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Simian virus 40 as a vector: recombinant viruses expressing individual polyoma T antigens

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Abstract

We constructed simian virus 40 (SV40)/polyomavirus recombinants by replacing in SV40 the T antigen coding region with polyoma early region sequences, either cDNAs encoding small, middle or large T antigen or the wild-type sequence coding all three proteins. The recombinants maintained the SV40 late region and origin of replication and were propagated in COS cells yielding recombinant virus preparations with titers of 10^6–10^7 infectious particles per milliliter. These viruses were characterized in productive infections of COS cells by analyzing early and late mRNA levels and by following synthesis of polyoma early proteins. In the absence of viral DNA replication, i.e. in infected monkey or mouse cells, expression of the polyoma T antigens was weak. Further experiments indicated that this was mostly due to high genomic instability during amplification, to lower levels of cDNA transcripts as compared to spliced mRNA, and possibly also to lower infectivity of the recombinant virions. It remains to be determined, whether these handicaps are unique to SV40/polyoma recombinants or whether SV40 is in general inadequate as a viral vector.

Keywords: Simian virus 40; Polyomavirus; T antigens; Viral vector; Recombination

1. Introduction

Simian virus 40 (SV40) sequences have been among the first to be used for the construction of mammalian vectors to express heterologous proteins (Berg, 1981; Gething and Sambrook, 1981). Plasmids containing a functional SV40 origin of replication can be amplified in COS cells, monkey cell lines expressing constitutively SV40 early proteins (Gluzman, 1981). Therefore, SV40 recombinant virions (Sambrook and Gething, 1988) might be useful to deliver efficiently and smoothly foreign genes to animal cells which are difficult to transfect. However, to our knowledge this tool has been used only occasionally (Settleman and DiMaio, 1988; Strayer and Milano, 1996) and SV40 recombinant viruses have not been described in detail.

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We have been interested for some time in the roles of the polyoma early proteins for the induction of the mitotic response in quiescent cells. Today the essential functions of large (LT), middle (MT) and small (ST) T antigens are known. LT, a nuclear phosphoprotein, is required for viral DNA replication (Fried and Prives, 1986) and is the main effector for the mitogenic activity of these viruses (Weil, 1978; Türler, 1980; Gioerup et al., 1994). MT, a membrane-bound cytoplasmic protein, induces tumors in newborn hamsters and transforms some established cell lines by interacting with cytoplasmic tyrosine kinases (Kaplan et al., 1989) and SH2-domain containing proteins (Dilworth et al., 1994; Dilworth, 1995). ST is a cofactor for the induction of S-phase and progeny virus production, possibly through its interaction with phosphatase 2A (Pallas et al., 1990; Sontag et al., 1993). However, a number of studies revealed that for most biological activities of polyomavirus cooperation of two or all three T antigens is necessary. This has been shown for S-phase induction (Türler and Salomon, 1985; Templeton et al., 1986; Ogris et al., 1992), viral DNA replication (Berger and Wintersberger, 1986), transformation (Land et al., 1983; Cuzin, 1984; Noda et al., 1987) and tumor induction (Asselin et al., 1984).

To evaluate the contributions of individual T antigens for mitotic stimulation under conditions resembling virus infection, and because quiescent cells are particularly difficult to transfect, we decided to use SV40 as a vector and to construct recombinant viruses expressing polyoma T antigens under the control of the SV40 early promoter. We report here the characterization of such recombinants in productive infections of COS cells and infections of quiescent baby mouse kidney cells. Unfortunately, in absence of viral DNA replication expression of polyoma T antigens was so weak that the recombinants could not be used for the envisaged purpose. When we compared infections and transfections by recombinant virus or viral DNA to SV40, we realized how severely genomic stability and gene expression was affected by exchanging the early region of these two closely related viruses.

2. Materials and methods

2.1. Cells, virus, plasmids

COS-1D cells (ATCC CRL 1650; Gluzman, 1981) were provided by P. Beard (ISREC, Lausanne) and cultured in medium containing 5% foetal bovine serum (FBS). Cultures of monkey CV-1 cells and primary cultures of mouse kidney cells had been described previously (Türler and Beard, 1985; Türler and Salomon, 1985). Murine polyomavirus strain A2 originated from B.E. Griffin (Soeda et al., 1980), the cDNA variant coding LT (Zhu et al., 1984) was a gift by R. Kamen. The bp numbering of polyomavirus A2 and of SV40 is according to the GenBank sequences (Buckler and Salzman, 1986). Complete genomes of polyomavirus A2, its LT cDNA variant and SV40 were cloned into the *Bam*HI site of pAT153 or pUC8. The polyoma cDNA variants coding MT (Treisman et al., 1981) or ST (Zhu et al., 1984) cloned in pAT153 were given to us by E. Wintersberger. Plasmids of the pUC series were purchased from Pharmacia, pBS+ from Stratagene.

