A murine RNA polymerase I promoter inserted into the herpes simplex virus type 1 genome is functional during lytic, but not latent, infection

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The development of herpes simplex virus as a vector for neuronal gene delivery is dependent upon the identification and characterization of promoter elements capable of driving long-term expression during latency. The majority of RNA polymerase II (pol II) promoters studied are active during acute infection but silenced during latency. In order to investigate the potential of a murine RNA polymerase I (pol I) promoter to drive reporter gene expression during lytic and latent infection, we describe the construction and characterization of two recombinant viruses; SC16 LAT neo and SC16 US5 neo. These viruses contain a pol I–encephalomyocarditis virus internal ribosome entry site (EMCV IRES)–neomycin phosphotransferase gene (neo<sup>R</sup>) cassette inserted into the non-essential major latency associated transcript (LAT) and US5 regions respectively. Pol I promoter activity could be detected in the rodent BHK cell line, but not the primate derived Vero cell line – consistent with the known species specificity of such promoters. This activity was specific to a virus containing an active pol I promoter. However, in situ hybridization analyses of latently infected cervical dorsal root ganglia failed to detect pol I mediated transcription of the reporter gene indicating that the murine pol I promoter is silenced following the establishment of latency. Insertion of the pol I–EMCV IRES–neo<sup>R</sup> cassette into the major LAT locus resulted in the production of a hybrid LAT transcript during latency which was translocated to the cytoplasm of latently infected neurones.

Introduction

The ability of herpes simplex virus (HSV) to establish lifelong latent infection in neurones has stimulated a great deal of interest in the development and potential use of HSV-based gene delivery vectors for the expression of foreign genes in the nervous system (reviewed in Glorioso et al., 1995; Efstathiou & Minson, 1995). HSV, as part of its natural life cycle, establishes latent infection of sensory neurones of dorsal root ganglia (DRG) innervating the site of primary infection. Studies using experimental animal models have demonstrated that, during latency, viral DNA persists in a stable, non-linear state in both the peripheral and central nervous system (Rock & Fraser, 1983; Efstathiou et al., 1986) and that there is continuing transcription of only a single region of the viral genome encoding the latency associated transcripts (LATs) (Stevens et al., 1987). There is, however, no evidence of viral protein expression in latently infected cells and expression of LATs is not required for either establishment or maintenance of latent infections (reviewed in Ho, 1992). These transcripts have, however, been shown to have an important role in natural reactivation through an as yet uncharacterized mechanism (Hill et al., 1990).

The ability of replication defective viruses, which are unable to initiate the cascade of gene expression necessary for lytic infection, to establish latency makes HSV an attractive candidate as a potential neuronal gene delivery vector. Attempts to obtain long-term gene expression from promoters recognized by RNA polymerase II (pol II promoters) in HSV vectors has, however, proven problematic (reviewed in Glorioso et al., 1992). The majority of heterologous promoters tested to date have either given transient, high-level gene expression or low-level long-term gene expression in a small proportion of transduced cells (Bloom et al., 1995; Ecob-Prince et al., 1995). The mechanism of promoter silencing in the context of the virus genome during latency is unclear, although
it has been suggested that such silencing may be a consequence of nucleosomal repression (Bloom et al., 1995).

The continued transcription of LATs during latency has resulted in a detailed examination of those regulatory elements which result in long-term stable LAT expression in vivo with the aim of utilizing such elements for long-term gene expression, either singly or in combination with other promoter elements such as the MoMuLV LTR (Lokensgard et al., 1994; Bloom et al., 1995). Such promoter combinations have demonstrated long-term reporter gene expression in peripheral nervous system neurones. It is still unclear, however, whether such promoter constructs will operate efficiently in central nervous system neurones.

In this communication we describe the construction of replication competent recombinants of HSV which contain a murine promoter recognized by RNA polymerase I (pol I promoter) driving expression of the neomycin phosphotransferase (NPT) reporter gene (neoR) in either the non-essential LAT or US5 gene loci. RNA pol I is responsible for the transcription of ribosomal RNAs and is, therefore, a cell type independent, but species specific, promoter (Bell et al., 1990). Thus, we were interested to determine whether such a cell type independent promoter element would facilitate long-term expression when placed in the context of the HSV genome during latent infection of sensory neurones. Our data indicate that a murine RNA pol I promoter facilitates specific reporter gene expression during lytic infection when inserted in either the LAT or US5 gene loci, but is effectively silenced during latent non-productive infection of murine sensory neurones.

