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Using *in vitro* selection to direct the covalent attachment of human immunodeficiency virus type 1 Rev protein to high-affinity RNA ligands

(directed ligand evolution/RNA hairpins/photocrosslinking)

KIRK B. JENSEN*†, BRENT L. ATKINSON*†‡, MICHAEL C. WILLIS§, TAD H. KOCH¶, AND LARRY GOLD*§‖

*Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309; §NeXstar Pharmaceuticals, Inc., 2860 Wilderness Place, Boulder, CO 80301; and ¶Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309

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ABSTRACT **We have used an *in vitro* selection procedure called crosslinking SELEX (SELEX = systematic evolution of ligands by exponential enrichment) to identify RNA sequences that bind with high affinity and crosslink to the Rev protein from human immunodeficiency virus type 1 (HIV-1). A randomized RNA library substituted with the photoreactive chromophore 5-iodouracil was irradiated with monochromatic UV light in the presence of Rev. Those sequences with the ability to photocrosslink to Rev were partitioned from the rest of the RNA pool, amplified, and used for the next round of selection. Rounds of photocrosslinking selection were alternated with rounds of selection for RNA sequences with high affinity to Rev. This iterative, dual-selection method yielded RNA molecules with subnanomolar dissociation constants and high efficiency photocrosslinking to Rev. Some of the RNA molecules isolated by this procedure form a stable complex with Rev that is resistant to denaturing gel electrophoresis in the absence of UV irradiation. *In vitro* selection of nucleic acids by using modified nucleotides allows the isolation of nucleic acid molecules with potentially limitless chemical capacities to covalently attack a target molecule.**

The iterative, *in vitro* selection method systematic evolution of ligands by exponential enrichment (SELEX) (1) is a procedure allowing the isolation of high-affinity nucleic acid ligands to a particular target molecule from large libraries of randomized sequences. SELEX has been employed successfully to generate high-affinity oligonucleotide ligands to dozens of protein and small-molecule targets (2, 3). Nucleic acid ligands have been isolated by SELEX with dissociation constants for their protein targets as low as 100 pM (4). While this affinity rivals that of the best antibodies for their antigens (5), it should be possible to use *in vitro* selection to generate nucleic acid molecules which can bind a protein target with high affinity and specificity and can in addition, form a covalent link with the target. A nucleic acid molecule which could associate in such a manner with a target protein might be valuable for biochemical and structural studies of nucleic acid–protein interactions and possibly for therapeutic applications.

One approach to endowing RNA with the ability to covalently link to a protein target would be through the use of an RNA base analog with enhanced chemical reactivity. A suitable substance for this purpose is the uracil analog 5-iodouracil (5-IU), which can be incorporated into a randomized oligonucleotide pool used for SELEX. 5-IU is reactive under long-wavelength UV irradiation and has been used to generate regiospecific crosslinks between singly substituted IU RNAs and protein targets (6). Recently, an IU-substituted RNA ligand has been used to probe RNA–protein interactions by crosslinking to the human small nuclear ribonucleoprotein U1A (7). UV irradiation of 5-IU is believed to produce a uracil-5-yl radical, which is reactive toward the aromatic amino acids and cysteine (T.H.K. and O. C. Uhlenbeck, unpublished data).

The human immunodeficiency virus type 1 (HIV-1) Rev protein is required for the expression of the structural genes *gag*, *pol*, and *env*. Rev has been shown to specifically bind a 234-nt region in the HIV-1 RNA genome know as the Rev-responsive element (RRE) (8–10). The interaction of Rev with the RRE has been thoroughly investigated, and a single, high-affinity site within the RRE (the IIB stem) has been identified (11–13) and structurally described (14–16). Our laboratory (17–19) and others (20, 21) have previously carried out *in vitro* selection using HIV-1 Rev as the target, and the evolved RNA ligands show substantial similarities in both sequence and secondary structure to the RRE IIB stem.

