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Replication but Not Transcription of Simian Virus 40 DNA Is Dependent on Nuclear Domain 10

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DNA viruses from several families including herpes simplex virus type 1, adenovirus type 5, and simian virus 40 (SV40), start their transcription and replication adjacent to a specific nuclear domain, ND10. We asked whether a specific viral DNA sequence determines the location of these synthetic activities at such restricted nuclear sites. Partial and overlapping SV40 sequences were introduced into a β-galactosidase expression vector, and the β-galactosidase transcripts were localized by in situ hybridization. Transcripts derived from control plasmids were found throughout the nucleus and at highly concentrated sites but not at ND10. SV40 genomic segments supported ND10-associated transcription only when the origin and the coding sequence for the large T antigen were present. When the large T-antigen coding sequence was eliminated but the T antigen was constitutively expressed in COS-7 cells, the viral origin was sufficient to localize transcription and replication to ND10. Deletion analysis showed that only the large T-antigen binding site II (the core origin) was required but the T antigen was needed for detectable transcription at ND10. Large T antigen expressed from plasmids without the viral core origin did not bind or localize to ND10. Blocking of DNA replication prevented the accumulation of transcripts at ND10, indicating that only sites with replicating templates accumulated transcripts. Transcription at ND10 did not enhance total protein synthesis of plasmid transcripts. These findings suggest that viral transcription at ND10 may only be a consequence of viral genomes directed to ND10 for replication. Although plasmid transcription can take place anywhere in the nucleus, T-antigen-directed replication is apparently restricted to ND10.

The nucleus is a highly organized structure with chromosomes occupying discrete territories (19) and interchromosomal spaces demarcated by the presence of the interchromatinic granule clusters or speckles containing splicing components (29). The interchromosomal compartment also harbors ND10 (PML bodies or PODs), some of which are recognized as nuclear bodies (1, 24). The interchromatinic space is used by DNA viruses of several families as the intranuclear location to start transcription and replication (14, 21, 26). An immediate transcript environment has been defined that consists of ND10, where viral transcription takes place, and the speckles or SC35 domains, where the viral transcripts move before being dispersed throughout the nucleus (16). For human cytomegalovirus (HCMV), this immediate transcript environment is induced by individual viral genomes, implying that other DNA viruses, which also start their replication at these sites, must also be deposited there. The mechanism of genome deposition at ND10 is not clear, but two extreme possibilities can be envisioned. First, ND10 might contain specific proteins essential for virus transcription and replication, so that only viruses that randomly migrate to this site are able to transcribe. This idea is supported by the finding that input genomes are present at sites other than ND10 but are apparently not transcribed (16). Second, competent viral genomes might be transported to ND10, where they begin their transcriptional cascade and later replicate. Such transport requires an address on the genome either on the DNA or on the proteins bound to the viral DNA. In this study, we asked whether the ND10 targeting rests in a specific viral DNA sequence.

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Large DNA viruses such as herpes simplex virus type 1 or HCMV dismantle ND10 through the action of immediate-early proteins (18, 22, 34) or modify them by the E4ORF3 protein in the case of adenovirus type 5 (4, 10, 14). By contrast, simian virus 40 (SV40) retains recognizable ND10 during the early replication stages (14). SV40 also has a small genome and is therefore ideally suited for transfection experiments to determine the presence of a potential ND10-targeting DNA sequence. In addition to a limited number of genes, the SV40 regulatory sequences are compactly organized, with early and late promoters that overlap each other and the origin of replication (2). The SV40 T antigen protein (T-ag) is required for initiation of viral replication. Its DNA binding, melting, and helicase activities are mediated by three domains in the origin of replication: a perfect palindrome with four pentanucleotide repeats sufficient for tight origin binding of T-ag, the incomplete inverted repeat at the early flanking region of the palindrome that is melted by the T-ag, and a 17-bp A+T-rich segment at the late flanking side involved in the bidirectional progression of the replication bubble (25).

Our understanding of DNA replication has been greatly enhanced by the ability to replicate the SV40 genome in vitro (11, 12). However, such in vitro reactions are in contrast with the highly organized nuclear interior. Since the cellular components necessary for replication can be extracted from the nucleus and purified, they might be expected to diffuse freely in the nucleus. Thus, once viral genomes have gained access to the nucleus, they should transcribe and replicate at any point in the nucleoplasm. However, SV40 replication takes place at a few precisely localized sites (14). These obviously nonrandom replication sites must have properties that define and specifically localize the start of replication of SV40 and other DNA viruses. These properties are presently unknown.