Methods

- **Cells and viruses.** Viruses were assayed on baby hamster kidney (BHK)-21 cells and on Vero cells. Cells were grown in Glasgow Modified Eagle’s Medium (GMEM) containing 10% newborn calf serum (NCS) and 10% tryptose phosphate broth (TPB).

  All viruses were made on an HSV-1 strain SC16 background (Hill et al., 1975). Viruses were propagated at a m.o.i. of 0.01 p.f.u. per cell on Vero cells and virus titres determined by suspension assay.

- **One-step growth curves.** Monolayers of BHK and Vero cells were prepared in 6 cm dishes and infected with virus at an m.o.i. of 5–10 p.f.u. per cell. Virus was allowed to adsorb to the cells for 1 h, and then the monolayers were washed with 135 mM-NaCl, 10 mM-KCl, 40 mM-citric acid, pH 3-0 for 1 min to inactivate unadsorbed virus. The monolayers were then washed twice with medium and overlaid with GMEM–10% NCS–10% TPB.

  At each time-point the medium was removed from the monolayers and the cells were resuspended in 1 ml of GMEM and stored at −70 °C prior to assay. The cell suspensions were sonicated for 30 s and virus yield was determined by monolayer plaque assay on Vero cells.

- **Plasmids.** pMENA contains a functional murine RNA pol I promoter, and the encephalomyocarditis virus internal ribosomal entry site (EMCV IRES) linked to neoR. pMENA is a non-functional derivative of pMENA which contains an 11 bp deletion in the murine pol I promoter. These plasmids were obtained from B. McStay (Palmer et al., 1993).

  pSLAT I contains a 4.8 kb PstI–BamHI fragment derived from the HSV-1 SC16 BamHI B region, cloned into pBluescribe M13* (Stratagene). This cloned region represents nucleotides 118867–123460 based on the nucleotide sequence of HSV-1 strain 17 (McGeoch et al., 1991).

  pSLAT 2 contains a 1.6 kb PstI–HpaI fragment derived from the HSV-1 SC16 BamHI B region cloned into pBluescribe M13*. This cloned region represents nucleotides 118867–120301 based on the nucleotide sequence of HSV-1 strain 17 (McGeoch et al., 1991).

  pHD5 is a pBR322 based plasmid designed to allow recombination of foreign sequences into the US5 gene of HSV strain SC16. Its construction has been described previously (Balan et al., 1994).

  pSLAT 1 neo contains a murine pol I–EMCV IRES–neoR cassette cloned in place of the 168 bp HpaI fragment (nucleotides 120301–120469) in pSLAT 1. The murine pol I–EMCV IRES–neoR cassette was excised from pMENA as a 2.8 kb Xhol–NotI fragment. This was end repaired using the DNA polymerase Klenow fragment, and gel purified. This insert was then blunt end cloned into Hpal cut pSLAT 1 using standard protocols. A recombinant plasmid containing the murine pol I–EMCV IRES–neoR insert in the antisense orientation to the LAT transcripts was generated.

  pSLAT 1 Aneo is similar to pSLAT 1 neo, but contains the inactive pol I–EMCV IRES–neoR cassette derived from pMENA (Palmer et al., 1993) cloned into the HpaI deletion of pSLAT 1 in the antisense direction to the LAT transcripts.

  pHD5 neo contains the murine pol I–EMCV IRES–neoR cassette cloned into the EcoRv site of pHDS. The cassette was prepared as described above and blunt end cloned into EcoRv cut pHDS. A recombinant plasmid containing the murine pol I–EMCV IRES–neoR insert in the same orientation as the native US5 gene was generated.

  pSLAT lacZa and lacZb contain a 4.2 kb lacZ cassette derived from the vector pMV10 (Wilkinson & Ackrill, 1992) by HindIII digestion. This cassette was end repaired and inserted into a 168 bp Hpal deletion generated in pSLAT 1. The 4.2 kb lacZ cassette consists of the lacZ gene under control of the human cytomegalovirus (HCMV) IE1 promoter (bases −299 to +69 with respect to the IE1 transcription start site) and terminates on the 3’ side at the HCMV IE1 polyadenylation signal (bases +2757 to +3053). Recombinant plasmids containing the CMV IE–lacZ cassette inserted in the sense (pSLAT 1 lacZa) or antisense (pSLAT 1 lacZb) orientations relative to the direction of LAT transcription were generated.

  pLacZ4 contains a 3.6 kb BamHI fragment containing the lacZ coding sequence from MV10 (Wilkinson & Ackrill, 1992) cloned into pBS (Stratagene) such that the 3’ end of lacZ lies adjacent to the T3 promoter.

