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METHODS REPORT

# A dual-luciferase reporter system for studying recoding signals

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## ABSTRACT

A new reporter system has been developed for measuring translation coupling efficiency of recoding mechanisms such as frameshifting or readthrough. A recoding test sequence is cloned in between the renilla and firefly luciferase reporter genes and the two luciferase activities are subsequently measured in the same tube. The normalized ratio of the two activities is proportional to the efficiency with which the ribosome “reads” the recoding signal making the transition from one open reading frame to the next. The internal control from measuring both activities provides a convenient and reliable assay of efficiency. This is the first enzymatic dual reporter assay suitable for in vitro translation. Translation signals can be tested in vivo and in vitro from a single construct, which allows an intimate comparison between the two systems. The assay is applicable for high throughput screening procedures. The dual-luciferase reporter system has been applied to in vivo and in vitro recoding of HIV-1 *gag-pol*, MMTV *gag-pro*, MuLV *gag-pol*, and human antizyme.

**Keywords:** antizyme; frameshift; HIV; MMTV; MULV; readthrough; ribosome; translation

## INTRODUCTION

There are a number of cases where a single mRNA is translated into more than one protein by recoding where special signals in the mRNA instruct ribosomes to change their decoding rules. In some cases of recoding, special signals are far distant 3' on the message (Berry et al., 1993; Miller et al., 1997). For studying the great majority of known cases of recoding, where the signals are close to the recoding site, it is helpful to measure the synthesis of two protein products. This has typically been done by monitoring radioactive proteins separated by SDS gel electrophoresis or by some combination with enzyme assays. These studies would be aided by a convenient and rapid assay suitable for both in vitro and in vivo screening of products. Here we describe a dual reporter system that employs two luciferase proteins, firefly and renilla, that can be assayed independently in one reaction mixture. Sequences

coding for presumptive recoding signals are placed in a plasmid construct between sequences encoding the two reporters with appropriate arrangement of reading frames and stop codons to test the ability of the test sequences to redirect ribosomes from the upstream open reading frame (ORF) to the downstream ORF.

This assay is particularly useful for studying recoding, where the rules for decoding are temporarily altered through specific sites and signals built into the mRNA sequences (Brierley, 1995; Gesteland & Atkins, 1996). In mammalian cells: (1) Redefinition of stop codons to sense codons (readthrough) allows synthesis of selenocysteine-containing proteins (Bock et al., 1991; Low & Berry, 1996) and synthesis of elongated proteins in many RNA viruses, like Moloney murine leukemia virus (MuLV) (Yoshinaka et al., 1985). (2) +1 Frameshifting regulates expression of ornithine decarboxylase antizyme. The system is autoregulatory, dependent on the concentration of polyamines (Hayashi et al., 1996). (3) -1 Frameshifting is used to synthesize the GagPol precursor polyprotein in retroviruses that have *gag,(pro)* and *pol* genes in different reading frames (except spumaretroviruses, Enssle et al., 1996).

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Examples are the mouse mammary tumor virus (MMTV) *gag-pro* frameshift (Jacks et al., 1987; Moore et al., 1987) and the human immunodeficiency virus type 1 (HIV-1) *gag-pol* frameshift (Parkin et al., 1992).

Continued efforts to study the elements on messenger RNAs that signal recoding have led to the development of several reporter systems. In some studies, the efficiency of recoding is assessed by analysis of <sup>35</sup>S-met-labeled translation products. Constructs direct ribosomes to initiate in the zero frame to translate ORF1. However, before the terminator of ORF1, a recoding signal in a test sequence directs a proportion of ribosomes to bypass the terminator. In some systems, this occurs by shifting the reading frame, in others, an amino acid is inserted for the stop codon. In both cases, the chimeric product has the upstream reporter fused to the downstream reporter. After electrophoresis of the translation products on an SDS polyacrylamide gel, the ratio of the shorter, upstream product to the chimera reflects the recoding efficiency of the test sequence. Several enzymatic reporter assays have been developed for in vivo studies using chloramphenicol acetyl transferase (*cat*) (Martin et al., 1989) or firefly luciferase (Cassan et al., 1990; Reil & Hauser, 1990). If the test sequence contains a frameshift signal, its control construct contains a one-base insertion or deletion (in-frame control). If the signal specifies readthrough, in the control, the stop codon is changed into a sense codon by a one-base substitution. Transfection efficiencies were determined by comparison with *lacZ* gene product from a cotransfected construct.

