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Inhibition of vesicular stomatitis virus in cells constitutively expressing an antisense RNA targeted against the virus RNA polymerase gene

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To study the effect of virus-specific antisense RNA expression on vesicular stomatitis virus (VSV) infectivity in cultured cells, a HeLaS3 cell line constitutively expressing antisense RNA complimentary to a portion of the VSV large RNA-dependent RNA polymerase gene (L) was established (HeAntiL). At an m.o.i. of 0·01 or 0·1, the HeAntiL cell line was able to reduce virus titre and delay virus-induced cell death by 9 or 5 h, respectively, when compared to a HeLa cell line stably transfected with the expression vector devoid of antisense sequence. Ribonuclease protection experiments showed a 10–20-fold reduction of hybridizable virus L mRNA in infected HeAntiL cells compared to infected control cells at various times before cell death. These results indicate that the antisense RNA approach can significantly reduce VSV mRNA transcription and virus production for a reasonable period of time. The robust growth rate of VSV eventually overwhelms the available antisense RNA and leads to delayed cell death.

The use of antisense RNA technology to inhibit virus gene expression has the potential to be a safe and effective antiviral therapy. In theory, antisense RNA would bind specifically to its target virus mRNA and block virus infection without interfering with the expression of endogenous genes or the normal functioning of the cell (Baltimore, 1988). The success of the antisense RNA approach depends on factors such as the vector and promoter chosen for stable integration and high level expression of the antisense RNA, the site of integration in the host genome and the stability of the antisense RNA within the cell. The growth rate, location of replication and cytotoxicity of a virus also influence how effective antisense RNA is in inhibiting infection. The antisense RNA approach has been used to inhibit, to various degrees, infection of adenovirus (Miroshnichenko *et al.*, 1989), avian retrovirus (To *et al.*, 1986), human immunodeficiency virus type 1 (HIV-1; Chuah *et al.*, 1994; Sczakiel & Pawlita, 1991; Sczakiel *et al.*, 1992), human cytomegalovirus (HCMV; Monte *et al.*, 1996; Ripalti *et al.*, 1995), measles virus (Koschel *et al.*, 1995), polyomavirus (Ottavio *et al.*, 1992) and prototype minute virus of mice (Ramirez *et al.*, 1995).

The goal of this study was to determine whether replication of vesicular stomatitis virus (VSV) could be inhibited by expression of a virus-specific antisense RNA in mammalian cells. VSV, a rhabdovirus, packages within its virion an RNA-dependent RNA polymerase that transcribes the negative-sense genome RNA into five mRNAs in the cytoplasm of infected cells (Banerjee, 1987). The active polymerase complex consists of the large RNA polymerase (L) protein tightly associated with an activator phosphoprotein P (Banerjee & Barik, 1992). We chose to target the L mRNA with antisense RNA because it is the message transcribed in the least molar amounts and because it encodes the RNA polymerase protein which is essential for both virus transcription and replication.

The first 550 bases of the VSV Indiana serotype L gene were cloned, in the antisense orientation, into the eukaryotic expression vector pCSD (Fig. 1). pCSD is a composite vector containing a HCMV promoter for expression of an inserted sequence and a mutant dihydrofolate reductase transcription unit (Prochownik & Kukowska, 1986). The L antisense expression plasmid was called pCDAntiL. The predicted secondary structure of the antisense L RNA did not contain long stretches of base pairing that could impair transcription or stability.

The selectable marker pSV_2Puro and a fivefold molar excess of either pCDAntiL or pCSD were cotransfected into HeLaS3 cells. Single clones selected for puromycin resistance were replated in fresh medium containing methotrexate (MTX) and expanded for continuous growth. By plaque assay analysis,

