Targeted nuclear antisense RNA mimics natural antisense-induced degradation of polyoma virus early RNA

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Communicated by Mary J. Osborn, January 24, 1994

ABSTRACT We describe a general antisense strategy to inhibit target gene expression. The substitution of a cis-acting ribozyme for a polyadenylation signal in an antisense expression vector results in the nuclear retention of RNAs and the efficient degradation of their targets. We demonstrate the utility of this system in polyoma virus, where early-strand RNA levels are downregulated in the nucleus by antisense late-strand counterparts. We show that mutations destabilizing these naturally occurring antisense transcripts lead to increased levels of early-strand RNAs. Furthermore, expression in trans of nuclear antisense transcripts lowers early-strand RNA levels and quantitatively mimics the natural regulation.

Although artificial antisense techniques have been widely used as a means to repress gene expression, there are few reports in eukaryotic systems of endogenous antisense RNAs that regulate gene expression under natural circumstances (1–5). In these systems the mechanisms of inhibition in vivo are poorly understood.

Cornelissen (6) proposed two independent mechanisms to describe the action of antisense RNA. In the first mechanism, a reduction of sense RNA levels is achieved in the nucleus through sense–antisense interactions. Nuclear antisense transcripts might block transcription initiation or elongation, RNA processing, or mRNA transport. RNA duplexes might also induce an endogenous RNAse activity. In the second mechanism, antisense RNA exerts its effect in the cytoplasm primarily by affecting translation. In contrast to the effects of nuclear antisense RNA, cytoplasmic antisense RNA usually does not reduce mRNA levels (5).

Polyoma virus provides a useful model system to study the effects of nuclear antisense RNA. The polyoma genome is a small circular double-stranded DNA molecule whose early and late transcription units proceed in opposite directions from an intergenic regulatory region. Before viral DNA replication, or in the absence of DNA replication, the early genes (encoding the large, middle, and small tumor antigens) are preferentially expressed, and few late-strand messages are detected (7–15). After the onset of viral DNA replication, there is little increase in the level of early-strand RNA, while the concentration of late-strand transcripts increases rapidly, mirroring the increase in viral DNA copy number (8, 13–15).

At late times in infection the apparent downregulation of early RNA levels relative to what one would expect from the rate of accumulation of viral DNA molecules is not mediated by large tumor antigen. Large tumor antigen does not repress early gene expression in transient transfection assays (16), and nuclear run-on assays indicate that the relative transcriptional activities from the early and late promoters change little throughout infection (15). Regulation of early gene expression after transcription initiation is also consistent with the data of Farmerie and Folk (10), who observed early gene repression even in constructs in which this transcription was driven by a heterologous promoter lacking upstream large tumor antigen binding sites.

It has been shown by a number of laboratories that transcription termination of the late genes is inefficient during the late phase of infection, allowing RNA polymerase II to continue around the circular viral genome multiple times (10, 11, 17–22). The resulting giant primary transcripts are eventually processed by RNA splicing and polyadenylation, but unprocessed giant transcripts accumulate to high levels in the nucleus (21, 22). Owing to the circularity of the polyoma genome, giant late-strand primary transcripts contain sequences complementary to early-strand transcripts, serving as natural antisense RNA. These giant transcripts accumulate at the same time that early-strand RNAs become relatively less abundant.

In this report, we have explored the repression of polyoma early gene expression by nuclear antisense RNA. We show that destabilization of antisense-early transcripts results in a dramatic increase in the steady-state level of early RNAs. Furthermore, using ribozyme technology we have been able to express and accumulate antisense-early RNA molecules specifically in the nucleus. Overexpression of nuclear antisense-early RNA in trans dramatically reduces polyoma virus early RNAs in a dose-dependent manner and quantitatively mimics the natural regulation.

MATERIALS AND METHODS

Plasmid Construction. Antisense Constructs. aE-RZ contains the polyoma antisense-early region driven by the cytomegalovirus (CMV) promoter, with 3'-end formation by a ribozyme. E-RZ contains the same polyoma early region and the ribozyme sequence but no CMV promoter or polyoma replication origin. Py-CMV contains only the CMV promoter and polyoma replication origin. Each construct was made in pBluescript. The orientation of the polyoma early region is indicated by arrowheads in Fig. 2. The RNA probe Ribo-Ava is also shown. The parent plasmid, BS-RZ, was generated by inserting annealed oligonucleotides comprising a hammerhead ribozyme into pBluescript opened with EcoRV and Sma I. The ribozyme cassette consisted of annealed oligonucleotides containing the sequence 5'-AAATCGGCGCTTATCAGGGCCATGCACTGCGCGCCGCTCCGGCGCCGCGCCGCTGATGAGTCCGTGAGGACGAAACATGCAT-3'. For E-RZ, wild-type polyoma nt 359–2908 were inserted into the BamHI site of BS-RZ. The CMV promoter excised from pDNA 1/Amp (Invitrogen) with EcoRV and Dra I was inserted at the BamHI site to create Py-CMV. aE-RZ was created by inserting the Nar I/HindIII fragment of Py-CMV containing the polyoma replication origin and the CMV promoter into the Sma I site of E-RZ. Construct Ribo-Ava was created by inserting the Ava I

Abbreviation: CMV, cytomegalovirus.