We describe a method for using an IU-substituted RNA library to carry out a crosslinking-SELEX experiment. We have chosen the HIV-1 Rev protein as a target and have used a "biased randomization" RNA library substituted with 5-IU for the selection. We report the isolation of RNA sequences which can bind Rev with high affinity and can be crosslinked to the protein with high specificity and high efficiency by using long-wavelength UV laser light. In addition, some of these RNA ligands, in the absence of irradiation, can form a complex with Rev which is resistant to denaturing-gel electrophoresis.

MATERIALS AND METHODS

Protein Preparations and Nuclear Extracts. Recombinant HIV-1 Rev, HIV-1 Tat, and HIV-2 Rev were obtained from Intracel (Cambridge, MA). Nuclear extracts were prepared from the fetal lung human diploid fibroblast cell line IMR-90, obtained from the National Institute on Aging Cell Repository. Extracts were gratefully provided by Laurie Goodman and Gretchen Stein (University of Colorado).

Oligonucleotide Templates. The biased randomization template is based upon the 6a ligand isolated in the Rev SELEX indicated in ref. 18. The templates for *in vitro* transcription were prepared by PCR from the following DNA oligonucleotides: template strand, 5′-CCCGGATCCTCTTTACCTCT-GTGTGagatacagagtccacaaacgtgttctcaatgacccGGTCGGAA-GGCCATCAATAGTCCC-3′ (Lowercase letters in the tem-

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Abbreviations: HIV-1, human immunodeficiency virus type 1; SELEX, systematic evolution of ligands by exponential enrichment; 5-IU, 5-iodouracil; RRE, Rev-responsive element; ARM, arginine-rich motif.

†K.B.J. and B.L.A. contributed equally to this work.

‡Present address: Department of Medicine, Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, CO 80220.

‖To whom reprint requests should be addressed.

plate oligonucleotide indicate that at those positions a mixture of reagents was used during synthesis in which 62.5% of the nt in the synthesis reaction was the nt represented by the lowercase letter and the remainder comprised 12.5% each of the other three nt.); 5′ primer, 5′-CCGAAGCTTAATACGACTCACTATAGGGACTATTGATGGCCTTCCGACC-3′; and 3′ primer, 5′-CCCGGATCCTCTTTACCTCTGTGTG-3′.

The template DNA oligonucleotides used to produce the truncated RNAs are as follows: DNA-t3 (trunc-3), 5′-GAGTGGAAACACACGTGGTGTTTCATACACCCTATAGTGAGTCGTATTA-3′; DNA-t24 (trunc-24), 5′-AGGGTTAACAGGTGTGCCTGTTAATCCCCTATAGTGAGTCGTATTA-3′; and PTS-1 (T7 RNA polymerase promoter oligonucleotide), 5′-TAATACGACTCACTATA-3′. PTS-1 was annealed with DNA-t3 or DNA-t24 to produce a template for transcription by T7 RNA polymerase.

SELEX. The SELEX methodology has been extensively described (1, 22); experimental conditions and extensions particular for crosslinking SELEX are reported here. Transcription using phage T7 RNA polymerase *in vitro* transcription was performed as in ref. 1, except 5-iodouridine triphosphate (5-IUTP) (Sigma) was substituted for UTP in the reaction mixture. Final concentration of all NTPs was 1 mM.

Nitrocellulose filter-binding selections. All rounds utilized ≈20–100 nM pool RNA and 1–6 nM Rev in 1× BB (50 mM Tris·OAc, pH 7.7/200 mM KOAc/10 mM dithiothreitol). Reaction mixtures were passed over a Millipore 0.45-μm nitrocellulose filter and washed with 1× BB containing 0.1 M urea. RNA was recovered from the filter by incubation in 200 μl of 7 M urea/400 μl of phenol/130 μl of $CHCl_3$/130 μl of H_2O. The eluted RNA was then precipitated for reverse transcription and PCR.