Nuclear entry of plasmids containing SV40 has been ex-

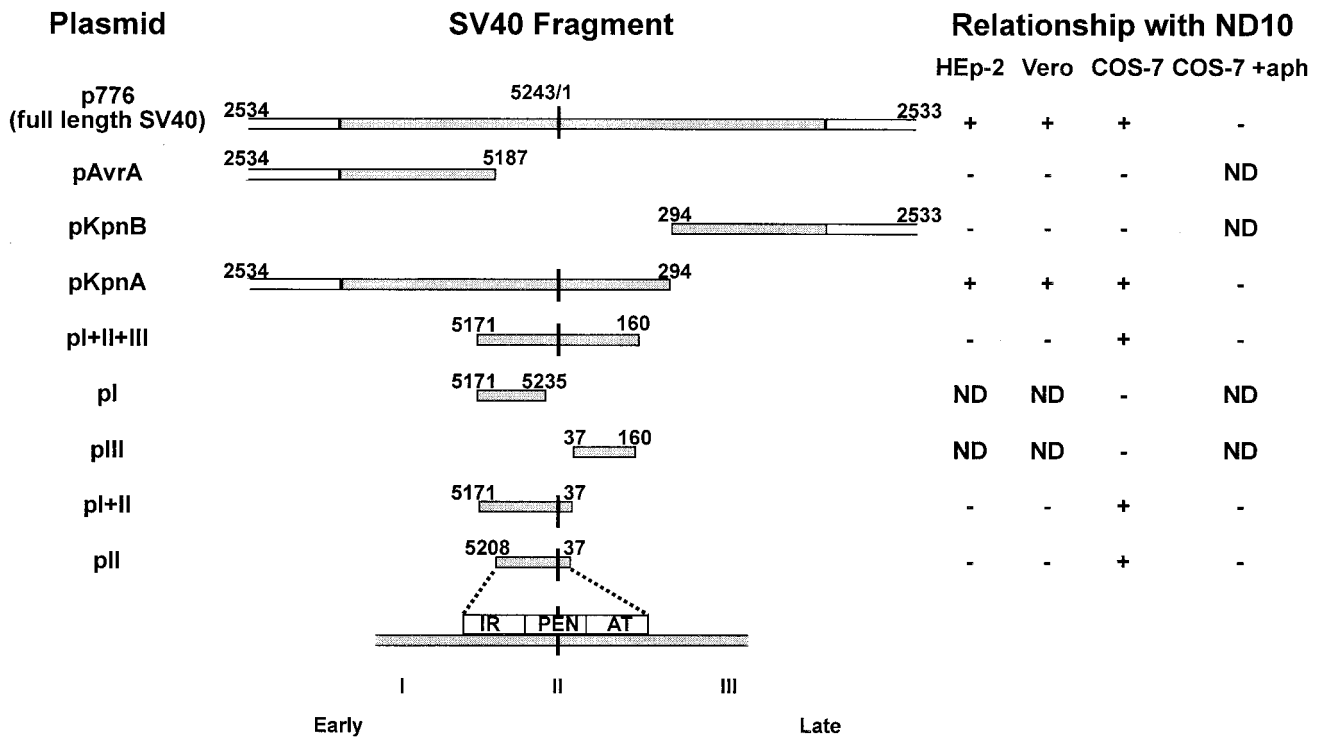

FIG. 1. Schematic diagram of SV40 fragments used and β-galactosidase transcript concentrations found in association with ND10. SV40 fragments present in the pRSVZ expression vector are shown relative to the origin of replication. Domains of the origin of replication according to the convention in reference 25 are given on the bottom line. The association of transcripts with ND10 for HEp-2, Vero, and COS-7 cells is shown on the right. +, association with ND10; −, no association (diffuse distribution); ND, not determined; IR, imperfect repeat domain; PEN, pentanucleotide palindrome; AT, adenine+thymidine-rich region; aph, aphidicolin.

plored, and sequences encompassing the origin of replication and the enhancer were found to be important in transport from the cytoplasm to the nucleus of microinjected or transfected plasmid DNA (7, 35). Binding of transcription factors to the viral sequences and transport directed by the nuclear targeting signals are thought to direct the plasmid transport through the nuclear pore complex. Migration within the nucleus might occur by diffusion or be partly guided along interchromosomal spaces (19). The latter possibility may lead to a selective movement that increases the probability that the plasmid DNA will reach the interchromosomally located ND10. We show here that plasmid transcription can take place throughout the nucleus but that the SV40 core origin of replication, in combination with expressed T-ag and the ability to replicate, is required for ND10-associated viral transcription.

MATERIALS AND METHODS

Antibodies and cell culture. ND10 were visualized using rabbit serum against Sp100 (17) and monoclonal antibody 138 directed against NDP55 (1). HEp-2, Vero, and COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For immunohistochemistry and in situ hybridization, cells were grown on round coverslips (Corning Glass Inc., Corning, N.Y.) in 24-well plates. Transfection was performed at 70 to 80% confluency with equal molar amounts of each plasmid, using the DOSPER transfection reagent (Boehringer Mannheim), as specified by the manufacturer. For β-galactosidase assays, cells were grown in six-well plates to 70 to 80% confluency prior to transfection.

