Cruciform structure of a DNA motif of parvovirus minute virus of mice (prototype strain) involved in the attenuation of gene expression

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It has previously been reported that the region between nucleotides 259 and 383 immediately downstream from the P4 early promoter of parvovirus minute virus of mice, prototype strain (MVMp) is responsible for transcriptional attenuation. Attenuation results from the premature pausing of RNA polymerase II within this sequence (designated to as att) and seems to depend on potential RNA secondary structure. To assess the attenuation capacity of att under near physiological conditions, the early transcription unit of MVMp was replaced by the chloramphenicol acetyltransferase reporter gene under control of the early P4 promoter, in the presence or absence of att. The resulting recombinant vectors were encapsidated in parvovirus particles and replicated in cells after co-infection with the wild-type virus. The att fragment reduced the rate of expression of the reporter gene by approximately threefold, confirming previously reported data from transfection experiments performed in the same cellular system. This attenuation factor is unexpectedly high, considering that the ‘readthrough’ fold of the nascent viral transcript is thermodynamically more stable than the ‘attenuation’ configuration. In an attempt to elucidate this point, we sought for the presence of secondary structures in the template DNA molecule. In vitro nuclease probing of viral dsDNA revealed that the att fragment had a cruciform configuration with both complementary strands folding into the computer-predicted stem-loop ‘attenuation’ structure. These observations lead us to propose that the secondary structure of the DNA template may prompt the formation of the ‘attenuation’ stem-loop in nascent mRNAs by bringing corresponding self-complementary sequences into close proximity.

Introduction

During the past few years, various reports have established that eukaryotic RNA polymerase II, similar to the prokaryotic RNA polymerase, can pause or prematurely terminate transcription within viral and cellular genes (reviewed in Spencer & Groudine, 1990). This phenomenon is termed attenuation. Although the lack of simultaneity of transcription and translation in eukaryotes excludes many of the proposed models of bacterial attenuation, prokaryotic attenuation mechanisms exist in which translation does not play a role (Yanofsky, 1988). Such mechanisms may be relevant to eukaryotic gene regulation. The model system examined in the present report is that of the parvovirus minute virus of mice, prototype strain (MVMp). Parvoviruses are small, non-enveloped lytic viruses which can infect a variety of animals from insects to humans (Tijssen, 1990). Parvovirus MVMp has an ssDNA genome of approximately $5 \times 10^3$ nucleotides (nt) and is bracketed by terminal palindromic regions that are essential for viral DNA replication (Astell, 1990). Similar to other paroviruses, MVMp depends extensively on host cell functions under developmental and proliferative control to accomplish its infectious cycle (Tattersall & Gardiner, 1990). Accordingly, transcription from the viral early and late promoters (designated as P4 and P38 and located at map units 4 and 38, respectively) is catalysed by the host RNA polymerase II (Cotmore, 1990).

It has been shown previously that RNA polymerase II pauses at a specific location, 142 to 147 nt downstream from the P4 promoter, resulting, both in vivo and in vitro, in the synthesis of short attenuated RNA fragments (Ben-Asher & Aloni, 1984; Resnekov & Aloni, 1989; Krauskopf et al., 1991; Spegelaere et al., 1991). Efficient pausing appears to require both RNA secondary structure (Resnekov & Aloni, 1989) and the absence of anti-attenuation factors provided by permissive cell extracts (Krauskopf et al., 1991). In keeping with the first
requirement, Ben-Asher & Aloni (1984) have suggested the occurrence of two alternative computer-predicted RNA structures. The 'readthrough' configuration would allow elongation, whereas folding into the alternative 'attenuation' structure would result in pausing of the polymerase complex. To date, however, little is known about the cis elements involved in the attenuation of P4-directed transcription. Studies performed with other viral or cellular systems presenting similar structural features, lead one to conclude that a stable stem-loop region followed by a uridine stretch is required for attenuation to occur (Hay & Aloni, 1984; Bentley & Groudine, 1988; Kessler et al., 1989; Resnekov & Aloni, 1989; Resnekov et al., 1989). However, orientation specificity of the stem-loop structure and directed mutagenesis experiments suggest that folding is not the only determinant and that polymerase pausing or termination may also involve sequence recognition within the folds (Toohey & Jones, 1989; Cheng et al., 1991; Resnekov et al., 1991). In a first attempt to identify the viral sequences responsible for the attenuation of transcription from promoter P4, we have placed the chloramphenicol acetyltransferase reporter gene (cat) under the control of this promoter (Spegeleare et al., 1991). The nt 259 to 383 fragment of MVMp DNA, termed att and transcribed into the above-mentioned RNA with potential alternative secondary structures, was placed between the promoter and the reporter gene. A control was produced in which att was absent. In the presence of att, a fourfold reduction of CAT activity was observed in transient expression experiments (Spegeleare et al., 1991). This figure is consistent with the attenuation factor measured by in vitro run-on assays performed with nuclear extracts from the same cell lines, suggesting that att may indeed be responsible for the observed polymerase pausing during P4-directed transcription (Spegeleare et al., 1991).

