Enhancement of ribosomal frameshifting by oligonucleotides targeted to the HIV gag-pol region

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ABSTRACT
The pol gene of all retroviruses is expressed as a gag-pol fusion protein which is proteolytically processed to produce all viral enzymes. In the human immunodeficiency virus (HIV), the gag and pol genes overlap by 241 nucleotides with pol in the -1 phase with respect to gag. The gag-pol fusion is produced via a -1 ribosomal frameshifting event that brings the overlapping, out-of-phase gag and pol genes into translational phase. Frameshifting occurs at a so called 'shift site' 8-10 nucleotides upstream of a hairpin loop which may play a role in the regulation of frameshifting. We have fused this region of HIV-1 to the 5' end of the firefly luciferase reporter gene in order to quantitatively measure ribosomal frameshifting both in cells and by in vitro translation. A series of 2'-O-methyl oligonucleotides was designed to specifically bind the sequences which flank the gag-pol hairpin. Ribosomal frameshifting is enhanced up to 6 fold by those oligonucleotides which bind the area just 3 to the stem. Oligonucleotides which bind 5' to the stem have no effect on frameshift efficiency. In addition, we have constructed a series of fusion genes which mimic the effect of the bound oligonucleotides with intramolecular hairpins. The results suggest that increasing RNA secondary structure downstream of the shift site increases the frequency of ribosomal frameshifting, and that this effect can be mimicked by antisense oligonucleotides.

INTRODUCTION
All retroviruses encode a short translation product, Gag, which gives rise to the viral core proteins (for recent reviews see 1-4). The Gag arises from the simple translation of an unspliced message. The same message also encodes a longer product, Pol, the precursor of the viral enzymes (5). Synthesis of the Pol protein is achieved via one of two mechanisms: stop codon readthrough and ribosomal frameshifting. Stop codon readthrough, which is seen in retroviruses such as murine leukemia virus (MLV), involves the periodic (5%-10%) insertion of a glutamine residue at the UAG amber termination codon which separates gag and pol (6,7).

The second strategy, which has been most thoroughly studied in RSV, and is also observed in HIV, requires a -1 ribosomal frameshift at an UUA codon of gag (8). In addition, some retroviruses, such as MMTV, require two -1 frameshifts to produce Pol proteins (1). It has been demonstrated that in all cases this frameshifting event occurs while the ribosome transverses a hepta-nucleotide sequence, referred to as the shift site (9).

The shift site is absolutely required for frameshifting, however it is probably not the sole determinant of frameshifting. Evidence for this comes from experiments in which MMTV shift sites transposed to a different context did not promote frameshifting when placed between two heterologous reading frames (10). The search for additional signals for frameshifting led to the identification of stable secondary and tertiary structures immediately 3' of the shift site (9-12). In RSV, mutational analysis showed that the hairpin 3' to the shift site was a critical component of the shift site (9). Disruption of base pairing in the stem by substitution in either the 3' or 5' arm eliminated frameshifting, while restoration of base pairing in the stem by specific re-stabilizing mutations rescued frameshifting. Further mutations in which the hairpin was left intact, but all sequences just 3' to it removed, substantially decreased frameshift efficiency, suggesting that the frameshift signal might extend beyond the hairpin. Similarly, deletion of certain bases downstream of the hairpin structure in IBV (avian infectious bronchitis virus, a coronavirus) also inhibited frameshifting. This led to the proposal that downstream bases interact with the hairpin to form a pseudoknot (13). Chamorro et. al. have shown by mutational analysis, that the pseudoknot structure in the MMTV gag-pro region is needed for efficient frameshifting and that shift sites from other viruses can function more efficiently in the context of MMTV RNA than in their native context (14).

Location of the RNA structure relative to the shift site is also important, since altering the distance between the shift site and the hairpin or pseudoknot by as little as three bases in either direction inhibits frameshifting (13). Furthermore, the putative structures are both highly stable and statistically significant relative to others in the gag-pol or gag-pro and pro-pol junction domains of RSV, HIV-1, BLV, HTLV-II, and MMTV. No other more stable or significant folding regions are predicted in these domains (15). It seems, therefore, that frameshifting requires a carefully positioned downstream hairpin or pseudoknot structure.

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for efficient frameshifting. It has been postulated that the structure may serve to pause translation long enough for the shift to occur. In fact, Jacks et al. report unpublished experiments in RSV showing that translating ribosomes pause at the shift site and that disruptions in down stream structures reduce this pausing (9).