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fragment spanning the cleavage site of the ribozyme of αE-R2 into pBluescript.

**Cell Culture, Transfection, and RNA Isolation.** Mouse NIH 3T3 cells were maintained and propagated as described (23). NIH 3T3 cells were transfected by the BES procedure with a total of 40 μg of DNA per 15-cm dish (24). Transfections were adjusted to 40 μg of DNA with pUC18 DNA. Virus constructs were released from plasmids with restriction enzymes and recircularized by dilute ligation. A single-step method using guanidinium thiocyanate (25) was used to isolate total RNA. RNA samples were then extracted twice with acidic phenol (12). Nuclear and cytoplasmic RNAs were isolated 48 hr after transfection as described (11). In protection experiments, 20 μg of total RNA was routinely used; for subcellular fractionation, the same percentages of nuclear and cytoplasmic RNA samples were used.

**RNase Protection.** Probes were made and protection experiments were performed as described (12). Quantitation was with a Betascope blot analyzer (Betagen, Waltham, MA), and results were corrected for uridine content. Control experiments confirmed that all RNase protection experiments were carried out in probe excess. Clones for generating late, early, and antisense-early RNA probes have been described (12, 26). After in vitro transcription by T3 or T7 RNA polymerase using [α-32P]UTP, DNA templates were removed by RNase digestion in combination with acid/phenol extraction. The labeled RNA probes were hybridized in excess to RNA samples at 57–60°C overnight (12). The resulting hybrids were digested with a T1/T2 mixture (27) at 37°C for 2 hr. Samples were resolved on denaturing 6% polyacrylamide gels. Quantitation of results was performed with a Betascope blot analyzer.

**DNA Replication Assays.** Episomal DNA was isolated as described (28, 29) and then digested with an excess of Dpn I, EcoRI, and BamHI. Replication assays were as described (28), with the probe being the 32P-labeled viral Ava I fragment (nt 673–1032). Bands were quantitated with a Betascope blot analyzer.

**RESULTS**

**Polyoma Mutants That Express Unstable Late-Strand RNAs Accumulate Higher Levels of Transcripts from the Early Region.** If antisense interactions were involved in the regulation of polyoma early-strand gene expression, then changes in the abundance of late-strand RNA species might alter the level of early-strand RNAs. To test this hypothesis, we examined the expression of early-strand RNAs from two mutants (Fig. 1A). Mutant 5'ssΔ carries a 6-bp deletion of a 5's splice site used in all late pre-mRNA splicing and expresses late RNA molecules that are rapidly degraded in the nucleus (30). Mutant ALM shows a similar phenotype but contains a nonoverlapping deletion (31). The promoter activities of both constructs have been shown by nuclear run-on assays to be unchanged from wild type (N. Barrett and G.G.C., unpublished data). Forty-eight hours after transfection into NIH 3T3 cells, total RNA was isolated and subjected to RNase protection assays with early, antisense-early, and late exon probes (25). As shown in Fig. 1B, both mutants are defective in late RNA accumulation (lanes 8 and 9), including the antisense-early region (lanes 5 and 6). However, early transcripts (and presumably early proteins) from these mutants accumulated to levels 8- to 10-fold higher than wild type, as judged from quantitation of band intensities (Fig. 1B, lanes 2 and 3). Quantitative DNA replication assays (28) (Fig. 1C) confirmed that both mutants replicate into wild-type levels, indicating that the increase in the level of early gene transcripts is not a result of a difference in the number of DNA templates. As the early promoter is not downregulated, these results suggest posttranscriptional regulation of early RNA levels. They further suggest that the level of early-strand RNA can be negatively regulated by complementary late-strand RNA. The phenomenon observed here is unlikely to be caused by a unique feature of either mutant because the deletions do not overlap. In addition, a number of other mutants with deletions or mutations in the late region also displayed the same phenotype of lower late-strand RNA levels and higher early-strand RNA levels (26, 32). Late proteins do not seem to be involved, since mutants that carry either deletions or frameshifts in the late coding region.