The present study was conducted to assess the attenuation capacity of the att sequence under natural infection conditions. With this aim in view, recombinant MVMp parvoviruses were produced, expressing the reporter cat gene under control of the early promoter P4, with or without the att fragment. The presence of att reduced P4-directed gene expression by approximately threefold. This decrease is consistent with the transcription attenuation data mentioned above, but is unexpectedly high considering that the 'readthrough' fold of nascent RNA is thermodynamically more stable than the 'attenuation' fold. We show that not only viral RNA, but also double-stranded att DNA exhibit a secondary structure resembling the 'attenuation' fold described by Ben Asher & Aloni (1984), and propose a model that may account for the efficient pausing of RNA polymerase within this region.

Methods

Plasmid construction and transfection. Plasmid pMM984 is a full-length infectious molecular clone of MVMp (Merchinsky et al., 1983). Plasmid pMVM/P4-cat1 was obtained by substituting the cat gene for the viral genes of pMM984 (Fig. 1). To this end, the complete cat sequence, flanked by eukaryotic 3' termination signals, was first connected to the initiation codon downstream from promoter P4, using the previously described pP4-cat(PCR1) and p-cat plasmids (Spegeleare et al., 1991). This allowed the cat sequence to be recovered as a Ncol–BglII fragment that was inserted between corresponding sites of pMM984. Plasmid pMVM/P4-att-cat1 was constructed by replacing the Ncol–Ncol fragment of pMVM/P4-cat1 (corresponding to the 5' portion of the cat gene) by the corresponding fragment from plasmid pM4-att-cat1 (Spegeleare et al., 1991), coding for a chimeric NS-CAT product (Fig. 1).

Calcium phosphate transfections were performed as described by Graham & van der Eb (1973). Cultures (8 × 10⁵ COS-1 cells) were cotransfected with 5 µg of pMM984 and an equimolar quantity of either pMVM/P4-cat1 or pMVM/P4-att-cat1. Three days after transfection, cells were lysed by two freeze-thaw cycles in PBS, and supplemented with 1 mM-MgCl₂ and 2 mM-CaCl₂. The lysates were centrifuged to clarify the supernatants from transfected cell lysates. Infected cells were washed three times with PBS, further incubated in 5 ml of complete medium for 48 h and processed for the measurement of replication centres, essentially as described by Russell et al. (1992). Briefly, nitrocellulose filters (Schleicher and Schuell BA85) were applied directly to the cultures, and transferred cell DNA was denatured by placing filters for 2 min, three times, on Whatman 3MM paper saturated with 0.5 M-NaOH-1M-NaCl. After neutralization with 1 M-Tri~l-5 M-NaCl-0.15 m-sodium citrate in the same way, denatured DNA was hybridized to a cat-specific probe (HindIII–BglII fragment of plasmid pBGU7; see Cornelis et al., 1988), and revealed by chemiluminescence, using the ECL direct nucleic acid labelling and detection system (Amersham).

