Revertants and pseudo-revertants of human immunodeficiency virus type 1 viruses mutated in the long terminal repeat promoter region

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The TAR domain is an RNA secondary structure element within the leader transcript of the human immunodeficiency virus type 1 (HIV-1) virus. TAR RNA forms the binding site for the viral trans-activator protein Tat and cellular co-factors that are involved in induction of the LTR transcriptional promoter. Here, we report that mutations in the single-stranded bulge- and loop-domains of TAR RNA impair the ability of the virus to replicate in T cell lines. Revertant viruses were isolated upon prolonged culturing and analysed through sequencing. The reversion data confirm the importance of both bulge and loop as sequence-specific recognition motifs. We also analysed the replication phenotype of a mutant HIV-1 virus with a substitution in the −19/−3 promoter region. This mutant displayed delayed infection kinetics compared to the wild-type virus, and revertants with increased replication potential could be isolated. Interestingly, all revertants had acquired an additional mutation at position −2. Primer extension analyses revealed that an upstream shift in transcription start site usage was induced by the −19/−3 substitution. This effect was compensated for by the nucleotide substitution near the RNA start site.

Introduction

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is essential for viral replication (reviewed by Cullen, 1991). Tat is a potent activator of viral transcription directed by the long terminal repeat (LTR), leading to increased expression of all viral genes. Tat binds to a specific RNA structure, termed the TAR element, found at the extreme 5' end of all HIV-1 mRNAs (Berkhout & Jeang, 1989; Dingwall et al., 1989). Although Tat is tethered to the transcription machinery by binding to the nascently transcribed TAR RNA (Berkhout et al., 1989), target sequences for Tat-mediated transcriptional activation are present in the upstream DNA promoter motifs (Selby & Peterlin, 1990; Southgate et al., 1990; Berkhout et al., 1990a; Kamine et al., 1991). Our current understanding of the HIV-1 LTR promoter comes primarily from transient LTR–CAT transfections in T cell lines or non-T cell lines (e.g. HeLa or COS). Although this approach has been useful for the initial characterization of important promoter motifs, recent data strongly suggest that LTR–CAT activity does not always accurately reflect the replication phenotype of an HIV-1 provirus carrying identical LTR mutations (Leonard et al., 1989; Ross et al., 1991; Jeang et al., 1993; Kim et al., 1993; Klaver & Berkhout, 1994c).

It is well documented that the Tat protein is absolutely essential for viral replication (Cullen, 1991; Dimitrov et al., 1993), but only limited infection data are available for TAR-mutated viruses. A large set of TAR-mutated HIV-1 proviruses was studied by Harrich et al. (1990) in transient transfection assays. Although expression defects were reported in some cell types, these experiments suggested that HIV-1 can replicate in a TAR-independent manner in phorbol ester stimulated T lymphocytes (Harrich et al., 1990). More recently, Tat was shown to efficiently activate a TAR-deleted LTR promoter through the NF-κB enhancer motifs in cells derived from the central nervous system (Bagasra et al., 1992; Taylor et al., 1992; Taylor et al., 1994). We therefore examined the functional defect of several TAR-mutated proviruses in a variety of T lymphocytic cell lines and show that both loop and bulge mutations dramatically interfere with HIV-1 replication. These results were confirmed by the analysis of revertant viruses obtained at late times after transfection.

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Methods

Cells, transfection and viral infection. Adherent HeLa and COS cells were grown in Dulbecco's modified Eagle's medium and DEAE-transfected as previously described (Berkhout et al., 1990b). All T cell lines and peripheral blood lymphocytes (PBLs) were maintained in RPMI-1640 medium containing 10% fetal calf serum. Infections with the HIV-1 virus were performed for 60 min at 37 °C with ~ 1 x 10^6 cells in 1 ml RPMI medium. The volume was subsequently increased to 10 ml. T cells were transfected with HIV-1 DNA by means of electroporation (Koken et al., 1992). Gag-p24 ELISA (Koken et al., 1992) and reverse transcriptase (RT) assays (Willey et al., 1989) were performed as described previously.

