Expression of Semliki Forest virus capsid protein from SV40 recombinant virus

Anu Jalanko

Recombinant DNA Laboratory, University of Helsinki, Valimotie 7, SF-00380 Helsinki 38, Finland

Received 6 March 1985; revised version received 25 April 1985

Here, the proteolytic processing of the Semliki Forest virus (SFV) capsid protein was studied in the absence of other viral functions. Two different fragments of the SFV messenger cDNA, coding for capsid protein and 174 and 38 extra amino acids from the envelope proteins, respectively, were cloned in the late region of the SV40 viral DNA. Cells infected with the SV40 recombinant virus stocks were analysed for the produ-

sis of the immunoprecipitated SFV capsid proteins showed that both recombinants yielded a labelled band equivalent in size to the SFV capsid protein. Thus the proteolytic processing takes place even under conditions where the capsid protein is the only virus-specified protein synthesized.

Semliki Forest virus *SFV capsid* *SV40 recombinant* *Proteolytic processing* *Expression*

1. INTRODUCTION

Semliki Forest virus (SFV) has a single-stranded genome (42 S RNA) of 11454 nucleotides. The genome itself serves as a messenger for 4 non-structural proteins, which are translated as a polyprotein [1,2]. A subgenomic RNA, the 26 S RNA, serves as a messenger for the 4 structural proteins, which are also formed by proteolytic processing of a common precursor. In this latter polyprotein, the hydrophilic capsid protein, which remains in the cytoplasm, is N-terminal followed by the 3 membrane proteins E3′, E2 and E1 in that order [3,4]. The capsid protein is cleaved from the nascent polypeptide. This is a prerequisite for the transport of the membrane proteins to the cell surface [5]. The cleavage of the capsid protein must consequently be fast, efficient and accurate. Aliperti and Schlesinger [6] have suggested that the cleavage is catalyzed by either the capsid protein itself or by a ribosomal protease. The wide host range of the SFV, in vitro translation assays [7] and the existence of a viral mutant with the cleavage blocked under restrictive temperature [8] suggest that the cleavage is a virus-coded function.

The virus replicates in the cytoplasm causing an efficient inhibition of the host cell protein synthesis [1]. The capsid protein may well play a role in this inhibition [9–11]. Pulse-chase studies in SFV infected cells have shown that the newly synthesized capsid protein first associates with the large ribosomal subunit and then with the viral 42 S RNA genome to form nucleocapsids [12].

Here, the expression and cleavage of the capsid from a truncated polyprotein were investigated in the absence of other SFV functions. The cDNA coding for the 5′-end of the SFV 26 S RNA has been positioned in a simian virus (SV40) cloning vector so that it is transcribed intracellularly as a part of the major late SV40 mRNA. Two different 26 S cDNA fragments, both extending beyond the 3′-end of the capsid gene, were transcribed, translated and cleaved into a capsid protein indistinguishable in size from its natural counterpart synthesized during SFV infections.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. MATERIALS AND METHODS

2.1. *Cells and viruses*

SV40 and mixed SV40 recombinant virus stocks (raised after DNA transfection) were cultivated in CV-1 cells as described [13,14].

2.2. *Bacteria and plasmids*

Escherichia coli HB 101 was used as a host for all plasmid propagations. The plasmid pBR322 was used as a bacterial vector in the subclonings. The SFV 26 S cDNA was recovered from a clone in the plasmid pBR322 [15]. SV40 DNA (Boehringer-Mannheim) was cloned into the *Bam*HI site of pBR322 prior to further constructions and the plasmid ptsA58Bam (hybrid of pBR322 and SV40 tsA58 DNA) was kindly provided by Professor Sherman Weissmann. All plasmids were purified as in [16].

2.3. *Construction of recombinant DNA molecules*

The restriction enzyme fragments were made blunt-ended by a fill-in reaction and the linkers were ligated as described [14]. The 26 S cDNA fragments with appropriate linkers were ligated into the plasmid pBR322 and transformed into *E. coli* HB 101. The colonies containing 26 S cDNA were selected by colony hybridization [17]. Plasmid DNAs containing the desired 26 S cDNA fragments were isolated [14] and their *Cla*I/*Bam*HI fragments inserted into the SV40 vector. The SV40 recombinant DNAs were propagated in *E. coli* after ligation to the *Bam*HI site of pBR322. Transfection of recombinant DNAs into CV-1 cells was performed as in [14,18].

