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A primer vector system that allows temperature dependent gene amplification and expression in mammalian cells: regulation of the influenza virus NS1 gene expression

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ABSTRACT

Influenza virus RNA segment 8 has been cloned into primer-vector pSLts1. This vector was designed to replicate in simian cells in a temperature dependent fashion by use of the SV40 tsA209 T-antigen gene. The oriented synthesis of cDNA on dT-tailed pSLts1 was performed on in vitro synthesized mRNA, and the second DNA strand was primed with an influenza-specific terminal oligodeoxynucleotide. Recombinant pSLVa232 contained the RNA segment 8 sequence directly fused to the SV40 late promoter contained in pSLts1, and followed by the SV40 polyadenylation signal. Expression of NS1 gene in transfected COS cells took place at a level comparable to that found in infected cells. When VERO cell cultures were transfected with recombinant pSLVa232, expression of the NS1 gene was temperature dependent. Close to one hundred fold increase in the amplification and expression of the cloned gene was observed after shift down of the transfected cells to permissive temperature. Vector pSLts1 and the cloning strategy described may be useful for the specific cloning and regulated expression of mRNAs of known 5'-terminal sequence.

INTRODUCTION

The possibility to prepare gene-specific probes via cDNA synthesis has been an important step for the structural analysis and expression of eukaryotic genes (1). In the case of viruses that contain RNA, cDNA synthesis is a mandatory step in the cloning of their genes. The availability of cDNA clones has led to a detailed understanding of the structure (2-5) variation (6,7) and some functional aspects of these viral genes (8-10).

The classical methods for cDNA synthesis and cloning (11) give rise to low yields of cloned containing full-length cDNAs, probably as a consequence of the trimming of 5'-terminal sequences by the S1 nuclease treatment of the DNA and the lack of any selection against the cloning of truncated copies. Alternative

methods have been recently published (12-15) that overcome such difficulties by using primer-vector systems for cDNA synthesis and cloning. Our laboratory is interested in the expression of influenza virus genes in a functional form to further develop the in vitro system for influenza virus RNA synthesis recently described (16). Here, we present the use of a simplified version of the Okayama and Berg technique on a new primer-vector, pSLts1, designed to replicate in a temperature-dependent fashion in simian cells, by the incorporation of the tsA209 T-antigen gene (17). The expression of the genes cloned under the late SV40 promoter in pSLts1 is regulated by the amplification of the recombinant upon temperature shift-down, in a similar way as in ts-COS cells (18, A. Portela, unpublished results), and also by the stimulation of the late promoter in the presence of a functional T-antigen (19,21). As an example of the results attainable with this system, we report the cloning of influenza virus segment 8 and the regulated expression of NS1 protein in simian cells.

MATERIALS AND METHODS

Viruses and cells

The A/Victoria/3/75 (H3N2) strain (clone Va3) of influenza virus was used for all experiments. Cell lines MDCK and VERO were obtained from the American Type Culture Collection. The COS-1 cell line (22) was a gift of Y. Gluzman. Culture of these cell lines was as described (23). The E. coli strains MC1061 (24) and HB101 (25) were used for cloning experiments.

Enzymes and reagents

The origin of most of the enzymes and reagents has been described (26,16). Anti-NS1 antiserum was kindly provided by P. Palese and J. Young. Anti-T antigen antisera were gifts of R. Carroll and A. Smith. Plasmid pRGM18 was provided by R.G. Martin. Influenza virus mRNA was synthesized in vitro and purified by oligo-dT cellulose as described (16).

Construction of plasmid pSLts1

The construction of pSLts1 plasmid is shown squematically in Fig. 1. Aliquots (10 µg) of pBR322 and pRGM18 DNAs were digested with AccI and Bam HI restriction nucleases, respectively. Digested DNAs were treated with 5 u of S1 nuclease for 1 h at 37ºC in

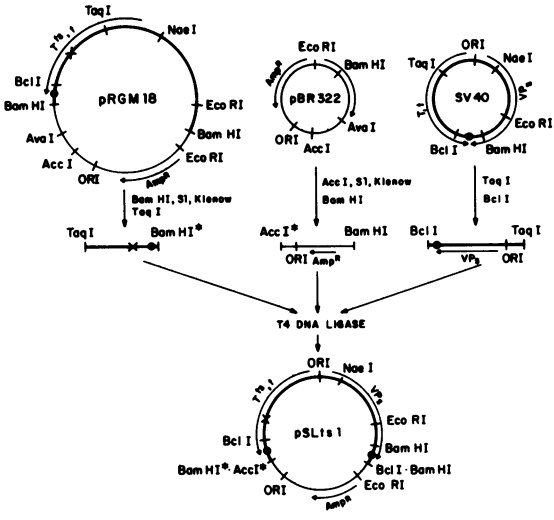

Fig. 1. Construction of vector pSLts1. The indicated DNA segments, isolated from pBR322, SV40 DNA or pRGM18, were ligated and the clone pSLts1 was characterized by restriction analysis as described in Materials and Methods. The heavy line denotes SV40 sequences and the thin line indicates pBR322 sequences. The black dots show the position of the SV40 polyadenylation signals and the cross indicates the approximate position of the tsA209 mutation. Asteriscs denote restriction sites that were lost during manipulation of DNA.