In order to select for revertant viruses, transfected cells were maintained for prolonged periods of time. Cultures were usually split 1:10 twice a week. Cell samples (containing integrated HIV-1 DNA) and supernatant samples (containing HIV-1 virus) were taken at intervals and stored at -70 °C for future analysis. Upon phenotypic reveration, as witnessed by the appearance of Env-protein induced cell syncytia, optimal viral replication was maintained by transfer of a supernatant sample (initially 100 µl, subsequently 0.5 µl) onto a 10 ml culture of fresh SupT1 cells.

Mutant HIV-1 genomes. The wild-type HIV-1 molecular clone pLAI (Peden et al., 1991) and the L5-L5 and B123-B123 double mutants have been described previously (Klaver & Berkhout, 1994a, b). The Xho-10 substitution was introduced into both 5' and 3'LTRs of pLAI following a multi-step cloning protocol as described by Klaver & Berkhout (1994a). All other TAR mutants used in this study (L1, L2, L3, L4 and BA) were introduced exclusively into the 5'LTR of pLAI.

Proviral DNA and viral RNA analysis. The proviral 3'LTR region was PCR-amplified from total cellular DNA, cloned and sequenced using published methods (Berkhout & Klaver, 1993). This protocol allowed for the analysis of the promoter region from position -220 to +80. The complete nef gene was sequenced in two clones containing a major deletion. For this purpose we used an antisense primer in the 3' region of the LTR (3' NRE-B, 5'TACCTAGGGTGTCCGCCACA-AGAGAGGA).

Primer extension reactions were performed to map differential start site usage among the mutant HIV-1 LTRs. Total cellular RNA was isolated by the hot phenol method and hybridized to a 32P-labelled TAR80/50 primer (Klaver & Berkhout, 1994a). cDNAs were resolved in denaturing polyacrylamide gels, visualised on X-ray films and scanned/quantified on a Molecular Dynamics β-imager.

Results

TAR bulge and loop mutations abolish HIV-1 replication in T cells

To study the effect of TAR sequences on viral replication, mutations were introduced into the 5'LTR promoter region of the pLAI molecular clone of HIV-1. Five constructs with a point mutation in the single-stranded TAR loop were made (L1 to L5, see Table 1). The TAR bulge was either substituted as in B123 (UCU → AAG) or deleted as in BA. For cloning purposes, the bulge variants contained a XhoI restriction site introduced into the -19/-3 region between the TATAA box (position -28) and the transcriptional start site at position +1 (Fig. 1b). It cannot be excluded that this Xho-10 substitution interferes with proper LTR function since this region encodes a putative initiator element and a low affinity binding site for the cellular transcription factor LBP-1. In order to study the effect of the -19/-3 substitution on HIV replication, we also generated the individual Xho-10 mutant (Fig. 1c).

The T cell line A3.01 was transfected with the various HIV-1 plasmids by electroporation. The spread of wild-type and mutant viruses was assayed by measuring Gag-p24 protein levels in the culture supernatant at day 6 post-transfection (Table 1, wild-type was set at 100%). We consistently measured a dramatic reduction in HIV-1 expression levels for the two TAR bulge mutants (B123 and BA) and most TAR loop mutants (L1, L2, L3 and L5). RNA analysis indicated a blockade at the transcriptional level (data not shown). In these transfection experiments, the phenotype of the L4 loop mutant and the Xho-10 mutant was typically intermediate between that of the wild-type and the other LTR-mutated genomes. To verify that Gag-p24 values in the culture medium accurately reflect the production of HIV-1 virions, we also performed an RT assay several days after transfection of A3.01 cells (Fig. 2). These results demonstrate that the TAR RNA bulge- and loop-domains both contain nucleotide sequences that are critical for efficient replication of the HIV-1 virus. Very similar results were obtained in transfection experiments with the SupT1 T cell line (Table 1). Since it has been reported that HIV-1 can replicate in a TAR-independent manner in phorbol ester stimulated lymphocytes (Harrich et al., 1990), we tested the various TAR mutants in PMA-stimulated SupT1 cells (Table 1). Direct comparisons of HIV production levels in stimulated versus untreated cells demonstrated no significant differences. In addition to these experiments in established T cell lines, similarly reduced replication rates of the LTR mutants were observed in PBLs (Table 1).