Plasmids and molecular cloning. For testing the ND10-targeting behavior of SV40 sequences, we used plasmid pRSVZ (obtained from American Type Culture Collection) as vector (20). This plasmid lacks any sequences of DNA viruses except for the SV40 polyadenylation signal and contains the β-galactosidase gene under control of a Rous sarcoma virus promoter. A series of SV40 fragments were cloned into the blunt-ended *Xba*I site of pRSVZ, resulting in the following plasmids (Fig. 1): pI, which contains T-ag binding site I; pII, which contains T-ag binding site II, i.e., the core origin of replication (5′-CCTCACTACTTCTGGAATAGCTCCAGAGGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAAAAAATTAGTCAGC-3′) (9); pIII, which contains T-ag binding site III; pI+II, which contains both T-ag binding site I and the SV40 core origin of replication; pI+II+III, which contains all known origin components including the DNA sequences of the origin of replication plus T-ag binding site I and three 21-bp repeats of the early promoter that overlap T-ag binding site III; pKpnA, which contains the T-ag coding sequence plus the core origin of replication; pKpnB, which contains SV40 late genes; pAvrA, which contains only the T-ag coding sequence; and p776, which contains the entire SV40 sequence. Fragments I, II, III, I+II, and I+II+III were derived from plasmids pOR1, pOR2, and pOR4 (9), whereas fragments KpnA, KpnB, AvrA, and p776 were subcloned from pW5BS, which contains the entire SV40 sequence (obtained from Stratagene, La Jolla, Calif.).

The enhanced green fluorescent protein (EGFP)-expressing vector pEGFP-C1 was obtained from Clontech (Palo Alto, Calif.). pGFP-NLS was constructed by adding a nucleus localization sequence to the EGFP coding sequence of pEGFP-C1 (15). pEGFP-Sp100 expresses a fusion protein of full-length human Sp100 and EGFP.

Inhibition of DNA replication. To inhibit DNA replication, cells were incubated with the DNA-DOSPER mixture for only 1 h and, after removal of the DNA-containing medium, incubated for 23 h in supplemented Dulbecco's modified Eagle's medium containing 10 μg of aphidicolin (Sigma [13]) per ml. This method minimized the reduction in the transfection rate induced by aphidicolin (see Results). Controls were performed in the same way but in the absence of aphidicolin. To quantitate β-galactosidase expression in the presence of aphidicolin, we had to normalize to the transfection rate of aphidicolin-free controls. To measure the decrease in the transfection rate caused by aphidicolin, HEp-2 cells were transfected in 24-well plates with equal molar amounts of pEGFP-C1, pEGFP-Sp100, or pEGFP-NLS and aphidicolin was added at 10 μg/ml immediately or 1 h after the start of transfection. When DNA-DOSPER-containing medium was removed 4 h later, aphidicolin was also added to the new medium, thus ensuring a continuous presence of the drug. Control cells were transfected in the absence of aphidicolin. At 24 h after transfection start, GFP-expressing cells were counted relative to the total cell number with a Leitz Fluovert inverted microscope.

Preparation of cell extracts and β-galactosidase assay. Extracts were made from HEp-2 and COS-7 cells after transfection as described previously (27). Briefly, cells were washed three times in phosphate-buffered saline (PBS) and resuspended in 250 mM Tris-Cl (pH 7.8). After the cell concentration was

determined, cells were disrupted by three cycles of freezing and thawing and the supernatant was used for the β-galactosidase assay. To determine the β-galactosidase activity, 10 μl of each cell extract was mixed in a 96-well plate with 1 μl of 0.1 M $MgCl_2$ containing 4.5 M β-mercaptoethanol, 22 μl of 4-mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside) in 0.1 M sodium phosphate (Na_2HPO_4, NaH_2PO_4 [pH 7.5]), and 67 μl of 0.1 M sodium phosphate (pH 7.5). The mixture was incubated at 37°C for 30 min, and the reaction was stopped by adding 167 μl of 1 M Na_2CO_3. The optical density was determined at a wavelength of 405 nm with a KC Junior Matrix system (Bio-tek Instruments Inc., Winooski, Vt.).