Crosslinking selections. Approximately 50–100 nM pool RNA was added to 0.2 (rounds 4–6) or 0.5 (round 7) μM Rev in 1× BB on ice and incubated for 10 min at 37°C. The samples were then irradiated at 37°C for 3 min at 308 nm by a XeCl excimer laser (round 4), 30 min at 325 nm by a HeCd laser (round 5), 10 min at 325 nm (round 6), or 1 min at 325 nm (round 7). The sample was heated in formamide loading buffer (50% final concentration of formamide) at 90°C for 4 min and separated by electrophoresis through a 7 M urea/8% polyacrylamide gel to partition the nucleoprotein complex from the free RNA.

For rounds 11–13, reactions were essentially the same as for the crosslinking selections, but 10 μM yeast tRNA was added to the reaction mixture to provide for binding competition during photocrosslinking.

Elution of RNA from Polyacrylamide Gels. The nucleoprotein-containing gel slice was crushed to a homogenous slurry in 1× PK buffer (100 mM Tris·HCl, pH 7.7/50 mM NaCl/10 mM EDTA). Proteinase K was added to a concentration of 1 mg/ml, and the mixture was incubated at 42°C for 30 min. Additional 15-min incubations at 42°C with increasing urea concentrations of approximately 0.7 M, 1.9 M, and 3.3 M were performed. The resulting solution was passed through DMCS-treated glass wool and a 0.2-μm cellulose acetate filter. The filtered solution was extracted twice with phenol/$CHCl_3$, and the RNA was precipitated.

RESULTS

Crosslinking SELEX. Prior to the selection for crosslinking ligands, an RNA molecule that had been previously selected for high-affinity binding to Rev, the 6a ligand, was tested for crosslinking with Rev (Fig. 1*B*; refs. 17–19). Long-wavelength UV irradiation of this molecule in the presence of Rev produced no crosslinked product. When this molecule was fully substituted with 5-IU, there was still no crosslinked product formed. This 5-IU-substituted molecule bound Rev with a K_d of 100 nM—1/100th the affinity of the unsubstituted parental ligand. Having established that the 6a ligand was not capable of crosslinking Rev, we began an *in vitro* selection to generate RNA which could bind Rev with high affinity and could form a covalent link with the protein.

For the crosslinking-SELEX experiment, we used a biased randomization DNA oligonucleotide library based upon the 6a ligand (see *Materials and Methods*). This library contained 35 randomized positions but was biased toward the sequence found in the 6a molecule (18). RNA for selection was transcribed from this oligonucleotide library by using 5-IUTP to generate fully substituted [5-IU]RNA.

The crosslinking-SELEX procedure alternated between two different selective pressures: one, for affinity, using standard nitrocellulose filter partitioning of Rev and bound RNA (rounds 1–3, 8–10); the second, for covalent association, using denaturing-gel partitioning after irradiation to separate the nucleoprotein complex from free RNA (rounds 4–7, 11–13) (Table 1). Each round of selection included a binding step with or without irradiation, physical separation of bound RNA followed by synthesis of cDNA from the recovered RNA, PCR amplification of the DNA, and *in vitro* transcription to generate a new pool of [5-IU]RNA.

To promote crosslinking between the RNA pool and Rev, a 325-nm HeCd laser was used to irradiate the protein–RNA sample before gel partitioning. UV irradiation of the sample produced a slowly migrating species on a denaturing gel; for the first round of photoselection (round 4) the amount of RNA found crosslinked to Rev was <1%. To recover the crosslinked RNA, the partitioned nucleoprotein species was eluted from the gel matrix and digested with proteinase K to release the RNA. In rounds 11–13, selection for affinity and photocrosslinking was performed simultaneously, by using tRNA as a competitor for binding in the reaction. The enrichment of this RNA pool for sequences which bound Rev with high affinity and crosslinked to the protein under 325-nm irradiation was about 30-fold, as shown in Fig. 2. Crosslinking required Rev, and the crosslinked complex was sensitive to proteinase K. Crosslinking also required the presence of 5-IU; the round 13 pool transcribed with UTP exhibited no crosslinking to Rev when irradiated (Fig. 2).