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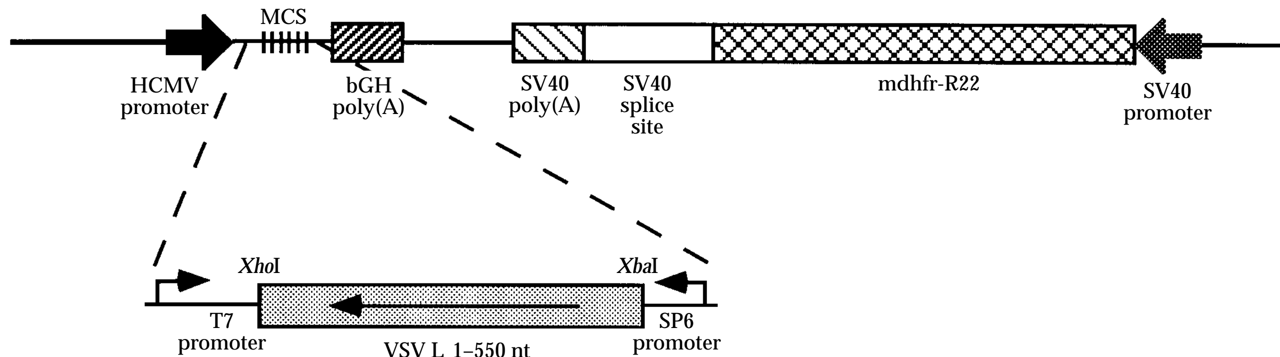

Fig. 1. Schematic diagram of the pCDAntiL plasmid. The top line shows the pCSD plasmid which contains a multiple cloning site (MCS) flanked by enhancer–promoter sequences from the immediate early gene of HCMV and a poly(A) signal and transcription termination sequences from the bovine growth hormone gene (bGH). pCSD also contains the mutant dihydrofolate reductase gene (mdhfr-R22) flanked by the simian virus 40 (SV40) early promoter, splice site and poly(A) signal sequences. The bottom line shows how the pCDAntiL plasmid was created by cloning the 5′ 550 nucleotides of L sequence from the Indiana serotype of VSV in the antisense orientation into the *Xho*I and *Xba*I restriction sites within the MCS of pCSD. *In vitro* transcription using the T7 or SP6 promoters and *Xba*I- or *Xho*I-linearized pCDAntiL plasmid produced antisense or sense L RNAs of 640 or 560 bases.

VSV infected the HeCSD cell line to the same extent as the untransfected HeLaS3 cell line (data not shown). This confirmed that MTX and integrated pCSD had no effect on the infectivity of VSV. Ten HeAntiL cell lines were tested for expression of antisense L RNA by RNase protection analysis and the cell line expressing the most antisense L RNA was selected for further study (data not shown).

To determine whether the HeAntiL cell line conferred any protective effect against VSV infection, both HeAntiL and HeCSD were infected with the Indiana serotype of VSV at m.o.i. of 0·01 or 0·1, and the progression of CPE was monitored. With an m.o.i. of 0·01, no visual difference between the HeAntiL and HeCSD cell lines was observed until 16 h post-infection (p.i.), when the control cell line HeCSD demonstrated some cell rounding (Fig. 2*a*). At 20 h p.i., 90% of the HeCSD cells were dead while the HeAntiL cells still looked healthy. It was not until 29 h p.i. that 90% of the HeAntiL cells rounded up and started to detach from the plate. Therefore, at an m.o.i. of 0·01, the presence of antisense L RNA was able to delay VSV-induced cell death by 9 h (Fig. 2*a*). At m.o.i. of 0·1, 90% of HeCSD cells were dead at 16 h p.i., while the HeAntiL cells only showed some cell rounding (Fig. 2*b*). By 21 h p.i., 90% of the HeAntiL cells were also dead. Thus, the presence of antisense L RNA was able to postpone cell death for 5 h at the higher m.o.i. (Fig. 2*b*).

To determine what effect the presence of antisense L RNA had on VSV replication, HeCSD and HeAntiL cells were again infected at the above stated m.o.i. and plaque assays were performed at various times after infection. For an m.o.i. of 0·01 and 0·1, HeCSD cells were dead at 20 and 16 h p.i. with a VSV titre of $1{\cdot}4\times10^{8}$ and $3{\cdot}4\times10^{8}$ p.f.u./ml, respectively. In contrast, the HeAntiL cells were dead at 29 and 20 h p.i. with a titre of $2{\cdot}1\times10^{7}$ and 5×10^{7}, respectively. These data demonstrate that the presence of antisense L RNA resulted in at least one log lower VSV titre at the time of cell death. It also underscored the ability of VSV to produce a high virus titre in a relatively short period of time.

