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Two Synthetic Sp1-Binding Sites Functionally Substitute for the 21-Base-Pair Repeat Region To Activate Simian Virus 40 Growth in CV-1 Cells

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The 21-bp repeat region of simian virus 40 (SV40) activates viral transcription and DNA replication and contains binding sites for many cellular proteins, including Sp1, LSF, ETF, Ap2, Ap4, GT-1B, H16, and p53, and for the SV40 large tumor antigen. We have attempted to reduce the complexity of this region while maintaining its growth-promoting capacity. Deletion of the 21-bp repeat region from the SV40 genome delays the expression of viral early proteins and DNA replication and reduces virus production in CV-1 cells. Replacement of the 21-bp repeat region with two copies of DNA sequence motifs bound with high affinities by Sp1 promotes SV40 growth in CV-1 cells to nearly wild-type levels, but substitution by motifs bound less avidly by Sp1 or bound by other activator proteins does not restore growth. This indicates that Sp1 or a protein with similar sequence specificity is primarily responsible for the function of the 21-bp repeat region. We speculate about how Sp1 activates both SV40 transcription and DNA replication.

The 21-bp repeat region activates simian virus 40 (SV40) early transcription and modulates late transcription (1, 3–5, 9, 16, 19, 22, 23, 25, 29, 30, 34–36, 40, 41, 61, 76, 78, 85, 96, 100) as well as DNA replication (6, 11, 13, 20, 29, 33, 37, 39, 42, 43, 45, 50, 64, 67). Sequences within the 21-bp repeat region are recognized by Sp1 (22), LSF (49, 61), ETF (58), Ap2 (77, 103), Ap4 (75), GT-1B (104), the single-stranded DNA binding protein H16 (31), the tumor suppressor protein p53 (2, 95), and the SV40 large tumor antigen (SV-LT) (72, 99). These proteins differ in structure and function, and the extent to which each participates in the promotion of SV40 growth is unknown.

Sp1 is a zinc finger protein present in the nuclei of mammalian cells as glycosylated and phosphorylated 95- and 105-kDa polypeptides. It promotes transcription from the viral early and late promoters through glutamine-rich domains as well as through its carboxyl terminus (10, 23, 34, 35, 52, 53, 57, 80, 82, 85). Within the SV40 21-bp repeat region, six GC motifs (GC motifs I to VI) have the potential to bind Sp1, but with very different affinities (22, 34, 35). LSF is a 63-kDa protein that stimulates SV40 late transcription and binds a bipartite site between GC motifs II and III, as well as another site upstream of the enhancer (49, 61). ETF also stimulates transcription from TATA-less promoters such as the SV40 late promoter (58), but its binding site in the 21-bp repeat region has not been precisely identified. Ap2 recognizes partial sites (5′-CCCA-3′) within the 21-bp repeat region (4). Transcriptional regulation by this 52-kDa protein is positively and negatively modulated by retinoic acid, cyclic AMP, and 12-*O*-tetradecanoyl-13-acetate and by SV-LT (68, 77, 103). GT-1B binds a site in GC motif III, but little is known about its structure or function (104). The 21-bp repeat region also contains partial sites for the transcriptional activator Ap4 (5′-CAGTTC-3′) (48) and two sites for H16 (31), and it is weakly bound by SV-LT and p53, by which viral transcription and DNA replication might be modulated (2, 95, 99).

Little or nothing is known about how most of these proteins affect SV40 gene function or replication. We have attempted to resolve their importance by substituting synthetic sequences capable of binding individual proteins in place of the 21-bp repeat region and then measuring their effects upon virus growth in CV-1 cells. Our results indicate that two high-affinity Sp1 motifs serve nearly as well as the intact 21-bp repeat region in activating SV40 early gene expression and, concomitantly, DNA replication and plaque formation. In contrast, binding sites for several other protein activators do not substitute for the 21-bp repeat region. Because much is known about Sp1 structure and function, we speculate about how Sp1 binding to the 21-bp repeat region might promote both DNA replication and DNA transcription.

MATERIALS AND METHODS

Plasmid constructions. Standard procedures were employed in cloning experiments (86) with *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.). The construction of key plasmids is described below.

(i) pCV21-0. Since the 21-bp repeat region is not flanked by unique restriction enzyme sites, we devised pCV21-0 to facilitate subsequent constructions (Fig. 1B). Cloning vector pCV21-0 contains the SV40 genome (776 strain) with the 21-bp repeat region replaced with prokaryotic filler DNA. The DNA fragments used to construct pCV21-0 were derived from pX113 and pS312 (29) and pBRX, a derivative of pBR322 in which the *Ssp*I site was converted into an *Xho*I site by the insertion of an 8-bp *Xho*I linker. The gap that results from the removal of the filler DNA (after double digestion with *Sal*I and *Xho*I) is referred to as the cloning site of pCV21-0.

(ii) pWTSV40. pWTSV40 contains the 776 strain of SV40 cloned at *Eco*RI into the same vector as pCV21-0.

(iii) p21-0. p21-0 was constructed by removing the filler DNA in pCV21-0, which yielded the sequence shown in Fig.

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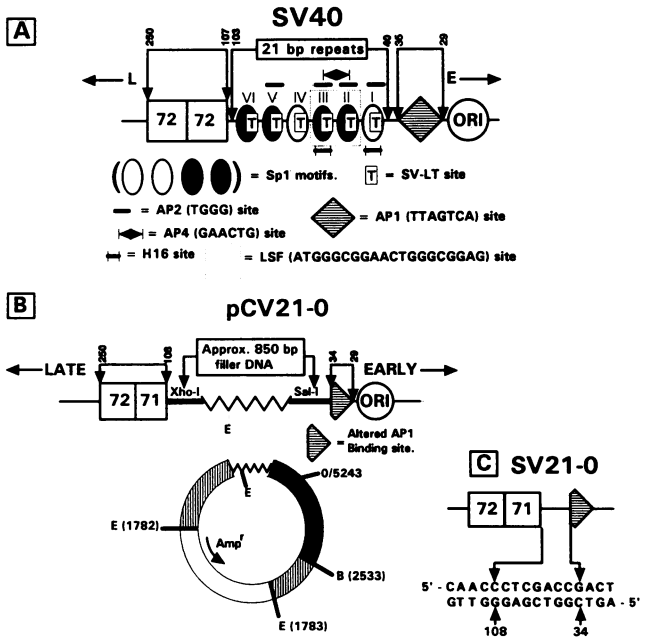

D 21 bp repeats oligo

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5'-TCGAGTCCCGCCC..... // .....CCGCCCCAG
   CAGGGCGGG..... // .....GGCGGGGTCAGCT-5'
          99                    46
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FIG. 1. (A) Schematic of the SV40 control region. The 72-bp repeats, the 21-bp repeat region, and the Ap1 site bordering the origin of DNA replication (*ori*) are indicated by vertical arrows. SV40 nucleotides that border these sequences are numbered according to The Genetic Sequence Data Bank, release 67.0, March 1991 (The Los Alamos Laboratory). GC motifs are depicted as ovals, and the Sp1 motifs within these are identified by roman numerals I to VI. An increase in the darkness of an oval represents an increase in the affinity of the site for purified HeLa Sp1 in vitro (22, 34). The relative directions of early (E) and late (L) transcription are shown by horizontal arrows. The figure is not drawn to scale. (B) Schematic of pCV21-0. The 2.3-kb carrier vector (from pX113) is identified by the open pattern, with SV40 sequences identified by vertical hatching. pS312-derived DNA is depicted in black, and filler DNA is indicated by the jagged line. Restriction endonuclease sites are identified by the letters E (*Eco*RI) and B (*Bam*HI). (C) Sequence between SV nt 34 and 108 in wt SV40 and in SV21-0. (D) Sequence of 21-bp repeat oligonucleotides.

