.BH10 encodes an infectious HIV-1_{HXB2} molecular clone derived from the IIIB strain of HIV-1.[16] After 48 h, viruses were isolated from the cultured supernatants and their gRNA was extracted, electrophoresed on a nondenaturing agarose gel, and visualized by Northern blotting with a ^{35}S-labeled HIV-1 riboprobe, followed by autoradiography and densitometric analysis.

Mutating Palindrome 2 Moderately Impairs gRNA Dimerization Despite an Active DIS; Compensatory Mutations Do Not Support a TAR−TAR Kissing Mechanism. To examine the TAR−TAR kissing hypothesis, we first studied the impact of mutations G35A and G33U+G35A on HIV-1 gRNA dimerization. These mutations are very destabilizing: they increase the predicted ΔG°_{37} of TAR palindrome duplex formation from −14.2 to −4.9 and 5.1 kcal/mol, respectively (Table 1). [Such ΔG°_{37} values oversimplify the actual interaction within a loop, but the differences in ΔG values ($\Delta\Delta G^\circ_{37}$) should reliably portray the expected impact of a mutation on duplex formation (legend of Table 1).] Consistent with the kissing-loop hypothesis, the G35A and G33U+G35A mutations reduced the level of gRNA dimerization to 82 and 75% of the WT level, respectively (Figure 2, lanes 1−3; Table 1). To verify the effect of compensatory mutations, we prepared suppressor mutation SUPC2: it transforms palindrome 2 of G33U+G35A HIV-1 into GGUGAUCACC40, which has a WT-like ΔG°_{37} of duplex formation (−15.3 kcal/mol vs −14.2 kcal/mol in the WT). However, the SUPC2 mutation did not significantly improve gRNA dimerization. The dimerization yield of gRNAs prepared from SUPC2 virions was 80% of that of the WT (Figure 2, lane 6; Table 1). The data do not support a kissing mechanism to explain the apparent involvement of TAR palindrome 2 in HIV-1 gRNA dimerization but suggest an indirect role of palindrome 2. Note that gRNA dimerization is a robust measurement, partly because it is a ratio involving two identical molecules. Within the range of our experimental conditions, gRNA dimerization is independent of the level of HIV-1 expression, the efficiency of gRNA packaging, and the amount of gRNA electrophoresed (legend of Figure 2).

The similar and moderate dimerization impairments that were seen in G35A, G33U+G35A, and SUPC2 virions are fully consistent with the WT-like dimerization levels previously associated with mutations A34U, U37A, and M34M37, which targeted A34 and U37 of palindrome 2.[26]

Palindrome 2 Controls a Dimerization Site Other Than SL1. Mutating nucleotides 5′ or 3′ of SL1 can disturb SL1 activity. For example, some mutations in SL3 (Figure 1) can impair gRNA dimerization in the WT, but these SL3 mutations do not impair dimerization when SL1 is disabled.[27] Besides, deleting SL3 hardly affects gRNA dimerization,[27] even though this impairs gRNA packaging.[13,36] Inversely, sequences 5′ or 3′ of SL1 can make intrinsic contributions to gRNA dimerization that are concealed in the presence of SL1. For example, an active SL1 can conceal the role of dimerization or stabilization sites located 5′ or 3′ of SL1, such as the 5′ DLS[15] and the 3′ DLS.[37−39] The in vitro contribution of these two sites was not detectable in the presence of SL1 but revealed when the DIS was inactivated or weakened.[15,37−39] As another example, TAR deletion impairs gRNA dimerization more strongly when SL1 is inactivated.[26]

Accordingly, mutation G33U+G35A was studied in the context of an SL1 disabled by deleting the DIS (ΔDIS). In this context, the G33U+G35A mutation had larger adverse effects. Namely, the G33U+G35A+ΔDIS mutation reduced the proportion of gRNA dimers to 51% of the ΔDIS level (Figure 2, lanes 4 and 5). Because the G33U+G35A mutation had reduced this proportion to 75% of the WT level (above), it follows that the G33U+G35A mutation has an effect that is half-concealed when SL1 is active. This shows that impairing palindrome 2 disturbs a dimerization site other than SL1, and that this disturbance, as measured by electrophoretic analysis, is overshadowed in the presence of SL1. Mutations G33U+G35A and ΔDIS are synergistic because their joint effect, i.e., a dimerization level that is 41% of the WT level (Figure 2, lanes 1 and 5), is larger than the combination of their individual effects (75% × 80% = 60%). Note that concealments can be reciprocal.[26] The effect of the ΔDIS mutation is partly concealed in the presence of an active TAR: G33U+G35A +ΔDIS reduced the proportion of gRNA dimers to 54% of the G33U+G35A level (Table 3).

Mutating Palindrome 2 Severely Impairs gRNA Dimerization in a Protease-Inactive (PR-in) Context and Produces Largely Monomeric gRNAs If the DIS Is Also Disabled. Inactivation of the HIV-1 protease blocks maturation of Pr55gag into progressively smaller nucleocapsid (NC) proteins NCp15 (NCp7-p1-p6), NCp9 (NCp7-p1), and NCp7;[40] HIV-1 particles are released, but they are immature in appearance and contain exclusively immature gRNA dimers[1,31] of the low-mobility form when grown-up (and only monomers

Table 3. Effect of TAR Palindrome Mutations on gRNA Dimerization in the Context of WT, ΔDIS, Protease-Inactive, and ΔDIS Protease-Inactive HIV-1

construct	control	% gRNA dimerization relative to control[a]	
		without SQV	with SQV (protease-inactive context)
G35A	HXB2	82 ± 2	–
G33U+G35A	HXB2	75 ± 1	63 ± 2.5
SUPC2	HXB2	80 ± 1	74 ± 6
G33U+G35A+ΔDIS	HXB2	41 ± 2.5	32 ± 2
G33U+G35A+ΔDIS	ΔDIS	51 ± 6	49 ± 3
G33U+G35A+ΔDIS	G33U+G35A	54 ± 3.5	51 ± 3.5
CAC	HXB2	92 ± 1.5	–
SUPCAC	HXB2	83 ± 1	–
ΔUCU	HXB2	52 ± 2.5	27 ± 1.5
ΔDIS	HXB2	80 ± 7	65 ± 2

