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Increased levels of B1 and B2 SINE transcripts in mouse fibroblast cells due to minute virus of mice infection

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Abstract

Minute virus of mice (MVM), an autonomous parvovirus, has served as a model for understanding parvovirus infection including host cell response to infection. In this paper, we report the effect of MVM infection on host cell gene expression in mouse fibroblast cells (LA9 cells), analyzed by differential display. Somewhat surprisingly, our data reveal that few cellular protein-coding genes appear to be up- or downregulated and identify the murine B1 and B2 short interspersed element (SINE) transcripts as being increased upon MVM infection. Primer extension assays confirm the effect of MVM infection on SINE expression and demonstrate that both SINEs are upregulated in a roughly linear fashion throughout MVM infection. They also demonstrate that the SINE response was due to RNA polymerase III transcription and not contaminating DNA or RNA polymerase II transcription. Furthermore, expression of MVM NS1, the major nonstructural protein, by transient transfection also leads to an increase in both murine SINEs. We believe this is the first time that the B1 and B2 SINEs have been shown to be altered by viral infection and the first time parvovirus infection has been shown to increase SINE expression. The increase in SINE transcripts caused by MVM infection does not appear to be due to an increase in either of the basal transcription factors TFIIIC110 or 220, in contrast to that which has been shown for other viruses.
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Keywords: Minute virus of mice; Parvovirus; Differential display; SINE; Retroposons; Viral pathogenesis; Gene expression

Introduction

The parvovirus minute virus of mice (MVM) has served as a prototype for understanding how vertebrate parvoviruses infect and replicate in their cellular host. MVM consists of approximately 26 nm nonenveloped icosohedral viral particles, with each particle containing one copy of a single-stranded (negative-sense) DNA genome of approximately 5 kb (Agbandje-McKenna et al., 1998). The viral genome contains two major open reading frames (Pintel et al., 1983), which through alternate splicing of the viral RNA encodes for two nonstructural proteins, NS1 and NS2 (Cotmore et al., 1983), and two structural proteins, VP1 and VP2 (with VP2 being the major coat protein) (Tattersall et al., 1976). NS1 is a multifunctional protein required for MVM replication and has nickase (Nuesch et al., 1995), helicase, ATP hydrolysis (Wilson et al., 1991), and DNA binding (Mouw and Pintel, 1998) activities. This viral nonstructural protein also has trans-activation activity (Doerig et al., 1988; Lorson et al., 1998), can induce cell cycle arrest in S phase (Op De Beeck et al., 2001), and is usually cytotoxic to host cells (Legendre and Rommelaere, 1992).

The prototype MVM virus (MVMp) can establish a permissive infection in the mouse fibroblast cell line LA9 (or A9) resulting in cell death. Initial studies in this lab investigated changes in gene expression in the mouse fibroblast cell line LA9 during infection with MVMp using differential display analysis. By differential display analysis, the murine B1 and B2 short interspersed element (SINE)

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transcripts were observed to increase as a result of MVM infection. The goal of this study was to confirm and further characterize the SINE response to MVM infection.

SINEs are members of the retroelement family (meaning they replicate through an RNA intermediate) and are commonly called retroposons (Labuda et al., 1995). They exist in high copy numbers in various eukaryotic DNA genomes (in mice, there are approximately 1.5 million; Mouse genome sequencing consortium, 2002) of various SINEs, composing 8.3% of the murine genome). SINEs range in size from 50 to 500 nt and do not contain any coding sequence. They contain an internal RNA polymerase III promoter, a poly A tract at the 3′ end, and are flanked by direct repeats of 5–20 nt in size (a result of the integration process) (Weiner, 2002). B1 SINEs have a consensus sequence of approximately 135 nt and are thought to have been derived from 7SL RNA. B2 SINEs have a consensus sequence of approximately 209 nt and are thought to have been derived from tRNA (Schmid, 1998). SINEs are believed to replicate through an RNA intermediate. This intermediate is reverse transcribed and integrated back into the host cell genome through the help of a reverse transcriptase/nuclease encoded by a long interspersed element (LINE) (Weiner, 2002). Putative roles of SINEs are reviewed in Discussion.

