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Identification within the Simian Virus 40 Genome of a Chromosomal Loop Attachment Site That Contains Topoisomerase II Cleavage Sites

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We demonstrate that the simian virus 40 genome contains a single MAR (matrix association region) that maps within a large T-antigen coding region (nucleotides 4071 to 4377). This region contains topoisomerase II cleavage sites, exhibits sequence similarity with cellular MARs, and recognizes the same evolutionarily conserved, abundant nuclear binding sites seen by cellular MARs.

DNA within eucaryotic interphase nuclei and mitotic chromosomes is organized into topologically constrained looped domains ranging from 5 to 200 kilobases in length (3, 11, 35, 51). DNA sequences that mediate chromosomal loop attachment can be identified by using an in vitro assay that localizes MARs (matrix association regions) within cloned cellular genes (8). This approach can be complemented by a nuclear "halo" mapping procedure (31) which uses nuclear fractionation of endogenous sequences to identify scaffold attached regions (18). It is significant that both assays identify the same fundamental class of attachment site sequences (8, 22, 36). MARs (or scaffold attached regions) are A+T rich (ca. 70%), at least 250 base pairs (bp) long, contain topoisomerase II consensus sequences and other A+T-rich sequence motifs, sometimes reside near *cis*-acting regulatory sequences, are evolutionarily conserved, and their nuclear binding sites are abundant (>10,000 per mammalian nucleus) (1, 8–10, 18, 19, 22, 30, 32). Such sequences have been identified in specific genetic loci in cellular DNA derived from human (6, 23, 47), mouse (8, 10), hamster (15, 25), chicken (36), *Drosophila melanogaster* (18, 19, 22, 30–32), and yeast (1).

Previous studies have shown that simian virus 40 (SV40) minichromosomes are associated with the nuclear matrix in infected cells (4, 34). Furthermore, by using the nuclear halo mapping procedure, Prives et al. (38) have found that two specific regions in the SV40 genome mediate nuclear matrix attachment, independent of transcription or of whether the viral genome has been stably integrated into cellular DNA. Here, by using the in vitro assay we demonstrate that these SV40 sequences recognize the same evolutionarily conserved, abundant nuclear binding sites as those seen by cellular MARs. The major SV40 MAR resides within the large T-antigen gene, shares sequence similarity with cellular MARs, and contains topoisomerase II cleavage sites.

Initially we compared the matrix binding preference of linearized SV40 DNA with that of the MAR-containing mouse immunoglobulin κ gene and the MAR-lacking pBR322. Since T antigen appears to be localized, in part, in the nuclear matrix (12, 46, 50) and is known to bind to the SV40 origin (48), to simplify the interpretation of our results, we decided to determine whether SV40 DNA would specifically bind to nuclear matrices of noninfected cells. Fragments were ^{32}P end labeled and associated with matrices in the presence of increasing amounts of unlabeled *Escherichia coli* DNA, employing the standard competitive DNA binding assay used to identify MARs (8). Figure 1A shows that matrices isolated from several mouse cell lines (8, 39) each preferentially retain both SV40 and κ gene DNA fragments to a similar extent relative to pBR322. For example, under conditions where about 15% of the input SV40 genome binds, about 15% of the input κ gene segment also binds, while less than 1% of the input pBR322 is recovered (Fig. 1A, lane 3). We conclude that the SV40 genome specifically binds to nuclear matrices in vitro.

To localize the MARs within the SV40 genome, we performed additional binding studies. Figure 1B shows that the MAR resides on a 2,206-bp *Bam*HI-*Taq*I fragment, which exhibits 5- to 10-fold-greater retention than the other *Bam*HI-*Taq*I SV40 fragment (Fig. 1B, lane 1) (see map in Fig. 1G). Further mapping revealed that the MAR resides on a 1,097-bp *Dde*I fragment (Fig. 1C), a 766-bp *Hin*fI DNA segment (Fig. 1D), and a 823-bp *Bst*NI fragment (Fig. 1E). As shown in Fig. 1G, these sequences each predominantly reside within the larger 2,206-bp *Bam*HI-*Taq*I fragment and overlap one another by only 306 bp. Furthermore, the 306-bp *Bst*NI-*Hin*fI fragment encompassing this overlap region also preferentially binds to matrices (Fig. 1F). Thus, a region between nucleotides 4071 and 4377 of the SV40 genome (17), which resides within a large T-antigen exon, constitutes a MAR. A weaker secondary MAR was also identified, corresponding to a 673-bp *Bst*NI fragment (Fig. 1E) and a 398-bp *Bst*NI-*Hin*fI fragment (Fig. 1F), which is localized between nucleotides 3610 and 4008 (17). It is particularly significant that both the primary and secondary MARs identified here by the in vitro assay correspond to the two regions previously mapped by Prives et al. (38) using the nuclear halo technique.