After 13 rounds of selection, the RNA pool was cloned, and 52 sequences were analyzed. Two highly conserved sequence motifs emerged, which we have named class 1 and class 2 (Fig. 1*A*). Since the RNA pool used for crosslinking SELEX was not entirely random, we have chosen to align these sequences to the 6a sequence used for the biased randomization template construction. This type of alignment permits the evaluation of the several evolutionary pathways used to arrive at the two classes of ligands; the consensus motifs for both the class 1 and class 2 sequences are found in several different "reading frames" within the biased randomization pool. Furthermore, the class 1 sequences, on the basis of their relative positions within this reading-frame alignment, partitioned into three major subsets (1A, 1B, and 1C), along with several orphan sequences. Similarly, the class 2 sequences fell into two subsets.

Sequence analysis and computer folding algorithms (23, 24) predicted that all class 1 molecules (77% of the clones) share a stem–loop structure (Fig. 1*B*) formed by the conserved sequences of the class 1 motif. The two nucleotide pairs at the base of the stem (boxed nucleotides, Fig. 1*B*) always co-varied to achieve base pairing but did not sample every possible base pair combination. Possibly, the bias for these particular base pairs was forced by the evolution of the class 1 sequences in the three different reading frames (A, B, and C) within the biased randomization pool; alternatively, these preferences might reflect genuine sequence constraints necessary for high-affinity binding and crosslinking to Rev. The class 2 molecules contained a 10-base conserved region that, on the basis of the computer folding algorithm, is hypothesized to form a stem with the 5′ fixed region of the RNA template.