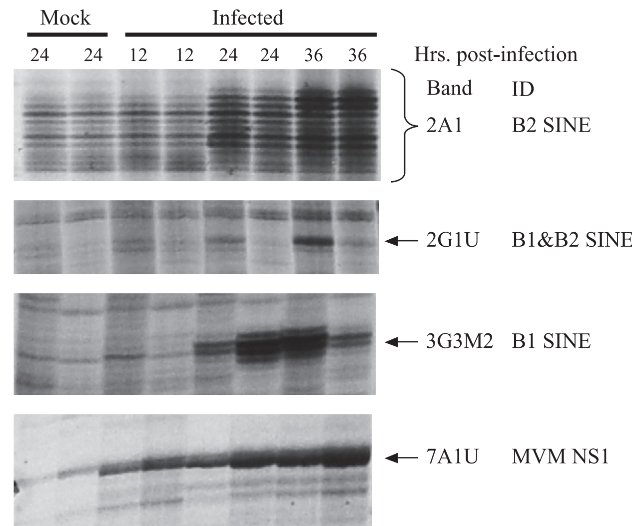

Fig. 1. Detection of increased SINE transcripts by differential display. RNA from MVMp-infected or mock-infected mouse fibroblast cells was reverse transcribed and amplified as per the differential display protocol (described in Materials and methods). The resulting radiolabeled cDNA transcripts were separated on a 6% denaturing acrylamide sequencing gel. Shown in the top three panels are three candidate bands encoding SINEs that showed altered transcription upon MVMp infection. A fourth candidate band encoding an MVM NS1 transcript fragment is also shown to illustrate the effectiveness of the technique. Each time point is composed of two duplicate PCR reactions.

Results

Detection of SINE transcripts by differential display analysis

The technique differential display (Liang and Pardee, 1992) was used to begin to profile changes in gene expression during cytopathic infection of murine LA9 fibroblasts with MVM. This method is an RT-PCR-based technique that amplifies a set of semirandomly chosen cDNAs (50–100 per PCR reaction) based on primer selection. Mouse fibroblast cells (LA9) were infected or mock infected with MVMp at an MOI of approximately 7–10 pfu/cell (almost 100% infection efficiency). RNA was isolated from infected or mock-infected cells at 12, 24, or 36 h postinfection. The RNA was screened for altered transcripts using differential display. Fig. 1 shows three bands that were upregulated as a result of infection compared with the same bands from fibroblasts that were mock infected and harvested 24 h postinfection. Cloning and sequencing of these bands identified them as containing B1, B2, or both B1 and B2 SINEs. This technique also identified the MVM NS1 transcript as being present during infection and absent in mock-infected cells, demonstrating the effectiveness of the technique (Fig. 1, lower panel). Out of the 15 altered bands detected by differential display, three contained SINE sequence.

The multiple bands seen in 2A1 and 3G3M2 (Fig. 1) are a common occurrence seen in differential display gels. It is thought to be due to the different mobilities of the two different strands of the PCR product and also due to Taq DNA polymerase occasionally adding a T to the 3′ end of the transcript (Bauer et al., 1993).

B2 SINE transcripts range from 200 to 600 nt

Northern blots were conducted on LA9 RNA separated on a 5% acrylamide gel using the differential display B2 SINE sequence as a probe. A band at approximately 190 nt was resolved along with a larger series of unresolved bands that ran from approximately 200 to 600 nt (Fig. 2A). Both the approximately 200- to 600-nt material and the 190-nt band were upregulated as a result of MVM infection, confirming the differential display analysis results. Both B1 and B2 SINEs lack a specific termination sequence; rather termination occurs when the polymerase encounters a run of Ts (Bogenhagen and Brown, 1981). This will result in a pool of transcripts of varying lengths, possibly the 200- to 600-nt material observed in the Northern blot. These results are similar to those seen in SV-40-transformed mouse fibroblasts (3T3 (Singh et al., 1985)). The distinct band at approximately 190 nt is presumably due to processed B2 SINE transcripts as it is similar in size to the B2 SINE consensus sequence of 209 nt. These results confirm that B2 SINE transcripts increase during infection.

B2 SINE transcripts are RNA and not DNA

Because of the high abundance of SINEs in the murine genome, it is possible, although unlikely, that the signal seen

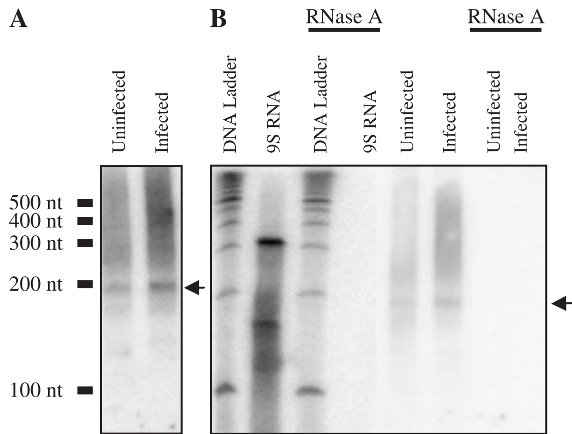

Fig. 2. (A) Resolving the size of the B2 SINE transcripts. Five percent acrylamide Northern blots containing 5 μg uninfected or MVM-infected total LA9 cell RNA per lane were hybridized to a B2 SINE probe (differential display fragment 2A1 radiolabeled by PCR). (B) SINE transcripts are RNA and not DNA. Five micrograms of uninfected or MVM-infected total LA9 cell RNA were treated or mock treated with RNase A, separated on a 5% acrylamide gel, blotted, and hybridized to a B2 SINE probe. As controls, a radiolabeled DNA ladder and bacterial 9S RNA (approximately 246 nt) were included. The arrow indicates the 190-nt SINE band. Uninfected: mock-infected LA9 mouse fibroblast cells harvested at 36 h post-mock-infection. Infected: MVMp-infected LA9 mouse fibroblast cells harvested at 36 h postinfection.