35 mM Na AcO, pH 4.6-0.2 M NaCl-1 mM $ZnSO_4$, and filled in with the Klenow fragment of E. coli DNA polymerase I for 2 h at room temperature in a reaction mixture containing 50 mM Tris.HCl, pH 7.5-50 mM KCl-10 mM $MgCl_2$-100 µM each dATP, dCTP and dTTP and 1 mM DTT. Then, the DNAs were digested with BamHI and TaqI nucleases, respectively. SV40 DNA (10 µg) was digested with both TaqI and BclI nucleases. The appropriate fragments were isolated and ligated in a reaction containing 50 mM Tris-HCl, pH 7.8-10 mM $MgCl_2$-20 mM DTT-1 mM ATP and 10^4 µ/ml T4 DNA ligase by incubation for 36 h at 4ºC. The ligation product was used to transform competent E. coli MC1061 cells and the transformed colonies were selected on ampicillin containing LB plates. The transformed colonies were tested by in situ hybridization (27) using as probe the SV40 DNA fragment described, labelled in vitro (28). The structure of one of the plasmids obtained, pSLts1, was confirmed

by restriction analysis and is shown in Fig. 1.

Cloning of influenza virus RNA segment 8

1) Elongation of pSLts1. Plasmid pSLts1 (10 µg) was digested with BamHI restriction endonuclease, filled in with E. coli DNA polymerase I (Klenow fragment) as above and tailed with dTTP in a reaction containing, in a volume of 66 µl, 100 mM Na cacodilate, pH 7.2-2.5 mM $CoCl_2$-1 mM dTTP-50 µg/ml gelatin and 30 u of terminal transferase. The reaction mixture was incubated for 30 min at 37ºC and the tailed DNA was purified by phenol extraction, chromatography on Sephadex G50.80 column and ethanol precipitation. Finally, it was digested with Nae I endonuclease and purified as above.

2) cDNA synthesis. The synthesis of cDNA was performed in a 40 µl reaction mixture containing 100 mM Tris-HCl, pH 8.3-10 mM $MgCl_2$-20 mM KCl-0.1 mM EDTA-1 mM DTT-0.8 mM each dGTP, dTTP and dCTP-0.4 mM α {^{32}P} dATP (760 cpm/pmol)-10 µg/ml actinomycin D-1 u/µl RNasin-1500 ng dT-tailed pSLts1-250 ng of in vitro synthesized mRNA and 6 u of reverse transcriptase. After incubation for 30 min at 42ºC, the reaction mixture was extracted with phenol and the product was filtered through a Sephadex G50.80 column and recovered by ethanol precipitation. The hybrid was resuspended in 10 µl of 10 mM Tris-HCl, pH 7.5-1 mM EDTA and incubated with 0.5 µg of RNase A for 10 min at 37ºC. To synthesize the second DNA strand, 1 µg of kinased AGCAAAAGCAGG oligodeoxynucleotide was added, the reaction mixture heated 10 min at 60ºC and completed to 50 µl with the following final composition: 100 mM Tris-HCl, pH 7.5-10 mM $MgCl_2$-50 mM KCl-1 mM DTT-15 µM each dGTP, dATP and dCTP -7.5 µM {^{3}H} dTTP (26,000 cpm/pmol) and 5 u of E. coli DNA polymerase I (Klenow fragment). After incubation for 3 h at 16ºC the reaction mixture was extracted with phenol and filtered through a Sepharose 4BCL column equilibrated with 10 mM Tris-HCl, pH 7.5-100 mM NaCl-1 mM EDTA and the DNA was recovered by ethanol precipitation.

Ligation was carried out for 24 h at 4ºC in a mixture containing 50 mM Tris-HCl, pH 7.5-10 mM $MgCl_2$-10 mM DTT-1 mM spermidine 1 mM ATP-50 µg/ml gelatin-1 µg/ml DNA and 10^4 u/ml T4 DNA ligase. The ligation product was used to transform competent E. coli MC1061 cells and plated on ampicillin containing LB plates. The

transformed colonies were screened as described (29) using as probe α ^{32}P-UTP labelled, in vitro synthesized influenza virus RNA (16).

