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Identification and analysis of antisense RNA target regions of the human immunodeficiency virus type 1

Karola Rittner and Georg Sczakiel*
Institut für Virusforschung, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

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ABSTRACT

Antisense RNA, transcribed intracellularly from constitutive expression cassettes, inhibits the replication of the human immunodeficiency virus type 1 (HIV-1) as demonstrated by a quantitative microinjection assay in human SW480 cells. Infectious proviral HIV-1 DNA was co-microinjected together with a fivefold molar excess of plasmids expressing antisense RNA complementary to a set of ten different HIV-1 target regions. The most inhibitory antisense RNA expression plasmids were targeted against a 1 kb region within the gag open reading frame and against a 562 base region containing the coding sequences for the regulatory viral proteins tat and rev. Experimental evidence is presented that the antisense principle is the inhibitory mechanism in this assay system.

INTRODUCTION

Antisense nucleic acids, i.e. single-stranded nucleic acids complementary to given single-stranded target sequences, can act specifically in vivo and in vitro as down-regulators of steps involved in gene expression (1–3) or in the replication of nucleic acids (e.g. plasmid replication (4,5)). Furthermore, steps in the life cycle of viruses can be inhibited by antisense nucleic acids in procaryotic (6) as well as in eucaryotic (7–10) systems leading to a reduction or prevention of infection of host cells. These results warrant a more profound analysis of the application of antisense nucleic acids as highly specific virus inhibitors. We have been studying the inhibitory effect of antisense RNA on the replication of HIV-1. Inhibition of this virus using antisense nucleic acids, mainly chemically modified antisense oligodeoxyribonucleotides or oligoribonucleotides has already been shown (11–16). However, the application of antisense RNA by constitutive intracellular expression (17–19) seems to be an alternative and promising approach since the continuous presence of HIV-1 targeted antisense RNA in permissive, non-infected cells might lead to 'intracellular immunity' (20) against subsequent HIV-1 infection.

Constitutive expression of antisense RNA in the nucleus and its accumulation in the cytoplasm, has been demonstrated in human T-cell clones with stably integrated antisense RNA expression vectors (18). These may lead to sufficient intracellular steady state concentrations of antisense RNA in those cell compartments where neutralization of target nucleic acids may take place. Moreover, antisense RNA transcripts covering long target regions can be expected to tolerate minor base exchanges, i.e. mutations in the target sequences which frequently appear in different HIV-1 isolates (21–24). In addition to these criteria, one of the most critical factors influencing the inhibitory efficacy of antisense nucleic acids in general, and most likely also in the case of HIV-1, is the subregion of the target sequence to which the antisense nucleic acid is complementary (11–14,25–27). The selection of the target sequence determines the biological step which can be affected during the viral life cycle and, in addition, its effectiveness is strongly influenced by the secondary and tertiary structures of the antisense RNA and its target.

In order to identify some of the critical factors influencing the effectiveness of intracellularly expressed antisense RNA against HIV-1 replication, we cloned eucaryotic vectors expressing antisense RNA complementary to ten different subregions of the HIV-1 genome and tested these for their antiviral activity in a transient comicroinjection assay in human cells described previously (28).

MATERIALS AND METHODS

Plasmids and cloning methods

The expression vectors used in this study, pKEX-2-XL and pKEX-2-XR, are derived from p2Hm (28) and are described in detail elsewhere (29). Briefly, the procaryotic sequences originate from plasmid pUC19, the constitutive transcription of heterologous sequences is directed by the human cytomegalovirus immediate early (HCMV-IE) promoter/enhancer element (pos.: −598 to +54;(30)) and terminated by SV40 t-splice and polyadenylation signals. Additionally, both vectors contain the hygromycin B resistance gene driven by the herpes simplex virus thymidine kinase (HSV tk) promoter. This does not interfere with HCMV-IE directed expression as was shown earlier (unpublished).

The HIV-1 fragments used for cloning of antisense RNA expression plasmids were derived from the two proviral clones pNL4-3 (31) and BH10 (32) as indicated in Table 1. The coding

* To whom correspondence should be addressed

sequence for chloramphenicol acetyltransferase (CAT) used in pKEX-2-XL-CAT was from plasmid pBLCAT2 (33).