in the previous Northern blot is the result of DNA contamination. To eliminate this possibility, RNA samples treated with RNase A were compared with untreated samples. As Fig. 2B demonstrates, treatment of RNA with RNase A results in elimination of the signal when the blot is hybridized with a B2 SINE probe. A DNA control (5′ end-labeled DNA ladder) and RNA control (radiolabeled 9S bacterial RNA) were included to demonstrate that only RNA was degraded.

B2 and B1 SINE levels are upregulated throughout infection

Primer extension using primers against the B1 and B2 SINEs (Liu et al., 1995) was conducted on MVM-infected LA9 cell total RNA. Fig. 3 shows the resulting products from the primer extension reactions separated on 5% acrylamide gels with the expected B1 and B2 SINE products indicated. In both cases, the abundance of SINE transcripts appears to increase by 24 h postinfection and continues to increase throughout the duration of the experiment in a more or less linear fashion. This is in contrast to an uninfected time course, where both SINEs showed no significant increase in expression until extremely late time points, presumably due to the cells becoming overgrown. Included in Fig. 3 are two mock-infected control samples at 36 and 48 h.

Primer extension was also used to measure 5S RNA expression as a loading control for the B2 SINE experiments (Fig. 3A). The B1 SINE and 5S primer extension products are too similar in size to be resolved on the same gel.

B2 and B1 SINE transcripts are transcribed predominantly by RNA polymerase III

To confirm the size of the major B1 and B2 SINE-amplified products (Fig. 3), primer extension products were

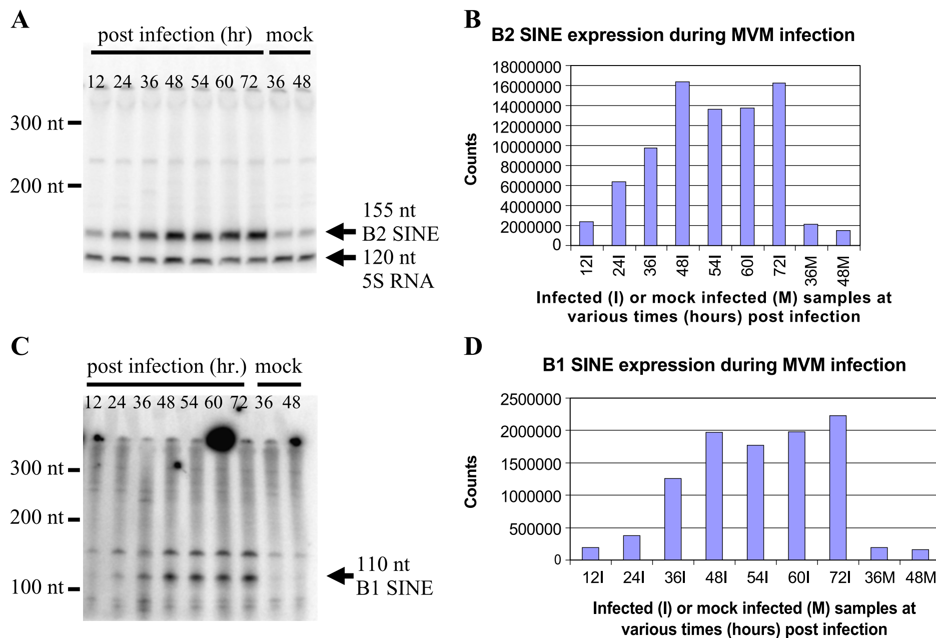

Fig. 3. B2 and B1 SINE transcripts are upregulated throughout MVM infection and are predominantly transcribed by RNA polymerase III. (A) Five percent acrylamide gel showing the products of primer extension with B2 SINE and 5S RNA specific primers. The B2 SINE product (155 nt) and 5S product (120 nt) are indicated by the bold arrows. (B) Radiometric quantification of the B2 SINE band in A. (C) Five percent acrylamide gel showing the products of primer extension with a B1 SINE-specific primer. The B1 SINE product (110 nt) is indicated by the bold arrow. (D) Radiometric analysis of the B1 SINE band (arrow) in C. M: mock-infected unsynchronized LA9 cells. I: MVM-infected unsynchronized LA9 cells.

separated on sequencing gels (not shown). In both cases, the major band was of the size of the expected RNA polymerase III transcript. These data further suggest that the majority of SINE transcripts are of RNA polymerase III origin, rather than another possibility in which the SINE is embedded in a polymerase II transcript. In the latter case, products larger than either the 110-nt (for B1 SINEs) or 155-nt (for B2 SINEs) band would be expected. Interestingly, in the B1 SINE primer extension experiment (Fig. 3C), a second band of 144 nt was also seen to be upregulated. Presumably this is due to a B1 SINE sequence embedded in another altered transcript.