Conditions for restriction and DNA sequence analysis were as described (26,30-32).

Transfection

Transfection of VERO or COS-1 cells was performed at 37ºC by the DEAE-dextran method (33). After transfection, cells were kept at 37ºC for 4 h in the presence of 100 µM chloroquine (34). Episomal replication of recombinant plasmids in transfected cells was assayed as described (35).

Analysis of RNA

Cytoplasmic RNA was isolated as described (36) and was further purified by two steps of oligo-dT cellulose chromatography. Messenger RNA was analyzed by electrophoresis on formaldehyde-agarose gels (37), transfer to nitrocellulose sheets (38) and hybridization to an in vitro labelled (28) NS1-specific probe.

Protein analysis

Cell cultures were labelled for 3 h with ^{35}S-methionine. Labelled extracts were used for solid-phase immunobinding (39) using anti-NS1 serum. The selected material was analyzed by SDS-polyacrylamide gel electrophoresis (40).

For indirect immunofluorescence, cells were fixed with cold acetone-methanol (3:1), incubated with anti-NS1 or anti-T antigen serum for 30 min at 23ºC and washed with PBS. A 1:50 dilution of fluorescein-conjugated goat anti-rabbit IgG serum was then added, the preparations were incubated for 30 min at 23ºC and washed with PBS.

RESULTS

Construction of vector pSLts1

To carry out direct cloning experiments aimed at the regulated expression of genes in mammalian cells, we constructed a pBR322-SV40 hybrid vector (Fig. 1). One of the SV40 DNA fragments used (coordenates 4739-2770) contained the origin-promoter regulatory sequences, the complete late region including the polyadenylation signal and part of the early viral region. The other SV40 DNA fragment (coordinates 2533-4739) was isolated from plas-

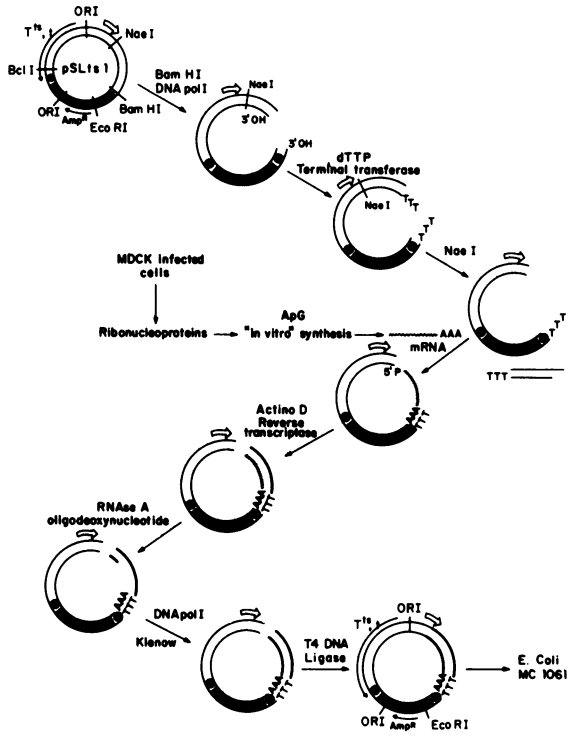

Fig. 2. Process for oriented cDNA synthesis and cloning in pSLts1 vector. The open regions indicate SV40 sequences and the filled ones show pBR322 sequences. The black dots show the location of the SV40 polyadenylation signals and the arrow denotes the origin and direction of transcription from the SV40 late promoter. The heavy line represents the in vitro synthesized cDNA.

mid pRGM18, that contains SV40 tsA209 DNA cloned into pBR322, and included the portion of the early region not present in the SV40 DNA fragment described above, with the tsA209 mutation and the polyadenylation signal. A third DNA fragment, obtained from pBR322, contained the plasmid origin of replication and the ampicillin resistance gene. Upon ligation the three DNA fragments, several recombinant plasmids were obtained, one of which, pSLts1, was characterized in detail (Fig. 1). It contains an unique Nae I site close to the late SV40 promoter and an unique BamHI site next to one of the two polyadenylation signals. The vector does not contain the toxic pBR322 sequences (41) and replicates in simian cells in a temperature dependent fashion (see below).