Restriction enzymes, alkaline phosphatase, T4-DNA ligase and Klenow fragment were from Boehringer Mannheim and were used following the laboratory manual of Maniatis et al. (1982, (34)).

Cell lines

The human epitheloid cell line SW480 (35) was maintained at 37°C/5%CO_2 in DMEM supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Human T-lymphoid MT-4 cells (36) were grown in RPMI 1640 medium at 37°C/5%CO_2 with the supplements described above.

Microinjection

The purification of plasmids, the preparation of plasmid mixtures and the microinjection of SW480 cells were performed as described (28).

HIV-1 antigen determination

HIV-1 specific antigens in dilutions of cell free co-culture supernatants with phosphate buffered saline (PBS) were measured using a commercial HIV-1 antigen ELISA (Organon-Teknika, Holland) which recognizes a series of different HIV-1 specific antigens, predominantly the gag p24 and env antigens. Relative antigen concentrations were calculated from the ELISA signals using a standard curve determined with recombinant p24.

RESULTS

Antisense RNA expression plasmids

For antisense RNA expression two expression vectors, named pKEX-2-XL and pKEX-2-XR, respectively, were used. All plasmids make use of the strong and constitutive immediate early promoter/enhancer element derived from the human cytomegalovirus. The capability of these vectors to express heterologous sequences was demonstrated earlier (18,28,29). Fragments of proviral HIV-1 DNA were cloned into this set of expression plasmids to give the HIV-1 antisense expression vectors pAR1,...,pAR10 as described in Table 1 and as shown in Figure 1A.

Inhibition of HIV-1 by antisense RNA expression plasmids

The inhibitory effect of each plasmid from the series of antisense RNA expression vectors listed in Table 1 on the replication of HIV-1 was measured in a transient microinjection assay with defined conditions (28). Briefly, nuclei of human epitheloid SW480 cells were co-microinjected with test plasmids together with the infective HIV-1 proviral clone pNL4-3 (10 ng/μl) at a constant molar excess of test plasmid of 5:1. Viral replication in these cells was amplified by coculturing the microinjected cells with human $CD4^+$ MT-4 cells which replicate HIV-1 virus efficiently. Virus release in cell free supernatants was measured by a commercial HIV-1 antigen ELISA (Organon Teknika, Holland).

The results, which have been derived from 12 independent comicroinjection experiments for each antisense RNA expression vector, are shown in Figure 1B. The strongest inhibition in this

Table 1. Description of the antisense RNA expression plasmids pAR1—pAR10 used in this work. The origins of the HIV-1 sequences (I) were the non-defective proviral HIV-1 clone pNL4-3 (N; (31)) and the defective clone BH10 (B; (32)). The expression vectors used (V) were either pKEX-2-XR (p-XR) or pKEX-2-XL (p-XL)(29). The full lengths of the HCMV driven antisense transcripts and the lengths of the HIV-1 portions are listed in the last column.

plasmid name	HIV-1 sequence	cloning procedure	total transcrpit length/HIV-1 portion (nuclotides)
pAR-1	N: HindIII 530-BssHII 710	I: N: HindIII, BssHII; Klenow; V: p-XR: SmaI; phosphatase	890/180
pAR-2	B: BssHII 255-SphI 990	I: B: BssHII; Klenow; SphI; T4-DNA-Pol; V: p-XR: SmaI; phosphatase	1445/735
pAR-3	N: BssHII 710-HindIII 1710	I: N: BssHII; Klenow; HindIII; V: p-XR: SmaI; phosphatse;	1710/1000
pAR-4	B: ApaI 1549-BglII 1640	I: B: ApaI, BglII;T4 DNA-Pol; V: p-XR: SmaI; phosphatase	801/91
pAR-5	B: NdeI 4704-SalI 5366	I: B: NdeI, SalI; Klenow V: p-XR: SmaI; phosphatase;	1372/662
pAR-6	B: SalI 5366-KpnI 5928	I: B: SalI, KpnI; V: p-XL: SalI, KpnI;	1272/562
pAR-7	B: SalI 5366-XhoI 8475	I: B: SalI, XhoI; T4-DNA-Pol; V: p-XR: SmaI; phosphatse	3819/3109
pAR-8	B: BglII 7198-HindIII 7719	I: B: BglII; Klenow; HindIII; T4-DNA-Pol; V: p-XR: SmaI;phosphatase;	1231/521
pAR-9	N: HindIII 8190-BamHI 8520	I: N: HindIII, BamHI; Klenow; V: p-XR: SmaI; phosphatse;	1040/330
pAR-10	B: XhoI 8475-KpnI 8595	I: B: XhoI, KpnI; V: pXR: XhoI, KpnI	830/120