[a]Dimerization level in mutant viruses relative to that of control viruses, multiplied by 100. Controls are HXB2, ΔDIS, and G33U+G35A HIV-1, depending on the lane. The percent dimer for each mutant sample represents the average from at least four independent experiments (i.e., independent virus preparations) ± the standard error. The percentage of gRNA dimers in HIV-1_{HXB2} was 81 ± 1% in the absence of saquinavir (SQV) and 57 ± 1.5% in the presence of SQV, i.e., 70 ± 2% of its level in the absence of SQV.

when newly released).[1] Low-mobility dimers are exclusively seen in newly released WT HIV-1[1] or in HIV-1 unable to process Pr55gag beyond the NCp15 stage.[12] Intermediate-mobility dimers are seen in 5 h old WT HIV-1[1] or in many HIV-1 forms with NC mutations.[12] Mature dimers are seen in grown-up WT HIV-1[1,31] or when Pr55gag matures to the NCp9 level.[12] (Grown-up viruses are defined as those produced by cells over a period of ≥12 h.[1,12])

If palindrome 2 promotes TAR−TAR kissing, mutation G33U+G35A should be ineffective in protease-inactive (PR-in) HIV-1 because TAR−TAR kissing is likely to require processed NC for two reasons. First, in vitro TAR dimerization requires NCp7,[30] in contrast to in vitro SL1 dimerization.[15] Second, palindrome 2 is situated partly in a helical region within the TAR upper stem (Figure 1); this probably makes it sterically inaccessible for TAR−TAR kissing unless the chaperone activity of NCp15, NCp9, or NCp7 is provided.[1,26,30] To produce PR-in HIV-1, transfected cells were incubated with 0.6 μM saquinavir. This inhibits viral protease activity to a level of >97%, as shown previously.[12]

Mutations G33U+G35A, SUPC2, G33U+G35A+ΔDIS, and ΔDIS were studied in PR-in HIV-1 (Figure 3A). Mutation G33U+G35A reduced the level of gRNA dimerization to 63 ± 2.5% of the control, which is the WT grown in the presence of saquinavir (Figure 3A, lanes 1 and 2); this was not appreciably corrected by SUPC2 (74 ± 6% dimers relative to the control) (Figure 3A, lane 5). These results do not support the TAR−TAR kissing hypothesis for two reasons. First, mutation G33U+G35A was effective despite the inactive viral protease. Second, suppressor mutation SUPC2 did not tangibly improve the small dimerization yield, suggesting that mutation G33U+G35A inhibited a dimerization process other than putative TAR−TAR kissing. Note that in the PR-in context, mutation G33U+G35A was as effective as ΔDIS (Figure 3A, lanes 2 and 4) and at least as effective as ∼50% of NC mutations studied by Jalalirad and Laughrea.[12]

Next, mutation G33U+G35A was studied in the joint context of a disabled SL1 and an inactive protease, to see if mutation G33U+G35A disturbed SL1 activity or if its effect was overshadowed by SL1 activity in PR-in HIV-1. Mutation G33U+G35A was more effective in the ΔDIS context: together with ΔDIS, it reduced the level of gRNA dimerization to 49% of the ΔDIS control (Table 3 and Figure 3A, lanes 3 and 4). This indicates that in the PR-in context, palindrome 2 controls a dimerization site other than SL1, can strongly stimulate formation of immature dimers without the assistance of NCp15, NCp9, or NCp7, and has an effect that is somewhat concealed in the presence of SL1. Indeed, the joint effect of mutations G33U+G35A and ΔDIS, i.e., a dimerization level that is 32% of the WT control (Table 3), is somewhat larger than the combination of their individual effects (63% × 65% = 40%). In brief, largely monomeric gRNAs were produced when the DIS, palindrome 2, and the protease were jointly disabled (Figure 3A, lane 3). This effect is similar to that caused by jointly disabling the protease and the nucleocapsid protein (see mutations ΔF1, S3E, and HC in ref 12).

The TAR Bulge Controls gRNA Dimer Yield and gRNA Dimer Conformation; No Evidence of Palindrome 1 Duplex Formation. The TAR bulge (UCU24) and adjacent nucleotides form a palindrome, termed palindrome 1, which is phylogenetically conserved among several HIV-1 subtypes.[41] Its sequence is CAGAUCUG25 in HIV-1_{HXB2} (Figure 1). Its ΔG°_{37} of duplex formation is −8.7 kcal/mol (Table 1), which is comparable to the ΔG°_{37} of DIS duplex formation (−10.4 and −7.6 kcal/mol for GCGCGC and GUGCAC, respectively). The TAR−TAR kissing potential of palindrome 1 was investigated by engineering three mutations: (i) CAC, to prevent formation of the palindrome 1 duplex (Table 1); (ii) SUPCAC, to introduce compensatory mutations that reconstitute an alternative palindrome with WT-like duplex stability (Table 1); and (iii) ΔUCU, to delete the TAR bulge and nullify the kissing potential of palindrome 1 (Table 1). We studied the effect of these mutations on gRNA dimerization in the context of WT and PR-in HIV-1. Care was taken to choose mutations that did not create mutant sequences complementary to palindrome 2.