The major nonstructural protein of MVM, NS1, induces increased B2 and B1 SINE levels

The major nonstructural protein of MVM, NS1, is known to be cytotoxic to LA9 cells and to induce nicks in host cell chromatin (Op De Beeck and Caillet-fauquet, 1997). It was hypothesized that the NS1 protein may also affect SINE levels. To test this hypothesis, LA9 cells were transfected with plasmids expressing either NS1–NS2 (pCMVNS1), NS1 only (pCMV1989, a gift from Dr. David Pintel, University of Missouri), or as a control β-galactosidase (pCMVβ-gal), and RNA was harvested at various times posttransfection. The pCMV1989 plasmid contains NS-1 coding sequence with a point mutation (A to C) that alters a splice acceptor site, preventing splicing of the NS1 (R1) transcript and expression of the NS2 (R2) transcript (Naeger et al., 1990). Primer extension analysis was used to quantify B2 SINE RNA expression in transfected cells (Figs. 4A and B). At 12 h posttransfection, there was no significant difference in B2 SINE levels in cells with the NS1-expressing plasmids compared with the pCMVβ-gal control. However, 24 h posttransfection, B2 SINE levels increased in cells transfected with either pCMVNS1 or pCMV1989. This trend continued at the 36-h time point, where an approximately 3-fold change in B2 SINE levels was seen for both pCMVNS1 and pCMV1989 compared with the pCMVβ-gal control. Interestingly, there appeared to be no difference between cells expressing NS1–NS2 or just NS1 alone. A similar trend was seen when B1 SINE levels were examined (Figs. 4C and D), with an approximate 2-fold change seen by 36 h. What is remarkable is that this approximately 2- to 3-fold increase in B1 and B2 SINEs was observed in cells in which the transfection efficiency was only 15–20%.

TFIIIC220 and TFIIIC110 protein levels do not increase during MVM infection

One way to increase the number of SINE transcripts is to increase the amounts of various basal RNA polymerase III transcription factors. It has been reported that murine fibroblasts (3T3 cells) transformed with SV-40 express