Parvovirus infection and titration by replicative centre assays. Cultures (4 × 10⁵ NBE cells) were washed with PBS and incubated for 1 h at 37 °C with 0.5 ml of 100- and 1000-fold dilutions of clarified supernatants from transfected cell lysates. Infected cells were washed three times with PBS, further incubated in 5 ml of complete medium for 48 h and processed for the measurement of replication centres, essentially as described by Russell et al. (1992). Briefly, nitrocellulose filters (Schleicher and Schuell BA85) were applied directly to the cultures, and transferred cell DNA was denatured by placing filters for 2 min, three times, on Whatman 3MM paper saturated with 0.5 M-NaOH-15 M-sodium citrate. After neutralization with 1 M-Tris-15 M-NaCl-0.15 m-sodium citrate in the same way, denatured DNA was hybridized to a cat-specific probe (HindIII–BglII fragment of plasmid pBGU7; see Cornelis et al., 1988), and revealed by chemiluminescence, using the ECL direct nucleic acid labelling and detection system (Amersham).

Transient expression and replication assays. Cultures were inoculated with virus from transfected cell lysates, as described above, incubated for 48 h and processed for the analysis of recombinant DNA expression and replication. With regard to expression, whole cell extracts were assayed for CAT activity, as described previously (Spegeleare et al., 1991). For the measurement of replication, low M r DNA was extracted from infected cells and analysed by Southern blotting according to Russell et al. (1992), using the same hybridization probe and detection system as in replicative centre assays.

Nuclease probing. The pP4-att-cat1 plasmid (Spegeleare et al., 1991) was used as a template to synthesize att DNA probes from flanking end-labelled oligonucleotide primers. The probes corresponding to transcribed and non-transcribed DNA strands were obtained after plasmid digestion with the Aps700 or Hhal restriction enzyme, and hybridization to the 32P-labelled primer 5'-TTAAAGTATATAAGCAACTACTGAAGTCAGTA-3' or 5'-TTTTCATTCCAGTTGA-
Results and Discussion

Production of cat-bearing MVMp recombinant virions

To construct parvoviral reporter vectors, we replaced the coding sequences of pMM984, an infectious molecular clone of MVMp (Merchlinsky et al., 1983), by the expression cartridges P4-cat or P4-att-cat described previously (Spegelaere et al., 1991). The resulting constructs were termed pMVM/P4-cat1 and pMVM/P4-att-cat1, respectively (Fig. 1). Viral and inserted sequences can be excised and packaged into recombinant virions, provided that the terminal palindromes remain intact and that viral proteins are available in trans (Astell, 1990; Russell et al., 1992). To this end, the MVMp-permissive simian cell line COS-1 was co-transfected with equimolar amounts of wild-type pMM984 and either of the recombinant constructs. The resulting virus preparations contained a large excess (100-fold) of wild-type virions (Russell et al., 1992; data not shown), that provided in-trans the factors necessary for replication of recombinant viruses in secondary infection experiments. Moreover it has been reported that transcriptional attenuation in permissive cells requires the expression of parvoviral products (Krauskopf et al., 1991). Therefore the presence of a large excess of wild-type virus in our preparations allowed us to assess the influence of the att sequence on the rate of P4-driven expression under near physiological conditions.

The att fragment exhibits attenuation properties in vivo

Transfected COS-1 cells were cultivated for 3 days and lysed. Detectable titres of either recombinant virus could be measured in transfected cell extracts by replicative
Table 1. Production of reporter recombinant viruses*

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Recombinant virus titres (replicative units/ml)</th>
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<tbody>
<tr>
<td></td>
<td>pMVM/P4-cat1</td>
</tr>
<tr>
<td>1</td>
<td>$3.5 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>$4.7 \times 10^4$</td>
</tr>
<tr>
<td>3</td>
<td>$7.2 \times 10^4$</td>
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<td>4</td>
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* Cultures were cotransfected with equimolar quantities of pMM984 and either pMVM/P4-cat1 or pMVM/P4-att-cat1. Cells were lysed 3 days after transfection, and recombinant viruses were titrated by replicative centre assays as described in Methods. Each titre is the average of four independent measurements (s.d. < 35%).
† Preparations pooled for subsequent infections.

Preparation used in subsequent infection experiments.