We found that CAC reduced the level of gRNA dimerization almost insignificantly [to 92% of the WT level (Table 1 and Figure 4A, lane 4)]. Suppressor SUPCAC further reduced the level of gRNA dimerization to 83% of the WT level (Table 1 and Figure 4A, lane 5). Both CAC and SUPCAC mutations reduced gRNA dimer mobility by 12%, which is characteristic of intermediate dimers;[1] this indicates that the gRNA dimer conformation in palindrome 1 mutants may be more extended than in the WT or palindrome 2 mutants. Mutation ΔUCU was much more striking: it reduced the level of gRNA dimer formation and blocked the transition from low-mobility dimers to intermediate and mature dimers. Specifically, it reduced the proportion of gRNA dimers to 52% of the WT level (Table 1 and Figure 4A, lane 3) and slowed dimer electrophoretic migration by 25%, i.e., to the level seen in PR-in HIV-1 (Figure 4A, lanes 1−3). Solely in terms of dimerization yield, and contrary to all previous mutations in palindromes 1 and 2, ΔUCU had harsher effects than inactivating SL1 (Figure 2, lane 4) or the viral protease (Figure 4A, lanes 1 and 2); the two preceding mutations reduced the level of dimerization to only 80 and 70% of the WT level (Table 3), respectively. Thus, ΔUCU hinders a dimerization site other than SL1. [Incidentally, MFold folding of the first 508 nt of HIV-1

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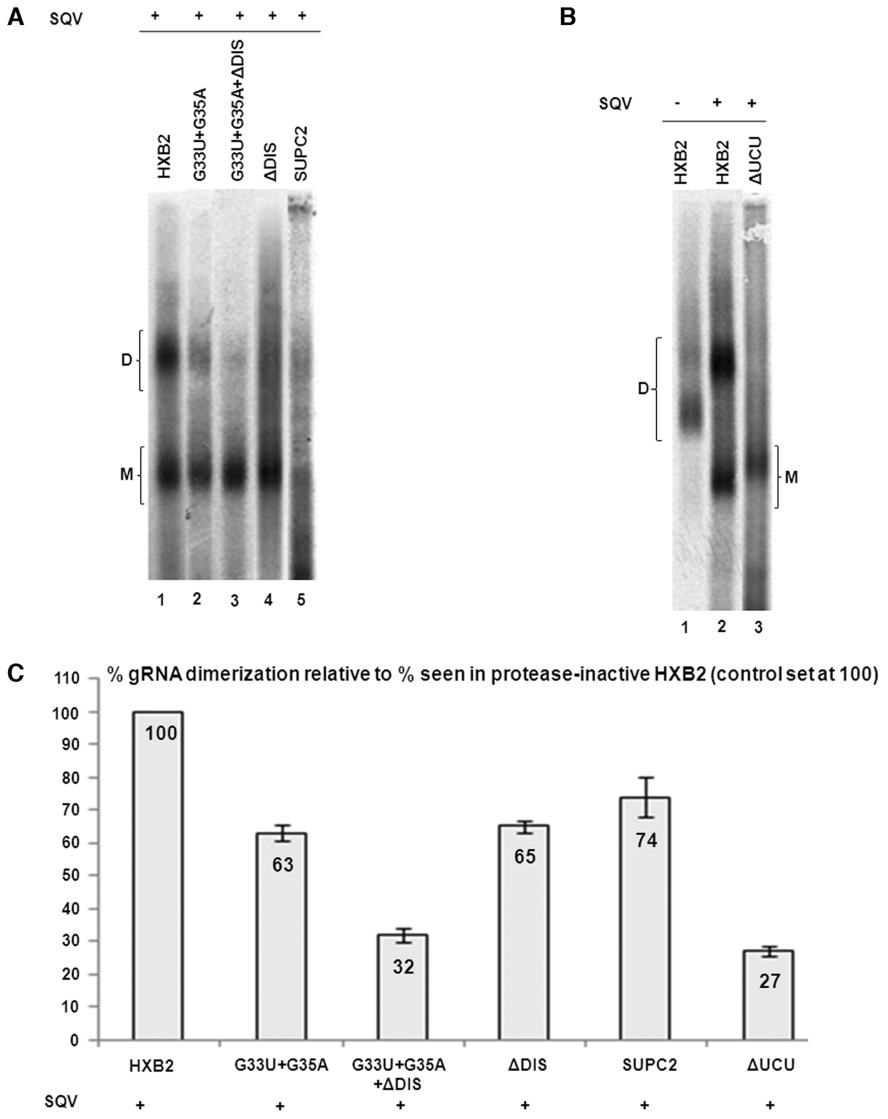

Figure 3. HIV-1 gRNA dimerization in a protease-inactive (PR-in) context. (A) Monomers are produced when palindrome 2 and the DIS are disabled, but there is no support for TAR−TAR kissing. (B) Monomers are produced when the TAR bulge is deleted. Dimerization level and electrophoretic migration of mutant gRNAs isolated from PR-in HIV-1_{HXB2}. In the + lanes, transfected cells were incubated with 0.6 μM saquinavir. Genomic RNAs were analyzed and bar graphs produced, as described in the legend of Figure 2. ΔUCU reduced the electrophoretic mobility of monomeric gRNA (B), indicating that the monomeric conformation was more extended in PR-in ΔUCU HIV-1 than in the control. However, this wast largely corrected when the viral protease was active (Figure 4, lane 3).

RNA (Materials and Methods) reveals that ΔUCU does not alter the secondary structure of SL1 or TAR (not shown).] Together, the results suggest that palindrome 1 plays an important role in gRNA dimerization but is not involved in a TAR−TAR kissing interaction. Together with those of Song et al.,[26] our results show that deleting the TAR bulge faithfully reproduces the impact of 5′ TAR deletion on gRNA dimerization. Mutation ΔUCU is unlikely to impair gRNA dimerization via impairing Pr55gag maturation, because it did not reduce the level of proteolytic processing of Pr55gag, as visualized by Western blotting using antibodies against capsid protein CAp24 (not shown). Recall that gRNA dimers from grown-up HIV-1 are fully mature even when Pr55gag is up to 70% unprocessed; immature dimers appear in large quantities only when Pr55gag is ≥90% unprocessed.[42]