The role of the hairpin which follows the shift site in HIV-1 is less well characterized. Deletion experiments reported by Wilson et al. have demonstrated that, unlike RSV, efficient frameshifting is observed in vitro and in yeast, even when the hairpin is deleted from reporter gene constructs (16). This led to the conclusion that this hairpin is not a contributor to frameshifting in HIV-1. However, it is possible that the in vitro systems do not accurately predict the in vivo situation. For example, it has been observed that host tRNA’s have an influence on frameshift efficiency (14), and Hatfield et al. have shown that the modification of tRNA’s involved in frameshifting in HIV and other retroviruses is altered as a result of viral infection (17). In addition, Reil et al. have observed variation in frameshift efficiency with reporter constructs introduced into different mammalian cell lines (18). Therefore, frameshifting may be differentially regulated based upon variation in in vitro or cellular translational components.

Recent experiments using an in vivo test system for the determination of HIV-1 frameshifting efficiency, have shown that while frameshifting does occur in the absence of the hairpin, the efficiency is reduced (19). Since the efficiency of −1 frameshifting of HIV-1 RNA determines the ratio of gag-pol gene products, a ratio which has been shown to dramatically affect retroviral assembly (20), any contribution that the hairpin does lend to frameshift efficiency in vivo may effect viral production. Alteration of the structure and surrounding sequences might further effect frameshift efficiency resulting in decreased viral production.

In the current study we develop a system to measure the effects of oligonucleotides upon gag-pol frameshifting. Both in vitro translation and cell culture systems have been utilized in which gag-pol was fused both in frame and −1 frame with luciferase. Antisense oligonucleotides were directed to the HIV-1 gag-pol frameshift site and hairpin region in an attempt to influence frameshifting. No effects were observed for oligonucleotides targeted to the shift site, directly to the hairpin, or to the region between the shift site and the hairpin. However, oligonucleotides which span the base of the stem or bind the sequences immediately 3’ of the hairpin, enhanced frameshifting in vitro by as much as 6 fold. We also constructed fusion genes in which a second RNA hairpin was introduced 3’ to the shift site where the active oligonucleotides bind. In these constructs, frameshifting efficiency occurred at rates of approximately 30 percent, compared to the native constructs where frameshifting occurred at a rate of about 5 percent. We conclude that RNA structure downstream of the HIV-1 shift site is an important contributor to frameshift efficiency and that oligonucleotides which increase structure may alter the frameshift frequency, which in turn may perturb virus production.

MATERIALS AND METHODS

Plasmid construction

For all plasmid constructs (−0) designates a fusion protein where gag-pol is in frame with luciferase, and (−1) designates a fusion protein which requires a −1 frameshift for luciferase expression to occur. pMGPL(−0) and pMGPL(−1), employ the MMTV LTR to drive the expression of a Gag-Pol/luciferase fusion protein. They were constructed as follows. The 117 bp synthetic piece of DNA shown in Figure 1, was ligated into the vector pRA-4 (21) prepared by digesting with Nhe I and Hind III. The natural AUG and second codon of luciferase are replaced with a Hind III site in pRA-4. The synthetic DNA insert supplies a translational initiation codon, followed by the gag-pol region, consisting of nucleotides 1619 to 1679 of HTLV-III (22), which covers the ‘shift site’ and hairpin. The base shown in parentheses was added to create the in frame (−0) constructs.

The plasmids pRGPL(−0) and pRGPL(−1) were constructed by ligating the Nhe I/Kpn I fragment of pMGPL(−0) and pMGPL(−1) into the vector pREP-4 (Invitrogen) prepared by digestion with Nhe I and Kpn I. This places the gag-pol/luciferase fusion under the transcriptional control of the RSV promoter.

The plasmids pT7GPL(−0) and pT7GPL(−1) were constructed by excising the Nhe I/Kpn I fragment of pMGPL(−0) and (−1) and ligating into the plasmid pBluescript KS, prepared by digesting with Xba I and Kpn I. This places the gag-pol/luciferase fusion under the control of the T7 RNA polymerase promoter.

The plasmids pT7GPEL(−0) and pT7GPEL(−1), in which a second intramolecular hairpin immediately follows the native stem/loop, were created by digesting the vectors pT7GPL(−0) and pT7GPL(−1) with Hind III and Nar I to completion. This releases a 33 bp fragment 11 bases downstream of the end of the native hairpin structure. This fragment was replaced with a 60 bp. synthetic double stranded DNA with compatible sticky ends, prepared by annealing the oligonucleotides 5’-AGCTTG-ACG CCACAAAGCT AGAAAAATTCC GAAAGCCCGA AAAACATATAA AAGGGCCCCGC-3’ and 5’-GGCGGGCGCCT TTTTATATGT TTTTGGCGTC TTCGGAATT TCTAAGCTTTGTCGA-3’.

The plasmids pRGPEL(−0) and pRGPEL(−1) were constructed by cutting pT7GPEL(−0) and pT7GPEL(−1) with Kpn I and Not I and isolating the gag-pol/luciferase fusion fragments. These fragments were ligated into pREP-10 (Invitrogen) prepared with Kpn I and Not I to give gag-pol/luciferase with double hairpins under the transcriptional control of the RSV promoter. The nucleotide sequence of the gag-pol/luciferase region (from Nhe I to Nar I) was confirmed by dyeoxy sequencing for all plasmid constructs.