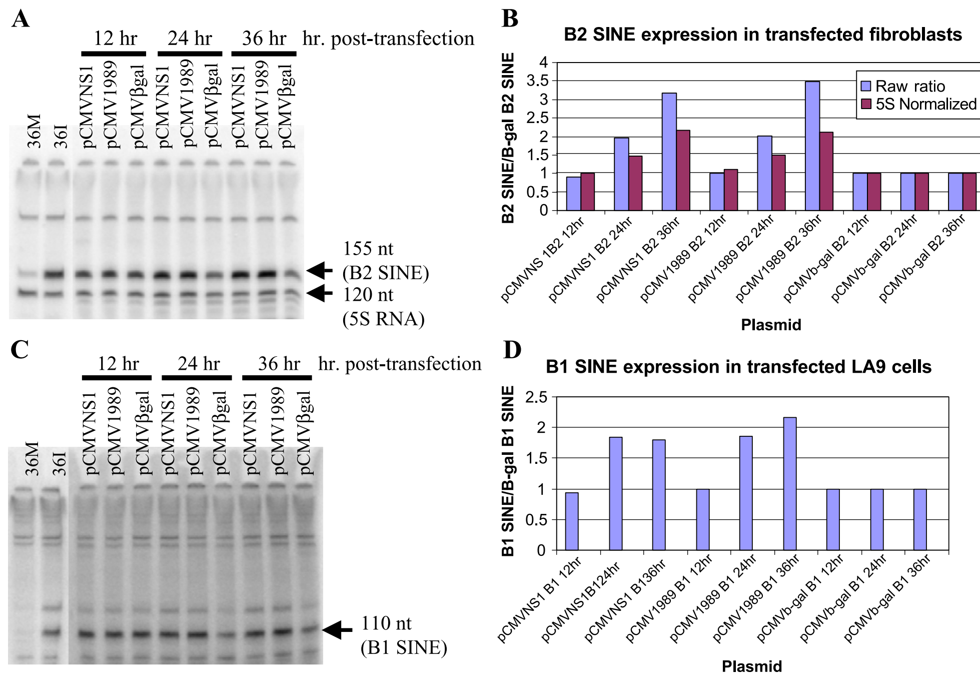

Fig. 4. Transient expression of MVM NS1 induces increased B2 and B1 SINE expression. (A) Five percent acrylamide gel containing the products of B2 SINE primer extension from LA9 cells transfected with pCMVNS1 (expresses NS1 and NS2), pCMV1989 (expresses NS1 only), or pCMVβ-gal (expresses β-galactosidase) at various times post-transfection. (B) Radiometric analysis of the 155-nt band in A. Data are expressed as fold change compared to the β-galactosidase control. 5S Normalized: data normalized relative to the 5S RNA signal (B2 SINE/5S) to correct for variable loading. (C) Five percent acrylamide gel containing the products of B1 SINE primer extension from LA9 cells transfected with the same plasmids as in A at various times post-transfection. (D) Radiometric analysis of the 110-nt band in C. Data are expressed as fold change compared to the β-galactosidase control. 36M: mock-infected unsynchronized LA9 cells 36 h. 36I: MVM-infected unsynchronized cells 36 h postinfection.

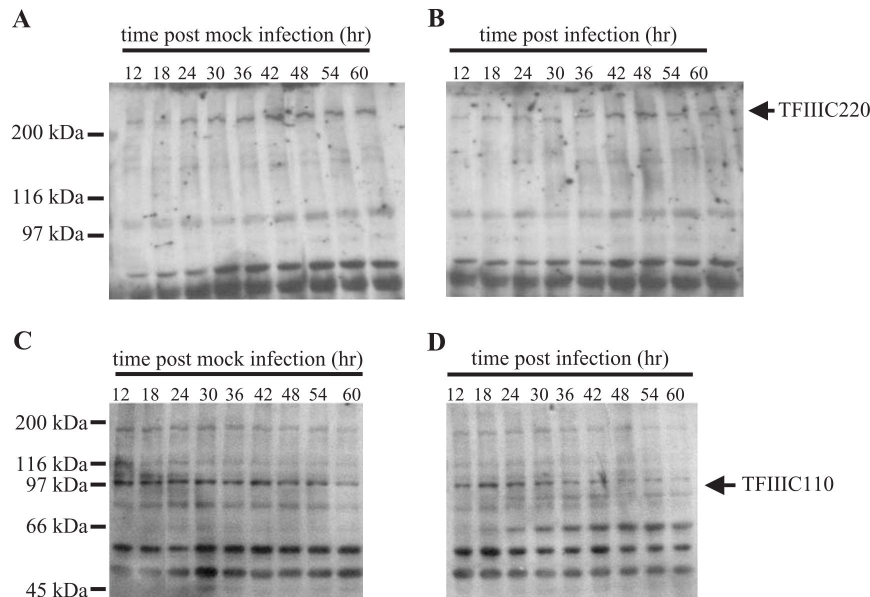

Fig. 5. The TFIIIC220 and TFIIIC110 proteins do not increase during MVM infection. Western blot analysis of total protein from unsynchronized uninfected LA9 (A and C) or MVM-infected (B and D) cells harvested at increasing times after a mock infection probed with anti-TFIIIC220 (A and B) or anti-TFIIIC110 (C and D). Actin was used as a loading control. Protein concentration was determined by the BCA assay. Arrows indicate TFIIIC220 and TFIIIC110 bands.

higher levels of TFIIIC220 and TFIIIC110 (Larminie et al., 1999). Adenovirus infection has also been shown to cause upregulation of TFIIIC110 in HeLa cells (Sinn et al., 1995). To determine if this could explain the increase in abundance of the SINE transcripts in MVM-infected LA9 fibroblast cells, expression of TFIIIC220 and TFIIIC110 proteins was examined. Western blots of both mock infected and infected unsynchronized LA9 cells were probed with anti-TFIIIC220 (a gift from Dr. Arnold Berk; (Shen et al., 1996)) or anti-TFIIIC110 (a gift from Dr. Robert White (Winter et al., 2000)), as shown in Fig. 5. In our studies, TFIIIC220 appeared to decrease but only at late time points post-infection (Figs. 5A and B). This may be due to general protein degradation occurring at these time points. Similarly, TFIIIC110 appeared to decrease as the infection progressed (Figs. 5C and D). The identity of the band at 66 kDa that appears to increase in MVM-infected cells (Fig. 5D) is unknown and not reproducible.

These results suggest that SINE upregulation is likely not due to an increase in the basal RNA polymerase III transcription factors TFIIIC220 or TFIIIC110. However, we cannot rule out upregulation of other polymerase III transcription factors, although it is known that TFIIIC110 and TFIIIC220 play crucial roles in transcription and are important targets by several viruses and in cell transformation (Jang and Latchman, 1992; Jang et al., 1992; Larminie et al., 1999; Sinn et al., 1995; Winter et al., 2000).