Immunohistochemistry and fluorescent in situ hybridization. For the simultaneous detection of ND10 and specific DNA or RNA sequences, we first carried out immunostaining for ND10 proteins and, after refixing the cells to cross-link the bound antibodies, performed fluorescent in situ hybridization in a second step (23). Cells grown on coverslips were first fixed in 1% paraformaldehyde (10 min at room temperature) at 24 h after transfection, permeabilized in 0.2% Triton (20 min on ice), and incubated with primary and Texas Red-labeled secondary antibodies (Vector Laboratories, Burlingame, Calif.) for 30 min each (all solutions in PBS). Cells were treated for 1 h at 37°C either with RNase-free DNase I (Boehringer; 200 U/ml in PBS containing 25 mM $MgCl_2$) for detection of RNA or with RNase (Boehringer; 100 μg/ml in PBS) for detection of DNA. After refixation in 4% paraformaldehyde (10 min at room temperature), specimens were equilibrated in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dehydrated in an ethanol series (70, 80, and 100% ethanol for 3 min each at −20°C), air dried, and incubated overnight at 37°C with the hybridization mixture. As probe we used pRSVZ which was labeled with biotin-11-dUTP by nick translation. The DNase concentration was adjusted to yield probe DNA with a fragment length of 200 to 500 bp. Probe DNA was dissolved at 10 ng/μl in Hybrisol VII (Oncor, Gaithersburg, Md.) containing 100 ng of salmon sperm DNA (Gibco BRL) per μl, 1 μg of yeast tRNA (Sigma) per μl, and 0.5 mg of cot1 DNA (Gibco BRL) per μl. For DNA detection, the probe and cells were simultaneously heated at 94°C for 4 min to denature the DNA. To detect RNA, only the probe DNA was denatured at 94°C for 5 min. After hybridization, specimens were washed at 37°C with 55% formamide in 2× SSC (twice for 15 min) and without formamide with 2× SSC (once for 10 min) and 0.25× SSC (twice for 5 min). Hybridized probes were labeled with fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories; 1:500 in 4× SSC plus 0.5% bovine serum albumin) and signals were amplified using biotinylated anti-avidin (Vector Laboratories, 1:250) followed by another round of FITC-avidin staining. Finally, the cells were equilibrated in PBS and mounted in Fluoromount G (Fisher Scientific).

Confocal images of the cells were obtained using a Leica confocal laser-scanning microscope. The two channels were recorded simultaneously if no cross talk could be detected. When strong FITC labeling was present, sequential images were acquired with more restrictive filters to prevent possible breakthrough of the FITC signal into the Red channel. Both acquisition modes resulted in the same images. Leica enhancement software was used in balancing the signal strength, and the image was scanned eight times to separate signal from noise.

RESULTS

SV40-containing plasmids replicate and transcribe at ND10. We previously showed that SV40 replicates at ND10 in virus-infected cells (14). When HEp-2 cells were transfected with a plasmid containing the full genome of SV40 and tested for the presence of DNA by in situ hybridization and for ND10 by immunohistochemistry using antibodies to Sp100, SV40 DNA was found to be localized adjacent to ND10 (Fig. 2A). That this location of the viral DNA does not simply reflect the direct deposition of input DNA but, rather, shows SV40 DNA that has replicated was demonstrated by inhibiting DNA replication and by testing transfected cells at shorter incubation times, which showed no replicated DNA in the nucleus (shown for shorter incubation times in Fig. 2B). Transfected DNA was sometimes seen as large clumps beside the cells or inside the cytoplasm (Fig. 2B), whereas no such clumps were apparent inside the nucleus in our experiments. When cells were tested for the location of transcripts by eliminating the RNase treatment and the denaturing heat step, the bulk of the concentrated in situ hybridization signal was situated beside ND10, as in the case of DNA (Fig. 2C). Smaller in situ signals were found throughout the nucleus and may represent areas where the optical section does not include ND10, transcripts from plasmids not at ND10, or transcripts in transit. Such signals were absent when cells were pretreated with RNase (Fig. 2D). These images indicate that transfected SV40-containing plasmids start their replication and transcription at ND10 and suggest that none of the capsid proteins are necessary for such activity. The transcription assay also controls for any potential DNA recognition of input DNA.

In HEp-2 cells the origin of replication and the T-ag gene are necessary for transcription at ND10. To identify SV40 sequences essential for the appearance of concentrated transcripts at ND10, several large SV40 fragments were introduced into the vector pRSVZ, which does not contain any DNA virus sequences except the SV40 poly(A) signal (Fig. 1) (see Materials and Methods). The vector also contains the β-galactosidase reporter gene. When these vectors were transfected and the transfected cells were tested by in situ hybridization, the β-galactosidase transcripts of the pRSVZ vector were mostly diffusely distributed (Fig. 2E). However, in about 10% of cells transfected with the pRSVZ vector, transcripts were found at localized higher concentrations throughout the nucleus, like those containing the SV40 genome, but these concentrations of transcripts were not located at ND10 (Fig. 2F). Transcripts from these plasmids also appeared to disperse faster than those transcribed from the vector containing the full SV40 genome. When the segments containing the T-ag (AvrA fragment) or the late promoter region and capsid proteins (KpnB fragment) were transfected into HEp-2 cells, neither of them produced β-galactosidase transcripts concentrated at ND10. Instead, most transcripts were diffuse or concentrated at sites other than ND10 (shown in Fig. 2G for pAvrA only), similar to those shown for the pRSVZ vector (Fig. 2E and F). These findings show that (i) transcripts are produced from expression vectors containing partial SV40 sequences, (ii) such transcribing plasmids are not concentrated at ND10, (iii) transcripts are not secondarily placed at ND10, and (iv) the T-ag does not place the plasmid at ND10. Parallel control cultures were tested for the expression of β-galactosidase to ensure that the transcripts could be translated and to estimate the number of cells transfected.