To obtain a direct measure of efficiency, in two cases (Reil et al., 1993; Stahl et al., 1995) a  $\beta$ -galactosidase reporter gene was inserted in the upstream ORF frame with firefly luciferase downstream providing an internal control for initiating ribosomes. This removed the issue of monitoring transfection efficiencies. The advantage of an activity-based reporter system is that it allows estimation of the ratio between upstream and downstream reporters for the control construct, whereas protein gels only show one product, corresponding to the fusion product. The activity ratio of the positive control can be used to normalize values obtained from the corresponding recoding signal construct. The power of this system, when applied systematically to each construct, is demonstrated in a recent report that showed that the efficiency of HIV-1 frameshifting is directly related to the stability of the stem-loop (Bidou et al., 1997). Although  $\beta$ -galactosidase reporters are useful in vivo, they are not suitable for in vitro translation, due to the length of the *lacZ* coding sequence (3 kb).

We have designed a dual-luciferase reporter system for measuring recoding efficiencies in vivo or in vitro from a single construct (Fig. 1). The firefly luciferase gene (*fluc*) has been cloned behind the gene of renilla luciferase (*rluc*), into an altered pRL-SV40 vector. Expression features for initiation and termination of transcription

and translation, as well as the nature of the two reporter genes (short enough to be efficiently synthesized in an in vitro translational system), allow use of the reporter construct for both in vivo and in vitro applications. Between the 5' reporter (*rluc*) and the 3' reporter (*fluc*), we have inserted two alternative polylinkers (giving p2luc and p2luci, Fig. 1B). This assay combines rapidity of the reactions with very low background levels and provides a powerful assay. In vitro experiments can be performed in 96-well microtiter plates, in vivo experiments in 6-well culture dishes. This makes the dual-luciferase assay suitable for high throughput screening approaches.

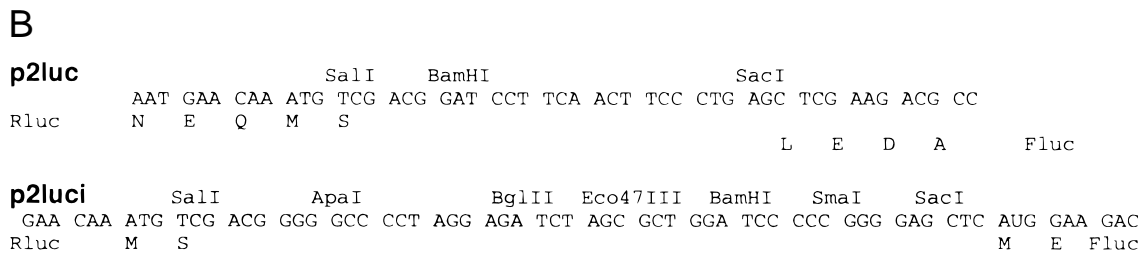
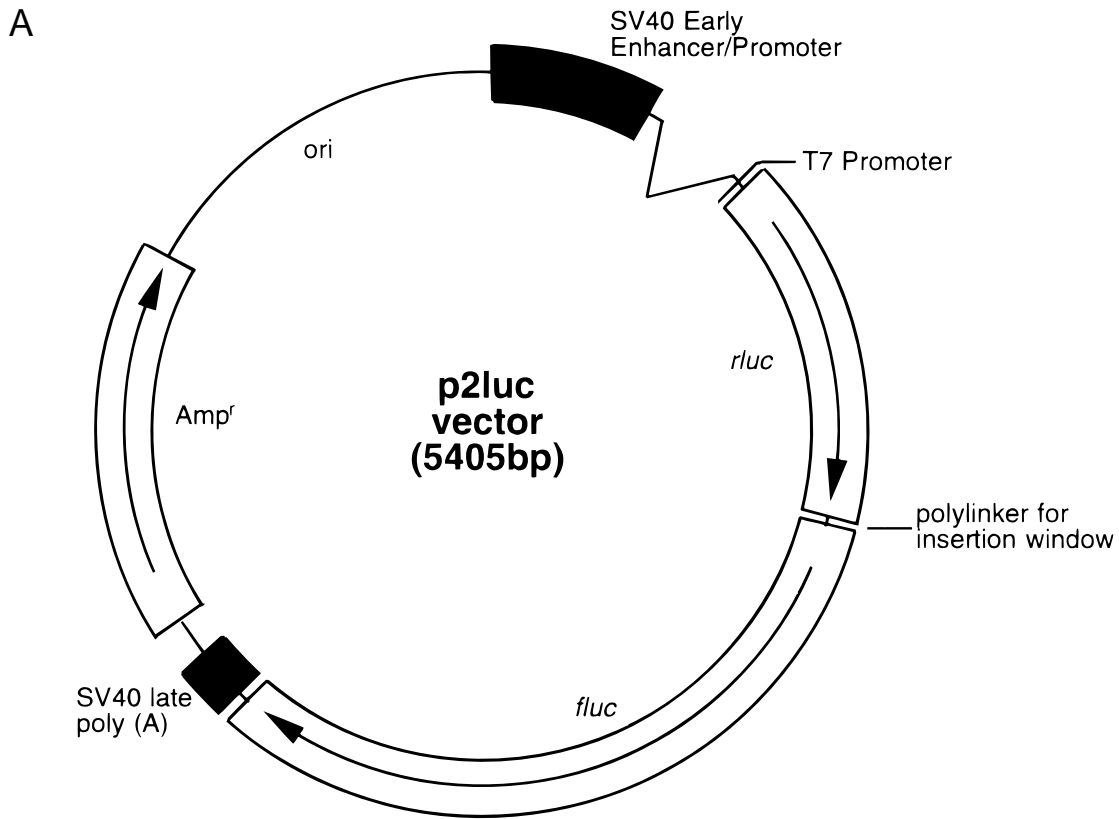
## RESULTS AND DISCUSSION