Inactivation of the LTR promoter will affect the synthesis of all HIV-1 proteins, but reduced expression of the Tat trans-activator protein is perhaps most detrimental to HIV-1 replication because it will further reduce LTR transcription levels. We therefore tested whether the TAR-defect could be partially complemented by expression of Tat protein in trans. First, we transfected the various HIV-1 constructs in the Jurkat-Tat cell line that stably expresses Tat protein (Table 1). Second, we co-transfected the HIV-1 plasmids with a pTat expression plasmid into HeLa cells, followed by co-culture with HIV-susceptible SupT1 T cells (Table 1). In both systems, the defect in replication of the bulge B123 and loop L5 mutants was not restored by exogenously expressed Tat protein. In fact, production of the partially defective L4 virus was significantly inhibited in the presence of additional Tat (Table 1). The combined data indicate that TAR defects cannot be simply rescued by overexpression of the Tat trans-activator.
Table 1. Virus production upon transfection of LTR mutants into a variety of cell types

<table>
<thead>
<tr>
<th>Mutant virus</th>
<th>SupT1†</th>
<th>HeLa-SupT1‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A3.01*</td>
<td>-PMA +PMA</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1000 (100)</td>
<td>1880 (100)</td>
</tr>
<tr>
<td>B123</td>
<td>0 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>L5 (G → U)</td>
<td>0 (0)</td>
<td>6 (0)</td>
</tr>
<tr>
<td>L4 (G → U)</td>
<td>110 (11)</td>
<td>880 (47)</td>
</tr>
<tr>
<td>Xho-10</td>
<td>410 (41)</td>
<td>1440 (77)</td>
</tr>
<tr>
<td>Ba</td>
<td>0 (0)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>L1 (C → A)</td>
<td>0 (0)</td>
<td>6 (0)</td>
</tr>
<tr>
<td>L2 (U → G)</td>
<td>1 (0)</td>
<td>6 (0)</td>
</tr>
<tr>
<td>L3 (G → U)</td>
<td>0 (0)</td>
<td>4 (0)</td>
</tr>
</tbody>
</table>

* Electrofection with 5 μg pLAI; Gag-p24 assay was performed at day 6 post-transfection.
† Electrofection with 5 μg pLAI, ± PMA (50 ng/ml) at day 1; Gag-p24 assay was performed at day 5 post-transfection.
‡ DEAE-transfection of HeLa cells with 5 μg pLAI ± 1 μg pTat, co-culture with SupT1 at day 3; Gag-p24 assay was performed at day 6 post-transfection.

Genetic analysis of revertant viruses

We next tested whether the replication-defective phenotype of the LTR mutants could be restored by the acquisition of advantageous mutations upon prolonged culture. Although the singly mutated 5'LTR constructs can be used to transiently monitor HIV replication levels, they are inappropriate for long-term reversion experiments due to the presence of a wild-type 3'LTR copy. We previously observed rapid reversion to a fully
Fig. 2. Replication potential of wild-type HIV-1 LAI virus and LTR mutants in T cells. The SupT1 T cell line was electroporated with 5 µg of the plasmids indicated (top of figure) or mock-transfected. Culture supernatants were sampled at days 4, 6 and 10 for RT activity. The intensity of each spot is a direct reflection of the amount of RT.

Fig. 3. Prolonged culture of transfected cells in order to select for revertant viruses. SupT1 cells were transfected with 2.5 µg (a) or 5 µg (b) of plasmid DNA. Cell cultures were split 1:3 every 3 to 4 days. HIV Gag-p24 antigen was measured in the culture supernatants.

We used the defective L5 and B123 mutants for a prolonged culture experiment in order to select for revertant viruses. No replication of the L5 virus was detected in the first 3 weeks after transfection, but a rapidly spreading revertant was apparent at day 21 (Fig. 3a). Integrated HIV-1 proviral DNA was PCR-amplified from total cellular DNA at time points before and after the phenotypic reversion (days 11 and 21, respectively). We sequenced the -220 to +80 LTR region, and multiple clones were sequenced for both timepoints (Fig. 1a). These results demonstrate that the phenotypic reversion is due to restoration of the wild-type loop sequence. We observed very similar kinetics of reversion in three independent transfections and wild-type TAR loop sequences were present in all reversion samples analysed (data not shown).