We then inserted the remaining region containing the origin of replication into the pRSVZ expression plasmid and tested for the localization of transcripts originating from this plasmid. Again, transcripts from these constructs were mostly dispersed in the nucleus (Fig. 2H). Indeed, none of the individual viral DNA fragments we tested resulted in deposition at ND10. We then tested whether an overlapping region of the origin and T-ag genomic sequence would give the same results as seen with the intact viral genome. In situ hybridization analysis of the KpnA fragment containing the origin and the T-ag coding sequences showed a side-by-side configuration of ND10 and transcripts in almost all transfected cells (Fig. 2I). Such plasmids also replicated as shown when probed for viral DNA using RNA digestion and heat denaturation (Fig. 2J). This KpnA segment of the SV40 genome is rather large, raising the question whether the targeting sequence was present at the overlap of the origin and the T-ag gene or whether T-ag as a protein, but not as a genomic part of the plasmid, was essential for the transcript emergence at ND10.

The T-ag binding region of the origin represents the minimal ND10 deposition sequence. To test whether the SV40 origin alone can act as the ND10 targeting signal, we used COS-7 cells, which constitutively express T-ag in *trans*. We could therefore test whether the DNA segment overlapping with the origin region in the KpnA fragment was essential or whether only the presence of T-ag was essential. As control cells we used Vero cells, from which COS-7 cells were derived and which do not contain the T-ag. When plasmids containing the origin alone were transfected into COS-7 cells, the tran-