### Properties of the dual-luciferase reporter

The dual-luciferase assay is designed so that synthesis of the second reporter (firefly luciferase, F) is dependent on recoding. However, on its own, the amount of this reporter is not a direct reflection of the efficiency of recoding. In the absence of in-frame stop codons, a significant proportion of translating ribosomes disengage prematurely from the mRNA; this is often known as ribosome drop off (early results from this and other laboratories have shown that 50% or more of *Escherichia coli* ribosomes drop off during synthesis of  $\beta$ -galactosidase; Manley, 1978; Kurland et al., 1996). Ribosomes that drop off while decoding the firefly reporter will lead to an underestimate of the proportion of ribosomes that respond to the recoding signals unless a correction is made. The basis for a correction factor is the assumption that drop off during synthesis of the firefly reporter is proportional to completion of synthesis of this reporter. The correction factor is provided by a control in which all ribosomes that complete synthesis of the first reporter (renilla luciferase, R) continue translation by starting synthesis of the F reporter.

The fate of ribosomes is assessed by the level of their products. Let  $R$  be the product of translation of the renilla reporter coding sequence, with ribosomes terminating at the zero frame terminator located at, or within a short distance 3' to, the site of recoding;  $Rf$ , the products of translating ribosomes that responded to the recoding signal, but aborted before completing synthesis of the firefly reporter;  $RF$ , products exhibiting firefly luciferase activity (their synthesis requires complete translation of the coding regions for both reporters). Let  $p$  be the proportion of ribosomes that respond to the recoding signal that complete synthesis of the firefly reporter,  $p = RF/(RF + Rf)$ . Because the test sequence and its corresponding control are identical downstream of the recoding signal, we assume the proportion,  $p$ , is the same for both constructs:

$$p = RF_{test}/(RF_{test} + Rf_{test}) = RF_{cont}/(RF_{cont} + Rf_{cont}).$$



**FIGURE 1.** Vectors for the dual-luciferase reporter system. **A:** Vector p2luc: *rluc*, renilla luciferase gene; *fluc*, firefly luciferase gene; Amp<sup>r</sup>, ampicillin resistance; ori, origin of replication in *Escherichia coli*. **B:** Sequences of polylinkers of p2luc and p2luci.

Recoding efficiency can be expressed by the number of ribosomes that respond to the recoding signal divided by the number of ribosomes that reach the recoding signal (i.e., that have completed translation of the renilla reporter):

$$Rec-eff. = (RF_{test} + Rf_{test}) / (RF_{test} + Rf_{test} + R_{test})$$

$$= [(RF_{test}) / (RF_{test} + Rf_{test} + R_{test})] / p.$$

The measured firefly luciferase activity is given by the number of peptides that have firefly activity ( $RF$ ) multiplied by the specific activity of these peptides ( $\Phi$ ). Because the peptide sequences of a test sequence and its control are identical, the specific activity of mol-

ecules synthesized from the test and its control reaction are equal:

$$Fa_{test} = RF_{test} \times \Phi \quad \text{and} \quad Fa_{cont} = RF_{cont} \times \Phi.$$

The measured renilla luciferase activity is given by the number of peptides that have renilla activity multiplied by the specific activity ( $\Omega$ ) of the respective species:

$$Ra_{test} = R_{test} \times \Omega_R + Rf_{test} \times \Omega_{Rf} + RF_{test} \times \Omega_{RF} \quad \text{and}$$

$$Ra_{cont} = Rf_{cont} \times \Omega_{Rf} + RF_{cont} \times \Omega_{RF}.$$

In our experiments, the specific activity of renilla luciferase was not altered by C-terminal extensions of the different constructs (see below). Then:

$$Ra_{test} = (R_{test} + Rf_{test} + RF_{test}) \times \Omega \quad \text{and}$$

$$Ra_{cont} = (Rf_{cont} + RF_{cont}) \times \Omega.$$

The experimentally established value for the ratio of firefly over renilla luciferase activity for the test sequence can be described as:

$$(Fa_{test}/Ra_{test}) = [RF_{test}/(R_{test} + Rf_{test} + RF_{test})] \times [\Phi/\Omega];$$

and the luciferase activity ratio of the control construct as:

$$\begin{aligned} (Fa_{cont}/Ra_{cont}) &= [RF_{cont}/(Rf_{cont} + RF_{cont})] \times [\Phi/\Omega] \\ &= p \times \Phi/\Omega. \end{aligned}$$

It follows that:

$$\begin{aligned} Rec\text{-}eff. &= [(RF_{test})/(R_{test} + Rf_{test} + RF_{test})]/p \\ &= (Fa_{test}/Ra_{test})/(Fa_{cont}/Ra_{cont}). \end{aligned}$$

In other words, the activity ratio of the control construct can be used to normalize the activity ratio obtained from the test sequence for drop off occurring downstream from the recoding signal.