The TAR Bulge Contributes Dominantly to gRNA Dimerization in Protease-Inactive HIV-1, i.e., in the Absence of NCp15, NCp9, and NCp7. If ΔUCU impaired gRNA dimerization merely (or in part) by hindering Pr55gag processing, it would lose all (or some) potency in a protease-inactive context. The opposite was seen. ΔUCU reduced the level of dimerization to 27% of the control level in the PR-in context, even after conservatively accepting as dimers some material that could be considered as background (Figure 3B, lanes 2 and 3). This effect was much more severe than that of ΔDIS in the PR-in context [65% of the control (Table 3)] and as severe as the combined effect of mutation G33U+G35A +ΔDIS in the PR-in context [32% of the control (Figure 3A, lane 3)]. The effect of ΔUCU in PR-in HIV-1 was comparable to the effect of powerful NC mutations in the PR-in context. Examples of these powerful NC mutations are (1) deletion of the proximal zinc finger of NC, (2) inversion of the charge of its basic linker sequence, and (3) mutation of both zinc fingers (mutations ΔF1, S3E, and HC in ref 12). The effect of ΔUCU in PR-in HIV-1 was also larger than its effect in the WT (Table 3).

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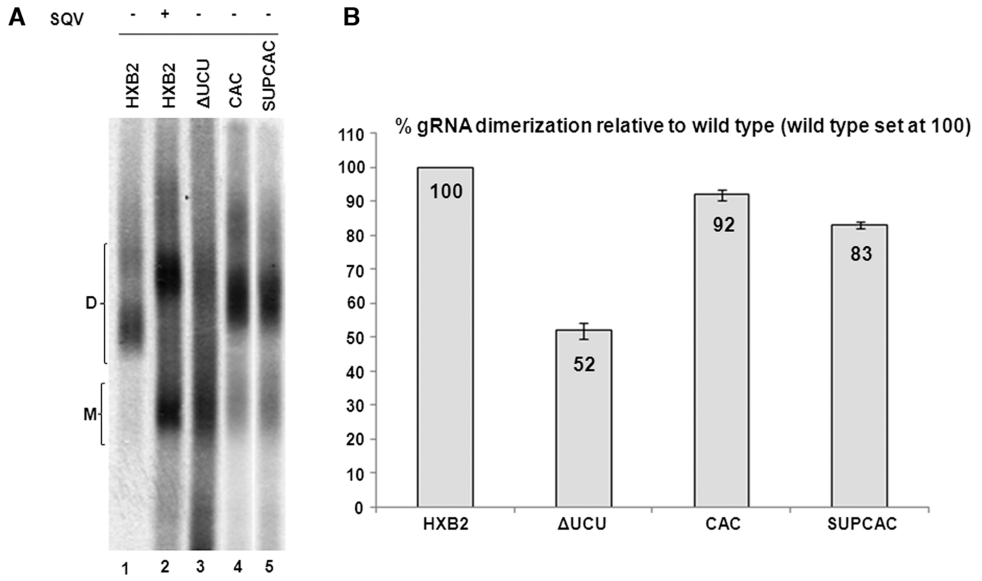

Figure 4. The TAR bulge controls gRNA dimer conformation and gRNA dimer yield but does not seem to be involved in TAR–TAR kissing. Dimerization level and electrophoretic migration of gRNAs isolated from HIV-1_{HXB2} virions mutated in palindrome 1. Experimental conditions for the gel and bar graph analysis were as described in the legend of Figure 2.

DISCUSSION

We have provided evidence that the TAR bulge and upper TAR stem–loop contribute enormously to HIV-1 gRNA dimerization, but not by a mechanism that involves TAR–TAR kissing. Deleting the trinucleotide bulge of 5′ TAR (UCU24) has dominant negative impacts on both gRNA dimer formation and gRNA dimer maturation, i.e., on dimerization yield and transformation of low-mobility (immature) dimers into high-mobility (mature) dimers. Trinucleotide deletion ΔUCU strongly inhibited the first process and blocked the other, making ΔUCU as effective as deletion of the 5′ TAR,[26] and more effective than deletion of the dimerization initiation site in SL1 (Table 3), inactivation of the viral protease (Figure 4A, lanes 1–3), or most severe mutations in the nucleocapsid protein (e.g., S3E and HC in refs 12 and 43). In a protease-inactive context, the impact of ΔUCU on gRNA dimer yield was as severe as inactivating both the DIS and TAR palindrome 2; in a WT context, it was almost as severe as inactivating both sites (Table 3).

It is striking that ΔUCU can reiterate the effect on gRNA dimerization of ΔTAR, a 57 nt deletion.[26] It is tempting to conclude that the role of large TAR mutations in gRNA dimerization is entirely assumed by the TAR bulge.

Palindrome 1. Though ΔUCU nullifies the kissing potential of palindrome 1 (Table 1), this neutralization alone should not affect gRNA dimer yield because mutation CAC equally neutralized the kissing potential (Table 1) without significantly reducing dimer yield (Figure 4 and Table 3). Thus, the kissing potential of palindrome 1 appears to be unrelated to dimerization yield. The major effect of mutation CAC was to impair the second step of dimer maturation: the mutation reduced dimer electrophoretic mobility to an intermediate level (Figure 4). By allowing the transition from low-mobility to intermediate-mobility dimer and by leaving dimer yield almost unchanged, mutation CAC shows that neutralization of the kissing potential generates milder effects than ΔUCU at the two levels of dimer yield and dimer maturation. (Intermediate dimers are the maturation products of low-mobility dimers and the immediate precursors of mature dimers.[1])

Could the blocked transition from intermediate to mature dimer reflect the lack of TAR–TAR kissing potential within palindrome 1 of the CAC mutant? Our results do not support this hypothesis because compensatory mutation SUPCAC re-creates an alternative palindrome 1 without correcting the mobility defect of mutant CAC (Table 1 and Figure 4).