Finally, to characterize the levels of both antisense L RNA and virus L mRNA present in VSV-infected HeAntiL and HeCSD cells, RNase protection experiments were performed. Single-stranded probes specific for sense or antisense L were obtained using linearized pCDAntiL and T7 or SP6 polymerase. In addition, an antisense 403 base probe was obtained using linearized pTRI-hGAPDH (Ambion) and T7 polymerase. This probe was used as an internal control and protects a 316 base fragment of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Total RNA was isolated from the antisense and control cell lines at various times p.i. After hybridization of the transcribed probes to the RNAs, samples were treated with ribonuclease and analysed on a denaturing gel (Fig. 3). Neither the sense (SP6) nor antisense (T7) L probes protected any RNA from the HeCSD control cell line in the absence of virus infection (lanes 3, 16, 18 and 27). The faint band seen at the 640 base position in lanes 6, 7, and 10 is the undigested L/T7 probe.

RNase protection results obtained with a m.o.i. of 0·01 are shown in Fig. 3 (*a*). The steady state level of antisense L RNA present in HeAntiL cells is shown in lanes 4, 8 and 12. Upon infection with VSV, the amount of antisense L RNA available to hybridize with the L/SP6 probe decreased at all three time points p.i. (Fig. 3*a*, lanes 5, 9 and 13). This is due to a portion of the antisense L RNA hybridizing to virus L mRNA and to VSV-induced inhibition of host cell RNA synthesis (Weck & Wagner, 1978). At 6 h p.i. there was no detectable virus L mRNA present in either HeAntiL or HeCSD cells (Fig. 3 *a*, lanes 6, 7). By 16 h p.i. there was a significant level of virus L mRNA present in HeCSD cells, but only 10% of this L mRNA was available to hybridize to the L/T7 probe in HeAntiL cells