2.2. Constructions

Using plasmids of the pUC series and current methods of molecular cloning (Sambrook et al., 1989) we constructed first a plasmid, pSVOL, with SV40 bp 5171 (*Hin*dIII) to 2774 (*Bcl*I) comprising the origin of replication, the promoter/enhancer region, the complete coding region for the capsid proteins and early and late polyadenylation sites. The SV40 sequence was flanked by *Sal*I sites and also contained a unique *Xba*I site for insertion of the polyoma early region (Fig. 1). From cloned polyomavirus wild-type DNA and from the cDNA variants coding LT, MT or ST we isolated the complete early region bp 96 (*Bgl*I) to 2966 (*Hin*cII). These sequences were made blunt ended and cloned into the *Sma*I site of pUC12. From plasmids with the appropriate orientation bp 96-2484 were isolated by *Xba*I digestion and inserted into pSVOL. The orientation of the polyoma sequence relative to that of SV40 was tested by digestion with *Kpn*I. Excision of the constructs

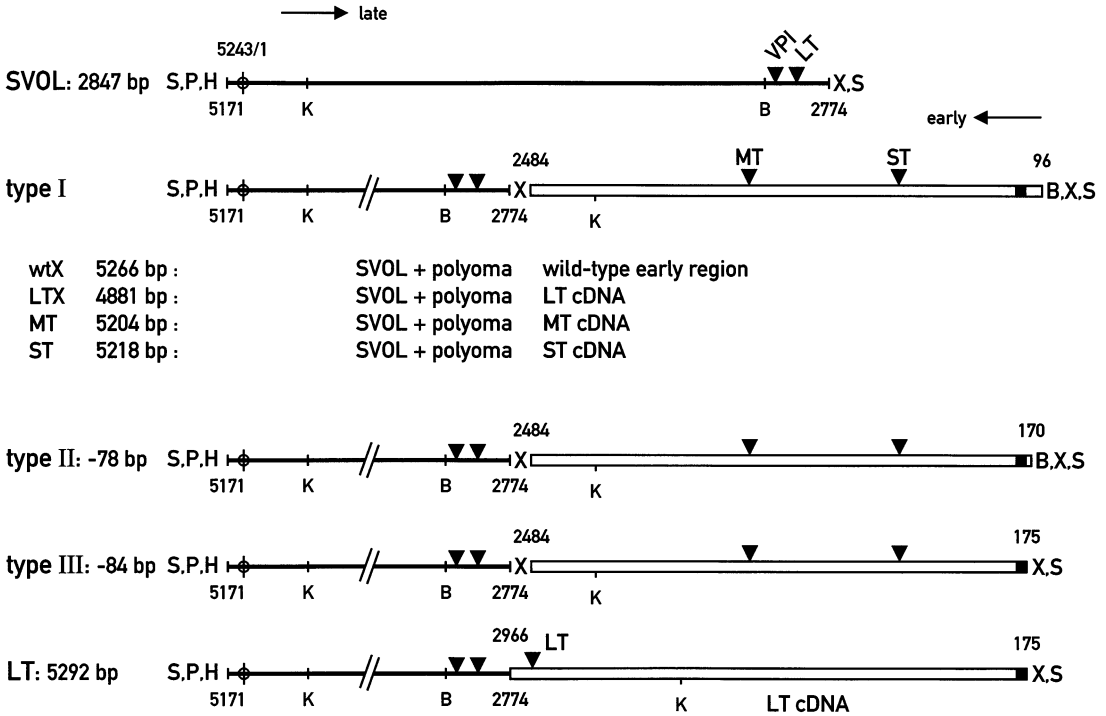

Fig. 1. Schematic representation of SV40/polyoma constructs (see Material and Methods). Only viral sequences are indicated: SV40 as single line, polyoma as double line. Nucleotide numbers are according to Buckler and Salzman (1986), for SV40 below the line, for polyoma above the double line. The maps represent type I, II and III wtX constructs. For LTX, MT and ST recombinants the wild-type early region was replaced by LT, MT and ST cDNAs, respectively. LT virus has LT cDNA of the entire early region. The wtX and LTX constructs code a truncated polyoma LT fusion protein composed of aa 1–641 of polyoma LT, glutamic acid and C-terminal aa 682–708 of SV40 LT. Landmarks: (ϕ) SV40 origin of replication; (■) translation initiation for polyoma T antigens bp 175; (▼) stop codons: SV40 VP1 2591, SV40 LT 2693; polyoma ST 808, MT 1500, LT 2915. Restriction enzyme sites: B: *Bam*HI; H: *Hin*dIII; K: *Kpn*I; P: *Pst*I; S: *Sal*I; X: *Xba*I.

with *Sal*I and circularization yielded hybrid viral DNA of type I (wtX, LTX, MT and ST, Fig. 1).

To remove the upstream, non-coding polyoma sequence (bp 96-174) type I subclones with polyoma bp 96-1565 (*Eco*RI site) were digested with *Bam*HI and *Bst*XI (bp 167–178). The *Bam*HI 5′-protruding and the *Bst*XI 3′-protruding ends were religated in the presence of synthetic pGATCCATC. Sequence analysis of the few plasmids obtained revealed two different sequences maintaining the translation start codon (nt 175) which differed by 6 bp and presence of a *Bam*HI site. With these sequences hybrid virus wtX, LTX, MT and ST of type II and type III were assembled (Fig. 1). For construction of LT virus, coding complete LT protein, we joined the *Sal*I-*Sac*I (bp 1375) fragment of LTX III with the *Sac*I (bp 1375)-*Hin*cII (bp 2966) fragment of polyoma wild-type DNA. After excision with *Hin*cII this sequence was inserted into the *Xba*I site of pSVOL made blunt ended (Fig. 1).