1C. p21-N and p21-R each contain a synthetic oligonucleotide with the sequence of the 21-bp repeat region between nucleotides (nt) 46 and 99 (Fig. 1D) in the cloning site of pCV21-0 in normal (N) and reverse (R) orientations. Clones p21-NSR and p21-N3D contain spontaneous point mutations in this sequence that were detected while p21-N constructs were screened. pSEGO21-R contains the 21-bp repeat region from pSEGO (3) cloned into pCV21-0. The SVGC3,5, SVGC1, SVGC2, and SVGC4 series were constructed by inserting synthetic DNAs (Fig. 2A) into pCV21-0, yielding viruses containing single and multiple inserts (Fig. 3).

DNA sequence analysis. Alkali-denatured double-stranded plasmid DNAs were sequenced (87) by using oligonucleotide primers (Fig. 2B) corresponding to SV40 nt 5195 to 5218 (sequencing primer 1) and SV40 nt 266 to 245 (sequencing primer 2) with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio). dITP was used in parallel runs with dGTP when compression was a problem.

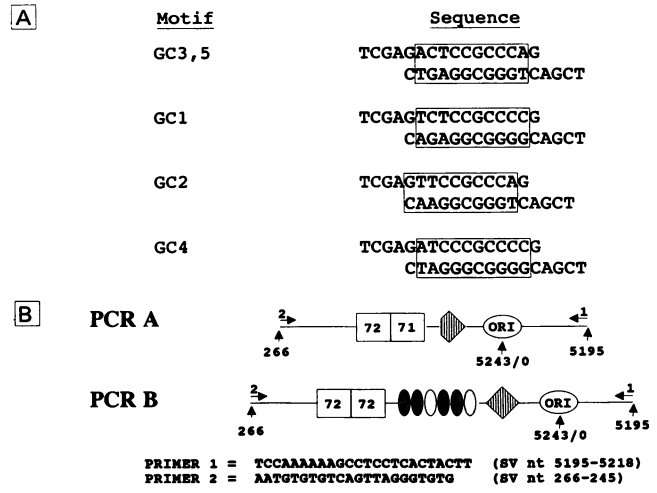


FIG. 2. (A) Sequences of synthetic Sp1 motif oligonucleotides (GC motifs). The 10-bp Sp1 recognition sites are boxed. (B) Schematic of competitor DNA PCR A and B used for mobility shift assays. Primer-binding sites (1 and 2) that were used to synthesize these double-stranded DNAs are identified by arrows.

Rapid preparation of viral stocks from plasmid DNA. Viral DNA was released from vector DNA by using *Eco*RI, purified and recircularized with T4 DNA ligase, and resuspended at a DNA concentration of 250 ng/μl. H_2O was added to 1 to 5 μg of the recircularized DNA to a total volume of 50 μl, and then 50 μl (50 μg) of Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, Md.) was added. The DNA-lipid mixture was incubated for 15 min at room temperature and then added to nearly confluent CV-1 cells in 60-mm-diameter dishes (26).

Cell culture and plaque assays. CV-1 cells (passage 31) were obtained from the American Type Culture Collection and passaged no more than 15 times as monolayers in minimum essential medium with Earle's salts (EMEM) supplemented with 10% fetal bovine serum (FBS). Hybridoma cells producing PAb 108 (38) were purchased from the American Type Culture Collection (TIB 230) and grown in

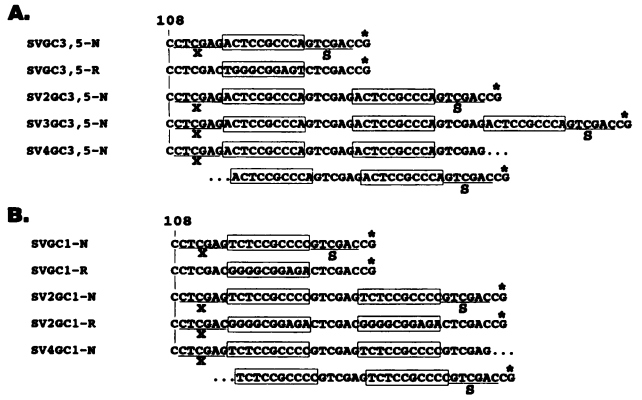


FIG. 3. Sequence between SV nt 34 and 108 in SVGC3,5 series viruses (A) and SVGC1 series viruses (B). SV nt 108 is identified by a vertical line, and an asterisk above the 3'-terminal G of each sequence identifies nt 34. Sp1 motifs are boxed. *Sal*I (S) and *Xho*I (X) sites are underlined.

Dulbecco's modified Eagle medium supplemented with 20% FBS.

For plaque assays, CV-1 cells were seeded into 60-mm-diameter dishes in EMEM plus 10% FBS. When the cells were confluent, the medium was changed to EMEM plus 5% FBS, and the cells were incubated for another 12 h. Virus samples were added in 2 ml of EMEM plus 1% FBS and incubated for 2 h, and the plates were rocked every 15 min. The medium was removed, and the cells were overlaid with 10 ml of autoclavable EMEM (Auto Pow; ICN Biochemicals, Inc., Costa Mesa, Calif.) supplemented with 5% FBS and 0.9% agarose. The cells were refed 8 days postinfection with 5 ml of overlay medium. A final overlay (5 ml) that contained 0.015% neutral red was added 12 days postinfection, and plaques were scored the following day. At least 50 well-isolated plaques of each virus were measured.

For DNA infectivity assays, constructs were linearized with *Eco*RI; the viral DNA was purified with Gene Clean II (Bio 101, Inc., La Jolla, Calif.), and its concentration was standardized by UV absorbance or by fluorescence in ethidium-stained gels. Serial dilutions of stock DNAs were mixed with 10 μg of carrier salmon sperm DNA and introduced into cells by calcium phosphate coprecipitation with a glycerol boost 5 h after the addition of the DNA. After 12 h, the cells were overlaid and treated as described above for plaque assays. A minimum of four plates per DNA sample were used, and each measurement was repeated at least five times.