Gel mobility shift assays

A template for the production of a 69 nucleotide fragment of gag-pol RNA, Gp90, which includes the hairpin and flanking sequences, was produced by PCR. Primers were designed to pT7GPL(−0) and pT7GPL(−1), such that the 5’ primer consisted of non-homologous sequences making up the 17 base T7 RNA polymerase promoter followed by 16 bases homologous to position +12 to +28 of the gag-pol/luciferase (Fig. 1), 3’-G-TACCTGTAAT ACGATCICACT ATAGGGCTAA TTTTTAAGG-3’. The 3’ primer consisted of 17 bases homologous to positions +63 to +80 of gag-pol/luciferase, 5’-GGCGGCAA-GC TTAGAAA-3’. This PCR product was then used as a template for a T7 reaction as previously described (23) to produce a 69 base RNA consisting of the hairpin and 3’ and 5’ flanking sequences, including the shift site.

Gel shift assays were performed by the addition of oligonucleotides at 5 μM, to 10 μl reactions containing 50 pM of the labeled Gp90, 90 mM NaCl, 3.3 mM NaCitrate (pH 7.5). Hybridizations were carried out for 1 hour at 37°C, then the gag-
pol RNA/oligonucleotide complex separated from unbound gag-pol RNA by electrophoresis on a 15% native acrylamide gel cooled to 15°C, in a running buffer consisting of 1×TBE plus 50 mM NaCl, at 25W for 3 hours. The gel was then dried and exposed to film. The exposed film was analyzed on a Molecular Dynamics Laser densitometer to determine the percent of target bound.

In vitro translation, cell culture and luciferase assays

In vitro synthesis of RNA. Capped transcripts for use as templates in in vitro translation reactions were generated from the plasmids pT7GPL(-0) and pT7GPL(-1) or pT7GPEL(-0) and pT7GPEL(-1) linearized at the Kpn I site. 10 μg of the linearized template was added to a 125 μl reaction using reagents supplied in a TransProbe T kit (Pharmacia), supplemented with cold UTP to 500 μM and m7GpppG as per the manufacturer’s directions, to generate capped RNAs. Following a 1 hour synthesis at 37°C and DNase treatment, the reactions were extracted once with phenol/chloroform/isooamyl-alcohol. The supernatant was passed over a Sephadex G-25 spin column to remove unincorporated nucleotides and DNA fragments, and the RNA was precipitated by the addition of NH₄OAc (pH 6.0) to 1M along with 2 1/2 volumes of 100% ethanol. The RNA yield was quantitated spectrophotometrically.

In vitro translation. For the in vitro translation reactions, 1 μg of the capped RNA was incubated in 10 μl of IVT buffer (20 mM KPO₄ and 2 mM MgOAc (pH 7.15)), for 2 minutes at 65°C. Oligonucleotides were then added to indicated concentrations and incubation continued for an additional 15 minutes at 37°C. The amino acid mixture (MET) supplied by the manufacturer (Promega) was added to recommended concentrations along with 35S MET (1200 Ci/mmole Amersham) or unlabeled MET at 50 μM, along with 10 μl of nuclease treated rabbit reticulocyte lysate. Reactions were then incubated 1 hour at 37°C. To analyze the size of the protein product produced, 5 μl aliquots were taken from the in vitro translation mixes containing 35S MET and separated on a 15% SDS/acylamide gel as described (24). Following electrophoresis, the gel was dried and exposed to film. The full length product was then quantitated using a Molecular Dynamics laser densitometer.

Luciferase assays. For luciferase assays, 50 μl of buffer I (1% Triton X-100, 25 mM Glycylglycine pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT) was added directly to the in vitro translation followed by 40 μl of buffer II (25 mM glycylglycine pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 15 mM KPO₄, 1 mM DTT, 2.5 mM ATP). The entire mixture was then transferred to a 96-well luminescence plate and luciferase activity read in a Dynatec multwell lumimeter which injected 50 μl of buffer III (25 mM glycylglycine pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 4 mM DTT, 1 mM luciferin) into each well, reading the light emitted (light units) over a 15 second interval.