2.3. Transfections

COS cells seeded at 10^5 cells/ml medium containing 5% FBS reached 70–90% confluence the next day. Transfections were done with 5–7 μg DNA and 500 μg DEAE-Dextran (Pharmacia) per ml Tris-buffered saline (TBS; Banerji et al., 1983). Of this mixture 0.6, 0.3 and 0.1 ml were used for 85 mm, 50 mm and coverslip (22×22 mm) cultures, respectively. After 1 h incubation at

room temperature the solution was removed and the cultures exposed to 25% dimethylsulfoxide in TBS for 2 min. The cells were carefully washed twice with TBS and then incubated in medium containing 1% FBS.

CV-1 cells were either transfected by the same procedure or by lipofection with DOTAP (Boehringer Mannheim) according to the manufacturers protocol. After lipofection cultures were incubated in medium with 1% FBS and 0.1 mM chloroquine for 6 h and thereafter in medium with 1% FBS.

2.4. Preparation of hybrid virus stocks

After excision and self-ligation the circular hybrid viral DNA was isolated by electrophoresis in agarose gels. COS cells were transfected with this DNA as described above and incubated for 3 days. Then, the medium was removed and used for infection of another culture of COS cells. After 3 days this procedure was repeated. The remainder of the first and second supernatants was saved for later amplifications. The third infected culture was incubated for about 1 week until it showed signs of lytic infection. The lysates were collected, frozen and thawed three times and then sonicated. After the removal of cell debris by low speed centrifugation, the supernatants were saved and tested. From good preparations larger virus stocks were prepared in 850 cm^2 plastic bottle roller cultures. Virus was concentrated by sedimentation at 80000 g for 3 h at 4°C. The virus pellets were resuspended in 10 ml medium and sterilized by filtration through a 0.2-μm disposable filter unit. After an addition of 0.5 ml FBS the hybrid viruses were stored at -20°C.

2.5. Other methods

For virus infections, extraction of viral DNA and agarose gel electrophoresis see Türler and Beard (1985). Immunofluorescent detection of polyoma early proteins was done by exposing fixed cultures on coverslips to hybridoma supernatants containing rat monoclonal antibodies αPy C4 recognizing LT, MT and ST or αPy LT1 recognizing LT (Dilworth and Griffin, 1982); the second antibody was fluorescein-conjugated goat anti rat IgG ('ImmunoPure', Pierce). Coverslips were mounted with 20% polyvinylalcohol in 50 mM Tris-phosphate pH 9 (Lenette, 1978). SV40 capsids containing cells were revealed with a rabbit anti SV40 capsid serum and rhodamine-conjugated donkey anti rabbit IgG (affinity purified, Bio-Science Products).

For electrophoretic analysis of polyoma T antigens cultures were labeled with 150 μCi ^{35}S-methionine (Expres ^{35}S, >1000 Ci/mmol, DuPont-NEN) in 2 ml methionine-free medium for 2 h. Polyoma T antigens were isolated by immunoaffinity chromatography using hamster anti polyoma T serum and protein A-Sepharose (Pharmacia) (Rey-Bellet and Türler, 1984).

To isolate cytoplasmic RNA cells were lysed as described by Buetti (1974), the nuclei were sedimented and RNA was extracted from the supernatant according to Favaloro et al. (1980). The RNA preparations were treated with 40 units RNase free DNase I (Boehringer Mannheim) per milliliter solution for 30 min at 37°C. To determine mRNA steady state levels aliquots containing 10 μg of RNA were subjected to electrophoresis in horizontal 1% agarose-formaldehyde gels in MOPS buffer, then transferred electrophoretically to GeneScreen membranes (DuPont-NEN) and crosslinked by UV irradiation (GeneScreen manual, DuPont-NEN). The membrane was stained with methylene blue to verify even loading and transfer and then prehybridized and hybridized in 50% formamide at 42°C according to the GeneScreen protocol. Radioactive probes were obtained by random-primed labeling (Boehringer Mannheim) of DNA fragments: to detect polyoma early mRNA *Pvu*II fragment 3 (bp 1147–2034), for SV40 early mRNA *Hin*dIII fragment 2 (bp 4002–5171) and for SV40 late mRNA SV40 bp 1783–2533.

DNA sequencing was done with the Sequenase Version 2.0 sequencing kit (United States Biochemical).

3. Results

3.1. Strategy

In principle altered polyomaviruses coding individual T antigens could be propagated in mouse cells expressing constitutively polyoma early proteins (Tyndall et al., 1981; Muller et al., 1983). To avoid recombinations between free viral DNA and integrated polyoma sequences which might regenerate wild-type virus, we decided to use SV40 as a vector (Sambrook and Gething, 1988) and COS cells (Gluzman, 1981) for its propagation. SV40 also has a wider host range for infection and expression of early proteins than polyomavirus. Chang and Wilson (1986) showed that the upper limit for packaging circular DNA into SV40 capsids is about 5500 bp. Insertion of polyoma cDNA coding ST, MT or LT in place of the SV40 T antigen coding sequence does not exceed this size. However, insertion of the complete polyoma early region, i.e. the wild-type sequence with the introns, would create a genome of about 5660 bp. Earlier and recent studies on polyoma LT showed that the domains responsible for immortalization and for induction of S-phase are located in the N-terminal third of the protein comprising about aa 1–260, while the C-terminal half is required for viral DNA replication (Gioerup et al., 1994). In a first series (recombinants type I, Fig. 1) the polyoma early region extended from the *Bgl*I site (bp 96) to the *Xba*I site (bp 2484). Later hybrid viruses without the 5′-untranslated region were constructed (type II, type III and LT virus; see below).

