Characterization of a full-length infectious cDNA clone and a GFP reporter derivative of the oncolytic picornavirus SVV-001

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Seneca Valley virus (SVV-001) is an oncolytic picornavirus with selective tropism for a subset of human cancers with neuroendocrine differentiation. To characterize further the specificity of SVV-001 and its patterns and kinetics of intratumoral spread, bacterial plasmids encoding a cDNA clone of the full-length wild-type virus and a derivative virus expressing GFP were generated. The full-length cDNA of the SVV-001 RNA genome was cloned into a bacterial plasmid under the control of the T7 core promoter sequence to create an infectious cDNA clone, pNTX-09. A GFP reporter virus cDNA clone, pNTX-11, was then generated by cloning a fusion protein of GFP and the 2A protein from foot-and-mouth disease virus immediately following the native SVV-001 2A sequence. Recombinant GFP-expressing reporter virus, SVV–GFP, was rescued from cells transfected with in vitro RNA transcripts from pNTX-11 and propagated in cell culture. The proliferation kinetics of SVV-001 and SVV–GFP were indistinguishable. The SVV–GFP reporter virus was used to determine that a subpopulation of permissive cells is present in small-cell lung cancer cell lines previously thought to lack permissivity to SVV-001. Finally, it was shown that SVV–GFP administered to tumour-bearing animals homes in to and infects tumours whilst having no detectable tropism for normal mouse tissues at 1×1011 viral particles kg⁻¹, a dose equivalent to that administered in ongoing clinical trials. These infectious clones will be of substantial value in further characterizing the biology of this virus and as a backbone for the generation of additional oncolytic derivatives.

INTRODUCTION

Seneca Valley virus (SVV-001) is a therapeutically promising oncolytic picornavirus with selective tropism for cancers with neuroendocrine features including small-cell lung cancer (SCLC) and a number of paediatric solid tumours (Morton et al., 2010; Reddy et al., 2007; Wadhwa et al., 2007). Although SVV-001 is advancing in clinical trials, little is known about the mechanism of tropism of the virus or the basis for the apparent lack of tropism for normal human cells and tissues. Tools for molecular genetics and derivative reporter virus strains have not been reported previously. Such tools would be valuable in better defining many aspects of the biology of this anticancer agent and in focusing its subsequent use in patients most likely to benefit from this therapeutic strategy.

The SVV-001 genome consists of a 7.3 kb positive-sense RNA that is translated into a single polyprotein by the host translation machinery subsequent to infection and uncoating of the virus particle (Hales et al., 2008; Reddy et al., 2007). In vitro RNA transcripts from cDNA templates of positive-sense RNA viruses can be transfected into permissive cell lines to rescue wild-type virus. To this end, infectious cDNA clones have been generated from picornaviruses, most notably poliovirus, for the purpose of genetic manipulation of the virus genome using standard molecular biology techniques (Kaplan et al., 1985; Racaniello & Baltimore, 1981; van der Werf et al., 1986). In the genera Aphthovirus, Cardiovirus, Erbovirus and Senecavirus of the family Picornaviridae, the viral polyprotein is cleaved co-translationally into the substituent capsid domain (P1) and replicative domain (P2) proteins by a conserved ribosome skipping sequence in the 2A peptide (Hales et al., 2008; Luke et al., 2008; Ryan & Drew, 1994). A sequence of the general form DxExNPG↓P in the
C-terminal region of the 2A peptide causes the ribosome to release the nascent peptide chain without ever catalysing a G–P peptide bond and to continue translation in frame (de Felipe et al., 2003, 1999; Donnelly et al., 2001b; Ryan et al., 1999). 2A ribosome skipping sequences have been exploited to create expression systems that produce equimolar quantities of two proteins from a single ORF and are favoured in situations where fusion to the 2A epitope is tolerated and sequence economy is desired (de Felipe et al., 1999; Osborn et al., 2005). We used the 2A protein from foot-and-mouth disease virus (F2A) to insert exogenous protein-coding sequences into the SVV-001 genome while retaining the native coding sequences of all SVV-001 proteins, an approach that has been used to express fluorescent proteins in poxvirus through transposon mutagenesis and in oncolytic adenoviral vectors (Szymczak et al., 2004; Teterina et al., 2010).