Oriented, specific cloning of influenza virus mRNA on pSLts1

Plasmid pSLts1 was used to clone influenza virus mRNA synthesized *in vitro* (Fig. 2). The BamHI site close to the SV40 polyadenylation signal was filled in an tailed with dTTP. The tailed DNA was cut at the unique NaeI site, generating two fragments, the largest of which is suited as a primer-vector for cDNA cloning, and the mixture of both was directly used to prime the synthesis of cDNA. Since the *in vitro* synthesized mRNA preparation also contains cellular and *in vivo* synthesized viral mRNAs, the cDNA product would reflect the heterogeneity of such template. The synthesis of the second DNA strand was primed with the dodecadeoxynucleotide AGCAAAAGCAGG, complementary to the 3'-end of all influenza virus RNAs (42) to specifically generate a library of full-length influenza cDNAs. This procedure should generate blunt-ended structures at both DNA termini of the synthetic recombinant molecules since the mRNA produced *in vitro* was primed with ApG. The ligation of these recombinants at low DNA concentration to generate circular DNAs constitutes a new selective step, since full-length recombinants are better substrates for T4 DNA ligase than incomplete or uncopied ones.

Characterization of recombinant pSLVa232

When the collection of colonies generated by the cloning of

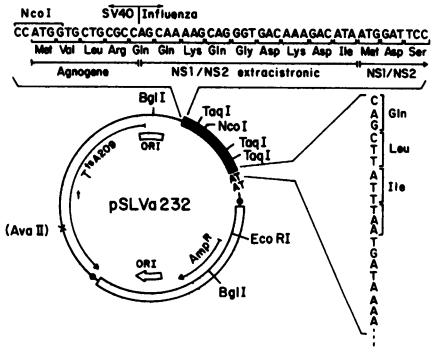

Fig. 3. Structure of the recombinant pSLVa232. Recombinant pSLVa232 was characterized by restriction analysis and sequence of the 5' and 3'-terminal regions of the RNA segment 8,including the fusions to the pSLts1 vector, as described in Materials and Methods. The absent AvaII site, shown in brackets, indicate the position of the ts A209 mutation.

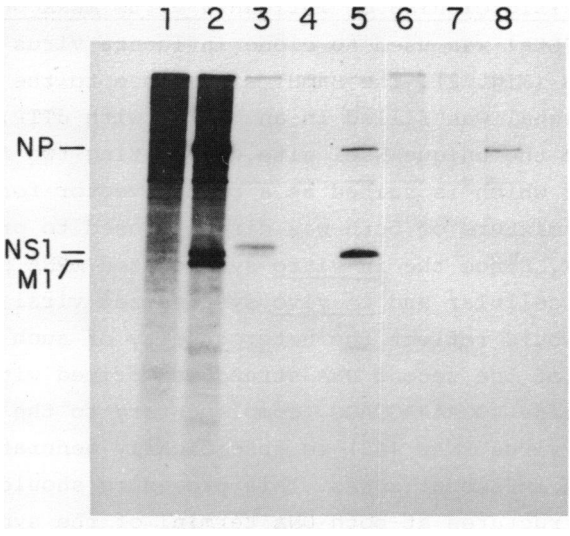

Fig. 4. Synthesis of NS1 protein in COS-1 cells transfected with recombinant pSLVa232. COS-1 cell cultures were infected with A/Victoria/3/75 virus, transfected with recombinant pSLVa232 or mock-transfected as described in Materials and Methods. At 72 hours post-transfection or 4 hours post infection, cells were pulse-labelled for 3 hours with ^{35}S-methionine and whole cell extracts were prepared. Solid-phase immunobinding was performed using either anti-NS1 or control antisera and bound protein was analyzed by SDS-polyacrylamide gel electrophoresis. 1,2. Whole cell extracts from mock-transfected or infected cells. 3,4,5. NS-1 specific material from transfected, mock-transfected or infected cells. 6,7,8. Material selected by control antiserum from transfected, mock-transfected or infected cells.

synthetic DNA obtained from 50 ng of mRNA was screened with *in vitro* labelled influenza virus RNA (16), 13 positive clones were obtained, among which two showed a restriction pattern compatible with having a complete segment 8 cDNA insert. One of such clones, pSLVa232, was further characterized by restriction analysis and sequence determination of the fusion regions. The results, shown in Fig. 3, indicate that clone pSLVa232 contains influenza virus segment 8 properly oriented and directly fused to the late SV40 promoter. The sequence of the fusion region between the SV40 promoter and the 5'-terminus of the segment 8 shows the presence of two in phase initiation codons, one corresponding to the SV40 agnogene (43) and the other to NS1/NS2 influenza proteins. Immediately after the segment 8 extracistronic

region, the recombinant contains a poly dA:dT region of about 100 nucleotides, consequence of the cloning strategy used.