assay was measured for three plasmids: Firstly, pAR3 (76% inhibition) which expresses antisense RNA complementary to a 1 kb region located within the gag open reading frame which encodes the p17 and p24 gag proteins (see Table 1 and Figure 1A). Secondly, pAR6 (78% inhibition) containing a 562 bp region (see Table 1 and Figure 1A) covering the first tat exon, the first rev exon and portions of the vpu and vpr open reading frames (see Figure 1A). This antisense RNA expression plasmid might inhibit the expression of the regulatory proteins tat and rev, which are essential for activation of viral gene expression (37,38) and for transport of viral mRNAs out of the nucleus (39–41). The

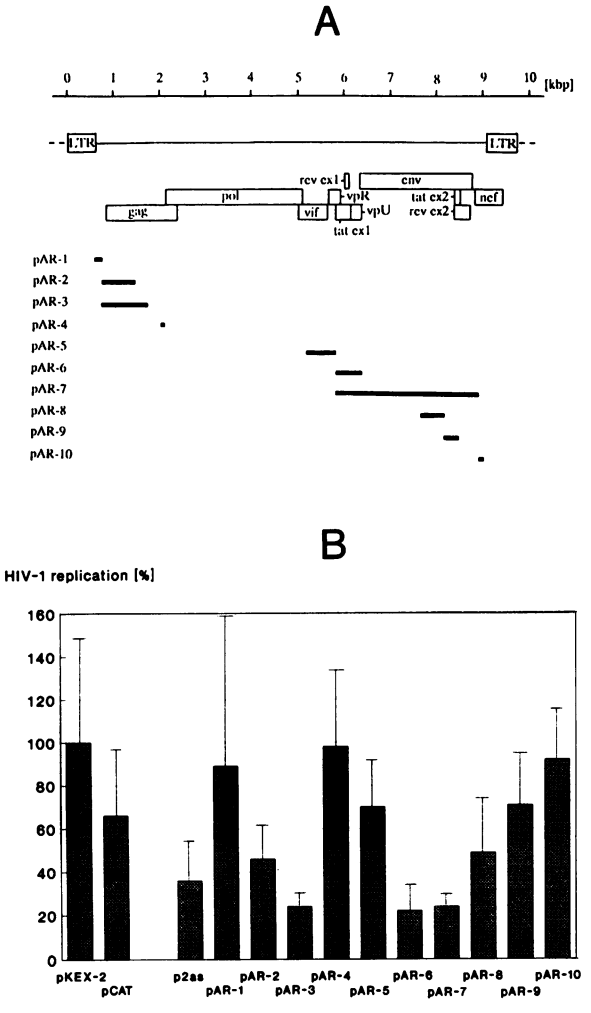

Figure 1. HIV-1 target regions and inhibitory effects of antisense RNA expression vectors pAR1-pAR10. **A)** Schematic representation of the HIV-1 genome. The bars below indicate the HIV-1 sequences being expressed from the antisense RNA expression vectors indicated on the left. **B)** HIV-1 replication in SW480 cells (filled bars) four days after comicroinjection with infectious proviral HIV-1 DNA (pNL4-3; conc.: 10 ng/μl) and a fivefold molar excess of the plasmids indicated below. The plasmid pCAT resulted from insertion of the coding sequence for chloramphenicol acetyltransferase (CAT) derived from pBLCAT2 (33) into plasmid pKEX-2. The plasmid p2as is identical to plasmid p2-Hm-HIV$_{410}$-as described elsewhere (28). It directs transcription of HIV-1 antisense RNA complementary to 407 bp of the 5'-leader/gag region (pos. 222–629; Ref). The error bars indicate the 1 × standard deviation of 12 independent experiments. HIV-1 replication was measured indirectly by determining the concentration of HIV-1 specific antigens in cell free coculture supernatants after a four days incubation period (using a commercial HIV-1 antigen ELISA). Relative antigen concentrations were calculated from the ELISA signals using a standard curve determined with recombinant HIV-1 p24 protein.

effectiveness of pAR6 could be explained by a block of both functions due to an antisense RNA-mediated down regulation of the expression of the genes coding for these viral factors in early steps of infection. Additionally, the expression of further potential viral regulator proteins (e.g. tnv (42)) encoded by the recently identified large number of spliced 'small' mRNAs (43) might be negatively affected. However it cannot be excluded that the pAR6 derived HIV-1 antisense RNA also affects the env coding 4.3 kb mRNA or the genomic RNA.