The methods reported in this manuscript provide tools for SVV-001 reverse genetics by insertion of exogenous coding sequences and site-specific mutagenesis, and saturating insertional transposon mutagenesis for forward genetic screens.

RESULTS

Cloning of full-length cDNAs and rescue of SVV–GFP

A cDNA encoding the full-length wild-type SVV-001 genome was cloned into the pGEM-4Z expression vector as described in Methods. To generate a recombinant reporter virus expressing GFP, a fusion protein of GFP and the F2A protein was cloned following the SVV-001 2A (S2A) protein in pNTX-09 to yield pNTX-11 according to the scheme detailed in Methods and depicted in Fig. 1. The F2A sequence was chosen over repetition of the S2A sequence to guard against unwanted recombination events between duplicated sequences. During translation of the SVV–GFP polypeptide, the ribosomes skips at TNPG ↓ P of the S2A sequence, continues in frame to produce a GFP–F2A fusion protein with one additional N-terminal proline from SVV-001 2B (S2B), skips a second time at the F2A SNPG ↓ P sequence and continues in frame a second time to translate the remainder of the SVV-001 polypeptide. One clear advantage of this strategy is that all SVV proteins produced retain their native sequence.

pNTX-11 was digested with SvaI and used as a template for in vitro transcription. RNA transcripts were transfected into ten 15 cm dishes of PER.C6 cells. A single GFP-expressing plaque was observed and purified. The low rescue efficiency compared with pNTX-09 suggested that SVV–GFP may contain a compensating point mutation or deletion that allows the virus to tolerate insertion of the GFP-coding sequence.

Plaque-purified and amplified SVV–GFP was used to infect SCLC H446 cells (Fig. 2a). A cytopathic effect (CPE) typical of wild-type SVV-001 infection was observed, as well as bright green fluorescence. Individual infected cells were bright enough to be detected easily over 4 logs by flow cytometry (Fig. 2b) or as plaques of varying sizes by fluorescence scanning (Fig. 2c).

Protein was extracted from H446 cells infected with SVV-001 or SVV–GFP and Western blotted for GFP, the F2A epitope and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Fig. 3). A strong signal for both GFP and F2A was detected at 30 kDa, corresponding to the GFP–F2A fusion protein. Another large band at 50 kDa was detected only in the GFP blot and may correspond to a GFP–F2A–S2B species, resulting from a failure of F2A to skip efficiently. Although the F2A core sequence is sufficient for ribosome skipping, sequence elements up to 30 aa upstream of the 2A/2B cleavage site enhance activity (Donnelly et al., 1997, 2001a). GFP–F2A ribosome skipping is less efficient than other GFP–2A fusions, perhaps due to loss of enhancer sequences found in 1D (Funston et al., 2008; Ibrahimii et al., 2009). No signal was detected in the same molecular mass range when blotting for F2A, suggesting that recognition of the F2A epitope may be lost when the peptide is located internally in a fusion protein.

Characterization of SVV–GFP viability

It is possible that introduction of a foreign fusion gene could adversely affect the replicative potential of SVV–GFP. To determine whether GFP insertion caused any replication defect in SVV-001, single-step growth curves were performed, which demonstrated that SVV–GFP growth rate was typical of that observed with wild-type SVV-001 (Fig. 4).

Whilst there was no apparent defect in the growth rate or spread of SVV–GFP compared with SVV-001, we hypothesized that, in the absence of selective pressure for retention of the SVV–GFP sequence, there was a risk of clonal loss of the introduced sequence from the virus genome. Indeed, we observed variable loss of GFP expression after numerous passages of SVV–GFP in culture without plaque purification. To characterize the mechanism for loss of expression in individual viral subclones, SVV–GFP RNA from infected cells was reverse transcribed and cloned into pNTX-11 for
Sanger sequencing. Several unique clones with common break points were observed in which GFP was lost but some N-terminal portion of the GFP along with the F2A sequence was retained without apparent deleterious effect (Fig. S1, available in JGV Online). No point mutations were observed in the GFP-coding sequence or the SVV genome immediately 5' or 3' of the GFP insertion site.