Cell culture assays. HeLa and NIH 3T3 cells were maintained in DMEM with 10% FCS. The day prior to the experiment, cells were seeded in 6-well dishes at 40% confluency. Plasmids were calcium-phosphate transfected as described previously (23). On the day of the experiment, 10 μg of plasmid and 8 μg of oligonucleotide (where indicated) were precipitated in 0.5 ml CaPO₄. A plasmid supplying glucocorticoid receptor was cotransfected with MMTV LTR driven constructs. Where indicated 5 μg of a plasmid expressing human placental alkaline phosphatase (25) was also included to monitor transfection efficiency. 150 μl aliquots of the precipitate were then added to each of three wells containing 1 ml of media. The precipitate was removed from the cells after 7 hours and fresh media added. Cells were incubated overnight at 37°C. Dexamethazone was added to the media of cells transfected with pMGPL(-0) and pMGPL(-1) at 0.25 mM to induce transcription from the MMTV LTR. The following morning cells were harvested in 150 μl buffer I. Subsequent steps for the determination of luciferase activity were as follows.

Calculation of frameshift efficiency. Frameshift efficiencies were calculated assuming that ribosomal shifting occurs at the same rate on both the GPL(-0) and GPL(-1) RNA; i.e., the GPL(-0) shifts out of frame at the same rate as the GPL(-1) shifts into frame. Therefore, the luciferase activity obtained for the GPL(-0) RNA can be considered to be representative of ribosomal readthrough efficiency, while the activity obtained for the GPL(-1) is a measure of frameshift efficiency. Percent frameshift efficiency can therefore be derived by the equation:

\[\frac{GPL(-1)}{GPL(-0) + GPL(-1)} \times 100 = \text{percent frameshift efficiency}\]

where GPL(-1) is equal to the luciferase activity in light units of the -1 frame RNA and GPL(-0) is equal to the activity of the in frame RNA.

Oligonucleotide synthesis

Unmodified oligodeoxynucleotides and phosphorothioate oligonucleotides were synthesized on an Applied Biosystems 380B DNA Synthesizer, as described previously (23). 2-O-Methyl B-cyanoethylisopropylphosphoramidites were purchased from Chemgenes (Needham, MA). 2-O-Methyl oligonucleotides were synthesized using the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. The 3 base bound to the CPG (control pore glass) used to start the synthesis was a 2-deoxyribonucleotide. After cleavage from the CPG-column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitation two times out of 0.5 M NaCl solution with 2.5 volumes of ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8M urea, 45M Tris-borate buffer, pH=7.0. Oligonucleotides were judged from acrylamide gel electrophoresis to be greater than 85% full length material.

Phenoxyacetyl protected phosphoramidates for RNA synthesis were purchased from BioGenex (Hayward, CA). The standard synthesis cycle was modified by increasing the wait step after the pulse delivery of tetrazole to 900 seconds. The bases were manually cleaved from the CPG with a 3:1 mixture of concentrated ammonia and ethanol. The PAC protecting group was removed by incubation in methanolic ammonia overnight at room temperature. Following base deprotection the oligonucleotides were dried in vacuo. The t-butyldimethylsilyl protecting the 2 hydroxyl was removed by incubating the oligonucleotide in 1M tetrabutylammoniumfluoride in tetrahydrofuran overnight. The RNA oligonucleotides were further purified on C18 Sep Pak cartridges, followed by ethanol precipitation.
Figure 1. General Structure of gag-pol/luciferase expression vectors. (A) pRA-4 contains the MMTV LTR driving the expression of luciferase. The natural initiation codon of luciferase has been replaced by a Hind III site. The synthetic DNA insert was designed to provide an in frame or −1 frame AUG, relative to the luciferase reading frame, as well as HIV-1 gag-pol sequences including the shift site and stem loop and sequences to rebuild the 5' UTR of MMTV. The gag-pol/luciferase fusions were then transferred to pBS SK− and pREP-4 as cassettes, for production of RNA by T7 RNA polymerase in vitro and constitutive expression in cells, respectively. (B) GPL(−1) constructs require a −1 frameshift to bring luciferase into the correct reading frame. GPL(−0) constructs have an adenosine inserted in the gag-pol sequence at position 31, bringing the AUG into frame with luciferase (numbering begins at the first base of the initiation codon).
RESULTS

In Vitro translation of a functional gag-pol luciferase reporter

The firefly luciferase reporter gene has previously been shown to be a quantitative measure of HIV-1 frameshifting in animal cell extracts (18). We constructed gag-pol/luciferase fusion genes (Fig.1) under the transcriptional control of the T7 RNA polymerase promoter, in order to test the effects of oligonucleotides upon frameshift efficiency in an in vitro system. We began by inserting a synthetic double stranded DNA encoding an AUG and the gag-pol shift site and stem/loop, in and out of frame of the luciferase reporter gene (26), into the vector pRA-4, to give pMGPL(−0) and pMGPL(−1). The gag-pol/luciferase cassettes from the pMGPL vectors were then transferred to pBluescript, to give pT7GPL(−0) and pT7GPL(−1) (Fig. 1). Capped RNA prepared from the vectors was quantitated and used as a template for in vitro translation. Translation efficiency was then measured by assaying for luciferase activity. Table I gives the luciferase activity for each RNA and the frameshift efficiency, calculated as described in Materials and Methods. The frameshift efficiency is in agreement with the results obtained by other investigators (18).