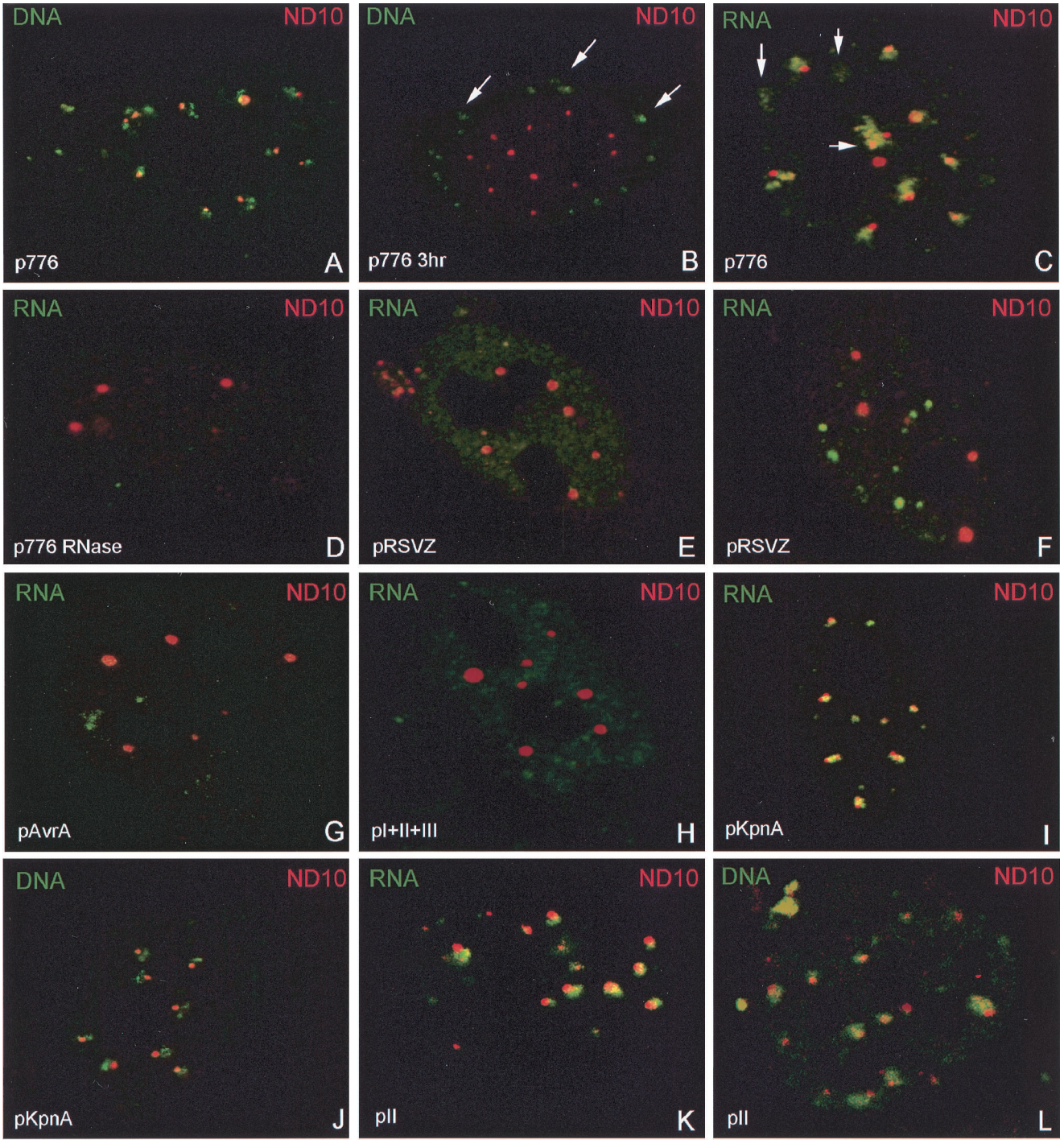

FIG. 2. In situ hybridization and immunohistochemistry of HEp-2 and COS-7 cells 24 h after transfection with various SV40 fragments spliced into pRSVZ expressing β-galactosidase. The different components probed for are indicated by their respective colors in the upper left and right corners. The in situ hybridization signal is always shown in green, and the ND10 signal is shown in red. Single representative nuclei are shown to retain a large magnification. (A) HEp-2 cell transfected with a plasmid containing the full-length SV40 genome (p776). In situ hybridization shows replicated small clumps of SV40 DNA beside ND10. (B) Same as panel A but only 3 h posttransfection, showing that no input plasmid DNA has entered the nucleus and that no replication has taken place. DNA is present in the cytoplasm (arrow). (C) HEp-2 cell transfected with a plasmid containing the full-length SV40 genome after DNase digestion and probed for transcripts by in situ hybridization. SV40 transcripts are located beside ND10. The yellow color is thought to come from the projection of transcripts and ND10 (horizontal arrow) within the depth of the optical section. The vertical arrow points to in situ signals without apparent ND10. (D) HEp-2 cell transfected with a plasmid containing the full-length SV40 genome after RNase digestion. In situ hybridization to transcripts shows no cross-reactivity with replicated SV40 DNA. (E) pRSVZ transfected into a HEp-2 cell and probed for β-galactosidase transcripts by in situ hybridization. The signal from the transcripts is present in a diffuse distribution excluding the nucleoli. (F) Same as panel E but showing the distribution of transcripts to be highly concentrated but not at ND10 (in a small number of cells). (G) HEp-2 cell transfected with the AvrA fragment of SV40 (pAvrA). Transcripts are present in a concentrated distribution but not at ND10. (H) HEp-2 cell transfected with the origin of replication containing all the T-ag binding sites (pI+II+III). No transcripts are found at ND10; instead, they are dispersed throughout the nucleus. (I) HEp-2 cell transfected with the origin and T-ag gene-containing segment of SV40 (pKpnA). Transcripts are found beside ND10; yellow parts are due to the projection of red and green signals within the optical section. (J) Same as panel I but probed for DNA. Replicated plasmid is found beside ND10. (K) COS-7 cell transfected with the SV40 core origin (T-ag binding site II) containing plasmid only (pII). Transcripts are concentrated at ND10. (L) COS-7 cell transfected with the SV40 origin-containing plasmid (pII) and probed for replicated DNA. DNA signal is found beside ND10.

scripts were found beside ND10. This localization was not seen in Vero cells. Thus, the T-ag itself rather than any genomic stretch of the T-ag is necessary in combination with the sequences at the origin of replication for ND10 localization.

There are three T-ag binding sites in the origin region (5, 8, 9). We asked if all are necessary for ND10 targeting. When tested, only constructs containing T-ag binding site II resulted in transcripts at ND10 (Fig. 2K) whereas neither T-ag binding site I nor T-ag binding site III showed this effect. The T-ag binding site II plasmid was also able to replicate in COS-7 cells (Fig. 2L). Thus, although all expression vectors could transcribe, transcripts and replication products were found only at ND10 when the core origin was present and T-ag was available for core origin binding.

The potential for replication is necessary to find transcripts at ND10. All of our in situ hybridization-based tests had been performed for transcripts and DNA. We had therefore recognized in parallel cultures that DNA could be replicated when transcripts were found at ND10. Inversely, when we did not find transcripts at ND10 but detected them spread throughout the nucleus, we did not find replicated plasmids. We therefore asked whether transcripts would be found at ND10 when DNA replication was inhibited. When DNA replication was inhibited with aphidicolin from the time of transfection, no increased concentrations of β-galactosidase transcripts were found at ND10 when either HEp-2 or COS-7 cells were used (data not shown). This observation indicates that either the accumulation of the core origin and T-ag produced a specific environment to confine the transcripts in a contained area and/or the larger number of replicated plasmids transcribed at a specific site increases the visibility of the transcripts.