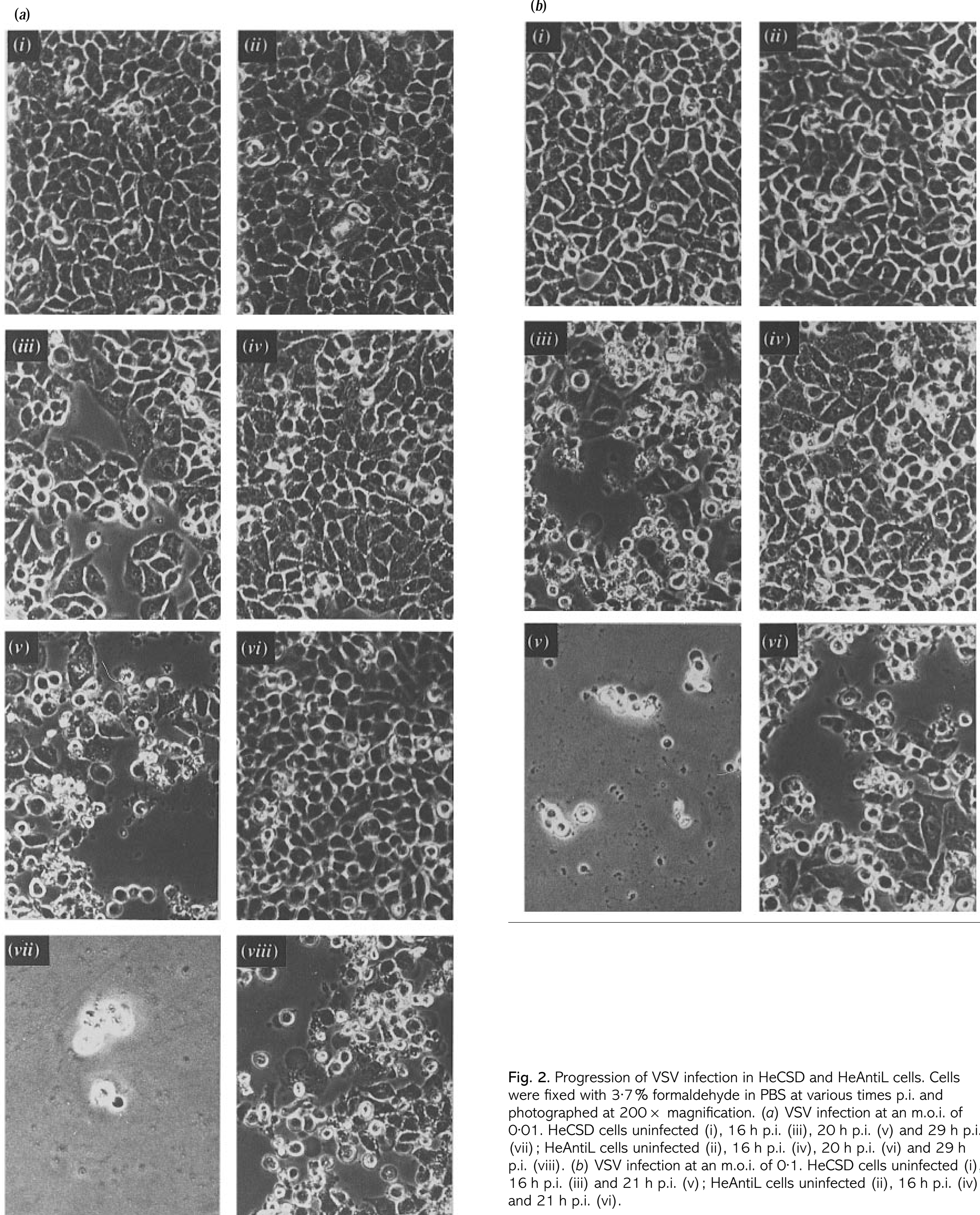

Fig. 2. Progression of VSV infection in HeCSD and HeAntiL cells. Cells were fixed with 3·7% formaldehyde in PBS at various times p.i. and photographed at 200× magnification. (*a*) VSV infection at an m.o.i. of 0·01. HeCSD cells uninfected (i), 16 h p.i. (iii), 20 h p.i. (v) and 29 h p.i. (vii); HeAntiL cells uninfected (ii), 16 h p.i. (iv), 20 h p.i. (vi) and 29 h p.i. (viii). (*b*) VSV infection at an m.o.i. of 0·1. HeCSD cells uninfected (i), 16 h p.i. (iii) and 21 h p.i. (v); HeAntiL cells uninfected (ii), 16 h p.i. (iv) and 21 h p.i. (vi).

Fig. 3. RNase protection analysis of total RNA extracted from uninfected and VSV-infected HeAntiL and HeCSD cells. Lanes 4, 8, 12, 19 and 23 contain RNA from uninfected HeAntiL cells hybridized with the sense L/SP6 probe. RNA from uninfected HeCSD cells was hybridized with either the sense L/SP6 probe (lanes 3 and 18) or the antisense L/T7 probe (lanes 16 and 27). At an m.o.i. of 0·01 (*a*), lanes 5, 9 and 13 contain RNA from infected HeAntiL cells harvested at 6, 16 and 20 h p.i. and hybridized with the sense L/SP6 probe, while lanes 6, 10 and 14 contain RNA from the same cells and times hybridized with the antisense L/T7 probe. Lanes 7, 11 and 15 contain RNA from infected HeCSD cells harvested at 6, 16 and 20 h p.i. and hybridized with the antisense L/T7 probe. At m.o.i. of 0·1 (*b*), lanes 20 and 24 contain RNA from infected HeAntiL cells harvested at 6 and 16 h p.i. and hybridized with the sense L/SP6 probe, while lanes 21 and 25 contain RNA from the same cells and times hybridized with the antisense L/T7 probe. Lanes 22 and 26 contain RNA from infected HeCSD cells harvested at 6 and 16 h p.i. and hybridized with the antisense L/T7 probe. All RNA samples were also hybridized with 20-fold less c.p.m. of the antisense GAPDH probe. The sizes of the undigested probes and protected antisense L or virus L RNA fragments are indicated in bases. All band intensities were quantified using a PhosphoImager. The L value was divided by the GAPDH value and expressed as a percentage of the L protected band in lane 4 or lane 19; 10 μg of total RNA was used in each hybridization reaction. Lanes 1, 2 and 17 contain 1000 c.p.m. of the indicated undigested, ^{32}P-labelled, *in vitro*-transcribed RNA probe.