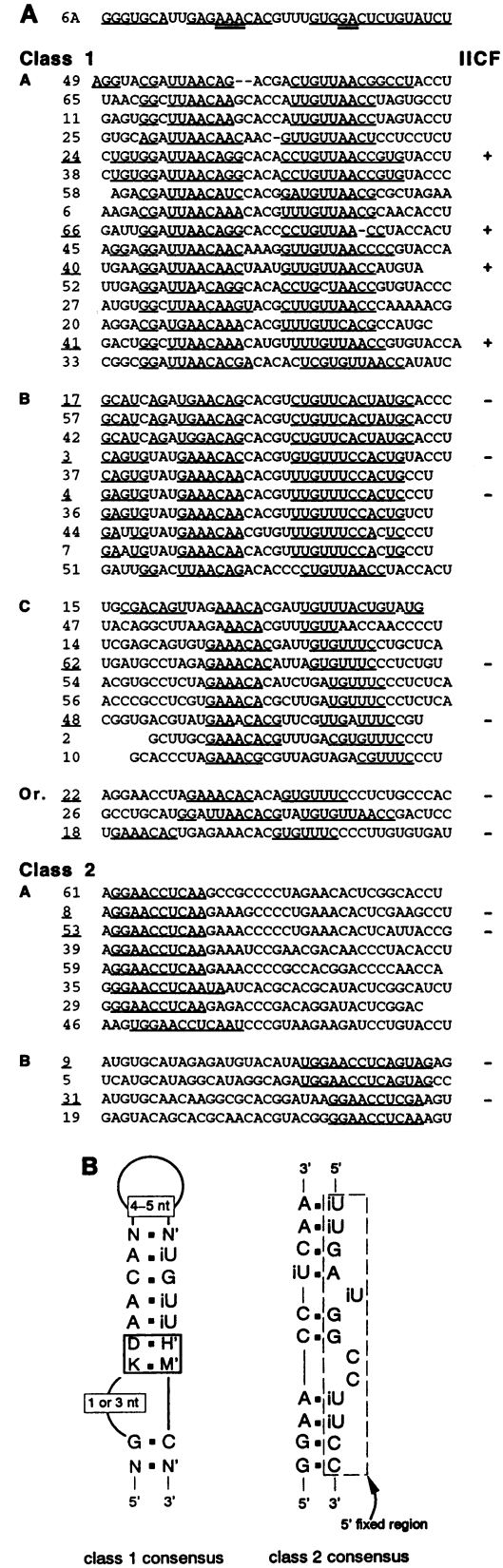

FIG. 1. (*A*) Round 13 sequences selected for photocrosslinking to HIV-1 Rev protein. All Us in the sequences represent 5-IU. The sequences are aligned to the parent 6a sequence; double underlining in the 6a sequence represents the region of proposed purine-purine base pairing (17, 18). Underlined nucleotides represent potential base pairing as indicated by the computer RNA-folding algorithm (23, 24); class 2 sequences are predicted to pair with the 5′ fixed sequence of each molecule. Fixed sequence regions of the RNA molecules are as follows: 5′ fixed region, 5′-GGGACUAUUGAUGGCCUUC-CGACC-3′; 3′ fixed region, 5′-CACACAGAGGUAAAGAGGAUC-CGGG-3′. Underlined clone numbers indicated those sequences tested for binding and crosslinking to Rev. IICF, irradiation-independent complex formation. + and − indicate that the sequence does or does not, respectively, exhibit irradiation-independent crosslinking. (*B*) Consensus secondary structures for class 1 and class 2 molecules, as predicted from computer folding algorithm. D·H′ is an A·U, U·A, or G·C base pair; K·M′ is a G·C or U·A base pair (25).

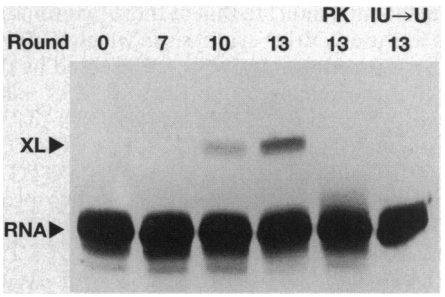

FIG. 2. Progression of the crosslinking SELEX. Photocrosslinking of ^{32}P-labeled, 5-IU-substituted pool RNA to HIV-1 Rev. Samples were irradiated with a 325-nm HeCd laser for 4 min at 37°C. RNA, free RNA; XL, nucleoprotein crosslink; PK, 1 μg of proteinase K per ml was added after crosslinking, with incubation at 65°C for 1 h; IU→U, round 13 pool RNA was transcribed with UTP substituted for 5-IUTP and crosslinked under conditions that were identical to those used for the [5-IU]RNA pools. For all samples: [pool RNA] = 100 nM, [tRNA] = 10 μM, and [HIV-1 Rev] = 500 nM.

The class 1 sequences tested exhibited biphasic binding to Rev, with high-affinity dissociation constants (K_ds) ranging from 1 nM to 10 nM. These class 1 sequences all crosslinked efficiently with Rev, with crosslinking yields of ≈30–40% (data not shown). Class 2 sequences tested showed monophasic binding to Rev with K_ds of ≈30–50 nM. The K_d of the starting [5-IU]RNA pool for Rev was 100 nM. Because of their low affinity for Rev, the class 2 RNA molecules were not further analyzed. However, the photocrosslinking yield of the class 2 clones tested (≈60–70%) was greater than that of the class 1 RNAs under saturating Rev concentrations (data not shown); it is perhaps this superior reactivity that allowed these sequences to be propagated throughout the selection.

Some of the Evolved RNA Ligands Can Form a Stable Complex with Rev in the Absence of UV Irradiation. Analysis of individual clones revealed a surprising result—several of the class 1A molecules tested formed a stable complex with Rev in the absence of 325-nm laser irradiation. The sequences which display this "irradiation-independent" complex formation all belong to the "A" subset of the class 1 sequences (Fig. 1*A*). As with irradiation-dependent crosslinking, irradiation-independent complex formation produced a slower migrating complex which was stable in denaturing loading buffers and during resolution on a denaturing gel. The irradiation-independent complex required Rev, was sensitive to proteinase K treatment, and required 5-IU.