![Gene expression from polyoma mutants producing unstable late-strand transcripts.](image-url)
display wild-type levels of early-strand RNAs (data not shown).

Expression of Nuclear Antisense-Early RNA from a Heterologous Promoter Inhibits Early-Strand RNA Accumulation. We next wanted to determine whether nuclear antisense RNA molecules, provided in trans, could mimic the natural polyoma antisense regulation. Eckner et al. (23) showed that substituting a polyadenylation signal with a cis-acting hammerhead ribozyme could lead to nuclear localization of the product RNAs. We therefore inserted a hammerhead ribozyme (33) immediately downstream of antisense DNA sequences transcribed from a strong, constitutive CMV promoter (construct aE-RZ; Fig. 2A). The antisense-early region includes polyoma nt 359–2908, spanning from ∼200 bp downstream of the early start site to a nucleotide 5 bp upstream of the early polyadenylation signal. A functional viral replication origin (nt 4636–96) allows amplification in mouse cells in the presence of large tumor antigen.

Subcellular distribution of RNA transcripts was determined in cells cotransfected with construct aE-RZ (the antisense expression vector) and mutant 5'ssΔ (the target plasmid). Mutant 5'ssΔ was used because it overexpresses early RNA and allows aE-RZ replication. Fig. 2B shows the results of a typical experiment. Nuclear and cytoplasmic RNA samples from cotransfected NIH 3T3 cells were analyzed with an RNA probe that detects antisense-early sequences from both constructs. Virtually all antisense RNA produced from construct aE-RZ remains in the nucleus. It is also evident that ribozyme cleavage is incomplete, with only ∼80% of the transcripts being cleaved. However, uncleaved transcripts also remain in the nucleus, as do the antisense-early transcripts from 5'ssΔ.

Importantly, the experiment shown in Fig. 2B revealed that, when cotransfected in a 9:1 molar excess over the target construct, aE-RZ expressed ≈3-fold more antisense-early RNA than construct 5'ssΔ. Wild-type polyoma also expresses ≈3-fold more antisense-early RNA than 5'ssΔ (Fig. 1). We next cotransfected a constant amount of construct 5'ssΔ into NIH 3T3 cells with increasing levels of aE-RZ and measured early RNA levels from construct 5'ssΔ by using an RNase protection assay. Fig. 3A (lanes 1 and 2) again confirms that mutant 5'ssΔ overexpresses early RNAs. However, when this construct was cotransfected into cells with a 9-fold excess of aE-RZ, early RNA expression (lane 3) was reduced to about the level seen with wild-type polyoma (lane 1). This result shows that nuclear antisense-early RNA expressed from construct aE-RZ quantitatively mimics the extent of inhibition observed in a productive viral life cycle. Control cotransfections using the same amounts (9:1 molar ratios) of control constructs E-RZ (a promoterless version of aE-RZ) and Py-CMV (lacking the antisense-early region) showed no decrease in early RNA levels from 5'ssΔ (lanes 4 and 5). A control plasmid containing a promoter and antisense sequences, but no polyadenylation signal or ribozyme, was not included for several reasons. First, it has been observed in a large number of laboratories that the lack of a 3' processing signal can lead to very low levels of accumulating RNA. Second, we have observed (Y. Huang, D.B.B., Z.L., and G.G.C., unpublished data) that in such a construct containing polyoma sequences, cryptic polyadenylation signals are used, resulting in cytoplasmic RNA localization and defeating the purpose of the intended experiment.

Fig. 3B shows the results of a dose–response analysis. As the amount of cotransfected aE-RZ was increased, striking diminution of 5'ssΔ early-strand RNA was seen. For example, at a 12:1 molar ratio, early RNA from 5'ssΔ was reduced by >6-fold. As construct aE-RZ expresses RNA localized to the nucleus, we conclude that nuclear antisense RNA is capable of reducing the accumulation of early-strand RNAs.

DISCUSSION

Polyoma virus gene expression is temporally regulated in productive infection. In the early phase of infection, early-strand gene expression dominates that of the late strand. The balance of early and late gene expression shifts after the onset of DNA replication; at late times, late-strand transcripts far outnumber early-strand transcripts. The late phase of infection is also characterized by significant levels of nuclear RNAs that could form antisense hybrids with early-strand pre-mRNAs. Previous run-on data from our laboratory (15) showed no significant difference in the distribution of RNA polymerase density along the early strand of the genome.