- **Construction of recombinant viruses.** The construction of the virus SAUS5lacZ, an HSV-1 SC16 based virus containing the lacZ gene under control of the HCMV IE1 promoter, inserted into the US5 locus, has been described previously (Balan et al., 1994).

  (1) **Construction of viruses SC16 C3b and C3b+.** pSLAT I lacZa or lacZb were linearized by HindIII digestion and 3 μg of this plasmid DNA was cotransfected into Vero cells with 10 μg of high molecular mass HSV-1 strain SC16 infected cell DNA by a CaCl2–DMSO boost method (Stow & Wilkie, 1976). After 3 days of culture infected cell monolayers were harvested, sonicated and recombinant progeny selected by overlaying Vero cell monolayers infected at low multiplicity with 1% agarose in medium containing X-Gal as described by Forrester et al. (1992). β-Galactosidase positive ‘blue’ plaques were picked and subjected to three rounds of plaque purification. Cloned viruses containing the lacZ gene in sense (SC16 C3b+) or antisense (SC16 C3b) orientation relative to the LAT transcription unit were isolated.
(2) Construction of viruses SC16 LAT neo and SC16 LAT Δneo. For the construction of SC16LAT neo and LAT Δneo, SC16 C3b was used as the parental virus. This allowed selection of recombinants by screening transfection progeny for β-galactosidase negative ‘white’ plaques on a ‘blue’ parental background. Co-transfections of sub-confluent monolayers of Vero cells were performed using 10 μg of high molecular mass SC16 C3b-infected cell DNA and 3 μg PvuI linearized pSLAT 1 neo or pSLAT 1 Δneo. Recombinant virus was purified from the transfection progeny by three rounds of picking white plaques under an X-Gal agarose overlay.

(3) Construction of the virus SC16 US5 neo. Co-transfections of Vero cells were carried out using 10 μg of high molecular mass SAUSSlacZ infected cell DNA and 3 μg of PvuI linearized pHD5 neo. White, β-galactosidase negative, progeny virus was plaque purified as described above.

The structures of all recombinant viruses were confirmed by Southern blot hybridization (data not shown).

■ Neomycin phosphotransferase assays. Assays for NPT activity were performed by modifying a standard protocol (Franke & Hruby, 1987). Monolayers of cells were prepared on 5 cm dishes. These were infected at an m.o.i. of 10 p.f.u. per cell. At each time-point after infection, monolayers were washed twice with PBS, then scraped off the dishes and pelleted by centrifugation. Pellets were resuspended in a volume of 0.25 ml, for Vero cells, or 0.5 ml, for BHK cells, in NPT buffer (67 mM-Tris, 42 mM-MgCl₂, 400 mM-NH₄Cl, 1.7 mM-DTT, pH 7.1 with maleic acid), and lysed by sonication for 30 s on ice. Cell debris was pelleted by spinning at 13 000 r.p.m. for 10 min at 4 °C in a microfuge, and aliquots of supernatant were frozen on dry ice. These aliquots were stored at −70 °C prior to assay.

Assays were performed by measuring γ-32P transfer from [γ-32P]ATP to kanamycin sulphate. The assay mix consisted of 10 μl cell extract, 10 μl NPT buffer, 2 μl 1 mg/ml kanamycin sulphate and 10 μl [γ-32P]ATP (10 μCi total γ-32P). The reaction mix was made up on ice, then incubated at 35 °C for 15 min. The reaction was then transferred to ice, and 38 μl ice-cold distilled water added. The mix was boiled for 3 min, and then spun at 13 000 r.p.m. for 2 min; 25 μl of supernatant was then transferred to each of two duplicate 1 cm² Whatman P81 filter papers. These filters were then washed in distilled water at 80 °C for 4 min. After this the filters were washed a further four times in distilled water at room temperature, and then left to dry. Bound radioactivity was measured under Optiphase ‘high-safe’ (Fisons) in a liquid scintillation counter for 1 min.