(Fig. 3*a*, lanes 10 and 11). This demonstrated that the antisense L RNA hybridized to L mRNA and reduced the amount of L mRNA available for hybridization to the L/T7 probe. At 20 h p.i. an even greater amount of virus L mRNA was present in HeCSD cells and there was a 20-fold reduction of available L mRNA in HeAntiL cells (Fig. 3*a*, lanes 14 and 15). These data indicated that the presence of antisense L RNA in the HeAntiL cell line could significantly reduce the amount of virus L mRNA available for translation up to 20 h p.i. We surmise that continuous production of more L mRNA, coupled with less antisense L RNA produced due to virus inhibition of cellular RNA synthesis, resulted in the L mRNA levels eventually overwhelming the antisense L RNA. This led to synthesis of polymerase proteins necessary for virus transcription and replication, which allowed high virus production and cell death at 29 h p.i. Numerous attempts to isolate intact total RNA from either cell line at 29 h p.i. were unsuccessful due to the large amounts of RNase present at late times in VSV infection.

At the higher m.o.i. of 0·1, the amount of antisense L RNA available to hybridize with the L/SP6 probe also decreased p.i. when compared to antisense L mRNA present in uninfected HeAntiL cells (Fig. 3*b* lanes 19, 20 and 23, 24). Again, a reduced level of L mRNA hybridized to the L/T7 probe in HeAntiL cells compared to HeCSD cells at both times p.i. (Fig. 3*b*, lanes 21, 22 and 25, 26). At 16 h p.i., there was an extremely high level of virus L mRNA present in HeCSD cells while HeAntiL cells had 10-fold less L mRNA available for hybridization to the L/T7 probe (Fig. 3*b*, lane 25 and 26). These data demonstrated that even at an m.o.i. of 0·1, the HeAntiL cell line could significantly reduce translatable virus L mRNA levels up to 16 h p.i. After this time, the L mRNA levels overtake the antisense L RNA levels, leading to cell death at 21 h p.i.

In this report, we have tested an antisense RNA approach against VSV to achieve constitutive expression of a short virus antisense RNA in mammalian cells, in an attempt to inhibit VSV infection. A portion of the L gene, in the antisense orientation, was cloned into an expression vector containing a mutant dihydrofolate reductase gene with an increased K_m for MTX (Simonsen & Levinson, 1983). This allowed for selective amplification of the integrated plasmid sequences in the presence of high concentrations of MTX and production of high levels of antisense L RNA sequence.

Our data show that the HeAntiL cell line does produce relatively high levels of antisense L RNA which can substantially inhibit the transcription and replication of VSV for a limited time after infection at low m.o.i. Other antisense RNA systems have shown a large excess of antisense RNA is required for sustained inhibition of gene expression (Kim & Wold, 1985). Complete inhibition of VSV infection was not attainable due to the increasing levels of virus L mRNA as the infection progressed. Since VSV replicates so quickly, the

concentration of target RNA will eventually be too high for inhibition by antisense RNA. Partial inhibition of virus replication by stable expression of an antisense RNA has been observed for adenovirus (Miroshnichenko *et al*., 1989) and the retroviruses Rous sarcoma virus (To *et al*., 1986) and HIV-1 (Sczakiel & Pawlita, 1991). Expression of antisense RNA failed to inhibit influenza virus, another fast-growing negative-stranded RNA virus (Leiter *et al*., 1989). Even with HCMV, which has a slow rate of virus production (Stinski, 1983), cell lines expressing antisense RNA did not always inhibit HCMV infection to 100% (Monte *et al*., 1996). Therefore, it is not surprising that our HeAntiL cell line delayed virus-induced cell death but did not totally inhibit VSV infection.

In addition to the speed of replication and the cytotoxicity of a virus, the effect of the virus on cellular transcription will also influence how successful the antisense approach will be in inhibiting infection. VSV rapidly shuts off cellular RNA synthesis, even at low m.o.i. (Weck & Wagner, 1978). The HCMV promoter used in our expression vector has been shown to be transcribed by the host cell RNA polymerase II *in vitro* (Thomsen *et al*., 1984), so it is possible that HCMV transcription was inhibited by VSV. We are now in the process of testing other promoters to see if we can find one that is not affected by VSV infection.

This is the first reported reduction of VSV infectivity using an antisense RNA stably integrated into the host genome. Taking into account the strength of VSV, we find our results very encouraging and plan to test for additional antiviral effects against VSV infection using other portions of the L gene cloned in the antisense orientation into expression vectors containing different promoters.

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