Palindrome 2. We have also provided evidence that TAR contributes to gRNA dimerization without involving palindrome 2 in TAR–TAR kissing (Table 3). A two-step experiment was required to reveal this evidence. First, we introduced point mutations that neutralized the kissing potential of palindrome 2 (Table 1). These mutations moderately reduced gRNA dimerization yield, consistent with TAR–TAR kissing. Second, we engineered compensatory mutations that restored WT-like kissing potential to mutant palindrome 2 (SUPC2 in Table 1). These suppressor mutations could not restore gRNA dimerization irrespective of whether the HIV-1 protease was active (Table 3). This suggests that palindrome 2 influences gRNA dimerization by means other than TAR–TAR kissing.

Mutations in palindrome 2 did not impair dimer maturation (Figure 2) but impaired only the initial step of gRNA dimer formation as assayed by gel electrophoresis. This suggests that they can be fully effective in the absence of Pr55gag processing, i.e., in a protease-inactive context. This is indeed what we found (Table 1). Thus, specifically nullifying the kissing potential of palindromes 1 and 2 has differential effects: impairment of the second step of dimer maturation (i.e., from intermediate to mature dimer) and reduction of dimer yield, respectively.

Beyond TAR–TAR Kissing. Together with ΔUCU and palindrome-inactivating mutations, our negative results with compensatory mutations introduced into palindromes 1 and 2 suggest that TAR contributes dominantly to gRNA dimerization, but not by means of TAR–TAR kissing implicating the bulge or upper TAR stem–loop. This suggestion is reinforced by the observation that ΔUCU impairs dimerization much more severely than nullifying the kissing potential of

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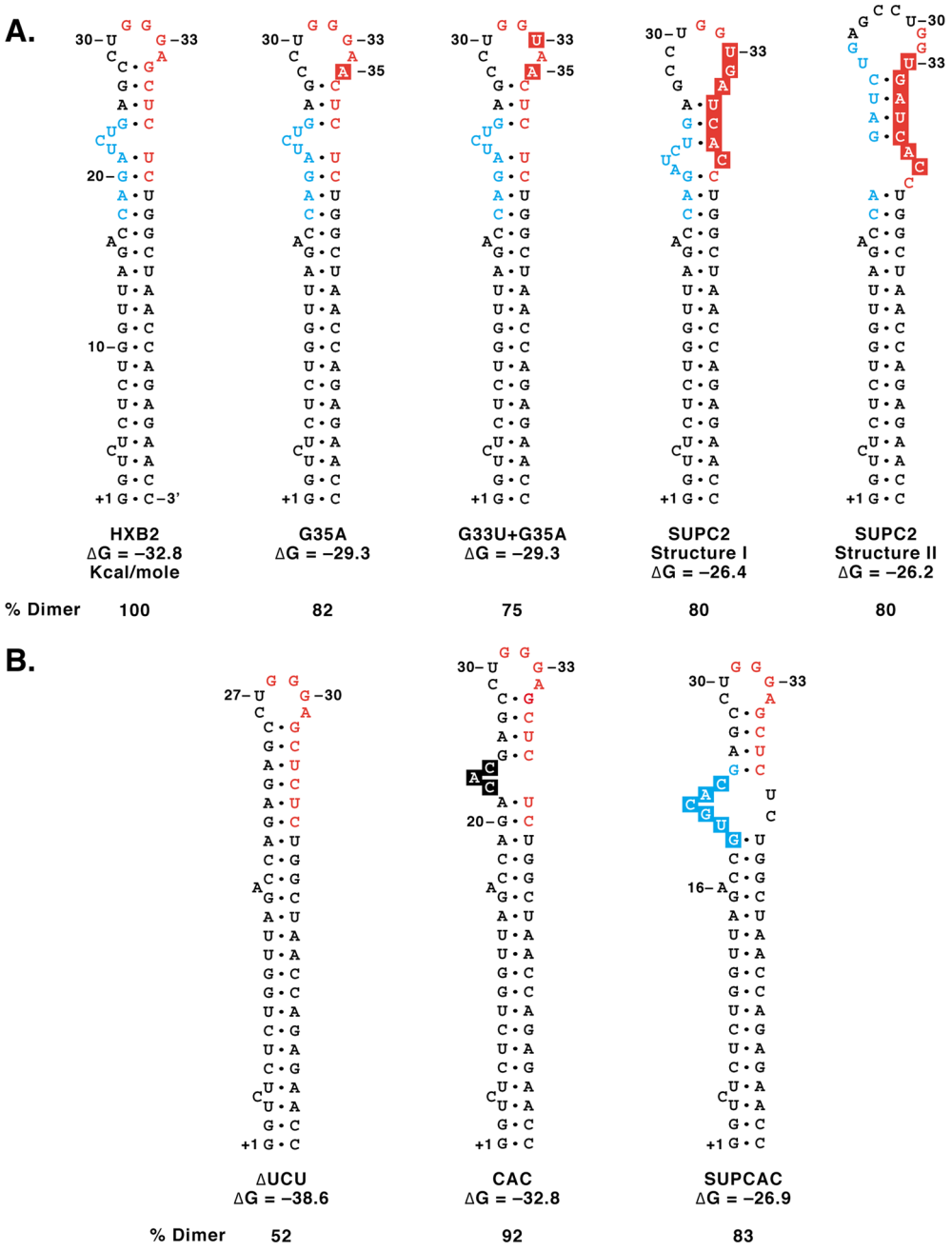