The above calculation of frameshift efficiency is based on the assumption that the specific activity of renilla luciferase is not altered by C-terminal elongation of the protein. Comparison of the radioactivity levels from <sup>35</sup>S-labeled translation products, electrophoresed on SDS polyacrylamide gels, with activity-assay values showed no significant alterations (data not shown). On the other hand, estimation of the amount of *Rf* by phosphorimaging is relatively imprecise, due to the large area of the gel containing *Rf* products. Another way to verify that the specific activity of renilla luciferase is not altered by a C-terminal extension is to establish a dose-response curve comparing reactions with different amounts of out-of-frame and in-frame mRNA. If the renilla activity is not altered by C-terminal extension, the firefly/renilla luciferase activity ratios (*Fa*/*Ra*) of the reactions should be linear and proportional to the amount of in-frame mRNA. If, on the contrary, C-terminal extension diminishes the specific activity of renilla luciferase, we would expect a nonlinear (concave) increase of the ratio.

Translation assays were performed in reticulocyte lysates with increasing amounts of the HIV in-frame control RNA (R-L), compared to the 2luc out-of-frame RNA (R) (Fig. 3). The firefly/renilla luciferase activity ratios (*Fa*/*Ra*) for different percentages of (R-L) mRNA were linear over two orders of magnitude, and were proportional to the reaction, containing 100% (R-L) mRNA.

## Application of the dual-luciferase reporter

p2luc and p2luci are vectors designed to allow recoding assays in vitro and in vivo. The plasmids contain major expression features of pRSV40: an SV40 early enhancer/promoter initiates transcription in eukaryotic cells. A chimeric intron increases transport of the messenger into the cytoplasm. The second reporter gene is followed by an SV40 late polyadenylation signal. Insertion of a T7 promoter in front of the renilla luciferase gene (*rluc*) and a blunt end restriction site behind firefly luciferase (*fluc*) provide the means to permit in vitro transcription. In vitro assays were performed four times with two independent RNA preparations. The ratio of luciferase activities in each reaction was determined twice. Standard deviation for determined activity ratios was 10% of the value or less.

The dual-luciferase assay was applied to four different recoding systems:  $-1$  frameshifting (HIV *gag-pol*, MMTV *gag-pro*), readthrough (MULV *gag-pol*), and  $+1$  frameshifting (antizyme). The sequences of the different constructs are shown in Figure 2B. The p2luc vector without any insertion has the firefly luciferase gene in the  $-1$  frame and ribosomes translating the upstream renilla luciferase gene are stopped at the sixth codon behind the *Sal*I cloning site. The HIV test construct contains a 307-nt long insertion, covering the entire overlapping sequence between *gag* and *pol*. Ribosomes that do not shift to the  $-1$  frame will stop at codon 95 of the test sequence. The in-frame construct has a one-base insertion leading translating ribosomes into the  $-1$  frame, and two single-base substitutions that destroy the slippery sequence and prevent frameshifting into the  $-2$  frame. The MMTV test sequence works very similarly. Ribosomes that do not shift are stopped at the fourth codon behind the recoding signal, but ribosomes that have shifted to the  $-1$  frame will translate into the firefly luciferase coding sequence. Again, the control construct has a one-base insertion and a destroyed slippery sequence. The MuLV recoding signal features an in-frame stop codon. Ribosomes that read through this stop codon continue translation and enter the firefly luciferase reporter. The control sequence has the stop codon, UAG, replaced with CAG, which codes for glutamine. The antizyme recoding signal contains an in-frame stop codon, which ribosomes can avoid by a  $+1$  frameshift. The control has an insertion of two bases, putting the stop codon out-of-frame and leading translating ribosomes into the  $+1$  frame.