2.4. *Analysis of RNA and proteins*

The cytoplasmic RNA was isolated from the recombinant and tsA58 infected CV-1 cells 48 h post-infection and poly(A)-containing RNA molecules were selected by oligo(dT)-cellulose chromatography [19]. Analysis of glyoxalated RNAs on 1% agarose gels, transfer of the RNAs to nitrocellulose filter and filter hybridization with nick-translated 26 S cDNA probe (0.5×10^6 cpm/ml of hybridization buffer) was done essentially as described by Thomas [20].

For immunofluorescence staining the infected cells were fixed with 3% paraformaldehyde and permeabilized with 0.05% Triton X-100. Indirect immunofluorescence staining was used (hamster T-antiserum for SV40 and rabbit anti-SFV capsid serum for the capsid protein) [14]. The recombinant virus stock titers were determined as immunofluorescence units (IFU) for T-antigen.

CV-1 cells (5×10^6) were infected with the recombinant virus and labelled with 50 μCi/ml of [^{35}S]methionine 48 h post-infection in methionine-free modified Eagles medium (MEM) for 5 h. Immunoprecipitation of the solubilized cells with anti-SFV nucleocapsid serum (diluted 1:100) was carried out as described [21]. The samples were then treated with electrophoresis buffer and 5×10^4 cpm of the supernatants were analyzed by SDS gel electrophoresis [22].

3. RESULTS AND DISCUSSION

3.1. *Construction of SV40 recombinants expressing Semliki Forest virus capsid protein*

For expressing the SFV capsid protein in mammalian cells, two different SV40 recombinants, svCI and svCII, were constructed from the 5'-region of the 26 S cDNA.

svCI: The 26 S cDNA was cleaved with *Eco*RI and the 1356 bp fragment containing the whole capsid and E3 genes, as well as 325 bp of the E2 gene, was isolated. *Bam*HI and *Cla*I linkers were ligated together into the fragment that had been made blunt-ended by a fill-in reaction with reverse transcriptase. After subcloning in pBR322, the clone having the *Cla*I linker at the 5'-end and the *Bam*HI linker at the 3'-end of the 26 S cDNA fragment was chosen. The fragment was inserted into SV40 late region between the *Hpa*II and *Bam*HI sites to form the construction svCI (fig.1).

svCII: The 26 S cDNA was cleaved with *Eco*RI, made blunt-ended whereafter a *Hin*dIII linker was added by ligation. This fragment was cleaved with *Xho*I and *Bam*HI linkers were added. The 948 bp fragment containing the whole capsid gene and 115 bp of the E3 gene was subcloned in pBR322. The 26 S fragment was cleaved from the vector with *Cla*I and *Bam*HI and inserted into the SV40 late region between the *Hpa*I and *Bam*HI sites (fig.1). The SV40 recombinant svCII obtained 5 bp of pBR322 sequences (the *Cla*I-*Hin*dIII fragment) in addition to the 26 S specific insert.

Both SV40 recombinants were then ligated to the *Bam*HI site of pBR 322 for DNA production in *E.*