The third antisense RNA expression plasmid, pAR7, which also exhibits significant inhibition (76%) includes the 562 bp HIV-1 fragment used in pAR6 in its 3109 bp long HIV-1 sequence. The inhibitory effect is, however, not greater than that measured with pAR6. Therefore it is possible that pAR7 either does not add any inhibitory sequence to that one contained in pAR6 or the technical restrictions of the assay system become relevant at equal or potentially more than 75% to 80% inhibition.

Evidence for the antisense principle

A set of data suggests that the 'antisense principle' plays a significant role in the antisense RNA expression vector-mediated inhibition of HIV-1 replication in human cells, although at present it cannot be decided whether the 'antisense principle' is causatively responsable for the inhibitory effects, e.g. hybrid formation between antisense RNA and target RNA was not analyzed. However, recently we showed that the inhibitory effects of the antisense RNA expression plasmid p2as (see: Figure 1 and (28)) on HIV-1 replication is sequence specific and can be assigned to the ability of the microinjected expression plasmid to transcribe HIV-1 antisense RNA (28).

Further evidence comes from this work:

Firstly, comparison of antisense RNA expression plasmids with the corresponding sense RNA expression plasmids as shown in Figure 2A. In six out of eight antisense/sense pairs, the antisense RNA expression plasmid leads to stronger inhibition.

Secondly, dose response experiments with the antisense RNA expression vector pAR3 show that the inhibitory effect on HIV-1 replication was 78% at 5:1 molar excess of antisense expression plasmid over HIV-1 proviral DNA (i.e. 22% remaining HIV-1 replication) and decreased to 52% at 2.5:1 molar excess (Figure 2B).

Moreover, the results shown in Figure 2B indicate that the pAR3 mediated inhibition of HIV-1 replication was specifically neutralized when the pAR3 corresponding sense RNA expression vector (pSR3) was comicroinjected with the same molarity as pAR3. The data shown in Figure 2B provide additional evidence that the pAR3 derived antisense RNA transcripts were the inhibitory molecules in this assay and not the microinjected double stranded DNA.

However, there remained an approximately 33% inhibition of HIV-1 replication with the mixtures pAR3+pSR3 and pCAT+pTAC (Figure 2B). This reduction of HIV-1 replication is in the same range as the inhibition measured with pCAT or pTAC (see Figure 1B and Figure 2B) or pSR3 (see Figure 2A) alone. Thus, one can conclude that there is no inhibitory effect of possible intracellular RNA double strand formation on HIV-1 replication.

Thirdly, the direct proof that the antisense transcripts are sufficient for inhibition of HIV-1 replication came from the observation that in vitro synthesized antisense RNA derived from the HIV-1 sequences also used in p2as and pAR6 cause a dose dependent and specific inhibition (Table 2).