3.2. Preliminary experiments for the isolation and characterization of hybrid viruses

The cloned viral constructs were excised from the plasmid, circularized and transfected into COS cells. The first lysates obtained after three consecutive infections with supernatant medium induced in COS cells synthesis of the expected polyoma T antigens and of SV40 capsid proteins detected by immunofluorescent stainings, however, viral DNA extracted from infected cultures was very heterogeneous in size. Subsequently, to minimize recombinations and rearrangements during amplification, circular, genome-size DNA purified by gel electrophoresis was transfected for the production of recombinant virus stocks.

Infection of COS cells with hybrid virus preparations yielded about 50% polyoma T antigen expressing cells after 30 h of infection. However, in infected mouse kidney cells only 1–5% weakly T antigen positive cells were observed. Furthermore, in hybrid virus infected COS cells polyoma early mRNA was detected only when viral DNA was replicating, but not when it was inhibited (not shown).

As a possible reason for the poor expression of the polyoma early proteins in absence of viral DNA replication we suspected the presence of a short stretch of non-coding polyoma DNA (bp 96–174) between the SV40 early promoter and the initiation codon for the T antigens. This sequence was therefore deleted in hybrid viruses of types II and III. Finally the series of recombinants was completed with LT virus coding the entire polyoma LT protein (Fig. 1).

From good virus stocks concentrated virus preparations were made and tested in productive infections of COS cells and by infecting CV-1 cells and quiescent baby mouse kidney cells in primary cultures.

3.3. Productive infection of COS cells with hybrid viruses

Analysis by gel electrophoresis of viral DNA synthesized in infected COS cells gave information on the homogeneity and thus on the quality of the hybrid virus used for infection (Figs. 2 and 3). MT and LTX viruses of both types I and III gave rather homogeneous DNA of the expected size. ST I, ST II and wtX III showed additional smaller molecules, but the majority of the viral DNA was of correct size. Only wtX I yielded a heterogeneous population with an important proportion of smaller DNA molecules.

Immunofluorescent stainings for polyoma T antigens showed only a slight increase of positive cells as compared to not concentrated lysates. At 30 h after infection between 50 and 60% positive cells were observed for most hybrid virus prepara-

tions. Using the same monoclonal antibody αPy C4 (Dilworth and Griffin, 1982) characteristic stainings were obtained for the different polyoma early proteins (Fig. 3). When we did double stainings for polyoma T antigens and SV40 capsids in the same cells, we observed with all recombinants up to 20% capsid positive cells that did not express polyoma T antigens (not shown). This indicated the presence of a substantial proportion of hybrid viruses that were defective for the expression of the polyoma early proteins. T antigen positive cells that did not express SV40 capsids, were less frequent. Endpoint dilutions of the virus stocks and assays for presence of infectious virus in the supernatant medium of infected COS cells indicated titers of 10^6–10^7 infectious particles per milliliter.

In time course experiments we determined levels of viral mRNAs and synthesis of T antigens in COS cells infected with hybrid viruses of types I and III (or II for ST). The results are shown in Figs. 4 and 5. They indicated that early and late mRNAs which were not detected 8 and 12 h after infection were essentially transcribed from replicated, amplified viral DNA. Synthesis of polyoma T antigens was detected at 20 h after infection, however the intensity of the bands varied for the different hybrid viruses. Maximal T antigen synthesis occurred probably between 30 and 40 h after infection. The protein gels for LTX and wtX also indicated that the truncated LT protein (see legend to Fig. 1) was not stable, its band remained rather weak and degradation products were observed. The MT protein was readily detected in immunoprecipitates of MT virus infected cells together with a number of additional bands which are due to cellular proteins associated with polyoma MT. In general, viruses of type III (or II for ST) gave somewhat higher levels of early mRNA and proteins than the corresponding viruses of type I suggesting that removal of the untranslated polyoma sequence improved slightly expression of the polyoma early proteins.

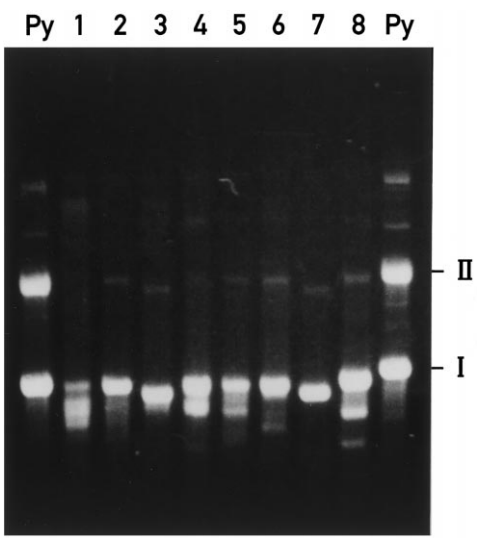

Fig. 2. Analysis by gel electrophoresis of hybrid viral DNA extracted from COS cells infected with concentrated virus stocks. One twentieth of the total yield from 85 mm cultures was loaded on a 1% agarose gel. Lane 1: wtX I; 2: MT I; 3: LTX I; 4: ST I; 5: ST II; 6: MT III; 7: LTX III; 8: wtX III. Py: 0.5 μg polyomavirus A2 DNA.