The protein content of each cell lysate was assayed colorimetrically using the Bradford protein assay (Bio-Rad), and the counts obtained in the NPT assay were divided by the protein content to give a standardized activity.

■ Mouse infections. Female BALB/c mice (Harlan), 5–6 weeks old, were infected with 2 × 10⁶ p.f.u. of either SC16 LAT neo or SC16 US5 neo in a volume of 20 μl GMEM by subcutaneous injection into the pinna of the left ear. At acute (5 days post-infection) and latent (1 month post-infection) time-points, mice were terminally anaesthetized with sodium pentobarbitone, and cervical DRG 2–4 were dissected and pooled prior to processing for in situ hybridization (ISH).

■ Generation of digoxigenin UTP-labelled riboprobes for ISH. Probes to detect LATs were made by T7 polymerase transcription of HinDIII linearized pSLAT2 using digoxigenin (DIG) as a detection system as described previously (Arthur et al., 1993). Plasmid (1 μg) was transcribed using Stratagene T7 polymerase under Stratagene’s recommended conditions. After transcription, the reactions were ethanol precipitated and the product was resuspended in 100 μl 10 mm-Tris pH 8, 1 mm-DTT with RNase inhibitor.

Probes for sense and antisense neoR transcripts were made by transcription from pMENA. EcoRI was used to linearize the plasmid for T3 transcription to make a probe to detect neoR mRNA. The probe for antisense transcripts was made by T7 transcription of BamHI linearized pMENA. Transcription reactions were carried out as described above.

Probes to lacZ transcripts were made by T7 polymerase transcription of Psfl linearized pLaCZ4, using the method described above.

ISH was performed as previously described (Arthur et al., 1993). The pooled ganglia were fixed for 1 h in periodate–lysine–paraformaldehyde at room temperature, then transferred to 50% ethanol and paraffin embedded. Sections (5 μm thick) were collected onto glutaraldehyde activated 3-aminopropyltriethoxysilane coated slides and de-waxed in xylene before use.

The sections were digested with 100 μg/ml proteinase K for 8 min at 37 °C. Overnight hybridization was carried out at 25 °C below the theoretical Tm (72 °C for the LAT specific probe, 62 °C for neoR specific probes and 60 °C for lacZ specific probes); 1–3 μl of DIG labelled riboprobe was used in each 100 μl of hybridization solution. The stringent wash in 0.1 x SSC, 30% formamide, 10 mm-Tris–HCl pH 7.5 was carried out at 10 °C below Tm (75 °C for the LAT specific probe, 65 °C for neoR specific probes and 63 °C for lacZ specific probes) for 30 min. Bound probe was detected with alkaline phosphatase conjugated anti-DIG Fab fragments, as per the manufacturer’s instructions (Boehringer Mannheim).

Results

Construction of recombinant viruses

In order to determine whether a murine RNA pol I promoter would facilitate gene expression in the context of the HSV genome during lytic and latent infection, three recombinant viruses were constructed. SC16 LAT neo contains a cassette consisting of the murine RNA pol I promoter linked to the EMCV IRES driving expression of neoR derived from pMENA (Palmer et al., 1993), inserted in the LAT locus of HSV-1 strain SC16. SC16 LAT Δneo contains an 11 bp deletion of the pol I promoter rendering it inactive. A cassette consisting of this non-functional pol I promoter linked to the EMCV IRES and neoR derived from pαMENA (Palmer et al., 1993) was also cloned into the LAT locus. SC16 US5 neo consists of the pMENA cassette used to construct SC16 LAT neo inserted into the non-essential US5 gene locus. A detailed description of the construction of these viruses is given in Methods and a schematic representation of the genomic structures of these viruses is shown in Fig. 1(b).