**Fig. 2.** Detection of GFP expression in infected SCLC H446 cells. (a) Bright-field and epifluorescence images of H446 cells infected with SVV–GFP showing a normal CPE and GFP expression. The percentage of SVV–GFP-positive cells (±SD) is indicated. (b) Bright GFP-positive cells were detected over 4 logs by FACS in SVV–GFP-infected H446 cells (filled histogram) versus uninfected cells (solid line). (c) Plaque assay in H446 cells using SVV–GFP. Robust GFP-expressing plaques were bright enough for detection by fluorescence scanning. Small plaques consisting of only a few cells that were too small to be counted by crystal violet staining could be detected and enumerated using SVV–GFP.

**Fig. 3.** Western blot analysis of H446 cells mock infected, infected with SVV-001 or infected with SVV–GFP to complete CPE with antibodies directed against GFP, F2A and GAPDH. The expected GFP–F2A fusion protein was detected by both GFP and F2A antibodies at ~30 kDa, the expected molecular mass. A second species that may represent a failure of F2A to skip resulting in a GFP–F2A–S2B species was observed only in the GFP blot. This may be due to failure of the F2A antibody to recognize an internal F2A epitope.

**Fig. 4.** Single-step growth curve of SVV-001 (●) vs SVV–GFP (□). There was no apparent significant defect in the production of SVV–GFP compared with the wild-type virus.
Characterization of SVV–GFP tropism in non-permissive SCLC cell lines

We previously characterized the spectrum of tropism of SVV-001 in a diverse panel of cell lines, demonstrating high-level permissivity among about half of all SCLC cell lines tested (Reddy et al., 2007). Recent studies have emphasized the relevance of intratumoral clonal heterogeneity (Gerlinger et al., 2012). Use of a fluorescent reporter virus allowed assessment of whether lines that were seemingly resistant to SVV-001 might contain subpopulations permissive for this virus. A panel of SCLC cell lines previously classified as non-permissive in cytolytic potency assays was infected with SVV–GFP at an m.o.i. of 1 viral particle per cell and observed by epifluorescence microscopy after 24 h. All SCLC cell lines tested were able to support virus infection in a subpopulation of cells ranging from ~1 to 21% of the total cells (Fig. 5). This observation was in contrast to non-malignant tissues and non-SCLC lines A549 and H460, in which SVV–GFP infection could not be detected at any level (data not shown).

In vivo characterization of SVV–GFP tropism

We next sought to explore the tropism, patterns of spread and specificity of the novel SVV–GFP virus in vivo. The permissive SCLC cell line H446 was used to generate subcutaneous xenograft tumours in immunosuppressed (nude) mice. Following intravenous administration of SVV–GFP, strong GFP fluorescence was observed in the H446 tumour xenografts when examined on serial pathological sections (Fig. 6). Spatial clustering of infected cells was observed at 24 h, representing initial points of virus extravasation into the tumour bulk from systemic circulation. GFP expression was limited largely to the xenograft; no GFP expression was observed in mouse organs including brain, heart, lung, kidney and spleen. Very dim GFP fluorescence was detected under high exposure in intracellular vesicles of liver sections and may represent clearance of lysed tumour cell debris. No strong cytosolic GFP fluorescence in liver cells of tumour-bearing animals infected with SVV–GFP was observed, consistent with initial clinical observations; immunohistochemical staining of liver sections from SVV-001-treated patients has not revealed evidence of virus replication in liver cells (Rudin et al., 2011). Representative images of GFP fluorescence and positive immunohistochemical staining observed using polyclonal anti-SVV–001 serum in sequential H446 tumour slides are shown in Fig. S2. No GFP fluorescence or viral staining was evident in tumour or organ tissues in control animals given a PBS injection.

DISCUSSION

In this report, we detailed the construction of a full-length SVV-001 infectious cDNA clone, pNTX-09, and documented its use for the production of a recombinant SVV–GFP

![Bright field GFP images](http://vir.sgmjournals.org)
reporter virus from a second clone, pNTX-11. We characterized the growth kinetics of SVV–GFP in the SCLC cell line H446 compared with wild-type SVV-001 in vitro and demonstrated no evident growth defect from the introduction of the GFP fusion gene. SVV–GFP is itself an important tool for studying SVV-001 biology but also represents an important proof of concept for insertion of foreign coding sequences into replication-competent SVV-001 without altering the sequence of terminally processed viral proteins. Interestingly, an F2A signal was not observed when the F2A epitope was located internally to a GFP–F2A–S2B fusion peptide that was the result of inefficient ribosome skipping, suggesting that the epitope is sensitive to location within the peptide.