Immunofluorescence assays. Monolayers of CV-1 cells infected with approximately 0.02 PFU per cell were washed twice with phosphate-buffered saline (PBS), trypsinized, and then seeded at the same density into dishes with coverslips and complete medium, which was changed 8 h postseeding. At various times, the coverslips were removed, washed in PBS, fixed in acetone (−20°C) for 5 min, and then incubated with PAb 108 for 45 min at room temperature and washed with PBS. Following a reaction with an empirically predetermined dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody in the dark for 45 min at room temperature, the cells were washed with PBS and mounted in a 1:1 mixture of PBS-glycerol.

DNA replication assays. Digestion with *Dpn*I was used to distinguish input DNA replicated in bacteria from DNA replicated in eukaryotic cells (81). Linear *Eco*RI-digested DNA was used for transfection, since it is easier to quantitate than in vitro recircularized DNA, which usually contains unwanted ligation products. Supercoiled pTZ18R DNA prepared in a *dam*-minus strain of *E. coli* was used as a nonreplicative internal control. Each sample (containing 1 μg of SV40 DNA, 0.4 μg of vector DNA, 10 μg of carrier salmon sperm DNA, and 1 μg of pTZ18R DNA) was transfected by calcium phosphate coprecipitation with a glycerol boost at 5 h into nearly confluent CV-1 cells in 60-mm-diameter dishes with EMEM that contained 10% FBS. DNA was extracted from the cells (47) at 48 h posttransfection, centrifuged for 1 h at 4°C at 9,000 × *g*, washed with 70% ethanol, and then resuspended in 100 μl of Tris-EDTA (pH 8). A portion (25 μl) of each sample was then digested with *Bam*HI and *Dpn*I and fractionated on a preparative 1% agarose gel. Southern blots (91) were probed at 68°C with randomly primed SV21-0 ^{32}P-labeled DNA (approximately 5 × 10^8 cpm/μg). Bands were scanned and quantitated by using a Molecular Dynamics Phosphor Imager.

Preparation of nuclear extracts. Nearly confluent CV-1 cells were scraped into PBS-EDTA and pelleted for 5 min at 290 × *g* at room temperature. The pelleted cells were then resuspended in 5 volumes of ice-cold buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]–KOH, 10 mM KCl, 0.3 M sucrose, 1.5 mM $MgCl_2$, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] [pH 7.9 at room temperature], 0.35% [vol/vol] Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mg of aprotinin per ml, 50 μg of tolylsulfonyl leucylchloromethyl ketone, and 100 μg of tolylsulfonyl phenylalanyl chloromethyl ketone). Aliquots were transferred to a Dounce homogenizer, and the cells were lysed with 10 strokes of the tight-fitting pestle and 12 strokes of the loose-fitting pestle. The nuclei were collected by centrifugation at approximately 350 × *g* for 10 min at 4°C, washed twice in ice-cold buffer A without Nonidet P-40, and then resuspended in 2.5 volumes of ice-cold buffer B (400 mM NaCl, 10 mM HEPES-KOH, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% glycerol [pH 7.9 at room temperature] supplemented as described above for buffer A). The mixture was placed on ice for 30 min and shaken gently at 5-min intervals. Subsequently, the nuclei were pelleted at approximately 3,800 × *g* for 15 min at 4°C. The supernatant was saved and mixed with an equal volume of saturated ammonium sulfate and stored for 4 h at 4°C. The precipitated proteins were collected by centrifugation at approximately 20,000 × *g* at 4°C for 1 h and resuspended in ice-cold storage buffer (10 mM HEPES-KOH, 75 mM KCl, 0.1 mM EDTA, 20% [vol/vol] glycerol, pH 7.9, at room temperature) supplemented with dithiothreitol, phenylmethylsulfonyl fluoride, tolylsulfonyl phenylalanyl chloromethyl ketone, and tolylsulfonyl leucylchloromethyl ketone (as in buffer A) prior to being used. Nuclear proteins extracted from cells harvested from 20 150-mm-diameter dishes were solubilized in 500 μl of buffer and then divided into 50-μl aliquots and stored at −70°C. Proteins were quantitated by the Bradford microassay (Bio-Rad Laboratories, Richmond, Calif.) (8) with bovine serum albumin as the standard.

Gel mobility shift assays. The procedure described by Garner and Revzin (32) and by Fried and Crothers (28) as modified by Martin et al. (70) was used with the following additional changes. Binding buffer consisted of 10 mM HEPES-KOH–150 mM KCl–1 mM EDTA–20% glycerol (pH 7.5 at room temperature); prior to being used, it was supplemented with Triton X-100 (0.1% vol/vol). The buffer contained 100 μM zinc sulfate when Sp1 samples eluted from wheat germ agglutinin columns were assayed. Assay buffers for Ap1 or Ap2 binding activity contained 4 mM dithiothreitol. Reaction mixtures typically contained 2 to 4 μg of nuclear extract, 1 ng of probe, and 1 μg of poly(dI · dC)-poly(dI · dC) in a reaction volume of 20 μl. Samples were preincubated for 15 min at room temperature prior to the addition of the probe and incubated for another 15 min at room temperature after the addition of the probe; they were then fractionated on a 5% native acrylamide (49:1 [wt/wt] acrylamide-bisacrylamide) gel. The DNA fragments used in competition assays were blunt ended with Klenow fragment prior to being used. Polymerase chain reaction (PCR) fragments used as competitors (Fig. 2B) were purified with the Qiagen PCR Purification Kit (Qiagen, Inc., Chatsworth, Calif.). Competition assays were performed with 1 ng of probe DNA and 500 ng of oligonucleotide competitor or 200 ng of PCR competitor. Immunodepletion assays were performed with polyclonal anti-Sp1 antibody and preimmune serum.

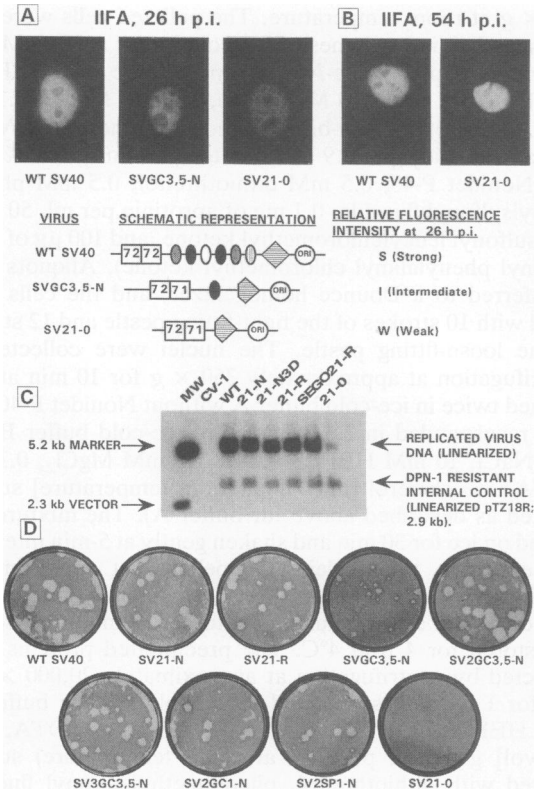


FIG. 4. (A) Examples of indirect immunofluorescence assays (IIFAs) of nuclei at 26 h postinfection (p.i.) with wt SV40 (which showed strong or bright fluorescence), SVGC3,5, and SV21-0. (B) Nuclei of SV21-0-infected cells and wt SV40-infected cells at 56 h p.i. (C) DNA replication assay of the 21 series viruses. The *Dpn*I-digested input DNA migrated ahead and is not shown. (D) Representative results of a plaque assay of virus constructs with natural or synthetic Sp1 sites in CV-1 cells. Samples were viewed 13 days p.i.; SV21-0 plaques are not detectable at 13 days p.i. in these cells.