By nuclease S1-mapping we determined the transcription initiation sites for early polyoma mRNA (data not shown). While SV40 early transcription starts at two distinct sites (Ghosh and Lebowitz, 1981; Salzman et al., 1986), we found for the recombinant viruses four to six major sites between SV40 bp 5224 and 20. These sites were closely similar for the different constructs and independent on expression of polyoma early proteins. In type I viruses we observed an additional site in the polyoma sequence (bp 154–156) which corresponds to the previously determined cap site of polyoma early mRNAs (Kamen et al., 1982; Salzman et al., 1986).

3.4. Infection of mouse cells with hybrid viruses (type III)

By immunofluorescence wtX and LTX virus gave only 3–5% positive cells at 30 h after infection. For wtX, but not for the other recombinants, positive cells increased up to about 10% after 2 days of infection. MT and ST virus infected cultures showed very few, about 1% weakly, but clearly positive cells. Determination of early mRNA levels showed that there was a significant difference between spliced wtX mRNAs and cDNA transcripts of LTX, MT and ST virus (Fig. 6). Despite of similar titers of ST,

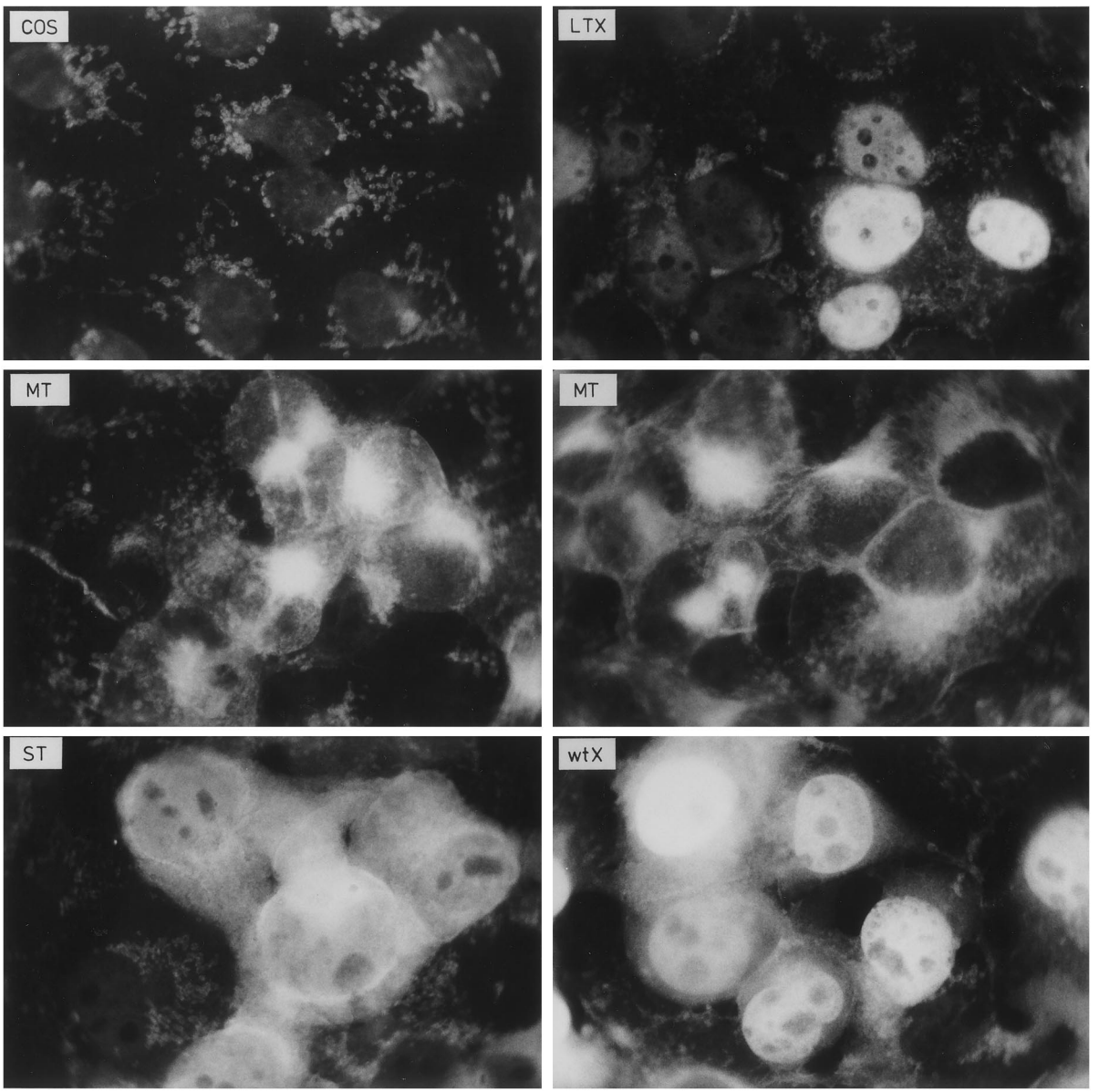

Fig. 3. Immunofluorescent stainings of polyoma T antigens expressed by hybrid viruses. COS cells grown on cover slips were infected with LTX III, MT III, ST II and wtX III and fixed 32 h after infection. Monoclonal antibody αPy C4 (Dilworth and Griffin, 1982) was used for all preparations. COS: uninfected cells; LTX: nuclear staining typical for polyoma LT; MT: cytoplasmic stainings with accumulation near the nucleus, often extending in weblike structures (Dilworth et al., 1986); ST: rather diffuse nuclear and perinuclear, occasionally also cytoplasmic staining; wtX: all three polyoma early proteins are expressed.

MT and LTX virus and similar mRNA levels in infected COS cells (Figs. 4 and 5), LTX transcripts were more abundant than MT or ST mRNA. When compared to SV40 infections, wtX early mRNA levels were comparable to those of SV40 early mRNA in cultures with about 25% T antigen positive cells and much higher than those observed in cultures infected with ten-fold diluted

SV40 with about 10% T antigen positive cells. However, it should be noted that SV40 early mRNA levels in mouse cells are highest between 9 and 15 h after infection and thereafter decrease gradually (Khandjian and Gauchat, 1988).