Transient expression of influenza virus NS1 protein from recombinant pSLVa232

The expression of NS1 protein from recombinant pSLVa232 was first assayed after transfection into COS 1 cells. Seventy two hours after transfection or 4 hours after viral infection, cultures were labelled with ^{35}S-methionine, total cell extracts were prepared and used in a solid-phase immunobinding assay that included anti-NS1 antibodies. The bound material was analyzed by electrophoresis and the results are shown in Figure 4. Extracts from cells transfected with recombinant pSLVa232 contained NS1 related material (Fig. 4, slot 3) with an apparent molecular weight 27,000, and a minor band with the same mobility as authentic NS1 protein found in infected cells (Fig. 4, slot 5). These proteins were specific of recombinant pSLVa232 since they were not found in extracts of mock-transfected cells (Fig. 4,slot 4). They were also absent when the immunobinding was performed with control antisera (Fig. 4, slots 6-8).

The intracellular location of the NS1 protein was determined by indirect immunofluorescence with anti-NS1 antiserum. Cultures were either infected or transfected as described above, fixed and processed for immunofluorescence. Transfected cells showed a pattern of nuclear immunofluorescence indistinguishable of that found in infected cells (Fig. 5). These results are in agreement with those previously reported (44).

All together, these data indicate that recombinant pSLVa232 contains the complete genetic information for influenza virus NS1 gene and that vector pSLts1 and the cloning strategy used are suited for the expression of this protein in transfected cells at levels comparable to those found in infected cells.

The expression of influenza virus NS1 from pSLVa232 can be regulated by temperature

Vector pSLts1 was designed to replicate in a temperature dependent fashion by the presence of the tsA209 mutation in the T-antigen gene. To study whether recombinant pSLVa232 showed a ts phenotype for replication, VERO cell cultures were transfected with either pSLVa232 DNA or pSEK1 DNA (a recombinant plasmid

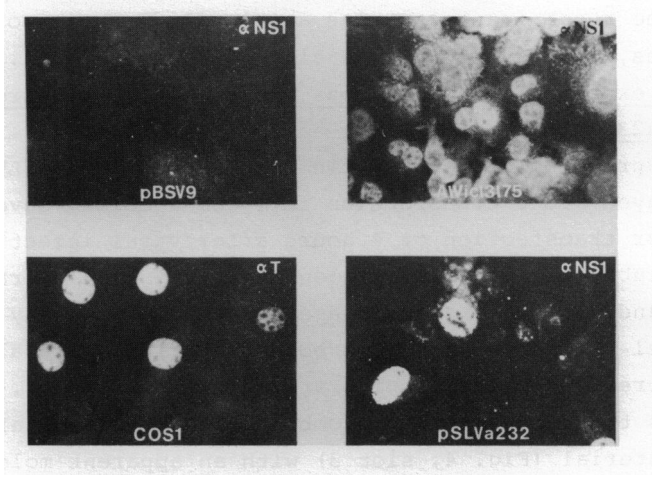

Fig. 5. Intracellular location of NS1 protein in infected and transfected cells. Cultures of COS-1 cells were infected with virus, transfected with recombinant pSLVa232 or mock-transfected with vector pBSV9 as indicated in Figure 4 and processed for immunofluorescence with anti-NS1 or anti T-antigen serum as described in Materials and Methods.

that contains the origin of replication and the early region of wt SV40 DNA) and incubated at either 33.5 or 40.5ºC. Low molecular weight DNA was isolated at several times post-transfection and plasmid replication was monitored by the appearance of form I DNA. As shown in Fig. 6A, accumulation of newly synthesized pSLVa232 DNA occurred at 33.5ºC, but not at 40.5ºC, whereas pSEK 1 DNA replicated more efficiently at 40.5ºC than at 33.5ºC. Furthermore, temperature shift-down of cultures transfected with pSLVa232 DNA and incubated initially at 40.5ºC for 48 hours gave rise to sharp increase in the amount of pSLVa232 DNA. The quantitation of these results is shown in Fig. 6B and indicates that pSLVa232 DNA is present in concentrations about 100 fold higher at 33.5 than at 40.5ºC.