RESULTS

Activator elements in the 21-bp repeat region enhance SV40 early gene expression, DNA replication, and virus production. The 21-bp repeat region interacts in vitro with the transcriptional activator proteins Sp1, ETF, LSF, Ap2, Ap4, and GT-1B and with SV-LT, the single-stranded binding protein H16, and the tumor suppressor protein p53, and it adjoins sites to which Ap1 binds (Fig. 1A). To reduce the complexity of this region and to determine whether binding of only one or several of these proteins plays a dominant role during viral growth, we constructed SV40 genomes in which the 21-bp repeat region was replaced with its subelements or with alternative activator sequences, and we employed three types of assays to measure the effects of these sequences in vivo. An indirect immunofluorescence assay with anti-SV-LT as the primary antibody was used to detect viral early gene expression (representative results are shown in Fig. 4A and B); the resistance of intracellular DNA to *Dpn*I was used to measure viral DNA replication, which relies upon viral early gene expression and *ori* function (representative results are shown in Fig. 4C); and plaque assays were used to determine viral infectivity and virus production, which rely upon early gene expression, DNA replication, and late gene expression (representative results are shown in Fig. 4D).

TABLE 1. Specific infectivities and titers of viral constructs

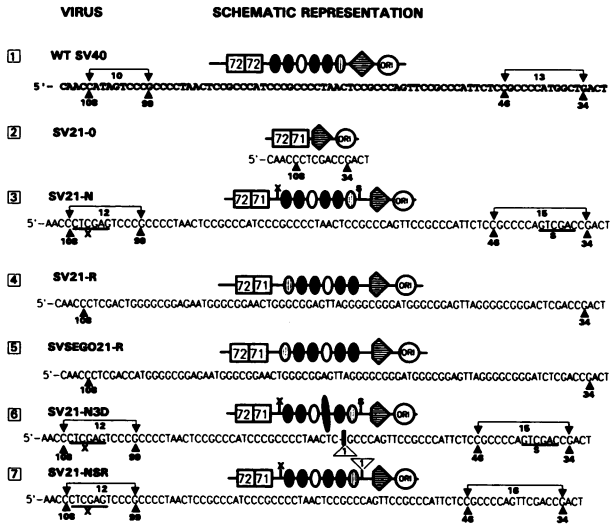
Virus	Infectivity[a] (10^3 PFU/μg)	Titer[b] (10^7 PFU/ml)
WT SV40	9	4
SV21-N	8	6
SV21-R	8	3
SVGC3,5N	8	4
SVGC3,5R	7	4
SV2GC3,5N	7	4
SV3GC3,5N	9	4
SV4GC3,5N	8	3
SV2SP1-N	8	6
SV2SP1-R	9	5
SV21-0	8	0.008

[a] Average values from one experiment, performed with purified linear virus DNA.
[b] Average values from three experiments.

Viruses lacking the 21-bp repeat region (SV21-0) are readily distinguishable from wild-type (wt) SV40 in CV-1 cells by these assays. Indirect immunofluorescence assays revealed that the quantity of early gene products expressed by wt SV40-infected CV-1 cells was maximal by 26 h after infection, but early gene expression in SV21-0-infected CV-1 cells was delayed, and maximal amounts of SV40 early gene products occurred at about 54 h after infection (Fig. 4A and B; also data not shown). CV-1 cells infected by SV21-0 accumulated about 10-fold less viral DNA by 48 h than cells infected by wt SV40 (Fig. 4C), and SV21-0 formed plaques that were detected at 15 days postinfection in CV-1 cells, whereas wt SV40 plaques were detected at day 8. The plaques formed by SV21-0 were consistently smaller than those formed by wt SV40 (Fig. 4D), and accordingly, virus titers in supernatants of cells infected by SV21-0 were 2 to 3 orders of magnitude lower than those obtained with wt SV40 (Table 1).

These results indicate that deletion of the 21-bp repeat region slows the rate of growth of SV40 in CV-1 cells. A primary cause of the limited DNA synthesis and virus yield is undoubtedly reduced early gene expression, which depends upon the 21-bp repeat region (3–5, 19, 22, 25, 29, 30, 35, 40, 46, 96, 100); however, replication and late gene expression are also directly affected by deletion of the 21-bp repeat region, even in the presence of SV-LT (1, 6, 9, 11, 13, 20, 33, 36, 37, 39, 41–43, 45, 61, 64, 78, 85, 100). Previous work has suggested that deletion of the 21-bp repeat reduced the specific infectivity of SV40 (42). However, we observed that the infectivity of SV21-0 DNA was virtually the same as for wt SV40 DNA (Table 1). A likely explanation for this different conclusion is that we measured plaque formation starting at 13 days posttransfection, whereas Hartzell et al. (42) measured plaque formation at 10 days posttransfection, which is too early to detect the small plaques formed by viruses lacking the 21-bp repeat region.

Insertion of the 21-bp repeat region into SV21-0 restores growth. In anticipation of studying the ability of synthetic DNA fragments to substitute for the 21-bp repeat region, we asked whether SV21-0 growth was restored by insertion of the natural 21-bp repeat region. A variety of constructs (termed the 21 series) differing in the orientations or boundaries of the inserted 21-bp repeat region were tested, and for each, early gene expression, DNA replication, and plaque size appeared to be nearly normal (Fig. 5). SV21-N contains the 21-bp region in its normal orientation and grew nearly as



IIFA	DNA REPLN.	PLAQUE SIZE,mm
26 h	48 h	day 13
S	100	3.5
W	12	0
S	95	3.3
S	91	3.3
S	92	3.3
S	93	3.3
S	ND*	3.3

*NOT DONE

FIG. 5. Structures of viruses in the 21 series and a summary of their properties. Numbers below the sequences identify reference nucleotides relative to those of wt SV40. Numbers within the brackets are a measure of the distances between reference nucleotides. S and X indicate unique *SalI* and *XhoI* sites, respectively. SV21-N3D has a deletion in GC motif II which alters both an Sp1 site and an LSF site. SV21-NSR contains a 1-bp insertion at its *SalI* site. Like SV21-R, SVSEGO21-R contains the 21-bp repeats in the reverse orientation; however, neither the nucleotides that flank the 21-bp repeats nor the distance to reference SV40 nt 34 or 108 is the same. DNA replication assays are average values from three separate experiments. Plaque sizes are average values from at least three separate experiments.

well as wild-type virus; SV21-R and SVSEGO21-R contain the 21-bp repeat region in the opposite orientation and accumulated slightly less DNA at 48 h, but their early gene expression was nearly normal and their plaques were nearly wild-type size. The infectivities and viral titers in culture supernatants of SV21-N, SV21-R, and SVSEGO21-R were those of wt SV40 (Table 1). Other viruses of the 21 series having single-base changes, such as SV21-N3D and SV21-NSR, or slight changes at the boundaries of the 21-bp repeat region yielded similar results (Fig. 5; also data not shown). Additionally, the properties of SV21-R and SVSEGO21-R indicate that the 21-bp repeat region functions in a bidirectional manner, which agrees with results obtained in vitro with similar constructs (3, 45, 100).