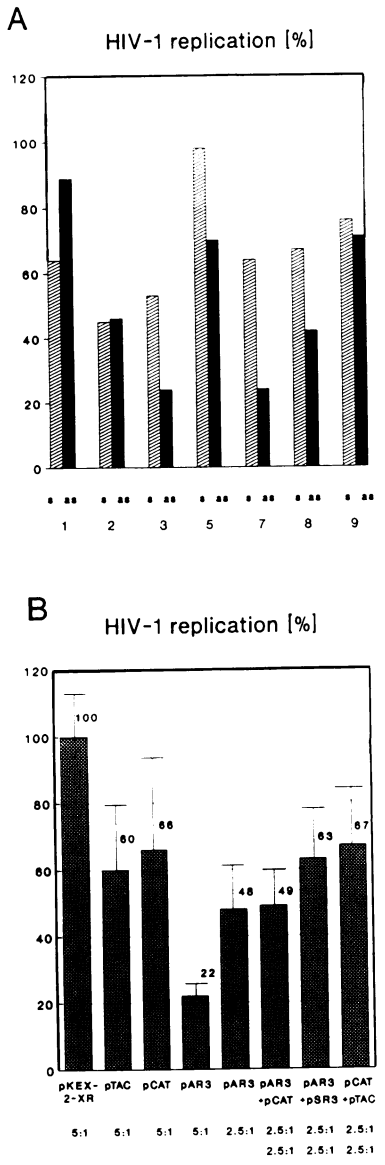

Figure 2. A) Comparison of the effects of corresponding antisense RNA and sense RNA expression vectors on HIV-1 replication in the comicroinjection assay described above. HIV-1 replication was standardized with comicroinjected plasmid pKEX-XR-CAT (100%). The numbers (1, 2, 3, 5, 7, 8, 9;) below the pairs of bars indicated with s (sense) or as (antisense) respectively, correspond to the pAR-plasmid numbering in Table 1 or Figure 1A respectively. **B)** Replication of HIV-1 in SW480 cells comicroinjected with infectious proviral HIV-1 DNA and the plasmids or mixtures of plasmids indicated below. The error bars indicate the 1×standard deviation of 4 independent experiments. Plasmids not described in the text: pSR3: Analogous vector to pAR3, expressing the 1 kbp HIV-1 fragment in sense orientation; pCAT: pKEX-2-XL derived plasmid expressing the CAT coding sequence from pBLCAT2 (33); pTAC: Analogous vector to pCAT, transcribing the CAT coding sequence in antisense orientation.

In tissue culture systems inhibition of HIV-1 replication can be mediated by α-interferon (44,45). However, this does not necessaryly reflect the in vivo situation (45). Moreover, recently it was demonstrated that there is no induction of α-interferon expression in human cells in which RNA double strands can be formed by antisense RNA and HIV-1 target RNA (17). For this reason and for the fact that there was no altered HIV-1 replication when mixtures of the sense RNA and antisense RNA expression

Table 2. Dose response (A) and specificity (B) of antisense RNA mediated inhibition of HIV-1 replication. In the first column the sources and concentrations of the in vitro synthesized RNAs which were comicroinjected togehter with HIV-1 proviral DNA (pNL4-3, 10 ng/μl) are listed. The second column shows the mean production of relative HIV-1 antigen concentrations measured in four experiments.

comicroinjected RNAs conc.	relative HIV-1 replication (±1×standard deviation)
(A)	
none	100± 7
2as, 1 ng/μl	96± 18
2as, 10 ng/μl	70± 12
AR6, 1 ng/μl	50± 10
AR6, 20 ng/μl	38± 27
AR6, 100 ng/μl	29± 5
AR6, 500 ng/μl	25± 9
(B)	
CAT,AR6 (100 ng/μl, 4 ng/μl)	107±43
CAT,AR6 (4 ng/μl, 100 ng/μl)	37±29

plasmids pAR3+pSR3 and pCAT+pTAC respectively were added (Figure 2B) we do not think that there are major antiviral effects mediated indirectly by double stranded RNA.

The extent of inhibition mediated by the antisense RNA expression plasmids tested in this study cannot be compared directly with each other because intracellular half lives and distribution over cell compartments are not known. However, for expression of heterologous sequences from episomally present pKEX plasmids (e.g. after transfection or microinjection) it is known that transcription is strong and not dependent on the inserted fragment (data not shown). In particular, in human T-cell lines stably transfected with pAR plasmids (e.g. p2as, pAR3, pAR6 or pAR7) the intracellular steady state levels of the transcribed HIV-1 specific RNAs are within the same range as was shown by Northern analysis with total cytoplasmic RNA (unpublished). This indicates that the intracellular stability of pKEX derived antisense transcripts is not particularly dependent on the HIV-1 portion. Thus, the antiviral efficacy of different pAR plasmids does not seem to be simply due to differences in intracellular concentrations. It seems more reasonable to assume that other HIV-1 subregion-linked parameters have influence on the strength of the inhibitory effects.