In order to establish that luciferase activity is an accurate measure of translational efficiency, 30 µl in vitro translation assays were carried out in the presence of 35S-methionine. 25 µl of each reaction were used for enzymatic assays of luciferase activity while 5 µl were analyzed by SDS PAGE. The results, shown in Figure 2, indicate that the data obtained by measuring luciferase activity are in close agreement with the amount of full length protein produced. Both methods of measurement give a frameshift efficiency of between 4 and 5 percent (compare lanes 1 and 4).

Effects of oligonucleotides on gag-pol/luciferase expression

Oligonucleotides were targeted to the initiation of translation to demonstrate the utility of the in vitro translation system. Many investigators have observed significant antisense activity with oligonucleotides targeted to this region of a message (27–29). The oligonucleotides were made as phosphodiester, phosphorothioate, RNA, or 2'-O-methyl, and were complementary to position −17 to +3 of the target (initiation of translation, +1). The results, shown in Figure 3, demonstrate sequence specific activity for the phosphodiester, the RNA and the 2'-O-methyl. The phosphorothioate gives complete inhibition of activity with both the specific and the sequence scrambled control oligonucleotide. We have consistently observed non-specific inhibition of in vitro translation with phosphorothioate oligonucleotides. The activity of the 2'-O-methyl, 3561, was confirmed by measuring the amount of full length 35S labeled protein produced in the in vitro translation assay (Fig.2, lanes 3 and 6). Luciferase produced by both the GPL(−0) and GPL(−1) RNA was decreased approximately 60% (compare Fig.2, lane 1 & 3 and 4 & 6), suggesting that the observed inhibition is independent of the frameshift. Since the 2'-O-methyl analogs gave the greatest and most specific activity, oligonucleotides targeted to the frameshift region were synthesized only as 2'-O-methyls.

We postulated that disruption of the hairpin would lead to a reduced frameshift efficiency. Oligonucleotides were therefore designed with complementarity to the stem and loop. None of these oligonucleotides showed activity in the in vitro translation assay against either the GPL(−0) or the GPL(−1) RNA template (data not shown). Attempts to bind the target with these oligonucleotides, as determined by gel mobility shift assay, showed that only one was capable of binding the target, and only very weakly (Kd >> 10 µM).

Oligonucleotides (shown in Table II) were designed with complementarity to the 17 bases just 5' (4151) or 3' (4129) of the hairpin. An additional oligonucleotide (3746) was designed with complementarity to the 9 bases immediately 3' and the 8 bases immediately 5' of the hairpin, separated by a 3 base uridine spacer.

Table I. Luciferase activity and frameshift efficiency of gag-pol/luciferase constructs.

|        | −1       | −0       | Frameshift Efficiency |
|--------|----------|----------|-----------------------|
| pMGPL  | 0.095    | 0.180    | 5.0%                  |
| (0.005) |          | (0.039)  |                       |
| pRGPL  | 0.109    | 2.276    | 4.6%                  |
| (0.011) |          | (0.330)  |                       |
| pT7GPL | 0.060    | 1.271    | 4.5%                  |
| (0.005) |          | (0.253)  |                       |

pMGPL and pRGPL constructs were transfected into HeLa cells. A plasmid supplying glucocorticoid receptor was co-transfected with the pMGPL constructs which were induced with dexamethasone. Cells were harvested and luciferase activity quantitated as described in Materials and Methods. RNA was produced from pT7GPL constructs by an in vitro T7 RNA polymerase reaction. The purified and quantitated RNA was then translated in vitro and luciferase activity measured. All figures are given in light units. The numbers in parentheses are the standard error derived from triplicate points. Frameshift efficiency is calculated as detailed in Materials & Methods.

Figure 2. Luciferase protein production and activity in rabbit reticulocyte lysates. GPL(−0) and GPL(−1) RNA produced from in vitro T7 RNA ymerase transcription reactions were translated alone or in the presence of oligonucleotides at 5 µM. The 2-O-Methyl oligonucleotides 4129 and 3561, targeted as 3' to the frameshift site and initiation of translation respectively, 35S-MET was included in the reactions and 1/6 of the translation products analyzed on a 15% SDS acrylamide gel. Density of the 64 Kd, full length product (arrow) was quantitated using a laser densitometer. Luciferase activity (light units) for the remainder of each translation reaction was measured using a Dynatech multi-well luminometer as detailed in Materials and Methods. Data are summarized below the autoradiogram and are given as percent of the T7GPL(−0) control (lane 4) for both assays.
Figure 3. Effects of 20-mer oligonucleotides targeted to the initiation of translation. Antisense oligonucleotides complementary to position −17 to +3 (Fig. 1b) and scrambled controls were synthesized as phosphodiester (PO), phosphorothioate (PS), 2′-O-methyl (2′-O-Me), or RNA. Oligonucleotides, at a concentration of 5 μM, were hybridized to T7GPL(−0) RNA which was then translated in rabbit reticulocyte lysates for 1 hour. Luciferase activity was quantitated as described in Materials and Methods.