The temperature dependence of the accumulation of NS1 mRNA was studied by transfection of VERO cells cultures with recombinant pSLVa232 and incubation at both permissive and restrictive temperatures. As shown in Fig. 7A, the accumulation of NS-1 specific mRNA is apparent in the cultures incubated at 33.5ºC, but not detectable at 40.5ºC. Temperature shift-down of transfected

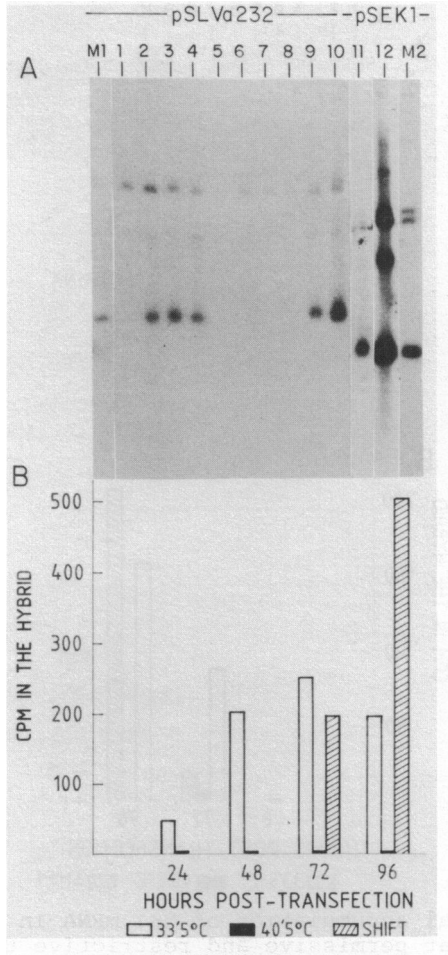

Fig. 6. Regulation of pSLVa232 amplification by temperature. A. Cultures of VERO cells were transfected with pSLVa232 or pSEK1 DNA and low molecular weight DNA was isolated at various times after transfection. After agarose gel electrophoresis and transfer to nitrocellulose filters, DNA was hybridized to a pBR322 labelled probe as described in Materials and Methods. 1-4. Low molecular weight DNA isolated from pSLVa232 transfected cultures incubated at 33.5ºC for 24,48,72 or 96 hours. 5-8. Low molecular weight DNA isolated from pSLVa232 transfected cultures incubated at 40.5ºC for 24,48,72 or 96 hours. 9-10. Low molecular weight DNA isolated from pSLVa232 transfected cultures incubated for 48 hours at 40.5ºC and then at 33.5ºC for 24 or 48 hours. 11-12. Low molecular weight DNA isolated from pSEK1 transfected cultures, incubated for 48 hours at 33.5ºC or 40.5ºC respectively. M1 and M2 purified pSLVa232 and pSEK1 DNAs directly applied to the gel. B. For quantitation of the results shown above, the pieces of nitrocellulose filter containing the form I pSLVa232 DNA were cut and counted in a toluene based scintillator.

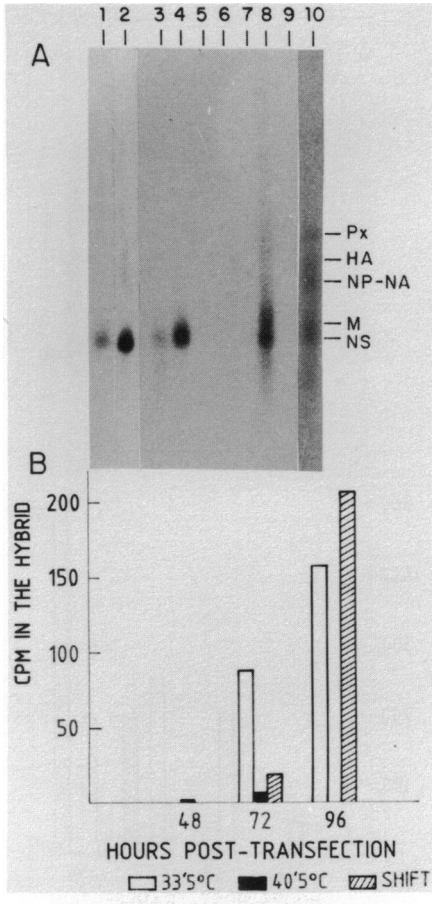

Fig. 7. Differential accumulation of NS1 mRNA in pSLVa232 transfected VERO cells at permissive and restrictive temperatures. A. VERO cell cultures were infected, transfected or mock-transfected as described in Figure 4. At various times of incubation at 33.5 or 40.5ºC cytoplasmic RNA was isolated, purified by oligo dT-cellulose chromatography and analyzed by blot-hybridization with a NS1-specific labelled probe after electrophoresis on agarose gel containing formaldehyde. 1. Messenger RNA isolated from infected cells. 2. Messenger RNA synthesized in vitro. 3,4. Messenger RNA isolated from cells transfected with pSLVa232 incubated at 33.5ºC for 72 or 96 hours (2 µg). 5,6. Messenger RNA isolated from cells transfected with pSLVa232 and incubated at 40.5ºC for 48 or 72 hours (6 µg). 7,8. Messenger RNA isolated from cells transfected with pSLVa232 and incubated for 48 h at 40.5ºC and then at 33.5ºC for 24 or 48 hours (2 µg). 9. Messenger RNA isolated from mock-transfected cells. 10. Messenger RNA synthesized in vitro (not hybridized). B. Quantitation of the results shown in A. was performed by cutting the regions of the nitrocellulose filter containing the hybrids and counting in a toluene based scintillator.