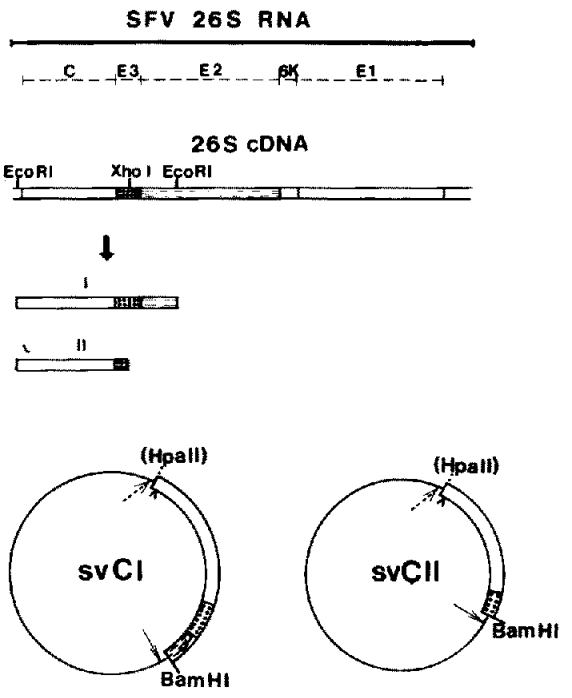

Fig.1. Schematic representation of the construction of the SV40 recombinants svCI and svCII. The Semliki Forest virus (SFV) 26 S cDNA *Eco*RI fragment (I, 1356 bp), with *Cla*I and *Bam*HI linkers, was inserted into the SV40 late region between the *Hpa*II and *Bam*HI sites to obtain the recombinant svCI. The 26 S cDNA *Eco*RI-*Xho*I fragment (II, 948 bp), with the ends altered to *Cla*I and *Bam*HI, respectively, was inserted between the *Hpa*II and *Bam*HI sites of SV40 late region to obtain the construction svCII. The solid black line in the recombinant circles represents SV40 sequences with the early region on the left side, the white bar SFV capsid gene and the small hatched bars E3 and E2 sequences, respectively. The stippled arrow points to the major 5'-end of the SV40 late RNAs, the solid arrow at the SV40 late polyadenylation site, and the arrowhead at the translation initiation codon at the capsid protein.

coli. The DNA constructions svCI and svCII are located in the SV40 late region replacing almost the whole coding sequence. They contain in frame 50 bp of the SV40 coding sequence downstream from the *Bam*HI cloning site. Both svC recombinants use the SV40 late promoter and polyadenylation signals, but lack the splice sites from the SV40 late region.

3.2. *Transfection of the SV40-SFV capsid recombinants into CV-1 cells*

The pBR322-svCI and pBR322-svCII recombinant DNAs were cleaved with *Bam*HI and religated to remove the pBR322 sequences. The DNA was then transfected into CV-1 cells together with the DNA of a SV40 early region mutant tsA58. Recombinant virus was collected 14 days after transfection and passaged 3 times in CV-1 cells to obtain a virus stock of about 5×10^6 IFU/ml.

Following infection with the virus stocks raised from svCI and svCII, about 5% of the cells (corresponding to a titer of 1×10^5 IFU/ml) expressed SFV capsid protein 48–72 h post-infection, as measured by immunofluorescence staining with SFV capsid antiserum. However, the SV40 T-antigen titers were about 5×10^6 IFU/ml for both stocks, suggesting that they contained much more of the tsA mutant than svC recombinant. This was also evident from analyses of the low-M_r DNA supernatants from Hirt extractions [23] by agarose gel electrophoresis.

Most of the DNA was found to be the tsA58 DNA (not shown). The restriction enzyme patterns of the two recombinant virus DNAs showed that the inserted 26 S cDNA fragments had retained the original structure. If the capsid protein actually inhibits host cell protein synthesis, as has been suggested [9–11], the poor production of the recombinant capsid protein is the expected result. Surprisingly, one of the svCII stocks (obtained from an independent transfection) yielded 70% of the infected cells capsid positive (corresponding to 1.4×10^6 IFU/ml). Whether this exceptionally good capsid producer recombinant perhaps contains a mutated capsid gene is an open question at present.

3.3. *Expression of SFV specific RNA*

Cytoplasmic poly(A)-containing mRNA molecules were isolated 48 h after infection of CV-1 cells with the recombinant virus stocks at a multiplicity of infection of 3–5 IFU/cell. Glyoxalated RNAs were separated on 1% agarose gels, transferred to nitrocellulose filter and hybridized with a ^{32}P-labelled pKTH 1003 DNA probe (containing the SFV 26 S cDNA in the pBR322 vector). As shown in fig.2, RNAs of 12.1 kb from svCI and 1.6 kb from svCII could be detected. A faint band at about 6 kb may represent contaminating DNA.