Figure 4. In vitro binding of 2′-O-methyl oligonucleotides targeted to GP_{pol} RNA. Oligonucleotides targeting the frameshift region as shown in Table II, were hybridized with GP_{pol} RNA produced from a PCR generated template as described in Materials and Methods. The RNA covers position +12 to +80 (Fig 1b). GP_{pol} RNA was 5′ end labeled to high specific activity and incubated at 100 pm for 1 hour in 100 mM Na+ buffer with 5 μM oligonucleotide. Bound RNA was then separated from unbound by electrophoresis on a 12% native acrylamide gel in a running buffer of 1×TBE plus 50 mM NaCl at 10°C.

Table II. Oligonucleotides targeted to gag-pol stem-loop flanking sequences.

| Oligo # | length | Sequence | Target Region | Pred. ΔG | %GP_{pol} bound @ 5 μM |
|---------|--------|----------|---------------|----------|------------------------|
| 3734    | 22     | UCU UCC CUU AAA AUA UAG CUU G | 10−31    | −37.0 | 100 |
| 4151    | 17     | AUC UCC CUU AAA AUA U | 16−32    | −24.9 | 100 |
| 4130    | 8      | AUC UCC C | 25−32    | −13.9 | <10 |
| 3746    | 20     | GAA AAI UCC UUU AUC UUC C | 25−32; 59−67 | −27.7 | 100 |
| 4152    | 9      | GAA AAI UCC | 59−67  | −13.3 | <10 |
| 4129    | 17     |CAA GCU UAG AAA UUC | 59−75  | −27.5 | 100 |
| 4289    | 16     | GCG UCA AGC UUA GAA A | 64−79  | −29.1 | 100 |
| 4610    | 14     | UUG GCG UCA AGC UU | 69−82  | −26.4 | 100 |
| 4490    | 17     | UU UGU UUU UGG CGU CA | 74−90  | −27.4 | n.d. |
| 4615    | 20     | GCC UUU UUU UAU UGU UUG GG | 79−98  | −30.2 | n.d. |
| 4489    | 17     | GGG CUC UUU UUG AUG UU | 84−100 | −28.6 | n.d. |
| 4288    | 14     | CCG GCC CUU UCU UU | 89−102 | −26.3 | n.d. |
| random  | 17     | AGA GAC CUC CCA GGC UC | none  | 0 | n.d. |

Identification number, length, and sequence is given for each 2′-O-methyl oligonucleotide. The target region is given relative to the numbering system shown in Figures 1 and 6. ΔG’s were calculated at 100 mM Na+ using OLIGO primer analysis software, which calculates free energy of the structures based upon nearest-neighbor ΔG values (29). The percent of GP_{pol} RNA bound at 5μM was determined by gel shift assay. This data was not obtained for oligos 4490, 4615, 4489, or 4288, since they extend beyond the 3′ end of GP_{pol}.

To bridge the base of the hairpin stem (complementary to bases 25−32 and 59−67; Fig. 1), the 5′ 8-mer (4130) and the 3′ 9-mer (4152), which comprise 3746, were also synthesized. The base composition of these oligonucleotides and ΔG’s, calculated for binding to the GPL(−1) target, are also given in Table II. The oligonucleotides were checked for in vitro binding to the target by gel mobility shift assay. For this assay, a 69-nucleotide RNA fragment, GP_{pol}, covering the shift site and hairpin, plus flanking sequences on either side, was produced as described in Materials and Methods. The shorter oligonucleotides, 4130 and 4152 showed only weak binding to the target RNA (Fig. 4 lanes 6 & 7), whereas the others all bound the target completely at 5 μM (Fig. 4 lanes 2−5 & 8−9).

The 5′ oligonucleotide, 4151 had no effect on luciferase production in in vitro translation reactions with the GPL(−0) and GPL(−1) RNA templates (Fig. 5). However, luciferase production for the GPL(−0) RNA template decreased in the presence of oligonucleotides 3746 and 4129, while production of luciferase from the GPL(−1) template increased. These results suggest that these oligonucleotides are able to enhance frameshifting approximately two and five fold respectively when hybridized to the target RNA, with the GPL(−0) shifting out of frame at approximately the same rate as the GPL(−1) shifts in to frame. The activity of 4129 was confirmed by measuring full length 35S labeled protein produced (Fig. 2, lanes 2 and 5), and was in general agreement with the results obtained with the luciferase assay.