Discussion

SINE transcripts are upregulated in a variety of conditions, usually associated with cell stress. These include heat shock (Alu, B1, and B2 SINES) (Liu et al., 1995, 1999), ethanol treatment (B1 and B2 SINES) (Li et al., 1999), cyclohexamide treatment (Alu SINE) (Liu et al., 1995), DNA-damaging agents such as cisplatin, etoposide, and γ-irradiation (Alu SINE) (Rudin and Thompson, 2001) in 3T3 cells transformed by SV-40 large T antigen (B2 SINE) (Singh et al., 1985) and by viral infection including adenovirus infection (Alu SINE) (Panning and Smiley, 1993), herpes simplex virus (HSV) infection (Alu SINE) (Jang and Latchman, 1989), and HIV infection (Jang et al., 1992). To the best of our knowledge, the increased B1 and B2 SINE expression during MVMp infection is the first example of murine SINE expression being upregulated by parvovirus infection. It would be interesting to know whether MVMi-infected lymphocytes or other parvovirus-mediated infections, such as B19, AAV, or CPV, can also cause an increase in SINE expression.

Theoretically, SINE transcript levels can be increased in one of two ways: either SINE transcription is increased or SINE transcript degradation is decreased. In all of the viruses studied thus far, SINE induction has always been due to increased transcription (Jang and Latchman, 1992; Jang et al., 1992; Panning and Smiley, 1993). In our studies, nuclear run-on experiments to determine the increase in the number of B1 and B2 SINE transcripts have so far not been successful due to technical reasons. However, it seems unlikely that the increase in SINEs in response to MVM infection is due to decreased degradation. Due to the unique location of each integrated SINE and the lack of an RNA polymerase III termination signal, each SINE transcript has a unique 3′ end, which will influence RNA stability. Any method affecting SINE degradation would have to affect each individual SINE transcript. Furthermore, the decrease

in degradation would have to affect B1 SINEs and B2 SINEs equally and yet not global RNA levels. However, without data supporting an increase in transcription, decreased RNA degradation cannot be ruled out.

One potential method of increasing polymerase III transcription is to increase the basal RNA polymerase III transcription factors. This occurs during HSV infection (Jang and Latchman, 1992), HIV infection (Jang et al., 1992), adenovirus infection (Sinn et al., 1995) (although increasing just the RNA polymerase III transcription factors during adenovirus infection is not sufficient for SINE upregulation (Panning and Smiley, 1995)), and in cells transformed with large T antigen (Larminie et al., 1999). Specifically, transcription factor TFIIIC activity was increased and this was due to increased expression of TFIIIC110 and TFIIIC220 component proteins. These proteins are essential for RNA polymerase III type II promoter activation (Paule and White, 2000; Schramm and Hernandez, 2002) (B1 and B2 SINEs contain this type of promoter). Antibodies obtained from Dr. Robert White and Dr. Arnold Berk were used to measure expression levels of the TFIIIC110 and TFIIIC220 proteins. The level of neither protein was altered as a result of MVMp infection (Fig. 5), suggesting that increasing RNA polymerase transcription factors may not play a role in MVM-altered SINE expression. However, this does not rule out the possibility of altered expression of other TFIIIC or TFIIIB proteins.

Chromatin rearrangement can also mediate increased SINE expression presumably by exposing new SINE promoter sites for transcription and thus releasing SINE transcriptional repression. Increased chromatin accessibility on Alu SINE, alpha satellite DNA, and L1 LINE has recently been demonstrated in heat-shocked HeLa cells (Kim et al., 2001). Increased SINE expression in adenovirus-infected HeLa cells is also thought to occur by increased chromatin re-arrangement (Panning and Smiley, 1995). MVM infection causes DNA damage, which would be expected to cause changes in chromatin structure. This may be the reason for increased SINE numbers during MVM infection and chromatin re-arrangement assay (nuclease digestion of SINE containing chromatin) would be informative.

MVMp nonstructural protein NS1 alone can upregulate B1 and B2 SINEs (Fig. 4). As NS1 is responsible for introducing DNA nicks in both the host and viral genome (Op De Beeck and Caillet-fauquet, 1997), it is tempting to speculate that this function could be responsible for SINE induction (as suggested above). Repeating mouse fibroblast transfection/primer extension experiments with an NS-1 nickase-deficient mutant would be informative. It would also be interesting to determine if MVM structural protein VP1, which contains a phospholipase domain (Zadori et al., 2001), can also induce increased SINE expression. The phospholipase domain could stimulate an inflammatory response that in turn may stimulate SINE expression.