These results indicate that it is possible to construct chimeric viruses from pCV21-0, whose growth resembles that of wt SV40. The spacing of the 21-bp repeat region relative to *ori*, the early promoter and the enhancer-late promoter, and the sequences at the boundaries of the 21-bp repeat region, including the *ori*-proximal Ap1 site, appears to be somewhat flexible. This permits synthetic sequence motifs inserted into SV21-0 to be tested for their growth-promoting functions. These results contrast with those of studies with reporter genes in transient assays using human (HeLa) cells, in which early promoter function was markedly decreased when insertions or deletions to the nucleotide sequences that flank the 21-bp repeat region were made (3, 19, 56, 96).

Synthetic GC motifs functionally replace the 21-bp repeat region. To determine whether subelements of the 21-bp repeat region can restore growth, we introduced synthetic GC motifs capable of binding Sp1 in vitro (GC motifs in Fig. 2) (10, 65, 98) into SV21-0. In CV-1 cell nuclear extracts, Sp1 is the primary, if not the only, protein which binds to these GC motifs. A gel mobility shift analysis with GC3,5 (whose sequence is that of motifs III and V from the 21-bp repeat region) resolves several bands (Fig. 6). We believe that the one with the lowest mobility is a complex between the probe and Sp1, on the basis of the following: (i) preincubation of the cell extract with polyclonal anti-Sp1 antibody completely abolished the Sp1-specific band, whereas preincubation with

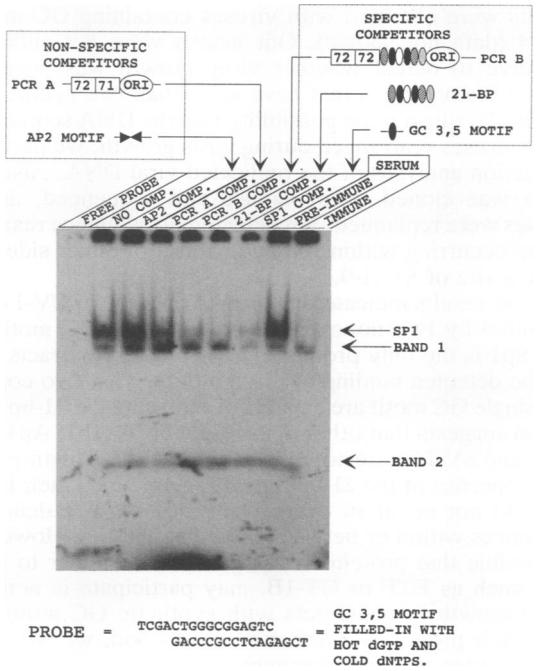


FIG. 6. Gel mobility shift analysis of Sp1-binding activity in CV-1 nuclear extracts.

preimmune serum had no effect, and (ii) a PCR fragment that contained the 21-bp repeat region and enhancer (PCR B) (Fig. 2B) acted as an effective competitor for formation of the complex, as did the synthetic 21-bp repeat oligonucleotide (Fig. 1). However, its formation was affected by neither a 500-fold excess of an Ap2 motif nor a PCR fragment derived from p21-0 that lacks the 21-bp repeat (PCR A) (Fig. 2B). The same band at approximately equivalent intensities was observed in a gel mobility shift assay with synthetic GC1, -2, -4, and -3,5, and these motifs were equally adept at competing for one another's binding sites (data not shown).

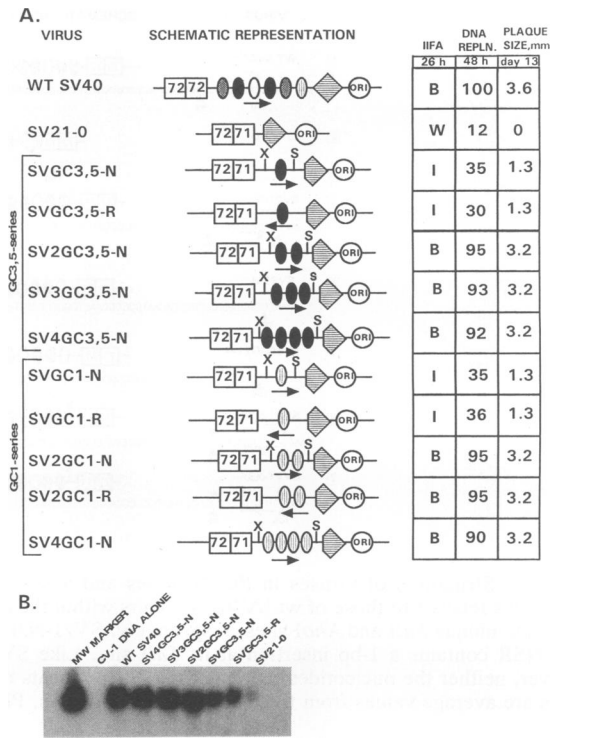
Of the two other bands observed in the gel in Fig. 6, band 1 is competed with by a large excess of motif GC3,5 and is not recognized by anti-Sp1 antibody. Formation of this complex is independent of the incubation temperature but is more pronounced when the probe is desiccated after ethanol precipitation (data not shown). We believe that this band consists of DNA with partial single-stranded character which is bound nonspecifically by non-Sp1 proteins (93). Band 2 is a complex of a protein with dGTP, for it is detected only when the probe is not completely separated from unincorporated [α-P^{32}]dGTP and it can be competed with by cold dGTP (data not shown).

UV cross-linking with CV-1 nuclear extracts was also employed to detect proteins which interact with the GC3,5 motif. An oligonucleotide with this motif was specifically cross-linked to a 97-kDa protein whose mass is very similar to that of Sp1. Cross-linking was abolished by preincubation of CV-1 nuclear extract with polyclonal anti-Sp1 antibody; furthermore, it was diminished by GC3,5 competitor DNA and did not occur when an Ap1 motif was used as the probe (data not shown).