Figure 5. Mfold-predicted secondary structures of mutant 5′ TARs studied in this paper. Palindromes 1 and 2 are colored blue and red, respectively. Mutated nucleotides are highlighted. For each structure, the predicted Gibbs standard free energy of TAR formation at 37 °C in 1 M NaCl is indicated (Materials and Methods), as well as the dimerization yield relative to that of the WT of HIV-1 gRNAs carrying the mutations. The predicted Δ*G* values for WT TAR and ΔUCU TAR are in good agreement with experimental Δ*G* values for similar TAR sequences using optical tweezers.[68]

palindrome 1 or 2. First, ΔUCU blocked the first step of the transition from low-mobility to higher-mobility gRNA dimers, whereas neutralizing the kissing potential of palindrome 2 or 1 allowed all transitions or blocked only the transition from intermediate to mature dimer, respectively. Second, ΔUCU reduced gRNA dimerization yield 2- and 5-fold more than neutralizing the kissing potential of palindromes 2 and 1, respectively. If TAR promoted dimerization by a TAR−TAR kissing mechanism involving palindrome 2 or 1, ΔUCU would have much milder effects on gRNA dimer formation and no effect on the transformation of immature dimers into intermediate dimers. Though it is conceivable that the suppressor mutations studied here happened to alter upper TAR stem−loop conformation in a way that makes reconstituted palindromes less accessible for kissing interaction, this does not explain the huge impacts of ΔUCU on gRNA dimerization.

By not uncovering a dimerization mechanism intrinsic to TAR and by verifying that TAR and SL1 mutations have synergistic effects on gRNA dimerization (see mutation G33U +G35A+ΔDIS in Table 3), our data strongly suggest that dimerization-impairing TAR mutations may have allosteric effects that hinder a dimerization site located 3′ of TAR yet not in SL1. (Song et al.[26] had also showed that TAR and SL1

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mutations have synergistic effects on gRNA dimerization.) Indirect effects of TAR mutations on 3′ sites are nothing new. Mutations in TAR often have indirect effects on 3′ sites that influence packaging, translation, reverse transcription,[44] and polyadenylation[45] of gRNA.

Mechanistic Considerations. The TAR bulge may promote gRNA dimerization in three ways without involving TAR−TAR kissing: (1) as an RNA binding site, (2) as a flexible promoter of TAR conformation(s) that leads to dimerization, and (3) as a protein binding site. Bulge−loop and/or bulge−helix tertiary interactions occur in self-spliced introns,[46] ribozymes,[47−50] rRNAs,[51,52] and the HIV-1 gRNA packaging sequence.[53] For example, trinucleotide bulge UGA of a self-spliced group II intron interacts with an apical loop.[46] It is therefore possible that ΔUCU prevents a tertiary interaction of the TAR bulge with gRNA regions 3′ of TAR. Such tertiary interactions may even cause RNA secondary structure rearrangements.[54]

The TAR trinucleotide bulge also serves as a flexible hinge between the upper and lower stems of TAR.[55−59] It endows TAR with a multitude of conformations in which the angle of the upper stem−loop relative to the lower stem (the interhelical angle) ranges from nearly 90° to nearly 0°,[56,60] and the interhelical twist ranges from −55° to 64°.[60] These correlated interhelical motions[59,61] are lost when the trinucleotide bulge is deleted, because the upper and lower stems then form one single helix, i.e., become perfectly coaxial.

By permitting the right conformers to form, the TAR bulge may facilitate interaction of the malleable TAR apical loop[62−65] with nucleotides located 3′ of TAR. The UCU bulge seems well suited to give considerable bending and torsional flexibility to the TAR upper stem−loop, because it destabilizes TAR more severely than other tripyrimidine bulges.[66] Though the TAR apical loop (CUGGGA34) is complementary to nucleotides UCCCAG1107 in the capsid-coding region of Gag, we note that UCCCAG1107 is protected from 2′-hydroxyl acylation within HIV-1 while the TAR loop is reactive.[67] This indicates that the two sequences do not usually exist as a duplex, at least in the context of HIV-1_{NL4-3} produced by 293T cells.

Consistent with the flexible hinge model, the TAR bulge is predicted to destabilize TAR by 6 kcal/mol (Figure 5). This is supported by experiments showing that the trinucleotide bulge destabilizes by 9.3 kcal/mol a TAR model that is free of an apical loop[66] and by up to 6 kcal/mol a TAR sequence similar to ours.[68]

The results with mutation CAC suggest that the sequence of the bulge plays a secondary role in gRNA dimerization. The primary role might belong to the peculiar kind of TAR flexibility that a trinucleotide bulge provides.[69] That precise kind of conformer display may be unavailable in SUPCAC TAR because it bears a 5 nt bulge [actually, a 5 nt/2 nt internal loop (Figure 5)]. A larger bulge is associated with less correlated interhelical movement; a larger internal loop increases the degree of interhelical overtwisting.[69] It is possible that ΔUCU locks TAR in a nonproductive perfectly coaxial conformation, that mutation SUPCAC generates ineffectively overtwisted TAR conformers, and that mutation SUPC2 generates inappropriately bent or overtwisted TAR conformers (Figure 5), while mutation CAC generates TAR conformers that are relatively more conducive to gRNA dimerization.

The lower stem and the upper stem−loop of TAR can be driven toward greater coaxiality by exposing TAR to argininamide[58] or high Mg^{2+} concentrations[70] or by replacing the deviant and unstable A21·U39 base pair[55,57,61] with a G·C base pair.[71] However, these nonidentical modes of coaxiality do not correspond to the rigid coaxiality expected in ΔUCU TAR. It may be interesting to verify the effect of the A21G+U39C mutation on gRNA dimerization, as it would give G21+C39 TAR a coaxiality approaching that of ΔUCU TAR without preventing the binding of bulge-interacting Tat protein.[72]

TAR Bulge and Upper TAR Stem−Loop as Protein Binding Sites. Perturbing the TAR bulge and/or upper stem−loop may impair several types of interactions between TAR and TAR-binding proteins (see below). This may have 3′ effects that modulate gRNA dimerization without affecting SL1. ΔUCU may impact gRNA dimerization mostly by impairing the interaction (transient or not) of a protein that stimulates gRNA dimerization or by stimulating the binding of a protein that hinders gRNA dimerization. Mutations CAC and SUPCAC would exert a milder form of prevention or stimulation. ΔUCU may be seen as preventing useful conformational changes in TAR, and possibly elsewhere in gRNA, that may modulate binding of such a protein.