Characterization of the in vitro growth properties of recombinant viruses and functionality of RNA pol I promoters

In order to determine functionality and species specificity of RNA pol I activity in the context of the viral genome during lytic infection in tissue culture, we chose to assay promoter activity in either a rodent (baby hamster kidney; BHK) cell line
Fig. 1. Schematic representation of the genome structures of recombinant viruses SC16 C3b, SC16 C3b⁺, SC16 LAT neo, SC16 LAT Δneo, SAUS5 lacZ and SC16 US5 neo. (a) Genomic map of HSV-1 with unique long (UL) and unique short (US) regions each flanked by inverted repeat regions (shown in shaded boxes). (b) Enlargements of the terminal repeats (containing the LAT region) and the region of the US5 gene are shown. H, HpaI restriction enzyme cleavage site. The sites of insertion and orientations of the CMV IE promoter-lacZ gene cassette and the murine RNA pol I promoter-EMCV IRES-neo R cassette are shown for the recombinant viruses SC16 C3b, SC16 C3b⁺, SC16 LAT neo, SC16 LAT Δneo and SC16 US5 neo (see Methods for details of construction).

or the primate (African green monkey) Vero cell line. Working stocks of each of the viruses SC16 LAT neo, SC16 LAT Δneo and SC16 US5 neo generated in Vero cells were assayed on both Vero and BHK cells to determine effective titres for each cell line before determining single-step growth curves. All viruses showed similar kinetics of growth in BHK cells (Fig. 2a). The growth kinetics of SC16 LAT neo and SC16 LAT Δneo were also similar in Vero cells; however, SC16 US5 neo replicated less efficiently in this cell line reaching titres an order of magnitude lower than either the SC16 LAT neo or Δneo viruses at 24 h post-infection, a phenotype which has also been observed with the parent of this construct, SAUS5 LacZ (A. Griffiths, personal communication). Though US5 has previously been defined as a non-essential gene both in vitro and in vivo (Balan et al., 1994) the reduced growth kinetics of SC16 US5 neo in Vero cells may reflect a requirement for this gene product in certain cell types. To assess the activity of the murine pol I promoter, high multiplicity time-course infections were performed in BHK and Vero cells with each recombinant virus. Cell lysates were prepared at various times post-infection up to 24 h, and NPT assays performed. Results from these assays indicate that NPT activity was observed in BHK cells (Fig. 3a) infected with either SC16 LAT neo or SC16 US5 neo. Enzyme activity was first detected 16 h after infection and increased through to 24 h post-infection and was similar for both recombinant viruses. Such late kinetics of expression is considered to be murine pol I promoter specific since SC16 LAT Δneo infection of BHK cells resulted in only approximately 10% of the activity of viruses carrying an intact and functional pol I promoter. In the case of SC16 LAT neo and US5 neo murine pol I promoter activity was not detected following high multiplicity infection of Vero cells (Fig. 3b), reinforcing the view that expression from pol I promoters is species specific.

From these data we conclude that a murine pol I promoter is active when placed at distinct loci within the HSV genome and this activity is observed at late times post-infection.

RNA pol I activity during latent infection of murine DRG

Having demonstrated murine RNA pol I activity in rodent cells in vitro using SC16 LAT neo and SC16 US5 neo, we were interested to determine whether such a promoter would
operate during latent infection of sensory neurones in vivo. BALB/c mice were infected in the left ear by subcutaneous injection with $2 \times 10^6$ p.f.u. of either SC16 LAT neo or SC16 US5 neo. At 5 days post-infection DRG were removed and assessed for transcription of the NPT gene by ISH with a neoR-specific DIG-labelled riboprobe. At the acute stage of infection, specific neoR hybridization could be detected in ganglia (Fig. 4a).

At 1 month post-infection mice which had been infected with SC16 US5 neo were sacrificed and latently infected sensory ganglia probed for LAT-specific transcripts using pSLAT2 DIG-labelled RNA probes (Fig. 4b). This virus established an efficient latent infection with an average of 5.3 LAT-positive neuronal profiles per ganglionic section (1082 LAT-positive neuronal profiles were detected in 203 ganglionic sections). Sections from the same paraffin embedded material were next examined for neoR transcription using a neoR-specific DIG-labelled riboprobe. Of 272 ganglionic sections examined no cells expressing the neoR transcript were detected. Ganglionic sections probed for anti-neoR transcripts were also consistently negative (data not shown). We conclude that despite the fact that SC16 US5 neo established ganglionic latency at high efficiency, as determined by in situ detection of LAT transcripts, no significant activity from an RNA pol I promoter inserted in the US5 locus could be demonstrated. Similarly, we were unable to detect neoR transcripts in sensory ganglia from mice latently infected with SC16 LAT neo (Fig. 4c).