The sequences which form this irradiation-independent RNA–Rev complex were stable when mixed 1:1 with either a formamide or an 8 M urea loading buffer and heated to 90°C for 4 min. However, unlike photocrosslinked RNA–Rev, the irradiation-independent RNA–Rev complex could be dissociated with the addition of a 10^4 molar excess of yeast tRNA (50 μM) or 0.3% SDS to the sample loading buffer before heating. Additionally, the class 1A sequences which showed this irradiation-independent complex formation could also be crosslinked to Rev by using UV irradiation; the irradiation-dependent nucleoprotein crosslink is fully resistant to the tRNA and SDS dissociative treatments.

Analysis of Truncated RNA Ligands. For further analysis of irradiation-dependent crosslinking, a DNA template was con-

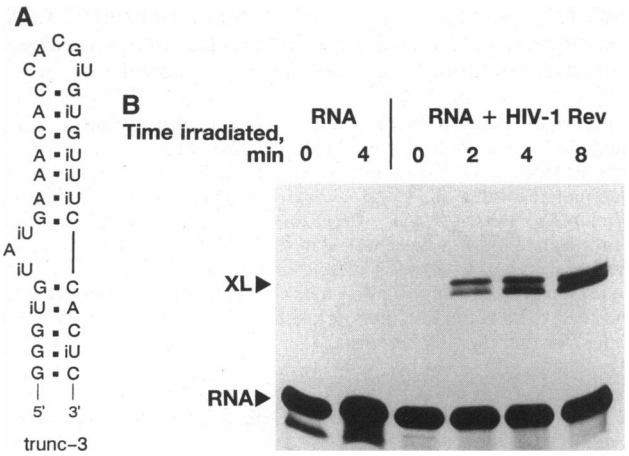

FIG. 3. (*A*) Sequence and predicted secondary structure of trunc-3. (*B*) Trunc-3 irradiation-dependent crosslinking behavior. Crosslinking reaction mixtures include 500 nM HIV-1 Rev protein (where indicated) and ≈5 nM ^{32}P-labeled trunc-3 RNA in 1× BB. Samples were mixed and then incubated at 37°C for 10 min. Irradiations were carried out by using 325-nm monochromatic light for the times indicated. Samples were mixed 1:1 with 8 M urea loading buffer, heated at 90°C for 4 min, and separated on a 7 M urea/8% polyacrylamide gel for analysis. RNA, free trunc-3 RNA; XL, nucleoprotein crosslinked product.

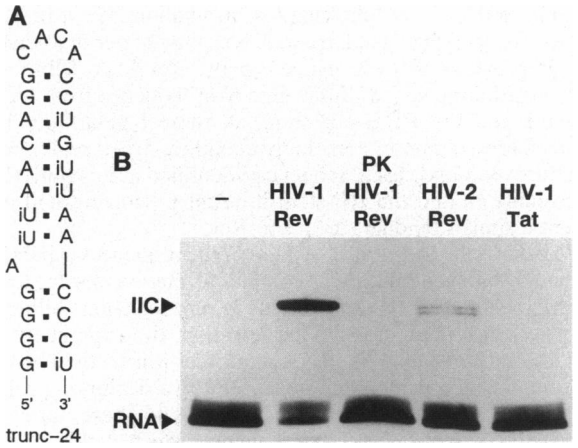

FIG. 4. (*A*) Sequence and predicted secondary structure of trunc-24. (*B*) Trunc-24 irradiation-independent complex formation behavior. Reaction mixtures include 500 nM protein (where indicated) and ≈5 nM ^{32}P-labeled trunc-24 RNA in 1× BB. Trunc-24 RNA and protein were combined and then incubated on ice for 10 min. Samples were mixed 1:1 with 8 M urea loading buffer, heated at 90°C for 4 min, and separated on a 7 M urea/8% polyacrylamide gel for analysis. PK, proteinase K treatment of sample (1 mg of proteinase K per ml was mixed with sample after crosslinking reaction and incubated at 37°C for 10 min); RNA, free trunc-24 RNA; IIC- irradiation-independent nucleoprotein complex.