Figure 4 shows an SDS polyacrylamide electrophoresis of products of an in vitro translation (Fig. 4A), compared to recoding efficiencies (Rec-eff.) in vitro (Fig. 4B) and in vivo (Fig. 4C) that were calculated from luciferase activity measurements. In vitro, renilla luciferase corresponded to a 40-kDa translation product on a protein gel and firefly luciferase activity was propor-



## A

SAA2luc CCATG**TCGAC**GGGGGCCCTAGGAAAAAGGGCT  
 B1Gpr GCAGGGATCCCTTTAGTTGCCCCCTATCT  
 BglIP1 GTTCGAAG**AGATCT**TCCCTTAAGAAGTTAGCCTG  
 fsAZ1 GGCCG**TCGACT**AGGGTTGCCCTTCATTGCTG  
 fsAZ2 GGCCGGATCCACTGTGATCCCGCTGACTGTT

**FIGURE 2.** Insertion of recoding "windows" into p2luc. **A:** Oligonucleotides for PCR synthesis of sequences from HIV-1 and antizyme. **B:** Sequences of cloned insertions. Restriction sites used for cloning are shown in bold characters.

## B

p2luc out of frame 5' G **TCG ACG GAT** CCT TCA ACT TCC CTG AGC TCG AAG ACG CCA AAA ACA TAA ...  
 rluc S T D P S T S L S S K T P K T \*

HIV 5' G **TCG ACG** GGG GCC CCT ... AAT TTT TTA GGG AAG ATC TGG CCT TCC TAC AAG GGA AGG CCA ... TAG GGG GGC AAC TAA AGG **GAT CC**  
 ...  
 rluc S T G A P ... N F L G K I W P S Y K G R P ...\*  
 F F R E D L A F L Q G K A R ... G G Q L K G fluc

HIV control 5' G **TCG ACG** GGG GCC CCT ... AAC TTC TTA AGG GAA **GAT CTG** GCC TTC CTA CAA GGG AAG GCC ... ATA GGG GGG CAA CTA AAG GGA TCC  
 rluc S T G A P ... N F L R E D L A F L Q G K A ... I G G Q L K G fluc

MMTV 5' G **TCG ACA** GCT GAA AAT TCA AAA AAC TTG TAA AGG GGC AGT CCC CTA GCC CCA CTC AAA AGG GGG ATA AGG **GAT CC**  
 rluc S T A E N S K N L \*  
 K K L V K G Q S P S P T Q K G D K G S ... fluc

MMTV control 5' G **TCG ACA** GCT GAA AAT TCG AAG AAG CTT GTA AAG GGG CAG TCC CCT AGC CCC ACT CAA AAG GGG GAT AAA **GGA TCC**  
 rluc S T A E N S K K L V K G Q S P S P T Q K G D K G S ... fluc

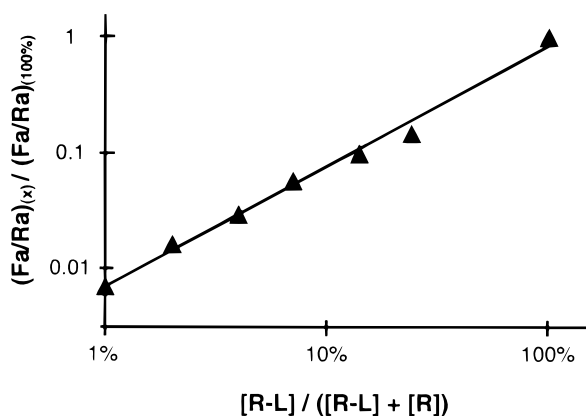
MuLV 5' G **TCG ACG** ACC CTA GAT GAC TAG GGA GGT CAG GGT CAG GAG CCC CCC CCT GAA CCC AGG ATA ACC CTC AAA GTC GGG GGG CAA **GGA TCC**  
 rluc S T T L D D \* G G Q G Q E P P P E P R I T L K V G G Q G fluc

MuLV control 5' G **TCG ACG** ACC CTA GAT GAC CAG GGA GGT CAG GGT CAG GAG CCC CCC CCT GAA CCC AGG ATA ACC CTC AAA GTC GGG GGG CAA **GGA TCC**  
 rluc S T T L D D Q G G Q G Q E P P P E P R I T L K V G G Q G fluc

antizyme 5' G **TCG ACT** AGG GTT GCC ... TGG TGC TCC TGA TGT CCC TCA CCC ACC CCT GAA GAT CCC AGG T...CA GCG GGA TCA CAG **TGG ATC C**  
 rluc S T R V A ... W C S \*  
 D V P H P P L K I P G Q R D H S G S fluc

antizyme control 5' G **TCG ACT** AGG GTT GCC ... TGG TGC TCC CCT GAT GTC CCT CAC CCA CCC CTG AAG ATC CCA GGT ... CGG GAT CAC AGT **GGA TCC**  
 rluc S T R V A ... W C S P D V P H P P L K I P G ... R D H S G S fluc

tional to a product of 100 kDa. Thus, activity of the firefly luciferase reporter was due to the expression of a fusion protein, which depended on the recoding efficiency of the different test sequences.