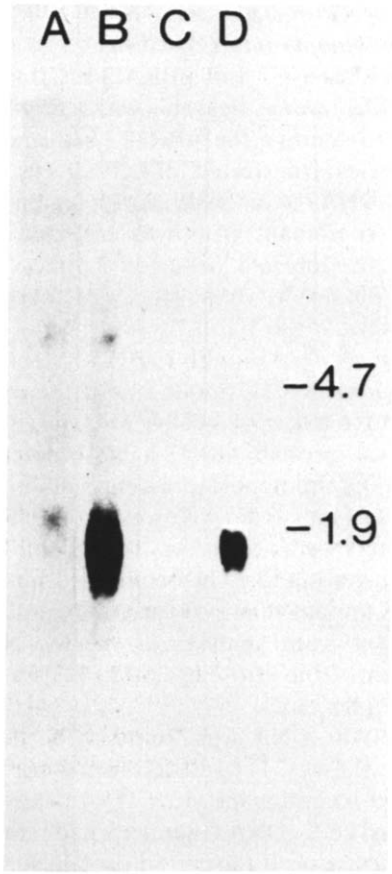

Fig.2. Northern blot analysis of RNA from svCI (A) and svCII (B,D) infected CV-1 cells. Poly(A)-containing cytoplasmic RNA was isolated, treated with glyoxal and electrophoresed on a 1% agarose gel. The RNAs were blotted to nitrocellulose filter and hybridized against nick-translated pKTH 1003 probe (plasmid pkTH 1003 contains the SFV 26 S cDNA in the pBR322 vector). The specific activity of the probe was 3.5×10^8 cpm/μg. (C) Cytoplasmic RNA from SV40 infected cells. The exposure time for lanes A–C was 3 d, D is the same as B but the exposure time was 12 h. The figure also demonstrates the striking difference in expression levels between the svCI stock compared to the efficiently replicating svCII stock.

3.4. *Intracellular location of the recombinant SFV capsid proteins*

Indirect immunofluorescence staining with SFV capsid antiserum was performed 48 h after infection of CV-1 cells with the SV40 recombinant virus. As shown in fig.3, the SFV capsid protein produced was mainly accumulating in the nucleus. In normal SFV infections the capsid protein rapidly forms nucleocapsids with the viral 42 S RNA genome and is not detectable as a free protein [24].

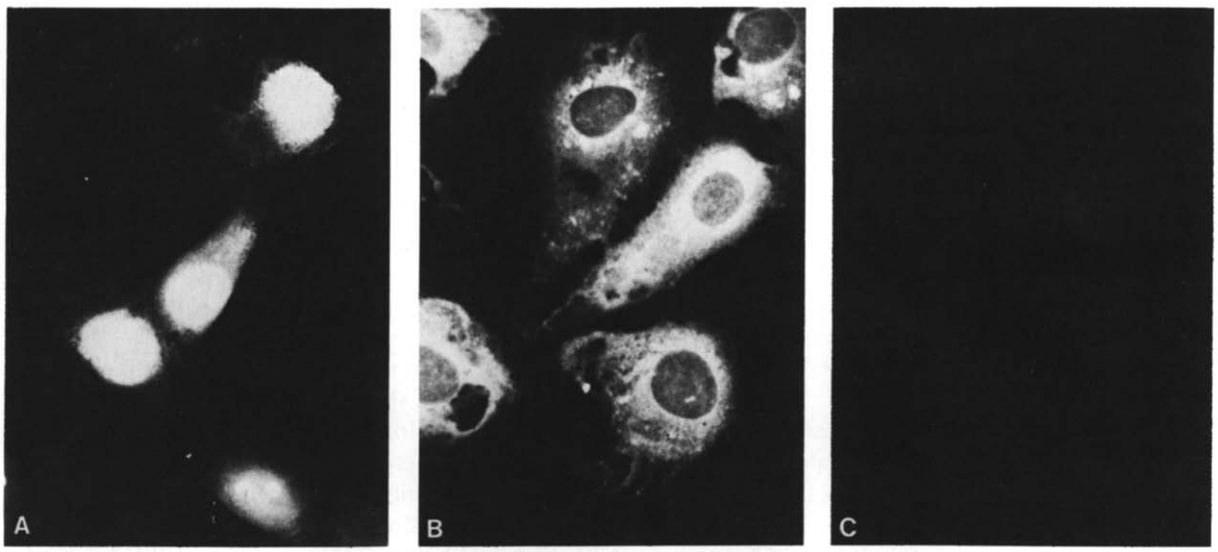

Fig.3. Immunofluorescence staining of CV-1 cells for SFV capsid protein produced by the SV40 recombinants. Indirect immunofluorescence staining for SFV capsid protein was performed 48 h post svCII recombinant virus infection (A) or 7 h post SFV infection (B). Capsid protein produced by the svC recombinant is located in the nucleus, in contrast to SFV infection where the fluorescence can be seen mainly in the cytoplasm. In SV40 infected cells (C) no SFV capsid specific fluorescence can be seen.