Using the SVV–GFP reporter virus, we further characterized SCLC cell lines reported previously to be non-permissive to SVV-001 infection and demonstrated the presence of minority populations of highly susceptible cells in these cell lines. The observed heterogeneity of viral infection in SCLC cell lines would be expected to reflect a similar heterogeneity of SCLC permissivity in patients, and has important therapeutic implications. Localized infection of a subset of tumour cells may be useful in a variety of anticancer strategies, including viral delivery of a toxin to the tumour microenvironment or selective intratumoral pro-drug activation by virus derivatives.

There are a number of possible explanations of the observation of SVV–GFP expression in a minority of non-permissive SCLC cells. The differential permissivity within these lines appeared fixed: even at a high m.o.i., it was not possible to increase the percentage of infected cells in the predominantly refractory cell lines (Reddy et al., 2007). This observation supports a model where differential infection is due to heterogeneity within the culture. SVV–GFP-positive cells may express an attachment molecule or receptor not present in the cells that do not become infected.

Another potential explanation could be differential expression of regulators of innate immunity, with the sensitive subpopulation having lost key antiviral response pathways. Further detailed analyses of the characteristics of permissive and non-permissive subsets of cells from clonal cell lines.
may be of particular utility in defining the determinants of successful oncolytic viral infection.

Finally, we used the SVV–GFP reporter system to infect H446 SCLC tumour-bearing mice. We were able to confirm active targeted infection of the tumour and, importantly, a lack of replication in various non-malignant tissues. Although it has been demonstrated that SVV-001 has a very high therapeutic index in non-transformed cell lines in excess of 10,000 viral particles per cell, it has not been demonstrated until this study that SVV-001 has no detectable tropism for normal mouse organ tissues, even at a dose of \(1 \times 10^{11}\) viral particles kg\(^{-1}\), equal to the highest dose currently used in clinical trials. Our findings are consistent with our previous clinical experience with SVV-001 demonstrating detectable virus replication in liver metastases but not in normal adjacent liver tissue (Rudin et al., 2011).

The infectious clone pNTX-09 and the recombinant reporter virus SVV–GFP will be important tools for forward genetics, for understanding the SVV-001 infection process and for elucidating the mechanism of tropism of this therapeutically promising oncolytic virus.

**METHODS**

**Cells and virus.** For plaque purification, PER.C6 cells (obtained through a license from Crucell) were plated at \(2 \times 10^6\) cells per well in a six-well tissue culture dish. After 48 h, the cells were infected with SVV-001 for 1 h and overlaid with 1% SeaPlaque agarose (Invitrogen). Two days later, clearly isolated plaques were picked or plates scanned on a Typhoon 9410 imager (GE Life Sciences). Plaque-purified virus was amplified in PER.C6 cells. PER.C6 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS (BioWhittaker), 10 mM MgCl\(_2\) (Sigma), 100 U penicillin (Invitrogen) ml\(^{-1}\) and 100 µg streptomycin (Invitrogen) ml\(^{-1}\). The infected cells were harvested when a complete CPE, characterized by rounding and detachment of cells from the dish, was observed and used as a source of the virus. The virus was purified by two rounds of CsCl gradients; a step gradient (CsCl density of 1.24 and 1.4 g ml\(^{-1}\)), followed by continuous gradient centrifugation (CsCl density of 1.33 g ml\(^{-1}\)). The purified virus was dialysed in 11 of cold dialysis buffer [200 mM Tris/HCl (pH 8.0), 50 mM HEPES, 10% glycerol (v/v)] using a Slide-A-Lyser dialysis cassette (Pierce) for a total of 4 h with an hourly change of buffer. Following dialysis, small aliquots of the virus were prepared in sterile 0.5 ml screw-cap tubes and stored at \(8^\circ\)C until use. The concentration of the purified virus was determined spectrophotometrically assuming 1 absorbance unit at 260 nm (\(A_{260}\)) was equivalent to 9.5 \(\times\) \(10^{12}\) particles (Porterfield & Zlotnick, 2010). Virus titres were determined by TCID\(_{50}\) and plaque-forming assays using PER.C6 cells.