Fig. 2. Replication of recombinant virus genomes in infected FREJ4 and NBE cells. Low M r DNA was extracted from cell cultures infected with either recombinant virus vector. One-tenth (MVM/P4-cat1) or one-fiftieth (MVM/P4-att-cat1) of the preparation was analysed by Southern blotting, using a chemiluminescent cat-specific probe. As illustrated in Fig. 2, this probe revealed both single- and double-stranded replicative forms of recombinant viral DNA at the expected M r values. Virions bearing att (MVM/P4-att-cat1) generated replicative intermediates of slightly but significantly higher M r than the vectors in which the P4 promoter was directly linked to the reporter cat gene (MVM/P4-cat1). The presence of the attenuator region did not significantly influence the amplification of the recombinant DNA template. Whole extracts of recombinant virus-infected cells were also assayed for reporter gene expression. The CAT activities were normalized for the titre of the respective recombinant viruses and for the reduced (60%) CAT specific activity of the NS-CAT fusion product encoded by the MVM/P4-att-cat1 vector (Spegelaere et al., 1991). As shown in Table 2, the yield of transcriptional attenuation resulting from the presence of the att fragment was of the order of two- to fourfold. These results are similar to those observed in transfection experiments (Spegelaere et al., 1991) and confirm the attenuation properties of att in the course of a physiological parvoviral infection. The analysis of viral RNAs from cells infected with wild-type MVMp, using RNase protection and run-on assays, showed that the att-induced reduction of gene expression primarily resulted from RNA polymerase pausing during the elongation step of transcription (Spegelaere et al., 1991; Krauskopf et al., 1991). Similar experiments could not be performed with the recombinant viral stocks obtained in the present work, owing to the low titres mentioned above and contamination by a large excess of the wild-type virus. Yet the conservation of promoter centre assays (Table 1). To verify that the reporter sequences were efficiently amplified in the infected host cells, we examined the yield of recombinant DNA after infection of permissive FREJ4 or NBE cell lines with the reporter viruses. Subconfluent cultures were infected with either recombinant virus preparations, at a multiplicity of 0.01 recombinant replicative units per cell. Two days post-infection, cells were harvested, and low M r DNA was extracted and analysed by Southern blotting, using a cat-specific probe. As illustrated in Fig. 2, this probe revealed both single- and double-stranded replicative forms of recombinant viral DNA at the expected M r values. Virions bearing att (MVM/P4-att-cat1) generated replicative intermediates of slightly but significantly higher M r than the vectors in which the P4 promoter was directly linked to the reporter cat gene (MVM/P4-cat1). The presence of the attenuator region did not significantly influence the amplification of the recombinant DNA template. Whole extracts of recombinant virus-infected cells were also assayed for reporter gene expression. The CAT activities were normalized for the titre of the respective recombinant viruses and for the reduced (60%) CAT specific activity of the NS-CAT fusion product encoded by the MVM/P4-att-cat1 vector (Spegelaere et al., 1991). As shown in Table 2, the yield of transcriptional attenuation resulting from the presence of the att fragment was of the order of two- to fourfold. These results are similar to those observed in transfection experiments (Spegelaere et al., 1991) and confirm the attenuation properties of att in the course of a physiological parvoviral infection. The analysis of viral RNAs from cells infected with wild-type MVMp, using RNase protection and run-on assays, showed that the att-induced reduction of gene expression primarily resulted from RNA polymerase pausing during the elongation step of transcription (Spegelaere et al., 1991; Krauskopf et al., 1991). Similar experiments could not be performed with the recombinant viral stocks obtained in the present work, owing to the low titres mentioned above and contamination by a large excess of the wild-type virus. Yet the conservation of promoter
and att-containing leader sequences between recombinant and wild-type viruses makes it very likely that att also interfered with RNA elongation when present in the context of the recombinant viral genome.