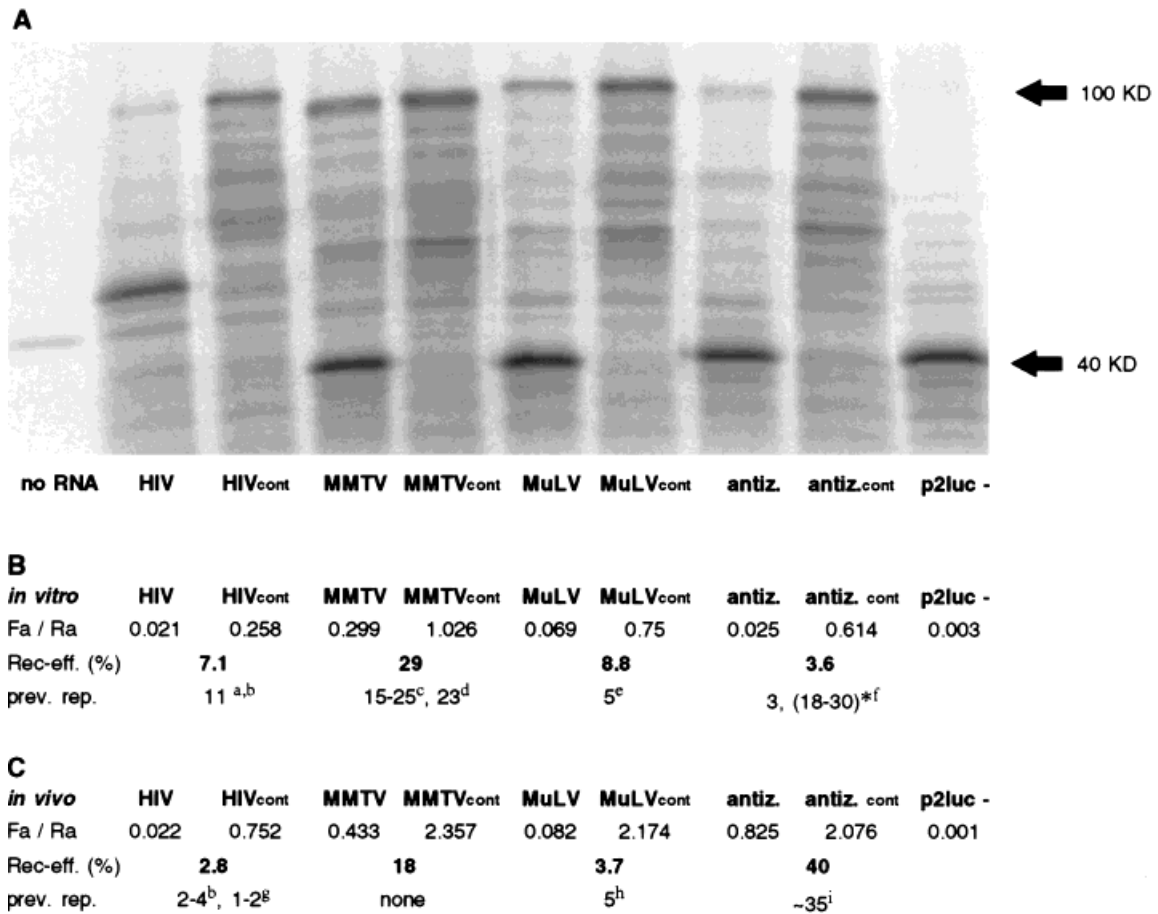


**FIGURE 3.** Reporter activities are proportional to in vitro translation of their mRNAs. In vitro reticulocyte lysate reactions were performed as described above in the presence of 1  $\mu$ g of RNA mixtures containing different percentages of HIV-1 in-frame control RNA (R-L) and the out-of-frame control p2luc-RNA (R). Firefly/renilla activity ratios ( $Fa/Ra$ ) were normalized to the reaction containing 100% (R-L) RNA.

Determined recoding efficiencies by the dual-luciferase reporter were very comparable to previously reported values (prev. rep). In vitro, HIV *gag-pol* frameshifting (7.1%) was a little lower than what was reported (11%). MMTV *gag-pro* (29%) and MuLV *gag-pol* (8.8%) recoding values were somewhat higher than previous data (15–25% and 5%, respectively). Antizyme frameshifting (3.6%) at the endogenous polyamine concentration of reticulocyte lysate corresponded well to what was reported (~3%).

In vivo, results obtained with the 2-luciferase reporter for HIV (2.8%) and antizyme (40%) were close to data reported by others (1–4% and 35% respectively). MuLV readthrough (3.7%) was slightly lower than reported previously (5%). For MMTV frameshifting in vivo, we obtained 18%. To our knowledge, no quantitative estimation for MMTV in vivo frameshifting has been published.