The potential to transcribe at ND10 has no impact on the amount of protein produced. We tested whether transcription at ND10 had an effect on the amount of protein synthesized. Since the potential amplification of the genomes at ND10 would make the comparison invalid, we tried to inhibit replication and measured the β-galactosidase activity. During the replication inhibition experiments, it became clear that aphidicolin severely restricted transfection. This restriction was quantitated by transfecting HEp-2 cells with plasmids expressing GFP with a nuclear targeting signal in the presence or absence of aphidicolin. The average transfection rate was approximately 17% in control cells but only 3% when aphidicolin was added at the time of transfection. When aphidicolin was added at various times after transfection, the rate increased to 9% after 1 h, 10% after 2 h, and 13% after 4 h. We used the 1-h interval for further experiments to ensure that no replication took place when the origin of replication and T-ag were present. The rate of cell transfection in the absence of aphidicolin was still twice that in its presence, and we needed to normalize by counting the number of transfected cells in each sample.

When HEp-2 cells were transfected with plasmids containing various SV40 segments and normalized for transfection rate, it became clear that an increase in the β-galactosidase activity was detected only when replication was possible (Fig. 3A). The activity was about twice that found in the absence of SV40 segments or fragments that could not replicate due to the absence of the origin or the T-ag. Blocking replication with aphidicolin reduced the β-galactosidase activity to the level found in the control plasmid (pRSVZ). The experiment was repeated with COS-7 cells. In these cells the core origin-containing plasmid provided a higher β-galactosidase activity. Aphidicolin reduced this activity to the level found in the control plasmid, indicating that the higher β-galactosidase activity produced was due to the replication of the template (Fig. 3B). These experiments show that the ability to replicate at ND10 had no transcriptional advantage that was measurable at the level of β-galactosidase activity.

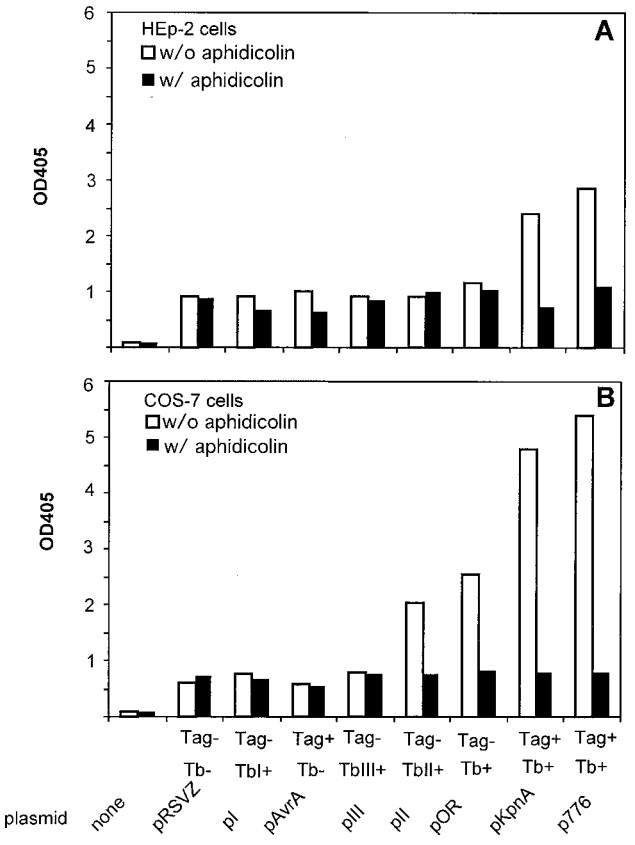

FIG. 3. Assay for β-galactosidase expressed from plasmids containing various SV40 regions. (A) Transfections in HEp-2 cells. (B) Transfections in COS-7 cells. Cells were transfected for 1 h and incubated with or without aphidicolin (10 μg/ml) for 23 h. T-ag and Tb indicate the presence or absence of T-ag and T-ag binding sites. Plasmid designations are as in Fig. 1. OD405, optical density at 405 nm.

DISCUSSION

DNA viruses from three families have been shown to start their transcription and replication at a specific nuclear domain, ND10. Two of the best-studied proteins of this domain, PML and Sp100, have interferon response elements and thus can be upregulated by interferon. Also, the interferon-induced GTPase MxA and an Mx-interacting protein kinase are deposited at these sites (32). A negative action of these domains with respect to virus replication had been anticipated and seems to be supported by a reduction in viral progeny when PML is present in excess (10) or when the viral proteins that destroy the integrity of ND10 are deleted from these viruses (3, 30). It is therefore remarkable that viral genomes are found to transcribe and replicate at these sites. Initial transport of virus genomes to the nucleus most probably occurs through a microtubule-mediated positioning at the nuclear pores followed by active transport of the viral genome into the nucleus (28). Further transport to or deposition of viral genome DNA at ND10 can be imagined to be an active process since HIV provirus DNA or plasmid DNA is not deposited at ND10 (P. Bell et al., unpublished data). A selective deposition implies

that a specific address is carried in the viral genome. Such an address may code for a specific protein binding site that allows the ND10 binding, or it may be a specific DNA sequence that binds to ND10-associated proteins, resulting in a capture mechanism at ND10. In either case, such a sequence could be mapped by splicing selected sequences into an expression vector that produces transcripts independent of the potential targeting sequence.