**Single-stranded and duplex att DNA fold into stem-loop structures**

Little is known concerning the mechanism that underlies polymerase pausing at the level of the att sequence, although RNA secondary structure seems to be required (Resnekov & Aloni, 1989). Krauskopf et al. (1991) suggested that in the absence of specific accessory elongation factors, nascent mRNA folds into the attenuation stem-loop structure, resulting in premature polymerase pausing. In the absence of viral products, this block to elongation would be alleviated by specific elongation factors present in permissive cells. This suggests that the regulation of attenuation is achieved through an ‘attenuation inhibitor’ rather than a factor that would stabilize the attenuation structure. However this model does not account for the low readthrough efficiency (20% to 30%), as in the absence of such stabilizing factor(s), the readthrough structure would be thermodynamically favoured over the attenuation structure (Ben-Asher & Aloni, 1984). Moreover, if transcription of the viral mRNA occurred on a linear dsDNA template, the readthrough stem (1 + 2)-loop structure would form first, as it involves sequences which are transcribed prior to the stretch 3 component of the alternative attenuation stem (2 + 3) structure (Fig. 3a). Indeed, it is well-established that RNA polymerase II-transcribed RNA remains associated with its template over 10 bases, at most (Gamper & Hearst, 1982) or even fewer according to a recent report (Rice et al., 1991). Outside this short stretch, nascent RNA is progressively displaced as a result of the reannealing of complementary DNA strands (Gamper & Hearst, 1982; Sluder et al., 1988). Therefore it is likely that shortly after transcription, stretch 2 would become available for hybridization to the already single-stranded attenuation stretch 1, to form a thermodynamically stable stem (Fig. 3a). Should this be the case, subsequent synthesis of stretch 3 would have little effect, as the complementary stretch 2 would already be involved in the lowest free-energy structure. In conclusion, although RNA secondary structure seems to be required for efficient polymerase II pausing, the attenuation loop would not form rapidly and efficiently enough to block elongation if its folding relied on a diffusion-dependent ‘scanning’ process and on its thermodynamic stability alone.

Parvoviral mRNAs are thought to be transcribed from dsDNA replicative forms. This prompted us to investigate whether duplex att DNA had a secondary structure which might influence the attenuation process. To this end, in vitro DNase I and S1 nuclease probing experiments were performed. It has been established previously that DNase I attack is targeted at the minor groove of duplex DNA structures (Moore, 1981) which are recognized through enzyme interactions with complementary strands (Suck & Oefner, 1986). In contrast, S1 nuclease is specific for single-stranded nucleic acids under the present experimental conditions (Vogt, 1980). To validate this approach, single-stranded att DNA was analysed in parallel. This probe is expected to fold into a stem-loop configuration, by analogy with corresponding transcripts for which attenuation was shown to require a stable RNA secondary structure (Resnekov & Aloni, 1989). Indeed the free energy of monocatenar DNA can be assumed to be minimized by intramolecular base pairings similar to those occurring in a homologous RNA strand. End-labelled monocatenar DNA probes were synthesized from the pP4-att-cat plasmid by repeated single primer extensions, partially digested with DNase I or S1 nuclease and analysed by sequencing gel electrophoresis. The ssDNA probes studied, of either plus (Fig. 4a) or minus (data not shown) polarity, were sensitive to both endonucleases, in agreement with their predicted folding. When referred to the model structures proposed by Ben-Asher & Aloni (1984) for nascent viral transcripts (equivalent to upper strands in Fig. 4b), most DNase I-induced cuts were located in stems A, B, 1, 2 and 3 (indicated by vertical bars on the right side of the autoradiogram). This result suggested that the ‘attenuation’ and ‘readthrough’ forms co-existed in solution. It should be stated that the putative loop regions of either structure are also single-stranded in the alternative fold, in agreement with their resistance to DNase I digestion. S1 nuclease attacked not only the putative loop regions (L1, L2 and L3) but also partly digested stretches within the putative stems 1, 2 and 3. This further supports the possibility of single-stranded preparations being a mixture of the readthrough and attenuation structures, as in this case, stretches 1, 2 and 3 should be partly single-stranded due to the equilibrium between the two alternating folds.

The duplex form of MVMp att DNA, as found in replicative intermediates, was also tested for its sensitivity to nucleases, using PCR products that were end-labelled on either strand. These probes exhibited a number of S1 nuclease-sensitive zones, suggesting the existence of a secondary structure with single-stranded regions (Fig. 4a). As in the case of the isolated strands, S1 nuclease attacked mainly the L1 and L3 regions, in agreement with their looping out from duplex DNA. In contrast, the L2 region proved to be resistant to S1 nuclease digestion. Since the L2 and L3 loops are specific for the readthrough and attenuation structures respectively, and
the L1 loop is shared by both forms, these results altogether support the hypothesis that the duplex form of MVMp att DNA may be found predominantly in the attenuation configuration. This is apparent from Fig. 4(b) in which the nuclease sensitivity data were plotted on the putative attenuation and readthrough structures, showing a fair agreement with the former but not with the latter. The regions nt 201 to 235 and nt 340 to 360 of the minus and plus strands, respectively, were beyond the resolution limits of the sequencing gel and therefore not