Here, where the SFV capsid protein is lacking its normal acceptor, it seems to be transported into the cell nucleus. It has recently been reported that there are defined sequence requirements for nuclear location of proteins [25,26]. SFV capsid protein has many clusters of basic amino acids lysine and arginine, typical for such 'transport signals', and one of these sequences may form a structural feature that allows the capsid protein to be transported into the nucleus. The biological significance of this finding is unclear at present.

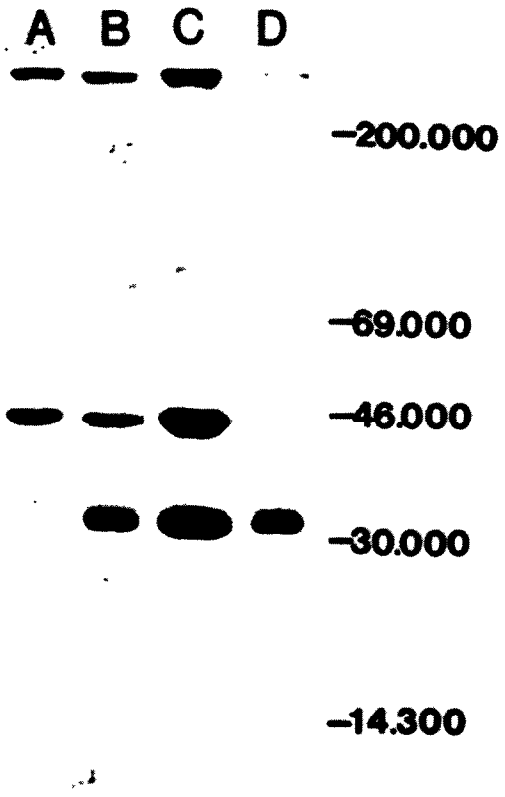

Fig.4. Analysis of SFV capsid proteins produced by the SV40 recombinants. CV-1 cells were infected with the recombinant virus and labelled with [^{35}S]methionine 48 h post-infection. The SFV capsid proteins were immunoprecipitated from the cell lysate with specific SFV capsid antiserum and electrophoresed on polyacrylamide gradient gels. Both capsid recombinants svCI (B) and svCII (C) yielded a 30 kDa protein corresponding to the immunoprecipitated SFV capsid protein (D). SV40 infected cells were used as a negative control (A).

3.5. *Processing of the recombinant SFV capsid protein*

CV-1 cells infected with the svC recombinants were analyzed for the sizes of the produced capsid polypeptides. SDS-polyacrylamide gel electrophoresis of the [^{35}S]methionine-labelled and immunoprecipitated capsid proteins showed that both recombinants yielded a labelled band of M_r 30000 equivalent in size to the SFV capsid protein (fig.4). A protein, which also can be seen in lane A representing the SV40 wild type-infected cells, is apparently nonspecifically bound to the antibody or to protein A-Sepharose. The SV40 control showed no band comigrating with the SFV capsid protein. If proteolytic processing of the recombinant gene products did not occur, the expected sizes of the fusion proteins would have been approx. 52 kDa (svCI) and 36 kDa (svCII). Products of these sizes would clearly have been separated from the 30 kDa band on the gels.

It has been previously suggested, based on in vitro translation studies with ts mutants [8], that the proteolytic cleavage of the capsid protein precursor may not be a host function. Here, in the absence of other SFV functions the capsid protein is still cleaved.

ACKNOWLEDGEMENTS

I wish to thank Mrs Ritva Rajala for excellent technical assistance and Drs Hans Söderlund, Leevi Kääräinen and Ismo Ulmanen for valuable discussions and reading of the manuscript.

REFERENCES

[1] Kääriäinen, L. and Söderlund, H. (1978) Curr. Top. Microbiol. Immunol. 82, 15–69.
[2] Strauss, E.G. and Strauss, J.H. (1983) Curr. Top. Microbiol. Immunol. 105, 1–98.
[3] Strauss, J.H. and Strauss, E.G. (1977) in: The Molecular Biology of Animal Viruses (Nayak, D.P. ed.) pp.111–116, Marcel Dekker, New York.
[4] Clegg, C. and Kennedy, J. (1975) Eur. J. Biochem. 53, 175–183.
[5] Garoff, H., Simons, K. and Dobberstein, B. (1978) J. Mol. Biol. 124, 587–600.
[6] Aliperti, G. and Schlesinger, M.J. (1978) Virology 90, 366–369.
[7] Glanville, N. and Ulmanen, I. (1976) Biochem. Biophys. Res. Commun. 71, 393–399.