When this approach was used, more than a specific DNA sequence in SV40 was necessary. None of the fragments of SV40 sequences alone allowed an ND10-associated transcript enrichment to be seen by using all possible overlaps. However, in the presence of T-ag, a minimal sequence corresponding to the T-ag binding site II at the core origin was determined. Other T-ag DNA binding sites flanking the origin did not have this effect. The T-ag alone did not accumulate at ND10, nor did a plasmid with the genomic sequence expressing T-ag transcribe in association with ND10. Most origin-containing plasmids not only transcribed but also replicated at this site when T-ag was present. This increase in template may have enhanced the transcription signal at ND10. However, the core origin, i.e., in the absence of the flanking T-ag binding sites I and III, replicates at negligible amounts compared with the wild-type virus at 3 days after transfection (9). Our assay was carried out 24 h after transfection, so that we could expect only minimal replication. We also tested the core region while suppressing DNA replication. In this case, no β-galactosidase transcripts accumulated at ND10 and the β-galactosidase levels were comparable to control levels. The trivial explanation is that the transcript accumulation is detectable only because of the large number of replicated and transcribing plasmids. It is likely that this replication contributes to the easier recognition of transcript accumulations. However, we have frequently seen transcript accumulations in the absence of the origin and T-ag, at early times after transfection, indicating that the higher concentrations are most easily recognizable when mRNA levels in the nucleus are low. Also, we have seen transcript accumulation at early times after transfection of the origin and T-ag gene-containing plasmids when the levels of replicative products should be low and with plasmids that contain the core origin, which replicates only marginally within 24 h (9). In addition, the increase in the β-galactosidase activity produced is not high. Therefore, we should consider that in the presence of the origin of replication and T-ag, an immediate transcript environment is created that depends on the ability to replicate. Initially the immediate transcript environment had been defined for HCMV (16), which has a delay of several hours between the start of transcription and replication. The specificity of the site for transcription was more remarkable in that about 80% of HCMV genomes were present at sites other than ND10.

Transcripts from plasmids without the SV40 origin of replication and from the control plasmid were observed mostly as a diffuse signal. Only a few such cells expressing β-galactosidase had the transcripts concentrated like those from plasmids containing the replication origin. In those cases, the concentrated transcripts were not found in association with ND10. Transcription from plasmids is therefore not dependent on their association with ND10, nor do templates that transcribe at ND10 seem to have a recognizable advantage or disadvantage. The presence of the core origin in association with T-ag and the capacity to replicate, as well as the results of the DNA synthesis inhibition assay, suggest that it is not transcription but the start of replication that is causally associated with a specific nuclear site.

The dependence of ND10-associated replication on the presence of the origin of replication and early proteins like T-ag is likely to be a common feature in papillomaviruses. Similar to our initial finding for SV40 (14), transient transfection of a plasmid carrying the origin of replication of the human papillomavirus (HPV) was found to replicate at ND10 in the presence of the early HPV proteins E1 and E2 (31). Whether the origin construct was able to replicate in the absence of E1 and E2, which are functionally equivalent to the SV40 T-ag, has not been reported. Interestingly, both proteins also localized at ND10 when transfected into cells together with the origin-containing plasmid. This localization pattern of E1 and E2 was found at a lower frequency in the absence of the origin (31). In contrast to HPV, in bovine papillomavirus only E2, but not E1, was found to be targeted to ND10 in infected cells. The deposition of E2 at ND10 was dependent on the expression of a late protein, the minor capsid protein L2. When E2 and L2 were coexpressed in cells, both proteins localized at ND10, whereas when expressed alone, only L2 but not E2 became ND10 associated (6). T-ag has also been reported to locate at ND10 (4); however, we did not see any enrichment at ND10 in untransfected COS-7 cells. However, the presence of T-ag can be explained by the replication of the plasmid used. It contains the origin of replication and T-ag similar to our pKpnA and will accumulate T-ag at the site of replication as previously shown (14).

Recently, the T-ag association with the core origin has been imaged at high resolution, and it appears that the total core origin DNA is contained within the two octameric T-ag tubes (33). Binding of the SV40 core origin DNA to an ND10-associated protein is therefore unlikely. With this in mind, we may construct the following scenario as a working hypothesis. Because expression plasmids without viral DNA sequences can transcribe in the nucleoplasm, T-ag might be transcribed any time after the virus enters the nucleus. Plasmids or viruses having T-ag bound to the origin might be deposited actively or arrive passively at ND10, and only those can begin replication. Other plasmids not localized at ND10 might be destroyed by nucleases or might be silenced by heterochromatinization, as expected for episomal DNA of herpes simplex virus or Epstein-Barr virus. Deposition and retention of the replication potential only at ND10 may come about through preventing genome degradation at these sites with a concomitant increase in the chance to replicate there. Such a possibility would suggest a protective mechanism for the viral genome, previously formulated as the nuclear depot hypothesis (21).

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