Fig. 3. Model for the influence of DNA secondary structure on the transcriptional attenuation of promoter P4. Viral mRNA synthesis is depicted on a linear (a) or folded (attenuation structure) (b) DNA template. Shaded and open boxes represent stem-forming DNA and RNA sequences, respectively. For the sake of simplicity, the stem-loop A-L1-B (A'-L1'-B') and the transcription complex are not shown. Base-pairing between the DNA template and nascent RNA is indicated by small bars. The poly(A)-poly(T) region constitutes the RNA polymerase pausing site. The large open arrow indicates the most probable RNA structure.
Fig. 4. Nuclease probing of the secondary structures of att. (a) Single-stranded (SS) or double-stranded (DS) att DNA labelled on the transcribed (−) or non-transcribed (+) strand, was digested with DNase I or S1 nuclease and analysed by electrophoresis on a sequencing gel. Lanes A+G, Maxam and Gilbert A+G sequence ladders. Bars on the right indicate the computer-predicted stems (1, 2, 3, A, B) and the observed RNA polymerase pausing site (PAUSE). (b) The distribution of DNase I- and S1 nuclease-sensitive sites, indicated respectively by bold lines and arrowheads, as determined from (a), are shown on the putative readthrough and attenuation configurations of double-stranded att. The digestion pattern of shaded sequences (◮) is beyond the resolution of the sequencing gel.
represented. Apart from the L1 and L3 loops, S1 nuclease-sensitive sites were detected at positions corresponding to nt 300 in both strands and A-T-rich stretches (in the region of nt 340 to 350 in the minus strand). Unlike single-stranded loops which were specifically cleaved by S1 nuclease, the region of nt 340 to 350 was characterized by its sensitivity to both S1 nuclease and DNase I. A preferential digestion of A-T-rich duplex DNA by S1 nuclease was previously reported (Hofstetter et al., 1976; Barrijal et al., 1992) and interpreted in terms of the tendency of such sequences to melt. This instability may be further enhanced by the presence of nearby cruciform structures in MVMp DNA. It is also noteworthy that the nt 340 to 350 region coincides with the observed RNA polymerase pausing site. The L3' loop was unexpectedly resistant to both nuclease and DNase I. A preferential digestion of A-T-rich stretches (in the region of nt 240 in the plus strand and nt 300 in both strands) and A-T-rich stretches (in the readthrough configuration, the stems-loops A-L1-L2-1 would be separated by 9 bp (Fig. 4b). In a regular β-helical structure, this corresponds to approximately one helix turn (10.5 bp) with both stems extending from the main helix at a distance of only a few nanometers. The resulting steric hindrance may induce partial melting of the 1 + 2 and 1' + 2' stems, in particular in the mismatched region at nt 270 of both strands, and should reduce the thermodynamic stability of the readthrough configuration, thus favouring the formation of the attenuation structure.

The observed folding of MVMp dsDNA into the attenuation structure in the att region may shed light on the basic mechanism of transcriptional attenuation. To account for the high ratio of attenuated versus readthrough transcripts, we propose that the secondary structure of the DNA template may influence the configuration adopted by the nascent mRNA. A possible model for this regulation is illustrated in Fig. 3(b). Briefly, the nascent RNA molecule is displaced during transcription, as a result of the reannealing of the non-transcribed and template DNA strands (Gamper & Hearst, 1982; Sluder et al., 1988). Such a displacement would not take place in the loops that are intrinsically single-stranded, leading nascent RNA molecules to remain hybridized to the loops longer than they do to the double-stranded regions of transcribed DNA. This could bring the complementary RNA stretches corresponding to the attenuation stem in close proximity, allowing subsequent stem formation through a ‘zipper-like’ mechanism. Such a ‘DNA structure-driven’ hybridization should proceed more rapidly and efficiently than a diffusion-dependent process. Indeed the latter would involve the pairing of short stretches of homologous sequences, followed by the annealing of the rest of the stem, and is expected to be slowed down considerably by the presence of a long intervening loop and intrastem mismatched bases. In this context, the kinetic advantage offered by the proximity of stems 2 and 3 should be a deciding factor in favour of the attenuation over the readthrough configuration of nascent RNA molecules.

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