The frameshift efficiency for antizyme was 10 times lower in vitro (3.6%) than in vivo (40%). In vitro frameshifting can be amplified up to 10 times by supplementing reticulocyte lysate with polyamines (Matsufuji et al., 1995). In vivo, antizyme frameshifting is high, presumably due to high intracellular concentration of polyamines. By controlling the intracellular polyamine concentration, similar dependence of antizyme frameshifting on polyamine concentrations has been ob-



**FIGURE 4.** Analysis of four recoding systems by the dual-luciferase reporter. Autoradiography (A) and firefly/renilla luciferase activity ratios (*Fa/Ra*) (B, C), after *in vitro* translations in reticulocyte lysate (A, B) or *in vivo* transient transfections in 293 human kidney cells (C). Out of frame control values from the p2luc-construct were subtracted from the firefly/renilla luciferase activity ratios (*Fa/Ra*). Values were then normalized to their corresponding control construct (cont), to calculate recoding efficiencies (Rec-eff.). Results are shown in comparison to previously reported (prev. rep.) values by other groups [<sup>a</sup>Jacks et al., 1988; <sup>b</sup>Reil et al., 1993; <sup>c</sup>Moore et al., 1987; <sup>d</sup>Jacks et al., 1987; <sup>e</sup>Wills et al., 1991; <sup>f</sup>Matsufuji et al., 1995; <sup>g</sup>Cassan et al., 1990; <sup>h</sup>Feng et al., 1990; S. Matsufuji, H. Takizawa, T. Matsufuji, S. Hayashi, R.F. Gesteland, & J.F. Atkins (pers. comm.)]. <sup>\*</sup>When supplemented with 0.8 mM spermidine.

served in transfected mammalian cells (S. Matsufuji, H. Takizawa, T. Matsufuji, S. Hayashi, R.F. Gesteland, & J.F. Atkins, manuscript in prep.).

For the three retroviral systems (HIV, MMTV, and MuLV), recoding efficiencies were observed to be lower *in vivo* than *in vitro*. The three systems contain a stimulatory secondary structure 3' to the recoding site, which might partially act by inducing ribosomal pausing (Tu et al., 1992; Somogyi et al., 1993). One possible explanation is that, under suboptimal *in vitro* conditions, the effect of ribosomal stalling has a higher impact than *in vivo*.

Luciferase activity ratios for the control constructs varied with the test sequence. Overall processivity of the 2-luciferase reporter system was significantly higher *in vivo* than *in vitro*. Nevertheless, variations of translation efficiencies, which we observed *in vitro*, were conserved *in vivo* (compare *Fa/Ra* values of control constructs in Fig. 4B and C). Specifically, the HIV frameshift window

significantly lowered translation processivity, which may be due to the length of the inserted sequence.

A general concern when using any reporter system is that artifactual results may arise occasionally from interactions between test and reporter sequences. Such artifacts can be controlled by comparing with the results from a different reporter system. The fact that previous and our results correspond gives confidence in the obtained results.

The 2-luciferase assay gave an *in vitro* background value for nonspecific recoding of about 0.3% for the out-of-frame control (p2luc-, Fig. 4B). The background *in vivo* (p2luc-, Fig. 4C) was much lower (0.1%).

In summary, the dual-luciferase assay allows us to obtain reliable numbers that are likely to quite accurately measure the efficiency of recoding for a given test sequence, both *in vivo* and *in vitro*. This is achieved by normalizing reporter activity ratios of recoding windows by their corresponding in-frame control. We show

linear activity ratios from 100% to less than 1%. The low background of the luciferase reactions allows quantitation of coupling of two translational cistrons in vivo and in vitro at efficiencies down to 0.1%. The rapidity and the accuracy of the assay make it a suitable tool for research on recoding signals, but also for applications in drug screening where translation recoding is targeted.