In the course of one L5 reversion experiment, we detected a unique virus with a major deletion in the nef gene. The PCR-amplified sample of the 3' region of the HIV-1 genome contained a substantially smaller fragment. This truncated fragment was cloned and subsequent sequence analysis indicated that part of the nef gene was deleted. We observed two very similar deletions in the 5' part of the nef gene (HIV-1 LAI coordinates 8968-9084 and 8968–9087). Both deletions are in frame with the nef open reading frame and will lead to synthesis of truncated forms of the Nef protein (lacking amino acids 42–80 or 42–81). It remains unknown whether Nef-truncation actually contributed to the reversion process because the clones carrying the deletion also contained the L5-reversion mutation. Furthermore, nef-deletion was observed in only one L5 reversion experiment. We note, however, that some previous studies reported inhibition of LTR-mediated transcription by the Nef protein (Ahmad & Venkatesan, 1988; Niederman et al., 1989; Yu & Felsted, 1992), which is consistent with the idea that nef-deletion may have supported LTR activity in the context of the TAR mutation.

As is shown in Fig. 3(a), replication of the B123 bulge mutant was dramatically delayed by nearly 6 weeks compared to wild-type HIV-1. In fact, we were unable to obtain revertants in two other transfections with the B123 mutant (data not shown). Sequence analysis of proviral DNA isolated as early as day 30 revealed the acquisition of the wild-type U nucleotide at the first bulge position (Fig. 1b). Transient transfection experiments confirmed that this bulge adaptation is sufficient for restoration of LTR-promoter activity in the presence

wild-type sequence in transfections with these plasmids (Klaver & Berkhout, 1994a). This reversion is due to a premature strand transfer that infrequently occurs during strong stop cDNA synthesis. For this reason, all reversion experiments were performed with doubly mutated HIV-1 genomes (e.g. L5-L5).

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of Tat (Fig. 5b; compare mutant B123 in lane 12 to revertants in lanes 13–16). Interestingly, an additional mutation was observed at position –2 of all bulge revertants. The –2T→G substitution was not stable either, as all progeny present at day 118 had undergone a subsequent –2G→A substitution. Revertant virus stocks obtained at days 39 and 74 and the wild-type HIV-1 virus were used to infect the SupT1 T cell line and virus production was assayed over time (Fig. 4a). Both viruses did replicate, although with reduced efficiency compared to wild-type HIV-1 LAI. The sequence changes at position –2 do not necessarily reflect a repair pathway induced by the TAR bulge mutation. In fact, it seems likely that this –2 effect is triggered by the relatively large Xho-10 substitution (–19/–3) present in these mutants (Fig. 1b). In order to test this hypothesis, we introduced the individual Xho-10 substitution into both LTRs of the molecular clone pLAI and selected for revertant viruses in prolonged tissue culture experiments.

The Xho-10 mutant established a productive infection in SupT1 cells, but we consistently observed slower kinetics of virus spread compared to wild-type HIV-1 (Fig. 3b). Results of replication assays in a variety of human T cell lines and PBLs were found to be similar to those in SupT1 cells (Table 1; data not shown). These results indicate that the –19/–3 substitution does have an appreciable effect on the ability of the virus to replicate. This defect may also partially explain the inefficient reversion observed for the B123 mutant. We also passaged the partially defective Xho-10 virus onto fresh T cells in order to select for spontaneous reversion mutations. Indeed, virus present at day 19 post-transfection did show markedly increased replication kinetics (Fig. 4b). The first mutation observed in LTR DNA isolated at day 19 was –2T→G, present in nine out of ten proviruses analysed (Fig. 1c). This revertant was stable upon prolonged culture up to day 62, although one variant with a –2 deletion combined with a +1G→C substitution was observed (–2 deletion was also transiently observed during the B123 reversion experiment, see Fig. 1b). The combined results of the B123 and Xho-10 reversion pathways strongly suggest that the mutation at position –2 is triggered by the –19/–3 substitution.