In order to determine the efficiency by which latency had been established in sensory ganglia from animals latently infected with SC16 LAT neo attempts to detect LATs were made. Despite the fact that in this virus the LAT region had been disrupted by insertion of the pol I neoR cassette, LAT-specific transcripts were detected using a LAT-specific probe (Fig. 4d). In contrast to the normal, nuclear localization of LATs which is observed during latency with viruses having an intact intron sequence, transcripts expressed in neurones latently infected with SC16 LAT neo were exclusively cytoplasmic, and were observed at a frequency of 0.12 positive neuronal profiles per ganglionic section (15 positive neuronal profiles from 123 ganglionic sections examined). In order to determine whether these cytoplasmic transcripts comprised a fusion between LAT RNA and antisense neoR RNA, we performed further ISH experiments using a riboprobe specific to antisense neoR transcripts (Fig. 4e). In this case we demonstrated the cytoplasmic localization of antisense neoR in 0.4 neuronal profiles per ganglionic section (22 positive neuronal profiles from 54 ganglionic sections examined). Thus both LAT RNA
Fig. 4. Light micrographs showing detection of neo<sup>R</sup>, LAT and lacZ transcripts in DRG by ISH. The sections have been photographed without counterstaining. (a) Section of DRG removed from a mouse 5 days after infection with SC16 LAT neo. ISH with a pMENA-derived probe specific for neo<sup>R</sup> mRNA detects viral nucleic acid in acutely infected ganglia. (b) Section of DRG removed 48 days after infection with SC16 US5 neo. ISH with a pSLAT2-derived LAT region probe detects nuclear LATs within neurons, demonstrating efficient establishment of latency. (c) Section of DRG removed 33 days after infection with SC16 LAT neo. ISH with a pMENA-derived neo<sup>R</sup>-specific probe failed to detect RNA pol I mediated transcription from latent genomes. (d) Section of DRG removed 33 days after infection with SC16 LAT neo. ISH with a pSLAT2-derived probe demonstrates transcription from the LAT region. These transcripts are cytoplasmic in localization rather than the characteristic nuclear localization of wild-type LATs. (e) Section of DRG removed 33 days after infection with SC16 LAT neo. ISH with a pMENA-derived probe specific for antisense transcripts from the neo<sup>R</sup> gene detects cytoplasmic transcripts. These are thought to represent hybrid transcripts transcribed from the LAT promoter extending through the pMENA insert within the major LAT coding sequence. (f) Section of DRG removed 30 days after infection with C3b<sup>+</sup>. ISH with a pLacZ4-derived probe specific for lacZ transcripts detects cytoplasmic transcripts. ISH with a LAT region probe gives a similar result, indicating that hybrid LAT transcripts have been exported to the cytoplasm.
sequences and antisense neoR RNA have a similar cytoplasmic localization. It seems likely that a hybrid RNA species consisting of both LAT RNA and antisense neoR RNA is being transcribed from the LAT promoter and that this hybrid RNA species is exported to the cytoplasm of latently infected neurones.

We consider it likely that the apparent difference in frequency by which latency is established by SC16 US5 neo (5-3 LAT-POSITIVE neuronal profiles per ganglionic section) and SC16 LAT neo (0-12-0-4 LAT-POSITIVE neuronal profiles per ganglionic section) reflects the relative signal intensities obtained by ISH using the LAT-specific riboprobe pSLAT 2. Thus, in the case of SC16 US5 neo the nuclear LAT signal was intense, reflecting the high stability of this transcript, and development times necessary to obtain such a signal were between 1 and 3 h. In the case of SC16 LAT neo, the intensity of cytoplasmic LAT signal observed after a 4 h development was low and positive cells were rarely observed. The examples of cytoplasmic LAT or antisense neoR detection shown in Fig. 4(d, e) represent sections which, following ISH with the relevant probes, were developed for 12–18 h. The effect on the cellular localization of LAT RNA does not appear to be specific to the pol 1 neoR cassette inserted into the major LAT locus since we have noted a similar distribution of transcripts in neurones of animals latently infected with SC16 C3b+ (Fig. 4(f)). In the case of this virus a cassette consisting of the CMV IE promoter linked to β-galactosidase has been inserted into the same LAT locus as that used for the construction of SC16 LAT neo.