**Synthesis and cloning of the full-length SVV-001 genome into a bacterial plasmid.** The genomic RNA of SVV-001 was extracted using TRIzol (Invitrogen). Briefly, 250 µl purified virus (\(\sim 3 \times 10^{12}\) viral particles) was mixed with 3 vols TRIzol and 240 µl chloroform. The RNA present in the aqueous phase was precipitated by adding 200 µl 2-propanol. The resulting RNA pellet was washed with 70% ethanol and suspended in sterile DEPC-treated distilled water. The quantity of RNA extracted was estimated by \(A_{260}\) measurements. To examine the quality of viral genomic RNA, an aliquot of RNA was resolved in a 1.25% denaturing agarose gel (Cambrex Bio Sciences Rockland) and the band visualized by ethidium bromide staining.

Synthesis of SVV-001 cDNA was performed under standard conditions using 1 µg RNA, oligo(dT) and avian myeloblastosis virus reverse transcriptase. Three cDNA fragments representing the full-length SVV-001 genome were amplified by three PCRs employing six sets of SVV-001-specific primers (Table S1). Turbo DNA polymerase (Stratagene) was used in the PCRs. First, a fragment representing the 5’ end of the SVV-001 genome was amplified with primers 5’-SVV-001-A and SVV-0011029RT-R1 and the resulting fragment was cut with ApaI and EcoRI and gel purified. The gel-purified fragment was ligated to Nde-I-SplSVV-001, an annealed oligonucleotide duplex containing an engineered NdeI site at the 5’ end, a T7 core promoter sequence in the middle and the first 17 nt of SVV-001 with an ApaI site at the 3’ end, and cloned into the NdeI and EcoRI sites of pGEM-3Z (Promega) by three-way ligation to generate pNTX-03. Next, a fragment representing the 3’ end of the viral genome was amplified by PCR with primers SVV-00116056 and SVV-0017309NsiI. The reverse primer, SVV-0017309NsiI was used to introduced a poly(A) tail of 30 nt in length and an NdeI recognition sequence at the 3’ end to clone into the Plb site of the pGEM-3Z plasmid. The resulting PCR product was digested with BamHI and gel purified. A fragment covering an internal portion of the viral genome was amplified with primers SVV-001911LI and SVV-0016157R. The resulting PCR product was digested with EcoRI and BamHI and gel purified. The two gel-purified fragments representing the middle and 3’ end of SVV genome were cloned into the EcoRI and Smal sites of pGEM-4Z by three-way ligation to generate pNTX-02.

To generate the full-length SVV-001 cDNA, pNTX-02 was digested with EcoRI and NsiI and the resulting 7.3 kb fragment was gel purified and cloned into the EcoRI and Plb sites of pNTX-03. The resulting full-length plasmid was called pNTX-04. pNTX-04 was further modified at both the 5’ and 3’ ends to facilitate in vitro transcription and rescue of the virus following RNA transfection into PER.C6 cells. First, a SwaI restriction enzyme site was inserted immediately downstream of the poly(A) tail to liberate the 3’ end of SVV-001 cDNA from the plasmid backbone prior to in vitro transcription and to provide a blunt end for termination. A PCR approach was used to insert the site utilizing the primer pair SVV-00161656S and SVV-0013SwaRev and pNTX-04 as a template. The reverse primer SVV-0013SwaRev contained 58 nt representing the 3’ end of the SVV-001 genome and recognition sequences for SwaI and SphI restriction enzyme sites. The resulting PCR fragment was digested with BamHI and SphI and used to replace the corresponding fragment from pNTX-04 to generate pNTX-06. Next, the four extra nucleotides present between the T7 promoter transcription start site and the 5’ end of the SVV-001 cDNA in pNTX-06 were removed using an annealed oligonucleotide duplex approach. The duplex oligonucleotides were engineered to contain a KpnI recognition site, a T7 core promoter sequence and the first 17 nt of SVV-001 with an ApaI site at 3’ end. The annealed oligonucleotides were used to replace the corresponding portion of pNTX-06 using KpnI and Apal sites to generate pNTX-07. Finally, a 2 bp deletion observed in the 3D polymerase-encoding region of pNTX-07 was restored by replacing the BamHI and SphI fragment with a corresponding fragment amplified from SVV-001 cDNA by PCR to generate pNTX-09.