We observed interesting mutations in the –19/–3 region of some revertants. For instance, one additional mutation (–17T→C) became fixed upon long-term culture of the B123 revertant (Fig. 1b). Other base changes were also detected in this region of individual clones (–15A→G, Fig. 1b; –17T→G, Fig. 1c). A hypothetical explanation for the appearance of these variants comes from inspection of the sequence of the Xho-10 insert. The insert encodes a T/A-rich motif that may interfere with transcription from the adjacent TATAA box by acting as a pseudo-TATAA element. It follows that any mutations in this TTA triplet will help to restore efficient LTR function.

Comparison of start site usage in LTRs with mutations at the –3/–2 position

The revertants obtained in infections with the Xho-10 mutant suggest that a minor adjustment of the sequences around the transcriptional initiation site can partially compensate for a defect imposed by the –19/–3 substitution. We were curious, therefore, as to whether the Xho-10 mutant and revertant LTRs used the same transcriptional start site. First, we performed primer extension analysis on transcripts synthesized by a wild-type and Xho-10-mutated LTR–CAT construct upon transient transfection in the absence and presence of the Tat trans-activator protein (Fig. 5a). We observed no quantitative difference in Tat response, but the Xho-10 promoter exhibited a more diffuse transcription start site
usage when compared to the wild-type LTR (Fig. 5a; lanes 4 and 2, respectively). The wild-type HIV-1 LTR initiates predominantly at the +1G, although some +2G initiated transcript can be detected (lane 2). Initiation was shifted approximately two nucleotides upstream in the Xho-10 context, with equal utilization of all four positions in the -2/+2 region. Next, we tested several Xho-10 variants with nucleotide substitutions at the -2 and -3 positions (Fig. 5b). Several lanes of the dried gel were scanned on a β-imager and the results are presented in Fig. 5(c). Comparison of the start sites for the different -3/-2 constructs suggests that the presence of the -2G nucleotide is decisive for restoration of the ‘wild-type’ +1/+2 initiation site (lanes 3 and 5; an RNA sample of HIV-infected T cells is included in lane 7 for comparison). These results strongly suggest that the upstream shift seen for Xho-10 is compensated for by substitution of the -2T into either G or A, as observed in several independent reversion experiments (Fig. 1b, c).

**Discussion**

In order to understand the functional role of the TAR RNA element in the life cycle of HIV-1, we changed the nucleotide sequence of the bulge- and loop-domains and analysed the replication potential of the mutant viruses in several cell types. With one exception, that is the L4 loop mutant, we found an absolute replication defect for all TAR mutants tested. Virus production observed for the L4 mutant was consistently slower compared to the spread of wild-type HIV-1 virus. These results are generally consistent with previous data on TAR-mutated viruses (Harrich et al., 1990; Berkhout & Klaver, 1993). The defect in viral replication, however, was recently
reported to be much less severe in activated PBCs (Harrich et al., 1994).

The replication defect of TAR-mutated viruses could not be restored by providing Tat protein in trans. Instead, replication of the partially defective L4 mutant was further reduced in Jurkat-Tat cells, which can efficiently support replication of wild-type HIV (data not shown). We offer two suggestions that may explain this seemingly contradictory result. First, the lack of a beneficial Tat effect may indicate that the LTR mutants are able to produce reasonable amounts of Tat protein themselves. Indeed, substantial Tat expression levels were previously reported for such LTR-mutated HIV-1 constructs (Koken, 1994). Second, a similar inhibition of LTR function at high Tat levels was previously observed in transient LTR–CAT transfections (Berkhout et al., 1990b). This decrease may correspond to a squelching effect of Tat, i.e. titration of its target factor, perhaps an intermediary molecule bridging either Tat and TAR RNA or Tat and the transcriptional machinery.