structed to allow transcription of an RNA ligand corresponding only to the evolved domain of the class 1B sequence 3, which displayed only irradiation-dependent crosslinking (Fig. 3*A*). In the absence of crosslinking, this RNA, termed trunc-3, showed monophasic binding and a K_d for Rev of 0.8 nM. Crosslinking required both Rev and laser irradiation, and crosslinking yield increased over time of irradiation (Fig. 3*B*). The nucleoprotein crosslinking yield reached ≈40% over extended periods of irradiation (data not shown). This suboptimal yield was possibly due to the photodamage of the multiply substituted [5-IU]RNA ligand. Two nucleic acid–protein crosslinks that differ slightly in mobility are seen in Fig. 3*B*; perhaps trunc-3 RNA can use one of two 5-IU nucleotides for crosslinking to Rev, which results in two differentially migrating crosslinked species.

The specificity of the trunc-3 photoreaction was examined by testing two similar "arginine-rich motif" (ARM) proteins (26, 27), HIV-2 Rev and HIV-1 Tat, for irradiation-dependent crosslinking (data not shown). Photocrosslinking of trunc-3 RNA to HIV-2 Rev, whose ARM region is very similar (65% amino acid identity) to HIV-1 Rev, under conditions identical to those described in Fig. 3*B* produced a well-defined nucleoprotein complex when visualized by gel electrophoresis. The crosslinking yield of trunc-3 with HIV-2 Rev was ≈1/5th that for HIV-1 Rev.

Laser irradiation of trunc-3 with 500 nM HIV-1 Tat (which contains a significantly different ARM region) showed a broad distribution of crosslinked products when analyzed by denaturing gel electrophoresis, indicative of nonspecific photocrosslinking. Titration of the photoreaction by tRNA prevented crosslinking with Tat but left trunc-3 crosslinking to HIV-1 and HIV-2 Rev intact. We believe that the promiscuity of crosslinking to Tat without competitor tRNA is due to the nonspecific binding of trunc-3 at 500 nM Tat, the presence of several photoreactive chromophores within each RNA molecule, and the free radical mechanism of the photoreaction.

Irradiation-independent, stable complex formation was further investigated by using another small RNA, trunc-24, based upon the irradiation-independent complex-forming class 1 sequence 24 (Fig. 4*A*). The apparent K_d of trunc-24 for Rev is 6 nM (monophasic binding), and stable complex formation between Rev and trunc-24 proceeds without laser irradiation (Fig. 4*B*). We tested the specificity of trunc-24 irradiation-independent complex formation with the ARM proteins HIV-2 Rev and HIV-1 Tat (Fig. 4*B*). HIV-2 Rev formed a stable complex with trunc-24, though with ≈1/10th the yield seen with HIV-1 Rev. HIV-1 Tat showed no stable complex formation.

Because of the superior specificity of irradiation-independent crosslinking and the possibility that such ligands would be useful *in vivo*, we decided to test the ability of trunc-24 to discriminate HIV-1 Rev in a complex mixture. Trunc-24 and human fibroblast nuclear extract were mixed together with HIV-1 Rev (Fig. 5). At 50 nM Rev and a 1:100 weight ratio of Rev to nuclear extract, a significant, specific complex was formed between trunc-24 and Rev. Nuclear extracts and trunc-24 alone yielded no observable, stable, irradiation-independent complexes.

DISCUSSION

The use of the photoreactive chromophore 5-IU in a randomized RNA library has allowed the *in vitro* selection of ligands which can bind a protein target with high affinity and can also efficiently

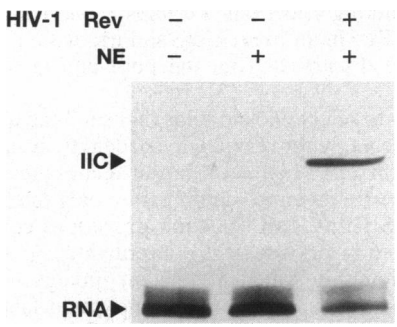

FIG. 5. Trunc-24 irradiation-independent complex formation with HIV-1 Rev in the presence of human nuclear extracts. Reaction mixtures contain 0.1 μg (50 nM) of HIV-1 Rev protein and 10 μg of human fibroblast nuclear extract (NE) (where indicated) and ≈5 nM ^{32}P-labeled trunc-24 RNA in 1× BB. Trunc-24 RNA, nuclear extract, and Rev protein were combined and then incubated on ice for 10 min. Samples were mixed 1:1 with 8 M urea loading buffer, heated at 90°C for 4 min, and separated on a 7 M urea/8% polyacrylamide gel for analysis. RNA, free trunc-24 RNA; IIC, irradiation-independent nucleoprotein complex.