Additional oligonucleotides were synthesized in an attempt to localize the frameshift enhancement effect. Six oligonucleotides were designed by shifting the region of homology of 4129 3' in 5 base increments, with lengths adjusted so the predicted hybridization free energy was in the 27 to 30 kcal/mole range (Table II). In in vitro translation assays, frameshift enhancement of GPL(−1) RNA could be observed through oligonucleotide 4615, which starts 20 bases 3' of 4129 (Fig. 6). Optimal enhancement was observed with oligonucleotide 4289, which is shifted 5 bases 3' of 4129. The activity of this oligonucleotide was found to be concentration dependent (data not shown). At concentrations below 50 nM little enhancement effect was observed. Dose dependent activity was observed up to 5 μM, where frameshift efficiency was increased 6 fold over the no
oligonucleotide control. This is equal to a frameshift efficiency of approximately 30 percent. Activity was not significantly increased at oligonucleotide concentrations above 5 μM. This correlates with the observation that the target is bound completely by the oligonucleotide at 5 μM as determined by gel mobility shift assays (Fig. 5). Additionally, at a concentration of 5 μM, 4289 decreases readthrough efficiency of the GPL(-0) RNA target by approximately 30 to 40 percent (data not shown). Frameshift enhancing is not seen with oligonucleotides which start more than 20 bases 3' of the hairpin (See Fig. 6, 4489, 4288), with short oligonucleotides (Fig. 6; 4152), or with oligonucleotides targeted to the region 5' of the hairpin (not shown).

To determine if the frameshift enhancement seen in the presence of oligonucleotides in \textit{in vitro} translation assays could be duplicated in cells, the \textit{gag-pol}/luciferase cassette was cloned in the context of the RSV LTR, a constitutive promoter, to give the plasmids pRGPL(-0) and pRGPL(-1) (Fig. 1). Table I shows the results when these constructs, and the MMTV LTR driven constructs, pMGPL(-0) and pMGPL(-1), were CaPO4 transfected into NIH 3T3 cells. While the MMTV driven constructs gave less total activity than the RSV driven constructs, the frameshift efficiency is for both sets of constructs is about 5 percent, suggesting that the frameshift is independent of the transcriptional promoter employed. Frameshift efficiency is slightly higher in HeLa cells (6–7 percent) than 3T3 cells.

3T3 or HeLa cells were calcium-phosphate transfected with pRGPL(-1) or pRGPL(-0) and oligonucleotides. The cells were harvested and luciferase assays performed the following day. None of the oligonucleotides which enhanced frameshifting in \textit{in vitro} translation assays, affected luciferase production in the cell culture assays. Attempts to increase oligonucleotide uptake into the cells by direct transfection of the oligonucleotides or by formulating with cationic lipid, were also unsuccessful.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Effects of oligonucleotides on \textit{gag-pol}/luciferase frameshift efficiency. Oligonucleotides were hybridized to either GPL(-0) or GPL(-1) RNA at 5 μM followed by a 1 hour \textit{in vitro} translation and determination of luciferase activity (light units). The numbers in parentheses represent the frameshift efficiency for GPL(-1) calculated as described in Materials & Methods.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Frameshift enhancement by oligonucleotides targeted to sequences 3' of the \textit{gag-pol} hairpin. 2'-O-methyl oligonucleotides complementary to T7GPL(-1) RNA as indicated above the graph and in Table II, were hybridized at a concentration of 5 μM to the message for 15 minutes, then \textit{in vitro} translation carried out for 1 hour as previously described. Luciferase activity was then quantitated. Each piece of data represents 3–6 reactions shown with standard deviations.}
\end{figure}
translated in vitro. The results are shown in Figure 7. The parent RNA's show a typical frameshift efficiency of 4.2 percent. However, a frameshift efficiency of over 32 percent is seen with the GPEL constructs. This is an almost 8 fold increase in frameshifting as compared to the parent constructs. These results correlate with those obtained with the GPL RNA in the presence of enhancing oligonucleotides, where a maximum 6 fold increase in frameshift efficiency was observed with oligonucleotide 4289.

To determine if the enhancement also occurs in cells, the double hairpin gag-pol/luciferase cassettes from pT7GPEL(−0) and pT7GPEL(−1) were ligated into the vector pREP-10, which contains the RSV promoter and eukaryotic processing signals, to give the plasmids pRGPEL(−0) and pRGPEL(−1). These plasmids, as well as pRGPL(−0) and pRGPL(−1), were transfected into NIH 3T3 cells along with a placental alkaline phosphatase reporter plasmid to normalize for transfection efficiency. Luciferase activity was determined 18 hours later as described in Materials and Methods. The double stem constructs, pRGPEL(−0) and pRGPEL(−1), gave a frameshift efficiency of 19.0 percent in cell culture, while the parent constructs, pRGPL(−0) and pRGPL(−1), gave a frameshift efficiency of 4.6 percent. This is approximately one half the enhancement observed with the pT7GPEL constructs in in vitro translation assays (Fig. 7).