Previous studies have shown that cellular MARs from diverse sources compete for similar binding sites in nuclear matrices (1, 8, 22, 36), and saturation binding experiments performed in the absence of competitor DNA reveal that these binding sites are abundant (8). To determine whether the SV40 MAR recognizes the same abundant binding sites

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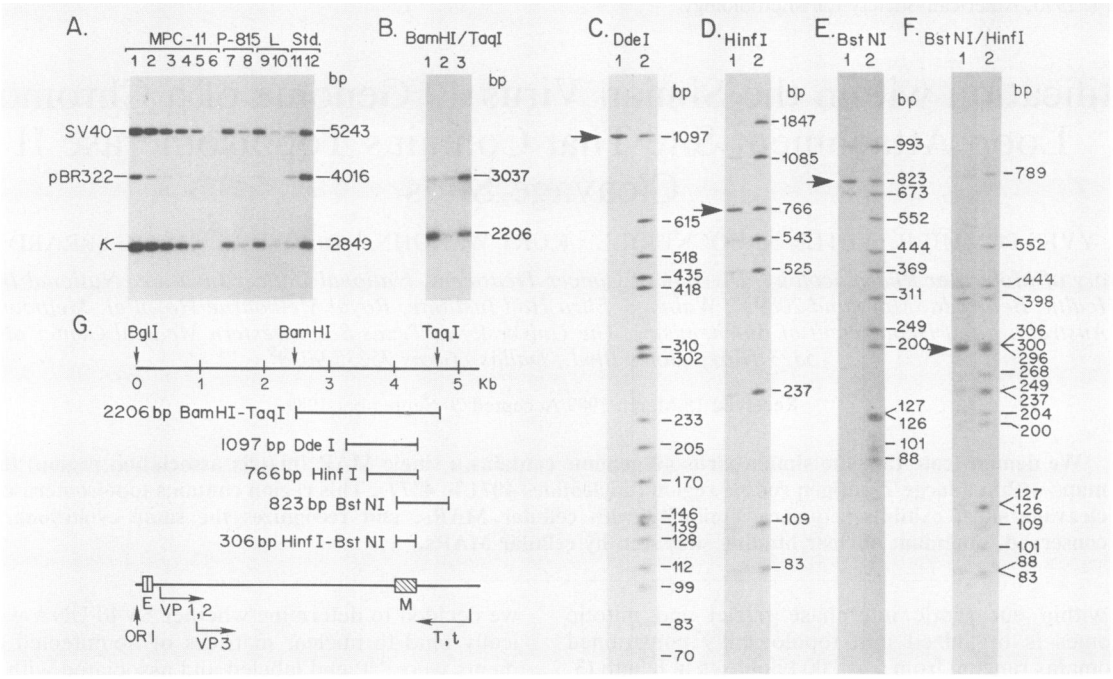
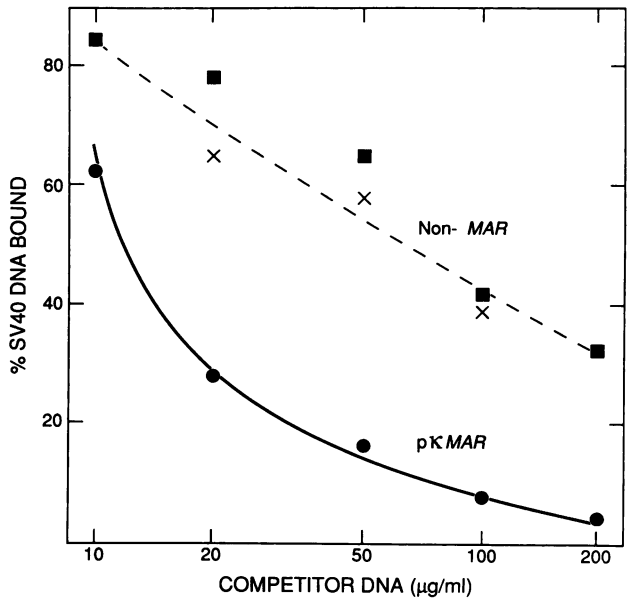