Viruses containing one copy of either the GC1 or the GC3,5 motif inserted in either orientation into the cloning site of SV21-0 expressed weakly augmented early gene transcription, DNA replication, and plaque size. However, insertion of two copies of either motif maximally activated DNA replication and plaque size (Fig. 7A and B). Similar results were obtained with viruses containing GC motifs 2 and 4 (data not shown). Our assays were not sufficiently sensitive to detect whether virus growth was augmented further in constructs that have more than two synthetic GC motifs. To rule out the possibility that the DNA sequences of these viruses rearranged during virus growth, we performed restriction analyses of the replicated viral DNA. Also, viral DNA was cloned from plaques and sequenced, and the viruses were replaqued. We detected no sequence rearrangements occurring within 100 nucleotides of either side of the cloning site of SV21-0.

These results indicate that growth of SV40 in CV-1 cells is activated by two nonoverlapping synthetic GC motifs and that Sp1 is the only protein in CV-1 nuclear extracts which can be detected binding to these motifs. That two copies of any single GC motif are capable of replacing the 21-bp repeat region suggests that other proteins, such as Ap1, Ap4, LSF, H16, and SV-LT, are not responsible for the growth-promoting properties of the 21-bp repeat region, since their binding sites do not occur in every GC motif but are defined by sequences within or between individual motifs. However, it is possible that proteins with specificities similar to that of Sp1, such as ETF or GT-1B, may participate in activating SV40 growth in constructs with synthetic GC motifs, and until their properties are better understood, we will not be able to judge their involvement.

The specific sequences recognized by p53 in the 21-bp repeat region are unknown, but we were unable to detect any



VIRUS	IIFA	DNA REPLN. 48 h	PLAQUE SIZE,mm day 13
WT SV40	B	100	3.6
SV21-0	W	12	0
SVGC3,5-N	I	35	1.3
SVGC3,5-R	I	30	1.3
SV2GC3,5-N	B	95	3.2
SV3GC3,5-N	B	93	3.2
SV4GC3,5-N	B	92	3.2
SVGC1-N	I	35	1.3
SVGC1-R	I	36	1.3
SV2GC1-N	B	95	3.2
SV2GC1-R	B	95	3.2
SV4GC1-N	B	90	3.2

FIG. 7. (A) Properties of the viruses of the GC3,5 and GC1 series. Arrows depict the orientations of the Sp1 motifs relative to those of wt SV40. (B) DNA replication assay of GC3,5 series viruses. Only the replicated DNA is shown.

interaction between p53 and the individual GC motifs in gel mobility shift assays (data not shown). As GC3,5 restores function but is weakly bound by p53 in the context of the entire 21-bp repeat region, it seems unlikely that p53 binding to the 21-bp repeat region has a significant effect upon virus growth; however, this remains to be clarified further.

Correlation of Sp1 binding with the growth-promoting activities of GC motifs. If Sp1 is primarily responsible for the growth-promoting effect of the GC motifs, the functions of these motifs might correlate with affinity for Sp1. To test this, oligonucleotides (GA and GT), whose binding by Sp1 is reduced because each contains an AT base pair in place of a central GC pair within the consensus Sp1 binding site, were synthesized (Fig. 8A). With the exception of a partial Ap2 site in motif GT (5′-TGGG-3′), these oligonucleotides lack other known activator-binding sites. A gel mobility shift assay assessed the relative affinity of Sp1 for these oligonucleotides with dilute nuclear extract and probes that had the same specific activity. Binding of Sp1 to oligonucleotide GC was similar to that observed with the other GC motifs, whereas oligonucleotides GA and GT bound Sp1 about three- to fivefold less well (Fig. 8B). These oligonucleotides were cloned into SV21-0, and viruses containing two inserts in the normal orientation (SV2GC-N, SV2GA-N, and SV2GT-N) (Fig. 8C) were assayed for their functions. We observed that SV2GA-N and SV2GT-N manifested properties between those of wt SV40 and SV21-0, with SV2GA-N promoting SV40 growth less well than SV2GC-N but better than SV2GT-N (Fig. 9A and B).

We also examined the growth of viruses that contained more than two of these motifs and observed that while a

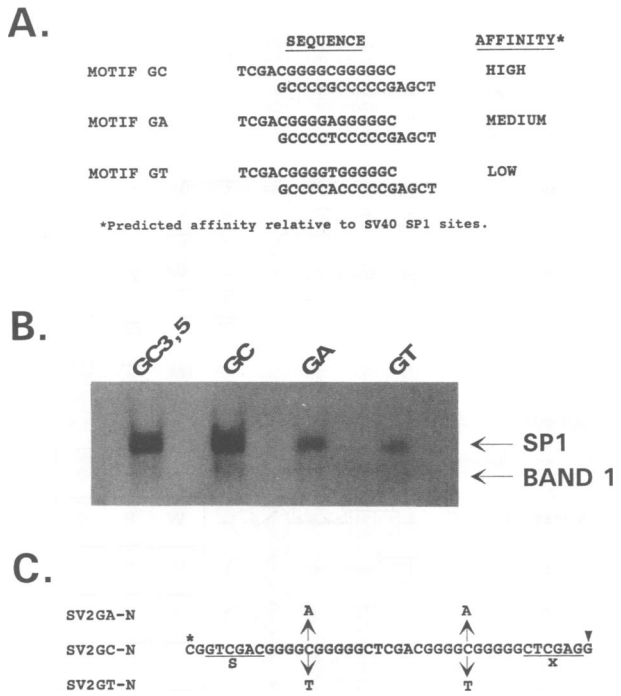


FIG. 8. (A) Sequences of motifs GC, GA, and GT. (B) Determination of relative affinities for Sp1 by motifs GC3,5, GC, GA, and GT. (C) The sequence of SV2GC-N between SV nt 34 (identified with an asterisk) and SV nt 108 (identified with an arrowhead). The *SalI* (S) and *XhoI* (X) sites are underlined.

construct with four copies of oligonucleotide GA approximated wild-type virus in its growth, a virus with five copies of oligonucleotide GT was still somewhat growth impaired (data not shown). Thus, unlike the situation encountered with constructs that contain high-affinity Sp1 sites, in which two sites sufficed for full growth promotion, more than two of the GT and GA motifs were needed for efficient growth in CV-1 cells. Presumably, this reflects the reduced occupancy by Sp1 of these sites in vivo. Even with the normal 21-bp repeat region, only late in infection is there substantial occupancy by proteins binding to the viral DNA in CV-1 cells (11). These observations support the suggestion that Sp1 is responsible for the growth-promoting activities of these oligonucleotides.