**DISCUSSION**

One of the objectives of this work was to determine if antisense oligonucleotides targeted to the gag-pol frameshift region of HIV-1 would effectively perturb frameshift efficiency and gene expression. To test this, we constructed an HIV gag-pol/luciferase fusion gene reporter system which quantitatively measured frameshifting in vitro and in transfected cells. We initially targeted the translational initiation codon and adjoining sequences with oligonucleotides synthesized as phosphodiester, RNA, phosphorothioate, or 2'-O-methyl. Several investigators have found that oligonucleotides designed to bind the translational initiation codon are capable of translational arrest (reviewed in 30). We used several different oligonucleotide analogs to compare their activities and specificities in in vitro translation. All modifications showed specific inhibition of luciferase expression in in vitro translation reactions (Fig 3), with the exception of the phosphorothioates, which showed high activity with both specific and randomized sequences. Other investigators have also observed non-specific activity with phosphorothioates in rabbit reticulocyte lysates (31). Therefore we used 2'-O-methyl oligonucleotides to target the frameshift region, as they gave the greatest amount of specific activity and, unlike phosphodiester and RNA, have been shown to be resistant to degradation in serum and cells (32).

Oligonucleotides were designed with complimentarity to the shift site, the hairpin, and the region in between the hairpin and shift site. Those which were targeted to the hairpin bound poorly or not at all, probably due to the high degree of stability of the stem. Oligonucleotides targeted to the shift site and surrounding sequences bound the target (Fig.4, 3734, 4151), but showed no activity against either in frame or −1 frame target RNAs.

Oligonucleotides targeted to the sequences 3' of the hairpin also bound the target with high affinity, but in contrast to oligonucleotides which bound 5' to the hairpin, increased frameshift efficiency in the in vitro translation assay when targeted to the GPL(−1) out of frame target (Figs. 5 and 6) in a dose dependent fashion. Conversely, when the GPL(−0) in frame
RNA was used as the target, luciferase production decreased (Fig 5). This suggests that the GPL(−0) construct shifts out of frame at approximately the same efficiency as the GPL(−1) construct shifts into frame when oligonucleotides are bound. Overall expression of luciferase seems to diminish slightly in the presence of these oligonucleotides, probably due to translational arrest of the ribosome as it encounters the area of the message bound by oligonucleotide. The frameshift enhancing effect was localized to the 40 bases immediately 3’ of the stem and was duplicated by the creation of a second intramolecular hairpin immediately following the native hairpin. Frameshif enhancement was also observed with the double stem constructs in cell culture. This effect was not as pronounced in NIH 3T3 cells (4 fold enhancement) as it was in in vitro translation assays (8 fold enhancement). This could be due to variations in the populations of the ‘shifty’ tRNA’s between lysates used in in vitro translation and cells (14) or to differences in the components of the translational machinery in the two systems.

The shift sites of nearly all retroviruses have been shown to be closely followed by highly stable hairpin structures (15). These structures are believed to function by causing the translating ribosome to stall as it transverses the shift site, allowing for slippage of the ribosome into the correct reading frame (33). Early experimental evidence suggested that this was not the case for HIV-1 (16), however more recent data have shown that the hairpin does contribute slightly to frameshift efficiency in vivo (19). Oligonucleotides which tightly bind the area 3′ of the native hairpin may enhance frameshifting by effectively increasing the structure following the shift site. This increased structure might cause increased ribosomal stalling and frameshifting and implies that the hairpin is an important component of the HIV-1 frameshift site. The observed enhancement effect seems to require antisense oligonucleotides with significant binding affinity, as the increase in frameshift efficiency was only observed for oligonucleotides with ∆G′ greater than −14 kcal/mole. Truncated versions (<10 nucleotides) of enhancing oligonucleotides gave no activity under our experimental conditions (compare Fig. 5; 4152 and 4129).

Other investigators have also found that antisense activity requires a minimum level of affinity, which, for many targets, translates to a minimum oligonucleotide length of 12–15 (21,30). Oligonucleotide modifications which increase binding affinity of the compounds would be expected to increase frameshift enhancement.

The frameshift enhancement effect also requires that the oligonucleotides bind within a roughly 40 base stretch following the hairpin (Fig. 6). It is possible that oligonucleotides which bind the sequences beyond this area are too far removed from the native hairpin to increase the overall stability of the structure encountered by the translating ribosome. Therefore, there is little impact on ribosomal pausing at the shift site.

The efficiency of −1 frameshifting of retroviral RNA determines the ratio of gag-pol gene products, a ratio that dramatically affects viral assembly (20) and therefore must be rigorously controlled. It follows that either inhibition or enhancement of frameshifting may affect viral production and that frameshift enhancing oligonucleotides might disrupt the HIV life cycle if successfully administered to infected cells.

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