Numerous proteins interact with the upper TAR stem−loop. They include Tat (transactivator of transcription), Cyclin T1, TRBP (TAR RNA binding protein), Vif, Staufen1, RNA helicase A, protein kinase R (PKR), NC, and Purα. It is possible that interaction between TAR and one of these proteins activates or alters a gRNA site that is responsible for SL1-independent gRNA dimerization. For example, formation of the U5-AUG duplex, 50 nt 3′ of TAR, is important for gRNA dimerization, but one does not know if the duplex is intra- or intermolecular.[26] Perhaps the interaction of some protein with the TAR bulge or upper stem−loop favors intermolecular U5-AUG duplex formation, among other effects. Proteins that would preferentially interact with mutant TAR stem−loop structures may exist but have not, to the best of our knowledge, been identified.

The TAR bulge is essential for high-affinity binding of viral protein Tat,[73] and Tat can be found inside HIV-1 particles.[74] Cyclin T1 interacts with both Tat and the apical loop of 5′ TAR.[75−78] It is found in both nuclear and cytoplasmic extracts.[79] The highest FRET value for Tat−Cyclin T1 interaction is seen in the cytoplasm.[80] The main binding site of TRBP on TAR is located in the upper TAR stem−loop, with emphasis on G25 in the duplex region and G32 in the loop itself (Figure 1).[81−83] TRBP is a cytoplasmic protein that inhibits the activation of protein kinase R.[84−86] Viral protein Vif binds to the upper TAR stem−loop with an affinity greater than that of Pr55gag and NCp7 for high-affinity sites such as SL1, SL2 and SL3 (Figure 1).[87] It promotes the formation of loose HIV-1 RNA dimers and impairs the formation of tight dimers.[88] TAR RNA is a preferred target of RNA helicase A,[89] which is specifically incorporated into HIV-1 particles in an RNA-dependent manner.[90] Much like TRBP,[83] Staufen1 binds TAR between the bulge and the apical loop.[91] Both overexpression[92] and depletion[93] of Staufen1 intriguingly led to a several-fold increase in the extent of gRNA packaging. PKR is a cytoplasmic protein[94] that binds to the upper stem of TAR via its first dsRNA binding motif and the lower TAR stem via its second dsRNA binding motif.[95] NCp7 protects the stem regions of TAR preferably to the apical loop, without protecting the bulge.[96,97] Purα is a single-stranded RNA and DNA binding protein that is found in HIV-1,[74] is ubiquitously expressed in cells,[98] binds HIV-1 TAR, and activates HIV-1 gene expression.[99] With regard to the possible role of cellular factors

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in gRNA dimerization, note that HIV-1 RNAs produced by HeLa and Cos-7 cells are ~20 and ~8% less dimeric, respectively, than those produced by 293T cells.[26]

General Context. Our results are supported by selective 2′-hydroxyl acylation analysis (SHAPE) performed on extracted gRNAs, on gRNAs inside native HIV-1 particles, and on gRNAs inside virions whose nucleocapsid−RNA interactions were chemically disrupted in situ.[97] This footprinting analysis shows that the 5′ half of palindrome 2 is highly reactive in all states, that the TAR bulge is highly reactive inside native particles, and that it is considerably reactive under the two other conditions. This indicates that the bulge is not base-paired inside HIV-1 (or at least inside HIV-1_{NL4-3} produced by 293T cells) and that it is actually further exposed in the presence of NC,[97] keeping in mind that there can be false positives during SHAPE analysis.[100] It also shows that the stem portion of the upper TAR stem−loop is unreactive in all states, confirming the secondary structure presented in Figure 1. Moreover, A21 and C23 of palindrome 1, as well as A34 of palindrome 2, are reactive with dimethyl sulfate both in H9 cells and inside HIV-1_{HXB2} produced by H9 cells.[101] These results do not support the idea that palindromes 1 and 2 are dimerization sites and fully support our evidence. In addition, they are not very supportive of the idea that the TAR bulge may act as an RNA binding site, keeping in mind that we studied HIV-1_{HXB2} produced by HeLa cells while 2′-hydroxyl acylation studies were performed on HIV-1_{NL4-3} produced by 293T cells. Most consistent with the available knowledge is the idea of the TAR bulge as a binding site for proteins or as a flexible promoter of TAR conformation(s) that leads to gRNA dimerization.

A transient C29·G33 cross-loop base pair exists within the TAR apical loop.[63,65,76] This cross-loop base pair is a further impediment to TAR−TAR kissing. However, both C29 and G33 were highly accessible in all of the 2′-hydroxyl acylation studies.[97] This stresses the transient nature of this base pair.

Severe mutations in TAR typically increase the level of packaging of spliced viral RNAs 4−7-fold, to a level of 15−20 mol % of total viral RNA.[102,103] This is similar to the effect of severe mutations in SL1, which increase the level of spliced viral RNAs to 10,[21] 20,[36] 32,[104] or ~50 mol %[13,105] of total viral RNA. This is also similar to the effect of severe NC mutations: they increase the level of spliced viral RNAs to 8−40 mol % of total viral RNA.[103,106,107] This increased level of packaging of spliced viral RNA is unlikely to complicate interpretation any more than in previous studies of the impact of mutations in SL1[1,13,16,21−23,27] and in NC[12,20,43] on gRNA dimerization. It is possible, here as in previous studies of mutations in SL1 and NC, that hypothetical binding of some of the spliced RNAs to gRNA contributes to the diffuse character of some mutant gRNA bands.