Growth-promoting activities of GC motifs are not positionally restrained. Constructs with two or more copies of GC motifs having high-affinity Sp1 sites are not quite fully wild type in their properties (Fig. 4 to 6). Is this because of the altered topology of Sp1 sites in these constructs? To study this, we made several changes in the spatial arrangement of the Sp1 sites, but we could detect no effect on function resulting from these changes (Fig. 10C and D). An example of such a virus is SV2SP1-N, which contains GC3,5 (Fig. 10). This construct differs from SV2GC3,5-N in the following ways. (i) The distance from the *ori*-proximal GC motif to the site of early transcription initiation was made more like that of wt SV40, since changes in the spacing between the 21-bp repeat region and the early promoter are reported to reduce early gene expression (3, 4, 19, 60, 96). (ii) The separation between the GC motifs was made equivalent to that of GC motifs III and V of wt SV40, placing the motifs on the same side of the DNA helix, which may affect their function (32, 35, 36). (iii) The distance from the distal GC motif to the enhancer was lengthened, so that it is the same distance from the late-transcription initiator as GC motif V is in wt SV40; this may affect late transcription (1, 35, 36, 41, 46, 78, 100).

As we were unable to fully restore the growth of viruses with only Sp1 sites to that of the wild type, it is likely that one or several other proteins besides Sp1 have a secondary role in the growth-promoting activity of the 21-bp repeat region. LSF is likely to be one such protein (61), and two other candidates are Ap2 and Ap1. However, it is unlikely that Ap2 contributes significantly to the function of the 21-bp repeat region, as motif GC4 promotes virus growth efficiently but should not be bound by Ap2. Additionally, we found that viruses with three or four Ap2 motifs inserted into SV21-0 exhibited growth characteristics that were unchanged from those of SV21-0 (Fig. 11). We also examined whether Ap1 binding on either side of the 21-bp repeat region promoted virus growth. This is of some interest, because Wang and Gralla have shown that Ap1 sites can efficiently cooperate with GC motifs to activate RNA polymerase II transcription (101). An Ap1 motif whose flanking nucleotides were those of the *ori*-proximal Ap1 site in SV40 was cloned into a construct with one GC motif (SVGC3,5-N) or two GC motifs (SV2GC3,5N), but its properties were indistinguishable from those of the parental DNAs (Fig. 11C), suggesting that the GC motifs function independently of Ap1 sites in promoting growth of SV40 in CV-1 cells.

DISCUSSION

Numerous studies have documented the importance of the 21-bp repeat region for activating SV40 early gene expression and for modulating viral DNA replication and late gene expression. Sequences in the 21-bp repeat region bind a wide array of cellular and viral proteins with differing activities; consequently, it is not readily apparent whether the functions of the 21-bp repeat region are attributable to a single

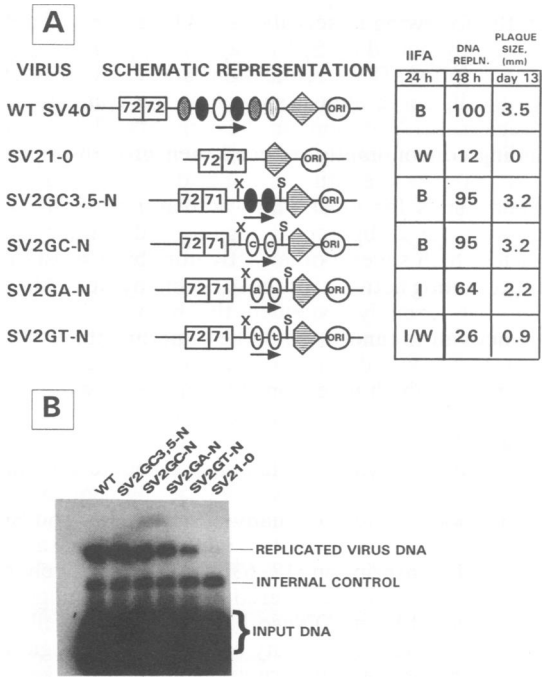


FIG. 9. Summary of the properties of (A) and DNA replication assay of (B) SV2GA-N, SV2GC-N, and SV2GT-N viruses.

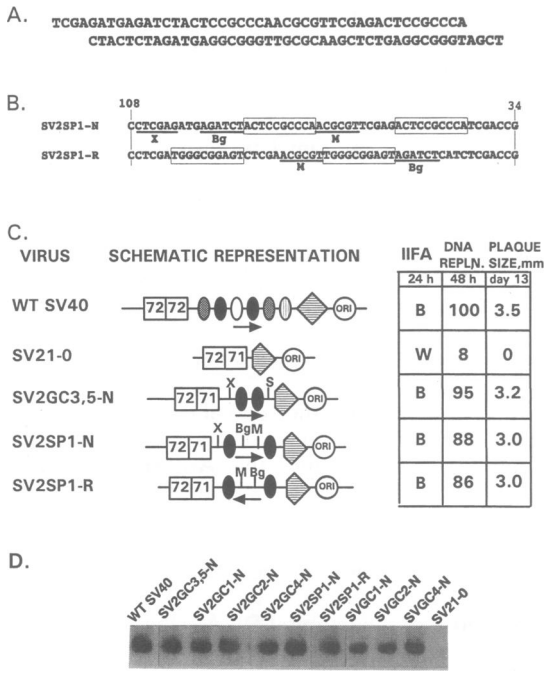


FIG. 10. (A) Sequence of GC2 Sp1. (B) Sequences of SV2SP1-N and SV2SP1-R between nt 34 and 108. *Bgl*II (Bg), *Mlu*I (M), and *Xho*I (X) sites are indicated below the sequences; Sp1 sites are boxed. (C) Summary of the properties of SV2SP1-N and SV2SP1-R viruses. (D) DNA replication assay, including members of the GC1, GC2, and GC4 viruses and GC_2 Sp1 viruses. Only the replicated DNA is shown.

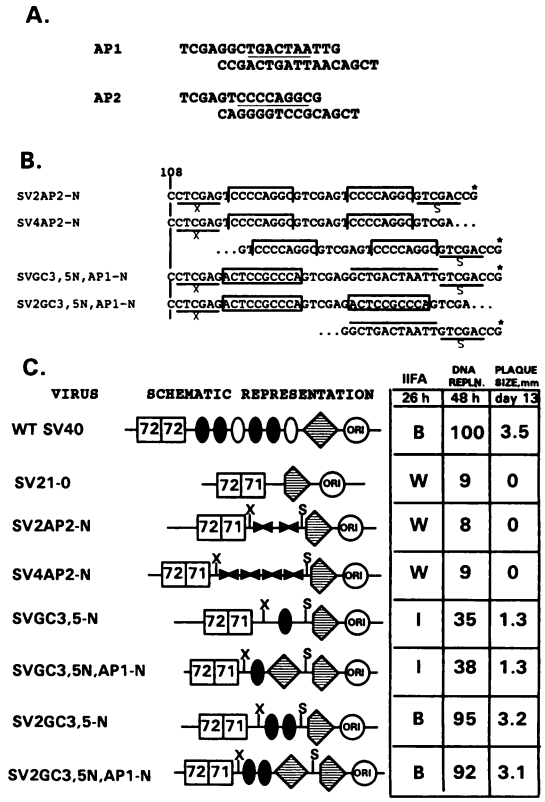


FIG. 11. (A) Sequences of Ap1 and Ap2 motifs, with the binding sites underlined. (B) Insert sequence between SV nt 34 and 108 in viruses of the GC3,5-AP1 and -AP2 series. Ap2 sites are bracketed, Sp1 sites are boxed, Ap1 sites are overlined, and *Sal*I (S) and *Xho*I (X) sites are underlined. SV nt 34 is indicated by the asterisk over the terminal G of each sequence. (C) Properties of these viruses.