crosslink to this target following UV irradiation. The affinity of the best crosslinking ligand, trunc-3, is slightly better than that of 6a, our previous SELEX ligand against Rev (17). While the photocrosslinking ligands differ greatly in sequence from the 6a molecule and the RRE IIB stem, all these high-affinity Rev ligands share a common stem–loop secondary structure. Possibly, both the class 1 and class 2 sequences identified in this work (Fig. 1) resemble 6a and the IIB stem in tertiary structure and may present a similar binding surface to Rev.

We believe the fortuitous isolation of the sequences capable of irradiation-independent, stable complex formation was due to the particular experimental design of the *in vitro* selection. Although conceived as a photocrosslinking selection, the experiment essentially asked for all [5-IU]RNA sequences which could bind to Rev and partition in a complex with Rev on a denaturing gel. In fact, there happened to be three solutions to these particular selective pressures: (*i*) the class 2 sequences which could bind Rev with a modest affinity and photocrosslink with high yield, (*ii*) the class 1 photocrosslinking sequences with high affinity and modest photocrosslinking yield, and (*iii*) the class 1A sequences which bind with high affinity and could form stable complexes with Rev in the absence of irradiation.

We have not yet determined the precise chemistry for either the irradiation-dependent crosslinking or irradiation-independent complex formation or the exact sequence or secondary structure requirements for each process, although we speculate that the irradiation-independent reaction proceeds via a Michael addition, which would require a nucleophilic attack by the protein at the 6 position of an [5-IU]pyrimidine ring (28). A Michael addition, like photoactivation, is another favorable chemical reaction for 5-IU (29), and Michael adducts have been postulated to occur in several RNA–protein interactions (30–32). Covalent Michael adducts can sometimes be isolated by using denaturing gel electrophoresis, but often the adducts are unstable during this separation process (28). However, what we observe as irradiation-independent complex formation might in fact reflect a large difference in the off rates for the subset of class 1 sequences—behavior which may or may not be due to a Michael adduct between the RNA and Rev.

Indeed, the reversible nature of the irradiation-independent complex probably was essential for its propagation throughout the SELEX; an irreversible irradiation-independent complex-forming RNA would have been discriminated against during the nitrocellulose filter partitioning phases of our selection. The design of nitrocellulose filter-binding SELEX did not anticipate the selection of irradiation-independent complex-forming RNA molecules—there was no step to explicitly remove an RNA covalently attached to a protein, as was done in the crosslinking selections. Perhaps RNA molecules which could attack Rev in an irreversible and irradiation-independent manner were discarded from the pool during filter-binding selection.

The ability to select nucleic acids that can bind and crosslink a protein target with very high specificity is a significant advance in current *in vitro* evolution techniques. While our experiments were meant to demonstrate only the feasibility of crosslinking SELEX, this selection procedure could be used for further study of Rev–RNA interactions, particularly in structural studies in which a covalent linkage would aid in obtaining a stable nucleoprotein complex. In fact, the structural properties of many nucleic acid-binding proteins could be investigated by using such crosslinking SELEX ligands.

This methodology also furthers the promise of using small nucleic acid molecules as highly specific inhibitors of disease processes *in vivo*. The possibility of selecting small nucleic acid molecules which can form a specific, stable complex (and possible covalent linkage) with a target without UV irradiation or any other activating process would allow the development of potent suicide ligands. Many chemically modified pyrimidine and purine bases (33) should serve as candidates for crosslinking SELEX, and we envisage further progress in the selection of nucleic acid molecules with distinct and useful chemical capabilities.

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