Fig. 2. (A) Antisense constructs. aE-RZ contains the polyoma antisense-early region driven by the CMV promoter, with 3'end formation by a ribozyme. E-RZ carries the same polyoma early region and the ribozyme sequence but no CMV promoter or polyoma replication origin. Py-CMV contains only the CMV promoter and polyoma replication origin. Orientation of the polyoma early region is indicated by arrowheads. The RNA probe Ribo-Ava is also shown. (B) Subcellular distribution of antisense RNA transcripts produced by aE-RZ. aE-RZ was cotransfected into NIH 3T3 cells along with mutant 5'ssΔ at a molar ratio of 9:1. Cytoplasmic (lane 2) and nuclear (lane 3) RNA samples were analyzed by an RNase protection assay with the RNA probe Ribo-Ava (lane 1). Lengths of protected probes are 421 nt for unprocessed antisense transcript containing the cis ribozyme, 337 nt for transcript cleaved by the cis ribozyme, and 305 nt for antisense-early transcripts deriving from mutant 5'ssΔ. Lane 4, molecular size markers with sizes (from top to bottom) of 501, 404, 353, 242, and 190 bp.
Throughout the infection, strongly suggesting posttranscriptional regulation. In addition, antisense-early RNA coexists with early-strand gene transcripts in the nucleus of infected cells. In this report, we have demonstrated that (i) reduced levels of antisense-early RNA in mutants with RNA stability defects lead to an overaccumulation of RNA from the early genes, and (ii) the presence of excess nuclear antisense-early RNA in trans can reduce the accumulation of early gene transcripts. From these data, we conclude that polyoma virus early transcripts are repressed in the nucleus by antisense RNA from the late transcription unit at late times of infection. This conclusion is supported by previous work suggesting that a posttranscriptional initiation mechanism is responsible for the repression of early genes (10, 15).

Antisense regulation is an efficient way for polyoma virus to achieve repression of early-strand expression because the antisense-early region of late-strand primary transcripts lies within a large intron that is normally removed by the nuclear splicing machinery. The efficacy of antisense inhibition of early-strand RNA accumulation may thus be closely related to the inefficiency of processing of late pre-mRNA molecules.

It has been shown in many artificial and natural systems that antisense RNA molecules or oligodeoxyribonucleotides can reduce the steady-state level of target RNA molecules and/or inhibit the translational activities of mRNAs (see refs. 1, 3, and 4 for reviews). The reduction in RNA levels by means of antisense polynucleotides most likely takes place within the nucleus (5, 6). The compartmentalized distribution of polyoma virus antisense-early RNA is consistent with this type of nuclear antisense effect. In most artificial manipulations to regulate gene expression by expressing antisense RNA, a small region or the full-length sequence of the gene to be targeted is inserted in the opposite orientation immediately downstream of a constitutive or inducible promoter. However, most commonly used expression vectors contain splicing signals and a polyadenylylation signal for 3'-end formation. These sequences allow the primary transcripts to be processed and exported to the cytoplasm, where inhibition of gene expression is primarily at the translational level.

Several models can be invoked to explain the mechanisms by which nuclear antisense RNAs induce the destruction of their targets posttranscriptionally. One explanation is the induction of double-strand RNase activities in the nucleus. However, there is little evidence for the presence of such an activity. RNA modification, such as the demonstrated double-stranded RNA "unwindase" activity reported by Bass and Weintraub (34) might play an important role in the response of cells to nuclear double-stranded RNA. Alternatively, RNA processing and export might also be vulnerable to interference by antisense RNAs (35). It was shown in vitro (35, 36) and suggested in vivo (37, 38) that antisense molecules complementary to splice junctions could strongly inhibit RNA processing. However, transport of the intronless herpes simplex virus thymidine kinase gene transcript in mouse L cells was shown to be blocked by antisense RNAs present in the nucleus (39).

Most artificial uses of antisense RNA in vivo have employed expression systems in which antisense transcripts are spliced and polyadenylylated (40). With such constructs, a 100- to 1000-fold excess of antisense RNA is typically needed for effective inhibition. Since there is little degradation of RNA–RNA duplexes in the cytoplasm (5), it is likely that inhibition would be more efficient if antisense RNAs were retained in the nucleus. Furthermore, such expression might eliminate the possibility of cytoplasmic induction of interferon by double-stranded RNA.

The more general applicability of the method described here has been demonstrated in recent work where we have shown that nuclear antisense RNA efficiently and specifically inhibits expression of the bacterial chloramphenicol acetyltransferase (CAT) gene in cultured cells. In this system, a 6-fold excess of antisense expression vector over target reduced CAT expression by 80% (data not shown).

We thank Nancy Barrett, Ellen Carmichael, Lisa Rapp, and Margery Szlachetka for helpful comments on the manuscript. This work was supported by Grant CA45382 from the National Cancer Institute.