While mutations that disrupt base pairing in the bottom or the middle of the TAR stem cause mild[108] to severe defects in gRNA packaging,[102,109,110] mutations in the upper TAR stem−loop, including ΔUCU[109] and mutations in palindrome 2, have little effect on gRNA packaging.[109,110] Though the 5′ TAR is important for maximal transcription from the viral promoter,[73,111−114] TAR-independent replication occurs in astrocytic glial cells,[115,116] inactivated T lymphocytes,[117] and H9 and U937 cells.[115]

Comparison with in Vitro and Electron Microscopy Data. Andersen et al.[30] studied the in vitro dimerization of RNAs 1−81 and 1−744 (the first 81 and the first 744 nt of HIV-1 gRNA, respectively). They found that TAR−TAR kissing occurs with great difficulty in vitro, even under favorable conditions such as short RNAs chaperoned by NCp7. RNA 1−81 does not dimerize spontaneously. Dimerization requires nucleocapsid protein NCp7 and is inefficient. Only ~40% of these RNAs dimerize in the presence of NCp7; deletion of A16 (Figure 1) eliminates dimer formation. RNA 1−744 dimerizes spontaneously only because it includes the DIS. If the DIS is inactivated (ΔGCGC262), RNA 1−744 does not dimerize; in the presence of NCp7, RNA 1−744 ΔGCGC262 hardly dimerizes (<5% dimers). To somewhat improve dimerization yield, mutationally destabilizing the TAR upper stem was required. In contrast, short RNAs containing the DIS need neither NC nor stem destabilization to dimerize, and their percentage of spontaneous dimerization is >95%.[14]

In summary, in vivo and in vitro experiments converge toward the idea that it is extremely difficult to coerce TAR into TAR−TAR kissing interactions; our experiments extend this idea idea further by showing that in vivo TAR−TAR kissing may not occur. In vivo and in vitro experiments also agree that TAR mutations can impair RNA dimerization, but they differ in two respects. (1) Mutations reducing the stability of the TAR upper stem can improve TAR dimerization yield in vitro, but none improved gRNA dimerization yield in vivo. (2) In the presence of an active DIS, several TAR mutations impaired gRNA dimerization in vivo but none impaired RNA dimerization in vitro. If virus replication requires TAR−TAR kissing interactions, a puzzle is why evolution did not produce palindromes 1 and 2 that are located in the middle of an apical loop or a bulge instead of being half-buried in a helical region and further restrained by a transient C29·G33 base pair. Our in vivo results do not generate this puzzle.

Electron microscopy characterization (EM) of gRNA isolated from HIV-1 shows pairs of gRNAs linked near their 5′ ends at two points separated by ~162 nt because gRNA dimers display a central loop of 323 ± 44 nt. One contact point was assumed to be the DIS, at nt 260, and the other was assumed to be the AAGCUU palindrome of the R-U5 stem−loop, at nt 80 (Figure 1).[118] However, it was later shown that the R-U5 stem−loop (polyA hairpin) can be inactivated without impairing gRNA dimerization in vivo, irrespective of whether SL1 was impaired.[20,26] Liberally interpretating the EM margins of errors, we find that the two linkage points might represent the DIS and palindrome 2 of TAR, which are separated by 223 nt (Figure 1). The electron micrographs of Hoglund et al.[118] display HIV-1 gRNAs that have been incubated for 1 h in a partially denaturing buffer (50% formamide and 2.5 M urea). A narrow window of denaturation was required; 40% formamide and 2 M urea left gRNAs too coiled and condensed, and 60% formamide and 3 M urea dissociated the dimers. One way to reconcile our in vivo data and the EM data is to suppose that the semidenaturing conditions required for the EM study created tertiary and secondary structure rearrangements that inaccurately reflect the in vivo situation.

CONCLUSIONS

Specific mutations in the TAR bulge and in the TAR upper stem−loop have dominant effects on gRNA dimerization; i.e., they impair dimerization even in the presence of an active DIS. Using DIS-independent mechanisms, they prove to be effective in the wild type and in protease-inactive virions. Our data disprove a dominant mechanism that involves TAR−TAR kissing via palindrome 1 because palindrome 1 could be

inactivated without affecting gRNA dimerization yield (mutation CAC). Though inactivation of palindrome 2 weakened gRNA dimerization, the data do not support TAR−TAR kissing via this palindrome because the tested suppressor mutation failed to rescue gRNA dimerization despite restoring WT-like palindrome duplex stability. Because there are 1024 ways for a decanucleotide to be palindromic (assuming all A·U and G·C base pairs are accepted; more ways if G·U pairs are also accepted, as in WT), many more inactivated palindromes and cognate suppressor mutations should be studied before declaring that TAR−TAR kissing via palindrome 2 is a disproved hypothesis. However, this hypothesis is refuted as a mechanism for explaining the effect of deleting the TAR bulge, because ΔUCU was more impairing than inactivating palindrome 2 in two respects: it reduced dimerization yield 2-fold more and blocked dimer maturation, while inactivating palindrome 2 left it unaffected. Because of the unique impact of deleting the TAR bulge on gRNA dimerization, and because we could not uncover a dimerization mechanism intrinsic to TAR in vivo, we conclude that deleting the TAR bulge impairs dimerization by disturbing an interaction of TAR with non-TAR and non-SL1 gRNA sequences, or by misdirecting the viral or cellular proteins that are affected by these TAR mutations. These mechanisms are proposed to account for the effect on gRNA dimerization of mutations in palindrome 2, keeping in mind that TAR−TAR kissing via palindrome 2 is not completely ruled out.

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ABBREVIATIONS

CAp24, capsid protein; DIS, dimerization initiation site; DLS, dimer linkage sequence; FRET, fluorescence resonance energy transfer; Gag, group specific antigen; gRNA, genomic RNA; HIV-1, human immunodeficiency virus type 1; klh, kissing-loop hairpin; NC, nucleocapsid protein; PBS, primer binding site; PKR, protein kinase R; Pr55gag, Gag polyprotein; PR-in, protease-inactive; SD, major splice donor; SL, stem−loop; SQV, saquinavir; TAR, transactivation response element; Tat, transactivator of transcription; TRBP, TAR RNA binding protein; UTR, untranslated region; Vif, virus infectivity factor; WT, wild type.

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