The role(s) of SINEs within cells is not fully understood. It is clear that SINE retroposition is a powerful mutational force. SINE integration can interrupt exons, alter splicing, alter promoter activity, alter or add poly adenylation sites, and can cause sequence duplications and deletions through unequal homologous recombination (Brosius, 1999; Ferrigno et al., 2001; Labuda et al., 1995). Alu SINE integration can cause genetic disorders and cancer in humans with examples including hemophilia, B-cell lymphoma, Tay-Sachs disease, thalassemia, and Lesch-Nyhan syndrome (Labuda et al., 1995). What is not clear, however, is whether SINEs are selfish parasites or if they convey a benefit to the host (for example, in a mechanism similar to adenovirus VAI RNA (Shenk, 2001)). Supporters of a beneficial role for SINEs argue that SINEs are upregulated in a manner similar to the heat shock genes following hyperthermic shock (Li et al., 1999), that the Alu SINE RNA can bind and antagonize PKR activation (Chu et al., 1998), and that the Alu, B1, and B2 SINEs can transiently stimulate translation of reporter constructs in a PKR-independent manner (Rubin et al., 2002). Furthermore, SINEs are found in G/C-rich (gene rich) DNA whereas LINEs are found in A/T-rich (gene poor) DNA (International human genome sequencing consortium, 2001; Mouse genome sequencing consortium, 2002). As SINEs and LINEs are believed to share the same integration mechanism, this suggests that there might be some sort of selective pressure on SINEs to remain in G/C-rich DNA that is absent on the LINEs.

Likewise, the role of SINEs in MVMp infection is also unclear. The increase in B1 and B2 SINEs may simply be a consequence of viral perturbations to the host cell. As such, they could act as indicators of stress within the cell, for example, chromatin re-arrangements or changes in RNA transcription or degradation. Alternatively, SINEs could be playing a protective function within the cell. SINEs could be maintaining protein synthesis by inactivating PKR. However, we are not aware of any reports that MVM infection leads to activation of PKR or of host cell protein synthesis shutdown. SINEs could also potentially bind the major MVM nonstructural protein, NS1, sequestering it away from the viral replication machinery. NS1 may not need to bind directly with SINE RNA as NS1 has been shown to bind cellular proteins that have RNA binding (Harris et al., 1999). Further understanding of the roles SINEs play within the host cell will be helpful in understanding their role in MVM infection.

In summary, through differential display, the murine B1 and B2 SINEs were found to be upregulated in mouse fibroblast cells in response to MVMp infection. These results were confirmed by Northern blot analysis and primer extension analysis. In the latter experiment, both B1 and B2 SINEs were found to increase in a more or less linear function as infection progressed and demonstrated that the SINEs were RNA polymerase III derived. Furthermore, RNase A treatment confirmed that the increase was not due to contaminating DNA. Transient transfection assays demonstrated MVMp NS1 protein alone could also cause an increase in both B1 and B2 SINE levels. Two basal RNA polymerase III transcription factor, TFIII220 and TFIIIC110,

levels were investigated and found not to increase with MVM infection.

Materials and methods

Cells, viruses, and transfections

Adherent LA9 mouse fibroblasts (Littlefield, 1964) were cultured in low glucose DMEM (Gibco BRL) supplemented with 5% fetal bovine serum as described (Brunstein and Astell, 1997).

Purified MVMp (Crawford, 1966) was prepared following a modified protocol of Tattersall et al. (1976). LA9 cells were grown in suspension in high glucose DMEM enriched with nucleotides (Gibco BRL), 5% FBS, 25 mM HEPES in an environment of 5%CO_2, 37 °C to a concentration between approximately 1×10^5 and 5×10^5 cells/ml, diluting as necessary. Cells were infected at low MOI (typically 0.01–0.001 pfu/cell) and monitored for cytopathic effects and cessation of growth. Cells were pelleted, washed with TNE buffer, taken up in low salt buffer (50 mM Tris–Cl pH 8.7, 0.5 mM EDTA, 0.1 M PMSF) and lysed by homogenization (VirTis 45") and sonication (Branson Sonifier 250). Cellular debris was pelleted and discarded. Virus particles were precipitated from the supernatant with 25 mM $CaCl_2$ and resuspended in viral uptake buffer (50 mM Tris–Cl pH 8.7, 20 mM EDTA) with gentle sonication. The virus preparation was purified by centrifugation in a CsCl gradient (SW-41, 28 000 rpm for 20 h) and full virus particles (ρ = 1.41 g/ml) were collected through the side of the tube using a syringe and subsequently dialyzed against TE pH 8.7. Host cell genomic DNA was removed from the virus preparation by addition of micrococcal nuclease (Pharmacia) to 20 μg (800 U)/ml and $CaCl_2$ (to 5 mM), and full virus particles were repurified through another round of CsCl gradient centrifugation and dialysis. MVM virus stock was tittered by plaque assay (Tattersall, 1972).

Dishes (60 mm) of LA9 cells (1.5×10^6 cells) were transfected with various plasmids using lipofectamine plus (Invitrogen) following the manufacturer's instructions using a solution of 8 μg plasmid DNA, 80 μl Plus reagent, and 20 μl of lipofectamine in DMEM lacking FBS. After 3 h, the medium with FBS was added back to the cells and the cells were harvested at the various times indicated.