## MATERIALS AND METHODS

### Plasmid constructions

General procedures for DNA recombination techniques were performed as described (Sambrook et al., 1989). The unique *Bgl*II site in pRL-SV40 (Promega, # TB239) was cut and filled in by T4 DNA polymerase. Blunt ends were religated, giving pG11. The PCR product of primers lucR (GGAAGATCTGTTTAAACGGATCCGTCGACATTTGTTCAATTTTGGAGAACTCG) and *Xcm*I (GGTGAAGTTCGTCGTCACAC), with pRL-SV40 as template, was digested by *Bgl*II and *Xcm*I and then inserted between the unique *Bam*H I and *Xcm*I sites of pG11. Thereby, the unique *Bam*H I site was replaced, and unique *Pme*I and *Sal*I sites were inserted (pG12). A *Bam*H I–*Pme*I restriction fragment out of pNewcite (G. Grentzmann, J.F. Atkins, & R.F. Gesteland, unpubl. results), containing the firefly luciferase gene, which was initially cloned from pBgalluc-1 (Reil et al., 1993), was inserted between the *Bam*H I and *Pme*I sites of pG12 (pG13). A blunt end linearization site behind the firefly luciferase coding sequence was added and the SV40 late polyadenylation signal was re-installed by cloning the PCR product of primers 2lucA1 (AAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTCCAAATTGTAACACGTGTAATTCTAGAGCGGCCGCTTC) and 2lucA2 (GACCGTTTAACTTATCGATTCCACATTTGT) applied to pRL-SV40 between the *Eco*N I and *Pme*I sites of pG13 (p2luc, Fig. 1A). A second version (p2luci) was constructed to establish supplemental cloning sites by inserting the complementary oligonucleotides luci1 (TCG ACG GGG GCC CCT AGG AGA TCT AGC GCT GGA TCC CCC GGG GAG CTC AUG GAA GAC GCC AAA AAC ATA AAG AAA GGC CCG G) and luci2 (CGC CGG GCC TTT CTT TAT GTT TTT GGC GTC TTC CAT GAG CTC CCC GGG GGA TCC AGC GCT AGA TCT CCT AGG GGC CCC CG) between the *Sal*I and *Nar*I sites of p2luc. Multiple cloning sites of p2luc and p2luci are shown in Figure 1B. The sequences of p2luc can be accessed on Gene Bank accession no. AF043450.

For constructs containing the recoding sequences of MuLV and MMTV, complementary oligonucleotides with *Sal*I and *Bam*H I compatible ends were synthesized on an Applied Biosystems model 380C synthesizer. For HIV-1 (strain HXB-2), PCR primers SAA2luc and B1Gpr, containing *Sal*I and *Bam*H I restriction sites, were used to amplify the wild-type sequence from pGEM3ZPS. An in-frame control was generated by amplifying pGEM3ZPS between SAA2luc and BgIP1. For cloning the antizyme test sequence, we used oligonucleotides fsAZ1 and fsAZ2, containing *Sal*I and *Bam*H I sites, to PCR generate wild-type and in-frame control sequences from AY103 and AYFC01, respectively (Matsufuji et al., 1996). Restriction-digested PCR products were cloned between the unique *Sal*I

and *Bam*H I sites of p2luc. The sequences of all constructs were verified by automated thermocycler sequencing. The sequences of PCR oligonucleotides and of the final test sequences, inserted into p2luc, are shown in Figure 2.

### Frameshift efficiency measurements

For in vitro transcription, *Pml*I linearized plasmids were used as templates for T7 RNA polymerase as described previously (Wills et al., 1991). For in vitro translation in reticulocyte lysate (Promega), 1  $\mu$ g of each RNA preparation was used per 7.5  $\mu$ L of reaction mixture, complemented at 130  $\mu$ M, with all amino acids except methionine, and 3.3  $\mu$ M (<sup>35</sup>S)-met (1,200 mCi/mM) was added. Samples were electrophoresed on SDS/15% polyacrylamide gels. Dried gels were scanned and quantified for radioactivity on a Molecular Dynamics PhosphorImager. For luciferase activity measurements, samples of 3  $\mu$ L or 0.6  $\mu$ L of reticulocyte lysate reaction mixtures were diluted in 100  $\mu$ L 1 $\times$  lysis buffer and analyzed for renilla and firefly luminescence activity, using the Dual-Luciferase™ reporter assay (Promega) on a Dynatech MLX Microtiter Plate Luminometer. For both reactions, light emission was measured between 2 and 12 s after luminescence substrate injection. In a first reaction, extracts were tested for firefly luciferase activity in the presence of beetle luciferin, O<sub>2</sub> and Mg<sup>2+</sup>, via a luciferyl-coA intermediate, which allows rapid enzymatic turnover. In a second step, firefly luciferase activity is quenched and the substrate of renilla luciferase (Coelenterazine) is added. To avoid contamination between the firefly reagent and its quenching reagent (in the second step), all 96 wells of one microtiter plate are tested for luciferase activity before starting measurement of renilla luciferase.

As controls, luciferase activity reactions were performed with and without 130  $\mu$ M of supplemented methionine (the endogenous concentration of methionine in reticulocyte lysate is 5  $\mu$ M). No differences in overall translational efficiency or recoding efficiency were observed. For in vivo translations, human kidney 293 cells (ATCC) were cultivated in minimum essential medium supplemented with 10% fetal bovine serum. Transient transfections were performed by lipofection (GIBCO BRL). In vivo expression of the reporter genes was dependent on cell density and transfection efficiency, but was consistent in a given series of transfection experiments. Luciferase activity was determined 24 h after transfection. One-hundred microliters of lysate was assayed by the Dual-Luciferase™ reporter assay as described above. In vivo recoding experiments were repeated four or six times in two or three different transfection series.

Renilla and firefly luciferase activities were assayed subsequently in the same tube. Standard deviations of established activity ratios are therefore independent of volume variations due to pipetting. Standard deviation for recoding efficiencies established in vitro and in vivo was less than 20% of the values.

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