We decided to select for revertant viruses by prolonged culturing of cells transfected with the L5 loop mutant (G→U) and the B123 bulge mutant (UCU→AAG). The analysis of revertant viruses is a powerful method to study structure–function relationships, and this genetic approach was previously used to study the HIV-1 Env protein (Willey et al., 1988; Fujita et al., 1992) and cis-acting regulatory sequences in the primer-binding site (Rhim et al., 1991; Das et al., 1994) and the lower TAR RNA stem (Klaver & Berkhout, 1994c). The genotypic analysis of TAR revertants obtained in this study indicates a strong selective pressure to restore the wild-type sequence in both the TAR bulge and loop. The L5 mutant did revert to wild-type (U→G) in several independent culture experiments. The B123 mutant restored replication to suboptimal levels by correcting the first bulge position (AAG→UAG), and this nucleotide change correlated with enhanced transcription from the LTR promoter. We have continued the reversion experiment beyond the sixth month of culture, but detected no further bulge adaptations. This result is consistent with transient transfection data and Tat-binding experiments that show a strong requirement for the U residue at the first bulge position (Berkhout & Jeang, 1989; Roy et al., 1990; Cahn et al., 1991). It cannot be excluded that additional changes elsewhere in the HIV genome contributed to the revertant phenotype.

We therefore sequenced the complete U3 region of the LTR promoter and the first coding exon of the tat gene in two clones obtained at day 153 in the B123 reversion, but did not detect any additional mutations. In conclusion, both the replication phenotypes of the TAR mutants and their reversion characteristics are in accordance with the idea that the TAR element is critically important in HIV-1 infection assays in T cells. It would be interesting to perform similar reversion experiments in activated PBCs or neural glial cells, which have been reported to support HIV replication in a TAR-independent manner (Bagasra et al., 1992; Taylor et al., 1992; Harrich et al., 1994; Taylor et al., 1994).

We tested one additional mutant with a 17 nucleotide substitution in the −19/−3 region of the LTR promoter. Delayed virus replication kinetics were measured for this mutant. Independent reversion experiments indicated that acquisition of an additional mutation at the flanking −2T position is correlated with partial restoration of this defect. These results suggest that virus replication can be supported by an LTR promoter that is mutated in the −19/−2 region. Consistent with this finding, we recently described an efficiently replicating HIV-1 variant with a deletion in this region (Klaver & Berkhout, 1994c). Interestingly, this region was previously reported to contain important DNA binding motifs for cellular proteins involved in the modulation of LTR promoter activity. For instance, this region contains a putative initiator motif (Du et al., 1993; Zenzie-Gregory et al., 1993) and the high-affinity binding site for the cellular UBP-1/LBP-1 protein (Garcia et al., 1987; Jones et al., 1988). Replication of the HIV-1 LAI isolate is apparently not dependent on these motifs that were identified either in transient transfections or in in vitro transcription assays with HeLa cells or their nuclear extract. The differences in experimental outcome may be due at least in part to differences in the cell types employed. Alternatively, transcription from an episomal plasmid may be mechanistically different from that of an integrated provirus (Jeang et al., 1993; Kim et al., 1993; Klaver & Berkhout, 1994c).

Analysis of the transcriptional start site usage of the wild-type and −19/−3 mutated LTRs suggests a sequence-specific contribution of the nucleotides at the RNA start site. Cellular protein factors that may be involved are the RNA Pol II complex and transcription factor TFIIIB, which were shown to be solely responsible for determining the start site of transcription (Buratowski et al., 1989; Li et al., 1994). Interestingly, in our reversion system we consistently observed the initial −2T→G substitution, although some revertants eventually preferred an A at this position (Fig. 1b). One can explain this typical order of events (T→G→A) if one assumes a two-step scenario. Whereas base substitution rates will largely determine the order of appearance of reversion-linked mutations, phenotypic selection criteria will eventually shape the virus population. Thus, T→G mutation should occur more frequently than T→A mutation and outgrowth of the −2A variant indicates that this sequence is most compatible with optimal LTR function. Indeed, in vitro
data indicate that among the RT-induced transversions, a $T \rightarrow G$ change is more frequent than a $T \rightarrow A$ change (Ji & Loeb, 1994) and a similar spectrum of mutations was seen in naturally occurring HIV sequences (Myers et al., 1992). It is currently unclear why $A$ is eventually selected at the $-2$ position. A possible explanation for the eventual $G \rightarrow A$ switch is based on a survey of eukaryotic promoter sequences. This survey indicates a strong bias for an $A$ nucleotide at the start position, with $G$ being the second most (Breathnach & Chambon, 1981; Bucher & Trifonov, 1986). Our $-2A$ mutant, however, did restore transcription initiation at the $+1$ position, suggesting that other sequence characteristics of the RNA start region play a role in start site selection.

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