Plasmids

Large-scale plasmid preparations were carried out by alkaline lysis/polyethylene glycol precipitation (Sambrook et al., 1989). Plasmids to be used in transfection experiments were further purified using a Qiagen plasmid maxi kit as per manufacturer's instructions. Plasmid concentrations were determined by A_{260}.

Three plasmids were used in the transfection studies: pCMVNS1 (Tam and Astell, 1993) contains the 2.3-kb fragment of the MVM genome cloned into the pCMV5 vector and expresses viral proteins NS1 and NS2; pCMV1989 (Naeger et al., 1990) also contains NS1 coding sequence but contains a point mutation in a splice acceptor site such that only the NS1 protein can be expressed; and pCMVβ-gal (Gibco BRL) expresses β-galactosidase.

Differential display

LA9 cells were infected at an MOI of 5 pfu/cell and harvested at 12, 24, and 36 h PI. As a control, LA9 cells were harvested 24 h after mock infection. Cells were directly lysed on the plate by the addition of 1 ml TRIzol reagent (Gibco BRL) per 100-mm dish. RNA was isolated according to the TRIzol procedure. RNA concentration was measured by absorbance at 260 nm in a Pharmacia Ultrospec 3000 spectrophotometer. To remove any residual DNA, RNA samples were further treated with DNase I (GenHunter) and purified by Phenol/Chloroform extraction. Differential display RT-PCR was carried out as described in the GenHunter's RNAimage kit. This was repeated twice for every possible H-T_{11}/H-AP primer combination for a total of 24 primer combinations. This is estimated to represent 15% of the actively transcribed RNA within the cell (Liang and Pardee, 1994). DNA products were amplified in a Perkin-Elmer 2400 Thermocycler. The resulting PCR products were separated in a 6% denaturing polyacrylamide sequencing gel visualized by exposure to Kodak Biomax X-ray film. Only bands that showed a reproducible change in two or more of the infected samples as compared to the control sample were analyzed further. These bands were cut out from the gel and the DNA eluted. The DNA present in the slice was re-amplified by another round of PCR using the same primer set. The PCR conditions were the same except that the radiolabel was omitted and the dNTP concentration was increased to 250 μM. The resulting DNA was purified through a 1.5% agarose gel. The correct size DNA product was cut out of the gel and purified using the MERmaid low molecular weight DNA purification kit (Bio-101) as per the manufacturer's instructions. The resulting DNA was cloned into the plasmid TOPO-TA pCR2.1 (Invitrogen). To compare clones, T ladder sequencing was conducted on inserts of the correct size. Clones that were confirmed to show altered expression patterns were sequenced and the data were used to search the NCBI databases using BLAST (Altschul et al., 1990).

Probe generation and primer extension

Primers against the murine B2 SINE (mouse B2: 5′-TACACTGTAGCTGTCTTCAGACA-3′, nucleotides 155–133), murine B1 SINE (mouse B1: 5′-CTGGC-TGTCCTGGAACTCACTG-3′, nucleotides 110–90), and murine 5S RNA (5S: 5′-AAAGCCTACAGCACCCGG-TATT-3′, nucleotides 120–99) (Liu et al., 1995) were labeled at the 5′ end with γ-^{32}P ATP using T4 polynucleotide kinase

and the DNA precipitated by addition of 95% ethanol, ammonium acetate (to 150 mM) with glycogen as a carrier. Radiolabeled primers were hybridized to 1 μg of total RNA at 60 °C for 5 min and chilled on ice. First strand synthesis was accomplished by the addition of MMLV reverse transcriptase (Gibco BRL), nonradioactive dNTPs, DTT, and buffer, followed by incubation at 37 °C for 45 min. The resulting RNA–DNA hybrids were then denatured by heating at 95 °C for 5 min and separated on a 5% urea polyacrylamide minigel (Triezenberg, 1992). Gels were dried (Biorad model 583 gel drier) and bands imaged and quantified using a phosphorimager. To accurately determine the size of the primer extension products, an aliquot of the labeled DNA was loaded onto a 6% urea polyacrylamide sequencing gel alongside a pUC19 sequencing ladder.

Northerns and Rnase A treatment

Five micrograms of uninfected or MVM infected (36 h postinfection) total LA9 cell RNA per lane was separated through a 5% acrylamide denaturing gel (Biorad) in 1× TBE (90 mM Tris-borate, 0.1 mM EDTA, pH 8.3), transferred to a Hybond N+ (Amersham Phamacia) membrane in 0.5× TBE at 80 V for 1 h, and UV cross-linked to the membrane. SINE sequences were detected by hybridization to a B2 SINE probe (differential display fragment 2A1 amplified and radiolabeled by PCR).

Samples were adjusted to 20 ng/ml Rnase A and incubated at 37 °C for 30 min.

Western analysis

Infected- or mock-infected cell lysates were subjected to SDS-PAGE and blotted onto PVDF membranes as described (Fan et al., 2001).

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