**Insertion of GFP-coding sequences into the full-length SVV-001 plasmid.** To insert a GFP-coding sequence fused to the P2A protein in between the SVV-001 2A and 2B-coding regions, overlap-extension PCR was used. Six primers were designed, each having overlap sequences to amplify three individual PCR fragments. The first PCR fragment (PCR a–b) was amplified using forward primer NI-03 binding upstream of 2A sequences and reverse primer NI-04 with 18 bp of 2A sequence, 3 bp of 2B sequence and 15 bp of the 5’ GFP sequence. A second PCR fragment (PCR c–d) with GFP-coding sequences was amplified with forward primer NI-05 having 9 bp of 2A sequence, 3 bp of 2B sequence and 29 bp of the GFP 5’ sequence and reverse primer (NI-06) with 21 bp of the GFP 3’ sequence and

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48 bp of the F2A sequence. A third PCR fragment (PCR c–f) was amplified with forward primer NI-07 containing 46 bp of F2A sequence and 24 bp of 2B sequence and reverse primer NI-08 binding 615 bp downstream of the SVV-001 2A sequence. PCR fragments a–b and c–d were fused by amplification using primers NI-03 and NI-06 to generate the PCR a–d fragment. Finally, the PCR a–d and e–f fragments were fused by amplification using primers NI-01 and NI-08 to generate the PCR a–f fragment. The PCR a–f fragment was digested with NheI and HindIII and inserted into the corresponding sites in pNTX-09 to generate pNTX-11, an SVV full-length plasmid containing GFP-coding sequences fused to F2A.

In vitro transcription and infectivity of RNA. The infectivity of in vitro-transcribed RNA was tested by first digesting pNTX-09 with Swai to liberate the 3′ end of the SVV-001 sequence from the plasmid backbone. The linearized plasmid was subjected to in vitro transcription using T7 RNA polymerase (Promega). To assess transfection of in vitro-transcribed RNA in SVV-permissive cells, PER.C6 cells were plated in six-well tissue culture dishes. On the following day, Lipofectamine reagent (Invitrogen) was used to transfect in vitro-transcribed RNA (1.5 μg) into the cells following the recommendations of the supplier. A CPE due to virus production was seen by 36 h post-transfection. The transfected cells were subjected to three freeze–thaw cycles, and the presence of virus in lysates was further confirmed by infecting PER.C6 cells.

Tumour cell lines and infection. All tumour cell lines used in the study were cultured in RPMI 1640 supplemented with 10% FBS. Infection efficiency mediated by SVV–GFP was determined by infecting a selected panel of cell lines at an m.o.i. of 1 viral particle per cell. Infected cells were incubated at 37 °C for 24 h to remove any bias introduced by the cell-cycle phase, and the cells were photographed and then analysed by flow cytometry on a FACSCalibur (Becton Dickinson). In vivo studies. Athymic female nude (nu/nu) mice aged 6–7 weeks were purchased from Harlan Sprague–Dawley. Mice were injected subcutaneously with 5 × 10^6 H446 cells in 50% phenol red-free Matrigel (Becton Dickinson Biosciences) into the flank using manual restraint. Tumours were measured in two dimensions, and volumes were calculated using the formula \( \pi/6 \times \text{W} \times \text{L}^2 \), where L is length and W is width of the tumour. When tumour volumes reached the appropriate size, mice were divided randomly into groups. Mice were injected with SVV-001 into the lateral tail vein at a dose of 1 viral particle kg\(^{-1}\) into the cells following the recommendations of the supplier. A CPE due to virus production was seen by 36 h post-infection. The transfected cells were subjected to three freeze–thaw cycles, and the presence of virus in lysates was further confirmed by infecting PER.C6 cells.

For GFP fluorescence, serial sections were cut at 6 μm from fixed tissues. After removal of the paraffin and rehydration, the sections were mounted in Vectashield medium (Vector Laboratories) containing DAPI for DNA staining. Mounted sections were examined by epifluorescence microscopy.

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REFERENCES