Table 1. Temperature dependent accumulation of NS1 protein in VERO cells transfected with recombinant pSLVa232

Cell line	Temperature ºC	Time after transfection(h)	Fluorescence intensity using antisera[a] Anti-NS1	Anti-T antigen
VERO	37	mock-transfected	N.D.[b]	N.D.
VERO	33.5	48	N.D.	7,8 ± 4,2(16,5)
VERO	33.5	72	17,7 ± 6,6 (182,3)	9,2 ± 8,9(19,3)
VERO	33.5	96	26,2 ± 7,4 (296,6)	20,5 ± 12,0(43,3)
VERO	40.5	48	N.D.	40,9 + 12,1(86,5)
VERO	40.5	72	N.D.	30,1 ± 15,4(63,4)
VERO	40.5-33.5	48/24	2,9 ± 8,7 (30,0)	36,9 ± 16,0(78,0)
VERO	40.5-33.5	48/48	23,4 ±10,5 (241,1)	52,5 ± 22,5(110,3)
VERO	37	6[c]	9,7 ± 2,5 (100,0)	N.T.
COS-1	37	mock-transfected	N.T.[d]	46,6 ± 21,5(100,0)
COS-1	33.5	72	16,6 ± 9,5 (170,7)	N.T.
COS-1	40.5	72	15,6 ± 6,4 (160,2)	N.T.

[a] Cell cultures were processed for immunofluorescence in parallel and fluorescence determination was performed in a Zeiss photomicroscope III equipped with a photometer head 03. Average fluorescence values for negative zones in each preparation were subtrated from the values obtained for each fluorescent nucleus. Average values were calculated on determinations of 20 fluorescent nuclei. The figures in parenthesis are the percentages calculated over the values of VERO infected cells (for anti-NS1 fluorescence) or mock-transfected COS-1 cells (for anti-T antigen fluorescence).
[b] No fluorescence detected over background when more than 1000 cells were screened.
[c] Hours after infection with A/Victoria/3/75 virus.
[d] Not tested.

cultures 48 hours after incubation at 40.5ºC resulted in the accumulation of NS1-specific mRNA to a level about 50 fold higher at 33.5ºC than at 40.5ºC (Fig. 7B). This preferential accumulation of NS1 mRNA at 33.5ºC was not due to its rapid turnover at 40.5ºC since the half-life of NS1 mRNA at 33.5ºC was only two times higher than that at 40ºC (see also Table 1 below).

The modulation of NS1 protein expression by temperature was studied by immunofluorescence. VERO cell cultures were transfected with recombinant pSLVa232, incubated at 33.5, 37 or 40.5ºC, fixed and processed for immunofluorescence using either anti-T or anti-NS1 sera. As shown in Fig. 8 and Table 1, cell cultures expressed T-antigen at comparable levels at every temperature tested, but NS1 protein was detected in cultures at 33.5 or 37ºC, but not at 40.5ºC. The determination of the relative fluorescence intensity in cells transfected or infected is shown in Table 1. The expression of NS1 protein in COS-1 cells was not temperature dependent, again suggesting that regulation is not due to differences in stability of NS1 protein or NS1 mRNA at restrictive and permissive temperatures, but associated to the presence of a ts T-antigen gene. The level of expression of NS1 protein