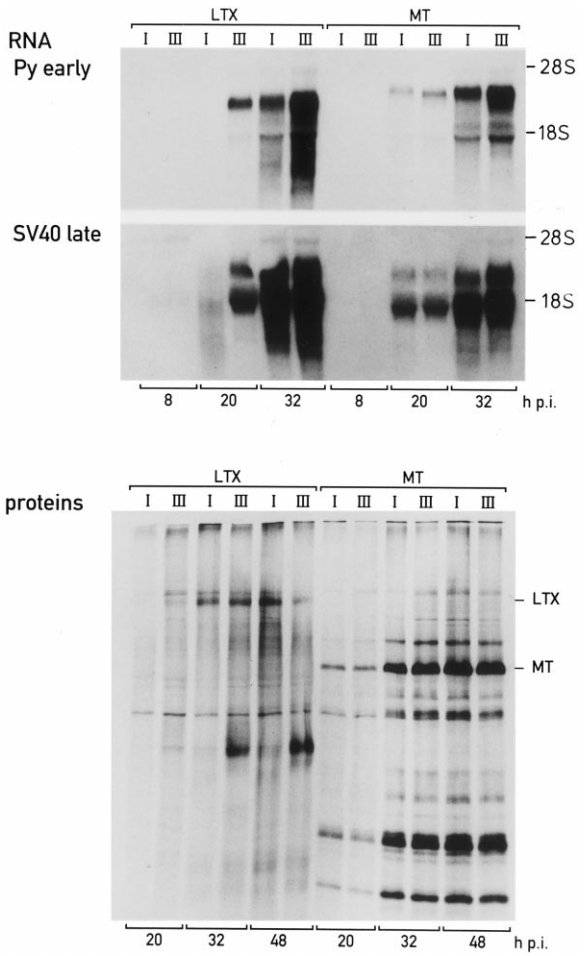

Fig. 4. Productive infection of COS cells with LTX and MT hybrid viruses types I and III. RNA: Northern blots of cytoplasmic RNA were probed for polyoma early mRNA (2×10^6 cpm/ml, exposed for 2 days) and for SV40 late mRNA (1×10^6 cpm/ml, exposed for 18 h). Proteins: SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine-labeled, immunoaffinity-purified proteins. A quarter of the sample was loaded on a 12% acrylamide gel. The dry gel was exposed for 5 days.

3.5. Comparison of infections and transfections by hybrid virus and viral DNA

To test early promoter strength of hybrid viruses we compared the infection of CV-1 cells by SV40 and hybrid viruses (type III) to the transfections with hybrid viral DNA and SV40 DNA cloned at the unique *Bam*HI site in the SV40 late region (see Fig. 1 and below). Transfections of CV-1 cells with hybrid viral DNA yielded significantly higher levels of polyoma early mRNA when compared to SV40 early mRNA in parallel cells transfected with SV40 DNA, even if DNA replication was not inhibited by ara-C (Fig. 7). As in infected mouse kidney cells, early mRNA was highest for wtX DNA and lowest for MT DNA transfected cultures. These results indicated that poor expression of polyoma early mRNA and proteins in hybrid virus infected cells and in absence of viral DNA replication was not due to reduced promoter activity. In CV-1 cells also, spliced transcripts were more abundant than cDNA transcripts, but we have no satisfactory explanation for the clear difference between LTX and MT early mRNAs.

The results were however different, when CV-1 cells were infected with SV40 or with hybrid viruses: SV40 early mRNA was readily detected, even when cultures were treated with ara-C, while polyoma early mRNA levels were much lower (Fig. 7). This corresponded to results obtained by immunofluorescent stainings: only 1–2% positive cells were found in wtX infected cultures and less than 0.5% in LTX infected cells. In this experiment SV40 infection yielded about 80 and 50% positive cells for undiluted and ten-fold diluted virus, respectively. Therefore, infection by hybrid viruses of CV-1 cells seemed to be inefficient compared to SV40. In this case the poor expression cannot be due to the heterogeneity of the hybrid viral DNA (see below), since this should not affect the levels of early mRNA significantly.