Discussion

Currently, there is a great deal of interest in the use of HSV based vectors for the delivery of genes to both central and peripheral nervous system neurones. This interest stems from the possible use of such vectors in the treatment of both inherited and acquired disease states, and in studies of basic neuronal function. Two major requirements for the generation of useful vectors are that they should be engineered such that they are unable to enter the lytic cycle and that transgene expression during latency should be stably maintained. Although considerable progress has been made in designing replication incompetent and therefore safe vectors, the identification of regulatory elements which are sufficient for long-term gene expression when placed in the context of the virus genome has proved more problematic (Glorioso et al., 1995). To date, all described approaches have investigated gene expression during neuronal latency from pol II promoter/reporter gene constructs.

In this study we have investigated murine pol I promoter activity during lytic and latent infection. RNA polymerase I drives transcription of ribosomal RNA in a species specific tissue independent manner (Palmer et al., 1993). Although RNA pol I promoters have been shown to produce abundant transcription when linked to a variety of open reading frames (Grummt & Skinner, 1985; Smale & Tjian, 1985; Lopata et al., 1986) protein expression is usually inefficient since RNA pol I transcripts do not have a trimethyl G cap at their 5’ terminus (Reeder et al., 1977). In order to overcome the poor utilization of pol I transcripts for efficient protein expression, Palmer et al. (1993) have shown that insertion of an EMCV internal ribosome entry site into the 5’ leader of an RNA pol I transcript efficiently overcomes this block to translation, and suggested that such promoter constructs may be useful for the expression of recombinant proteins in vector systems. In the studies described here, we demonstrate that RNA pol I promoters can be utilized to drive transgene expression in the context of the HSV genome during lytic, but not latent, infection. We have been unable to demonstrate any ongoing transcription from an RNA pol I promoter inserted into either the US5 or LAT loci during HSV latency. This is despite having demonstrated the presence of latent virus genomes expressing LATs in murine DRG. It is possible that there is some neoR transcription but that the message is present at levels below the sensitivity of detection of our in situ protocol. Thus, during latency, although there is continuing rRNA synthesis within the cell, an RNA pol I promoter inserted into the viral genome is apparently not efficiently utilized. During lytic infection HSV is known to cause nucleolar disintegration (Roizman & Sears, 1996). Since RNA pol I is localized within nucleoli such disruption could facilitate the formation of stable pol I promoter complexes on amplified HSV DNA templates. In contrast, during latent infection, no nucleolar disruption is evident and viral template amplification is not thought to occur. One explanation for our failure to observe pol I promoter activity during latency may be a result of an exclusion of HSV DNA templates from nucleoli and/or the inability of unamplified HSV DNA to assemble pol I promoter complexes due to the limitation of one or more factors necessary for stable complex formation (Lopata et al., 1986).

An unexpected observation from our current studies relates to the detection of hybrid LAT transcripts in the cytoplasm of neurones latently infected with SC16 LAT neo. This virus contains the pol 1–IRES–neoR cassette inserted in an antisense orientation within the major LAT intron at a site approximately 1-5 kb downstream of the LAT transcription start site. Our in situ data are consistent with the view that this transcript may represent a hybrid RNA derived from transcription from the latency associated promoter through the LAT region and pMENA derived insert. This putative hybrid transcript is transcribed to the cytoplasm and is detected by both LAT specific and neoR specific in situ probes. It has been noted previously that deletion of the HpaI fragment located at nucleotide positions 120301 and 120469 and insertion of a bacteriophage λ DNA insert of 440 bp results in reduced stability of the major LAT making it undetectable by ISH (Block et al., 1990). Our ability to detect a putative hybrid LAT in the cytoplasm of latently infected cells may be due to an
increased sensitivity of our in situ protocol which utilizes non-
radioactive DIG-labelled probes as compared to radioactive
probes used in the study of Block et al. (1990). In addition, the
size of insert used in the construction of SC16 LAT neo is large
(2·8 kbp) and may conceivably have a more drastic effect on
the processing and transport of LATs. The transcript we detect
appears to be present at lower amounts in the cytoplasm of
latently infected cells than that of wild-type major LATs in the
nucleus since longer incubation times are required for the
detection of hybridized DIG-labelled probes. Thus, our
inability to detect as many latently infected neurones as we
would expect may simply be due to a reduced sensitivity of
detection by our in situ technique. Experiments are currently in
progress to determine the cis-acting signals responsible for the
altered transportation of hybrid LAT transcripts.

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