DISCUSSION

The experiments presented in this study show that intracellular antisense RNA expression is capable of inhibiting HIV-1 replication in a sequence specific and dose dependent manner. The subregion of HIV-1 against which antisense transcripts are targeted has crucial influence on the extent of inhibition. In this study the antiviral effect is most pronounced (75% inhibition) when antisense RNA complementary to the gag p17 and p24 coding sequences or antisense RNA complementary to the coding sequences of the HIV-1 regulatory proteins (tat, rev, vpu, vpr, tev, ...) is expressed. A direct comparison of these data with those obtained with antisense oligonucleotides added to the culture medium does not seem to be reasonable for several reasons: Cellular uptake and intracellular stabilities cannot be controlled easily for extracellularly applied oligonucleotides. Further, secondary structures presumed to exist on the relatively long antisense RNA transcripts and presumably affecting the inhibitory properties, are most likely irrelevant for the relatively short oligonucleotides. However, using antisense oligonucleotides to

inhibit HIV-1 replication, it was found that antisense-oligonucleotides complementary to the LTR sequences and splice sites within the HIV-1 genome seem to be the most potent inhibitors (13).

The maximal extent of HIV-1 inhibition in this co-microinjection assay (75%) cannot be increased by application of higher molar excesses of antisense RNA expression plasmids. This is because unspecific inhibition of HIV-1 replication by the HCMV-IE promoter/enhancer element become effective at these higher ratios (28). Probably also the fact that only 'late' steps in the HIV-1 replication cycle, such as gene expression or packaging can be affected, contributes to the incomplete inhibition measured in this system. Since complete proviral HIV-1 DNA is applied directly into the nuclei of microinjected cells, early steps of the viral replication cycle, for example reverse transcription, second strand synthesis or transport of HIV-1 specific nucleic acids to the nucleus cannot be affected. However, a higher extent of HIV-1 inhibition was measured in another test system, namely human T-cells with stable antisense RNA expression derived from p2as. In these stably transfected cells HIV-1 replication was inhibited up to 89% by p2as derived antisense RNA (18), whereas p2as leads to only 65–70% inhibition in the microinjection assay (28). The increased antiviral efficacy measured with pAR6 in the microinjection assay (78%) is also reflected by an increased inhibition of HIV-1 replication in stable Jurkat clones with constitutive pAR6 derived antisense RNA expression (19). This relationship indicates that the microinjection assay is suitable to predict HIV-1 target sequences which are effective also in stably transfected human T-cell clones which may support the search for model systems for 'intracellular immunization' against HIV-1 infection (19,20).

The measurements of sequence specific and dose-dependent antiviral effects with in vitro synthesized antisense RNA (Table 2) as well as with antisense RNA expression vector pAR3 (Figure 2B) demonstrate that HIV-1 antisense RNA is the inhibitory molecule. This together with neutralization experiments (Figure 2B), in which the antiviral effects of the antisense RNA expression plasmid pAR3 can be abolished by co-microinjected corresponding sense RNA expression plasmid suggests that the antiviral mechanism is the antisense-principle.

However, some HIV-1 inhibition can also be measured with the sense RNA expression plasmids corresponding to p2as (26), pAR1 and pAR2. The fact that vectors expressing sense RNA from 5'-portions of the HIV-1 genome also show a certain degree of reduction (nos. 2 and 3 in Figure 2A) is in accordance with the observation that HIV-1 sense RNA expression of 406 bases from the 5'-leader/gag region (p2s, HIV-1 pos. 222–629 (32)) leads to a similar degree of specific inhibition as the corresponding antisense RNA expression plasmid p2as (28). It could well be that these specific inhibitory effects might be due to negative interference of the sense transcripts or hypothetical protein products (carboxyterminally truncated gag proteins) with virus replication. It has recently been shown for herpes simplex virus (46) and for HIV-1 (47,48) that expression of mutated viral proteins in host cells can reduce viral replication.

A further analysis of critical parameters for the antiviral efficacy of antisense RNA expression plasmids suggests that the local target region has crucial influence. So far, it cannot be answered what particular property of the antisense RNA / target RNA system is affected. For example it cannot be answered whether the selection of an effective subregion of the HIV-1 genome, e.g. the one cloned in pAR6, determines the intracellular