3.6. Amplification of hybrid virus generates multiple genomic rearrangements

In the original constructs the viral DNA was interrupted by the vector between the SV40 early

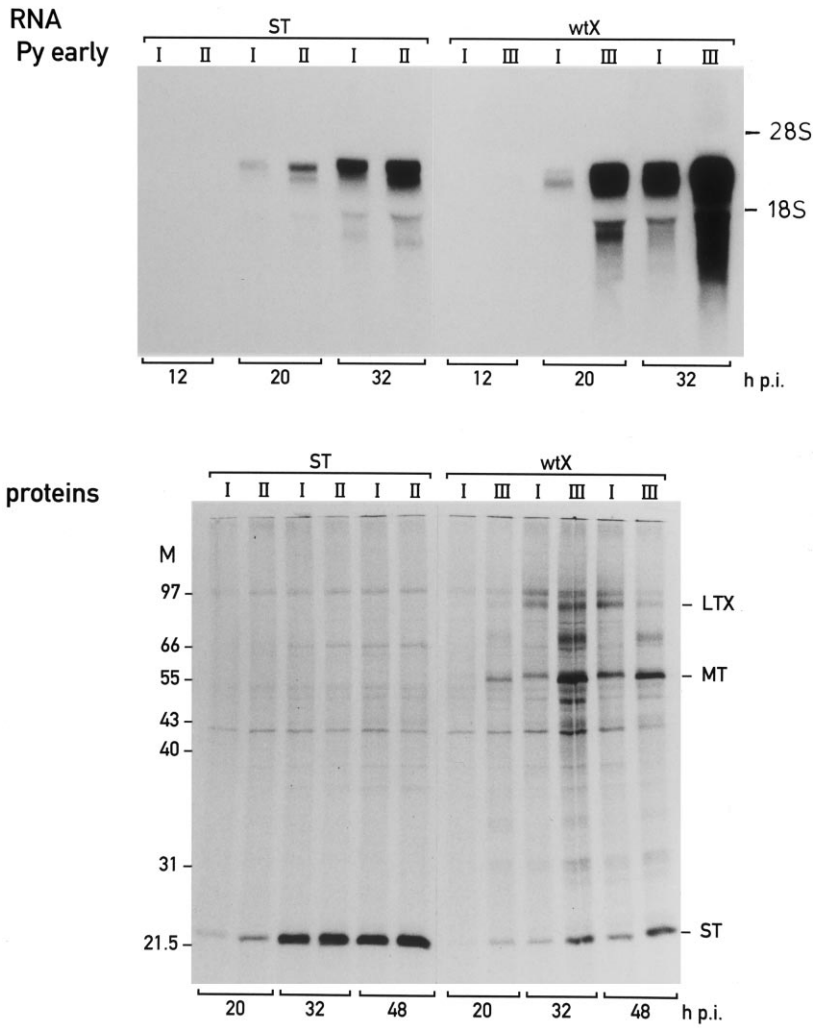

Fig. 5. Productive infection of COS cells with hybrid viruses ST I or II and wtX I or III. RNA: Northern blots of cytoplasmic RNA were probed for polyoma early mRNA (1×10^6 cpm/ml, exposed for 24 h). Proteins: SDS-polyacrylamide gel electrophoresis as in Fig. 4.

promoter and the polyoma sequence (Fig. 1). For the transfection experiments reported above we had to reclone the hybrid viral DNA. Doing this with DNA extracted from type III infected COS cells we became aware of the heterogeneity of the hybrid viral genomes. Plasmids with inserts of smaller size could easily be eliminated, but even apparently homogeneous LTX DNA (Fig. 2) yielded clones which were negative for T antigen expression in transfected COS cells. Therefore, hybrid viral DNA from individual plasmids was compared to the original constructs by digestion with restriction enzymes and tested for T antigen synthesis after transfection of COS cells. In all T antigen negative clones we found more or less important changes in the restriction enzyme fragments. The DNA of LT virus, for which we were unable to get a good virus stock, was particularly subject to deletions or rearrangements all over the genome. With 18 LT plasmids analyzed (not shown) we found 15 different patterns, only two of them were indistinguishable from the original

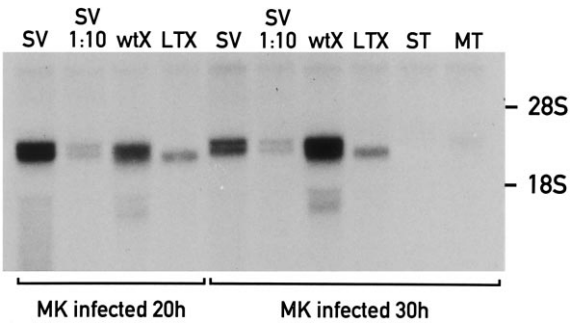

Fig. 6. Early mRNA levels in SV40 and hybrid virus infected mouse kidney cells. Primary cultures of mouse kidney cells were infected with SV40 (SV, about 5×10^8 to 1×10^9 plaque forming units per milliliter), ten-fold diluted SV40 (SV 1:10) or concentrated virus stocks of wtX III, LTX III, ST II, MT III. The membrane was hybridized simultaneously with probes for SV40 and polyoma early mRNAs (3×10^6 cpm/ml hybridization mixture each) and exposed for 4 days.

construct and only one expressed polyoma LT protein in transfected COS cells. Detectable rearrangements in the polyoma sequence were more frequent (seven plasmids) than in the SV40 late region (one plasmid); the other patterns could not be interpreted. Finally, partial sequence analyses of apparently intact clones (wtX, LTX and LT) revealed the occurrence of single basepair changes.