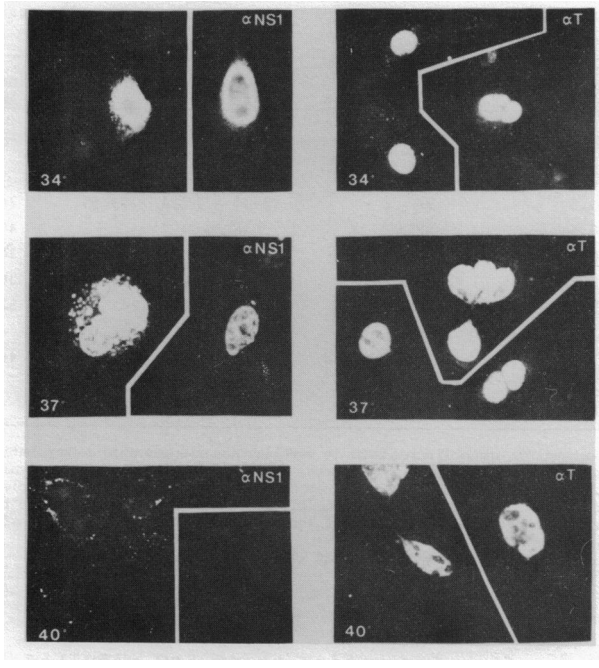

Fig. 8. Regulation of NS1 protein accumulation in pSLVa232 transfected VERO cells. Cultures of VERO cells were transfected with pSLVa232 and incubated at 33.5, 37 or 40.5ºC. At 72 hours post-transfection the cultures were fixed and processed for immunofluorescence, using either anti-T antigen or anti-NS1 serum as shown in the Figure.

in transfected VERO cells at permissive temperature was 2-3 times higher than in infected cells, whereas at restrictive temperature it was not possible to detect cells positive for immunofluorescence with anti-NS1 antiserum.

DISCUSSION

Plasmids for the modulable expression of cloned genes, such as pSLVa232, should provide a tool to specifically incorporate a gene into the cell chromosome and regulate the concentration of its gene product. Several biological problems would be rendered amenable to analysis by such constructs. Genetic variability of RNA viruses is a quantitatively prominent phenomenon (6,7,45) with largely unexplored molecular basis. *In vitro* systems for the synthesis of influenza virus RNA (16,46), in conjunction

with the regulable expression system described here, will allow gaining information on the viral RNA replication mechanism and its accuracy. One of the viral products that could be involved in RNA replication is NS1 protein. It accumulates in the nucleus of infected cells early after infection (47) and ts mutants in the NS1 cistron seem to be affected in late viral gene expression (A. Ishihama, personal communication). Transcription of NS1 and NS2-specific mRNAs and expression of NS1 protein in mammalian cells have been obtained by the use of SV40 defective recombinant viruses(44,48). Our purpose is to obtain high level production of influenza virus gene products in permissive cells, and hence a regulable expression system is highly desirable. The regulated expression of cloned genes in mammalian cells has been reported by the use of the metallothionein promoter, that allows a 10-12 fold induction by metal ions (49-51) and the MMTV LTR (52,53). Regulation in both expression systems operates at the transcription level (54-56). To attemp the regulated expression of influenza virus NS1 gene, we have used vector pSLts1 (Fig. 1), in a cloning procedure similar to that reported for vector pBSV-9 (57)(Fig. 2). The structure of pSLVa232 recombinant (Fig. 3) reveals the presence of two in phase initiation codons in the putative composite mRNAs that are transcribed from the major late SV40 cap sites (Fig. 7). From the size of these mRNAs, it can be suggested that they are polyadenylated either at the encoded dA:dT tract (Fig. 3) or at the SV40 signal. The NS1-specific translation products in COS-1 transfected cells show mobilities in polyacrylamide gel electrophoresis compatible with the use of both initiation codons (Fig. 4), suggesting the synthesis of agnogene-NS1 fusion protein as well as authentic NS1 protein.

The modulation of the expression of cloned genes by vector pSLts1 was studied at the level of gene amplification, transcription and translation. Upon shift-down from 40.5 to 33.5ºC, rapid gene amplification was observed, paralleled by an accumulation of NS-1 specific mRNA and NS1 protein (Figs. 6 to 8 and Table 1). The increase of NS1 mRNA concentration after temperature shift-down would be the result of two processes : gene amplification (Fig. 6) and stimulation of the late SV40 promoter by functional T-antigen (19-21). The differential expression levels obtained

with vector pSLts1 at permissive versus restrictive temperatures (50-100 fold) compare favourably with the regulation obtained with other systems like metallothionein or MMTV LTR promoters. Formally, regulation by vector pSLts1 may be considered as a combination of that obtained by runaway plasmids of E. coli (58) and by λ pL promoter in the presence of a ts λ repressor gene (59, 60), although the detailed regulation mechanisms are different. In addition to the regulation during the transient expression of the NS1 gene, it is conceivable to obtain simian cells stably transformed with the recombinant pSLVa232 at restrictive temperature, and induce a temperature dependent excision of the integrated replicon (61,62), leading to high level expression of the cloned gene. Using recombinant pSLVa232, we have recently obtained transformed cells that stably maintain the NS1 gene at restrictive temperature. These cells do not show detectable NS1 protein expression at restrictive temperature, but could be induced by temperature shift-down to express NS1 protein at levels comparable to those found in transfected cells (A. Portela, to be published).

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