protein or to many. In this study, we have established a clear correlation between SV40 growth in CV-1 cells and the quantity and quality of Sp1 sites in the 21-bp repeat region through the following observations. (i) One copy of a motif which is tightly bound by Sp1 is capable of promoting SV40 early transcription, DNA replication, and virus production, and two motifs serve almost as well as the complete 21-bp repeat region. (ii) Mutations in these motifs which reduced Sp1 binding concomitantly reduced their growth-promoting activities. (iii) These synthetic motifs did not contain obvious binding sites for most other activator proteins which interact with the 21-bp repeat region, and neither did sequences to which several other activators bind substitute in growth-promoting activity for those bound by Sp1. Although we have not formally excluded the binding of all other possible activators (and it would be experimentally difficult to do so), the preponderance of evidence suggests that the principal factor which is responsible for the growth-promoting activity of the 21-bp repeat region is Sp1.

How does Sp1 promote SV40 growth in CV-1 cells? It is likely in multiple ways. That histones compete for binding sites important for the activity of *cis*-acting sequences has been amply documented for many systems (27), and Sp1 is able to counteract H1-mediated inhibition of basal RNA polymerase II transcription (18, 63). Sp1 is very likely to be partly responsible for the altered chromatin configuration over *ori* (detected by its DNase I sensitivity) as well as for the heightened DNase sensitivity of the enhancer region (50, 56). This altered chromatin structure may help to keep the early and late promoters, *ori*, and the enhancer sequences accessible to *trans*-acting factors and also may help to order nucleoprotein complexes which initiate transcription and DNA replication (24).

Sequences adjacent to *ori*, including the 21-bp repeat region, facilitate SV40 DNA replication and transcription of templates from which nucleosomes have been stripped, so it is not solely through chromatin structure that Sp1 must act. The 21-bp repeat region promotes SV-LT-dependent DNA unwinding during the assembly of an initiation complex (6, 19, 33, 37, 39), and although SV-LT binds to several sites in the 21-bp repeat region, it is unlikely that such sites are essential for the function of these "auxiliary *ori*" sequences (43). Possibly, Sp1 binding to DNA helps to recruit SV-LT to the *ori* through protein-protein interactions in a fashion analogous to that shown to occur between Sp1 and the BPV E-2 protein (67) or the cellular OTF-1 protein (55). Alternatively, Sp1 may be capable of directly acting upon the *ori* to induce structural changes in DNA which accompany the formation of an initiation complex (7). The most likely target for its action would be the AT-rich segment which is distorted by SV-LT binding to site 2 in *ori* (7) and whose mutation can be compensated for by changes in the 21-bp repeat region (33). We are presently testing this notion.

Virtually all available data indicate that the 21-bp repeat region activates the early promoter; however, the control of the late promoter is less clear. Prior to the expression of SV-LT, the 21-bp repeat region appears to suppress late transcription (1, 78), but following SV-LT expression, the 21-bp repeat region activates late transcription (19, 36, 46).

The suppression of late transcription might result from the squelching of a mediator required for transcription (15, 83), while activation may result from LSF or Sp1 binding.

Sp1 activates transcription through multiple domains, and protein-protein interactions between oligomers bound to multiple sites result in synergistic transcriptional activation (10, 71, 80, 92). This synergism must occur through interactions with nascent transcription complexes rather than during DNA binding, since Sp1 molecules which lack the ability to bind DNA can still promote synergism (16). Sp1 interacts with specific cofactors (97) both to antagonize negative effectors and to potentiate positive effectors of the assembly of an initiation complex via TFIID (62, 73, 74, 82, 89). TFIID is required at both the TATA-containing SV40 early promoter and the TATA-less late promoter, and Sp1 should be capable of facilitating the assembly of initiation complexes at each (84, 90).

The specific infectivities of viruses lacking the 21-bp repeat region are not altered; this contrasts with the effect of eliminating the viral enhancer, which causes a marked reduction in specific infectivity (29, 44, 63a, 79). This suggests either a qualitative or a quantitative difference, or both, between the role in establishing a productive infection of the enhancer and that of the 21-bp repeat region. Considerable evidence suggests that the enhancer and the 21-bp repeat region can substitute for each other in promoting transcription and DNA replication (12, 36, 43, 45, 46), suggesting that there is perhaps only a quantitative difference in their functions within the SV40 genome, despite clear differences in their abilities to promote transcription from a distance (3, 5, 10). Consistent with this notion is our observation that constructs lacking the 21-bp repeat will express high levels of T antigen in rare cells at times long before the rest of the population (data not shown). This presumably reflects the establishment of stable transcription complexes on those genomes, as has been suggested to occur with SV40 genomes lacking enhancers (102). Similar arguments for the role of Sp1 in promoting transcription from TATA-containing and TATA-less promoters (62, 89), such as occurs in SV40, have been put forward. One qualitative difference between the SV40 enhancer-binding proteins and Sp1 may be their capacities to displace core nucleosomes already positioned on DNA. Sp1 appears unable to invade nucleosomes (63); while it is not known whether any of the proteins binding to the enhancer have this capacity, precedents for such exists (27). That core particles are preferentially localized on enhancer sequences assembled in vitro but not in vivo (14, 54, 88, 94) suggests the need for such a capacity. Several subelements within the SV40 enhancer which restore infectivity, albeit only weakly, to viruses lacking functional enhancers have been identified previously (59, 79) and are good candidates for binding sites of proteins that can displace nucleosomes.

Although two high-affinity Sp1 sites between the enhancer and *ori* suffice to nearly maximally activate SV40 growth in CV-1 cells, it is likely that sites in the 21-bp repeat region bound by other proteins may be used to modulate the activity of Sp1 or to promote SV40 growth in other cells and tissues in the animal host. These additional binding sites might have been created concomitantly with or after the duplication of Sp1 sites in the 21-bp repeat region, in some cases reducing the affinity of Sp1 for individual GC motifs. Nevertheless, while rapid growth may be advantageous to SV40 infecting CV-1 cells in culture, it may not be advantageous during infection of a host for which the establishment of a slow, persistent infection is favored. This prompts us to question whether SV40s freshly isolated from monkeys contain multiple Sp1 sites adjacent to *ori* or whether the formation of the 21-bp repeat region occurred during passage of the virus in cell culture. The closely related BK and JC viruses, when freshly isolated from humans, do not contain analogous repetitive sequences near *ori*, but their regulatory regions rapidly undergo sequence changes upon passage in cell culture. These viruses appear to have sequences which might function analogously to Sp1 in promoting virus growth (21, 69). This will be the subject of a subsequent report.

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