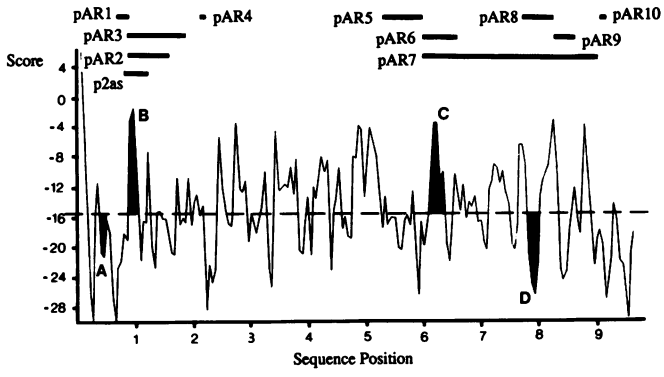

Figure 3. Distribution of the free energies (scores in kcal/mole) calculated by the algorithm of Zuker and Stiegler (51) of 100 nucleotide segments along the antisense strand of the HIV-1 genome with steps of 50 nucleotides. The HIV-1 sequence positions in kilobase pairs correspond to the proviral clone pNL4-3 (31). The bars above indicate the target region for antisense RNA expression plasmids. Peak labels indicate: **A**, high local folding potential corresponding to the TAR sequence (ca. 80 nucleotides, (54)); **B**, region of low local folding potential covered by p2as, pAR2 and pAR3; **C**, region of low local folding potential covered by pAR6; **D**, high local folding potential corresponding to the RRE (37) and UFR (38) respectively.

accumulation of antisense RNA transcripts, the biological step involved in HIV-1 replication or biochemical parameters (secondary structure, i.e. capability of base pairing).

One can assume that two complementary RNA strands can interact with each other via portions which do not participate in intramolecular interactions, (e.g. loop regions). This kind of relationship is well studied in procaryotic plasmid replication which is regulated by small antisense RNAs (4,5). Initial interactions and in regard to the kinetics of double strand formation over all rate limiting interactions between antisense RNA and target RNA are formed in the so called 'kissing complex' by three loops which are located on both RNAs and are complementary in sequence to each other (49,50). Further assuming a direct dependence of the antiviral efficacy of antisense RNA on the abundance of single stranded subregions, one would expect that higher degrees of intramolecular folding lead to reduced inhibitory effects of a given antisense RNA molecule. This would mean that the lower the potential of intramolecular folding of a given RNA molecule and the corresponding subregion of its complementary counterpart, the higher the probability of intermolecular interactions, i.e. inhibition of HIV-1 replication in this study.

Further, assuming that in vivo long range intramolecular interactions are less significant, we calculated 'local' secondary structures for relatively short stretches of long transcripts.

Secondary structures of 100 nucleotide stretches were calculated along the HIV-1 genome and the free energies were compared with the experimentally obtained antiviral effects with the antisense RNA expression plasmids summarized in Figure 1A. The lowest energy secondary structures of 100 nucleotide sequence stretches were calculated by the program of Zuker and Stiegler (51) with 50 nucleotide intervals on both strands of the HIV-1 genome. The profile of the lowest possible free energies of the calculated local secondary structures in dependence on the position on the HIV-1 sequence numbered according to (31)is shown in Figure 3. An analogous free energy distribution was obtained with intervals of 3 nucleotides. A similar

approach which was aimed to calculate parameters for the local folding potential lead to the identification of the highly folded rev responsive element (RRE) very close to the gp120/gp41 cleavage site in the env gene on the viral target strand (52). However, although there is no direct experimental support for the shown free energy profile, the algorithm used in this work was able to identify the RRE which is the same sequence as the recently calculated unusual folding region (UFR, (53), see also peak D in Figure 3). Furthermore the hairpin structured TAR sequence was also identified as a local maximum of the free energy (see also peak A in Figure 3).

The same location of the HIV-1 sequences transcribed by the most inhibitory antisense RNA expression plasmids p2as, pAR2 and pAR3 on the one subregion and pAR6 on the second subregion was found for the maxima of the local energy profile, i.e for the subregions with minimal local secondary structure potential (peaks B and C in Figure 3). Since a similar energy profile as was calculated for the antisense strand was also found for the viral target strand (unpublished data), we conclude that the most effective inhibition of HIV-1 replication is correlated with a high local energy state, i.e. only little secondary structure in a given subregion of the antisense RNA strand and of the target strand respectively.

This correlation between the experimentally measured antiviral efficacy of a given antisense RNA and the corresponding subregion might be of general significance for the prediction and selection of effective subregions for antisense RNA mediated inhibition of gene expression and viral replication.

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