4. Discussion

To investigate the roles of individual polyoma T antigens for mitotic stimulation of quiescent cells we tried to use SV40 as a vector to express these proteins. Despite of its limited packaging capacity SV40 seemed to be a convenient tool to introduce heterologous genes into animal cells, because of its wide host range and strong expression with its enhancer and early promoter. It was particularly attractive for us, because SV40 and polyoma virus infections of various cells had been studied for years in our laboratory. A viral vector with a broad host range would indeed be useful to introduce potential mitogenic genes into quiescent cells and to study cellular elements controling passage from G_0 to S-phase. For this purpose we invested a considerable amount of effort to construct and characterize SV40/polyoma recombinant viruses expressing individual polyoma T antigens.

It turned out that in the absence of viral DNA replication, i.e. in infected mouse or CV-1 cells, expression of polyoma T antigens was far too low for biochemical analyses of S-phase induction. The main drawbacks for the use of such recombinant viruses were genomic instability, low levels of early mRNAs and possibly also reduced infectivity. It remains to be shown, whether these drawbacks are unique to SV40/polyoma hybrids or also characteristic for other SV40 recombinants.

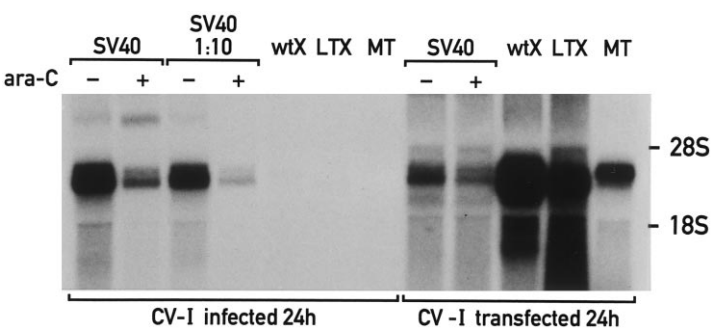

Fig. 7. Early mRNA levels in infected and transfected CV-1 cells. Primary cultures of mouse kidney cells were infected with SV40 (SV, about 5×10^8 to 1×10^9 plaque forming units per milliliter), ten-fold diluted SV40 (SV 1:10) or concentrated virus stocks of wtX III, LTX III, ST II, MT III. Transfections with cloned SV40 or hybrid viral DNA (type III) using the DOTAP transfection reagent (Boehringer Mannheim). The membrane was hybridized and exposed together with the membrane shown in Fig. 6.

Mutations of the genomes during virus amplification in COS cells were apparently quite frequent, despite several precautions taken, like using purified genome-size DNA for the initial transfections, doing several transfections in parallel and saving the first supernatants for further amplifications. Appearance of smaller molecules with deletions greater than 5% was easily detected, but attempts to eliminate defective DNA or virions by different methods were tedious and barely successful. Even apparently homogeneous viral DNA contained defective genomes and partial sequence analyses of cloned viral DNA also showed single basepair substitutions. The relative proportion of unaltered versus rearranged DNA in a given virus preparation was difficult to evaluate. Double immunofluorescent stainings for polyoma T antigens and SV40 capsid proteins and analysis of cloned viral DNA revealed that mutations in the polyoma sequence were more frequent than in the SV40 late region. It had been reported that polyoma LT and SV40 LT promote recombinations (Piché and Bourgaux, 1987; Stewart and Bacchetti, 1991; St-Onge et al., 1993; Laurent et al., 1995). In our experiments amplification of recombinant virus expressing the entire polyoma LT was particularly deleterious and we were unable to obtain good virus preparations with this recombinant. This could suggest that the C-terminal region (aa 640–785) contributes efficiently to the recombination promoting activity of polyoma LT. Whether the high genomic instability of our recombinants was due to the simultaneous presence of polyoma and SV40 T antigens during amplification in COS cells remains to be investigated.

The finding that recombinant viruses with cDNA sequences (LTX, MT and ST virus) yielded reduced levels of early mRNA as compared to spliced mRNA of wtX virus was not unexpected. It was surprising, however, to find a clear difference between LTX mRNA and MT or ST mRNA, since these mRNAs only differ by the nucleotide sequence at the exon junction. Finally, comparison of transfections and infections of CV-1 cells suggested that in these cells recombinant virions were much less infectious than SV40.

5. Conclusion

In conclusion, we started our project with a rather straightforward approach and a well defined system, due to the small size and the low genetic complexity of these two intensively studied viruses. Unfortunately, in our system SV40 turned out to be a bad viral vector. Apparently, the concise genomes of these small DNA viruses can hardly be manipulated without disturbing significantly infectivity and gene expression.

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