FIG. 1. Binding of segments of the SV40 genome to nuclear matrices. Reactions were performed with about 10^7 cell equivalents of nuclear matrices and 10 ng of each ^{32}P-labeled DNA species (approximately 10^4 cpm) as described elsewhere (8). (A) Comparative binding of *Taq*I-digested SV40 DNA and *Bam*HI-*Hin*dIII-digested recombinant plasmid pG19/45 (pBR322 containing a 2.8-kb mouse DNA insert [8]) to nuclear matrices of MPC-11 mouse plasmacytoma, P-815 mouse mastocytoma, and L-cell fibroblasts in the presence of a 2.5×10^2 (lane 1)-, 10^3 (lanes 2, 7, and 9)-, 2.5×10^3 (lane 3)-, 5×10^3 (lanes 4, 8, and 10)-, 10^4 (lane 5)-, and 5×10^4 (lane 6)-fold excess of *E. coli* DNA. As a reference, 3 and 15% of the input probe was loaded in lanes 11 and 12, respectively. An autoradiogram of a 1% agarose gel is shown here and in panel B (27). (B) Binding of *Bam*HI-*Taq*I-digested SV40 DNA to MPC-11 nuclear matrices in the presence of a 10^4-fold excess of *E. coli* DNA (lane 1). As a reference, 3 and 15% of the input probe was loaded in lanes 2 and 3, respectively. (C) Binding of *Dde*I-digested SV40 DNA to MPC-11 nuclear matrices in the presence of 2.5×10^3-fold excess of *E. coli* DNA. As a reference, 10% of the input probe was loaded in lane 2. An autoradiogram of a 5% polyacrylamide gel is shown here and in panels D through F (27). (D) Binding of *Hin*fI-digested SV40 DNA to MPC-11 nuclear matrices in the presence of a 10^4-fold excess *E. coli* DNA (lane 1). As a reference, 1.5% of the input probe was loaded in lane 2. (E) Binding of *Bst*NI-digested SV40 DNA to MPC-11 nuclear matrices in the presence of a 5×10^3-fold excess of *E. coli* DNA (lane 1). As a reference, 3% of the input probe was loaded in lane 2. (F) Binding of *Bst*NI-*Hin*fI-digested SV40 DNA to MPC-11 nuclear matrices in the presence of a 10^4-fold excess of *E. coli* DNA (lane 1). As a reference, 3% of the input probe was loaded in lane 2. (G) Summary diagram of SV40 DNA fragments that preferentially bind to nuclear matrices. The SV40 genome is depicted as a linear map from the *Bgl*I site (17). Fragments indicated by the arrowheads in panels C through F that specifically bind to nuclear matrices are depicted as solid bracketed lines. The origin (ori), enhancer (E), MAR (M), and transcription initiation sites (arrows) are indicated.



as those seen by cellular MARs, we performed a competition experiment. As shown in Fig. 2, binding of SV40 DNA to nuclear matrices was competed for 5 to 10 times more effectively by a recombinant plasmid containing a 365-bp κ gene MAR insert (pκMAR) relative to the degree of competition exhibited by the corresponding non-MAR-containing DNA controls. We conclude that the viral and cellular MARs recognize the same abundant binding sites in nuclear matrices.

Cellular MARs contain topoisomerase II consensus cleavage sequences (8, 19, 36, 40) and preferentially interact with the enzyme (41, 45). Furthermore, topoisomerase II is abundant in certain nuclear matrix preparations (5) and is

FIG. 2. Competition of SV40 DNA binding to nuclear matrices. Each reaction was performed with about 5×10^6 MPC-11 cell nuclear matrices, 5 ng of ^{32}P-end-labeled *Bam*HI-linearized SV40 DNA, and the indicated amounts of unlabeled linearized competitor DNAs in 100 μl as described elsewhere (8). The recombinant plasmid pκMAR consists of pUC19 containing the 365-bp *Hin*dIII-*Hin*fI fragment from pG19/45 (8) inserted into the *Sma*I site. Symbols: ●, pκMAR as competitor; X, pUC19 as competitor; ■, *E. coli* DNA as competitor.

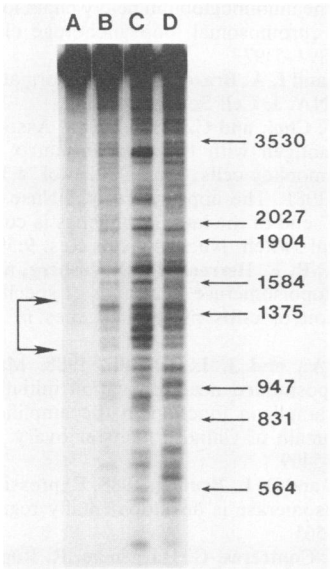


FIG. 3. Topoisomerase II-mediated cleavage in SV40 DNA. SV40 DNA was linearized by *Ban*I cleavage, end labeled with ^{32}P, and cut with *Hpa*II, resulting in nucleotide 298 of the Crick strand carrying the radioactive tag on the long DNA molecules. DNA (50 ng) was reacted with purified mouse L1210-cell topoisomerase II (4 U) in the absence or presence of antitumor drugs (24, 37). An autoradiogram of 1% agarose gel is shown (27). The gel was calibrated with the indicated DNA size markers, whose lengths are given in base pairs. Lane A, control DNA; lane B, topoisomerase II-treated DNA without drug; lane C, topoisomerase II-treated DNA with 10 μM 4′-(9-acridinylamino)methanesulfon-m-anisidide; lane D, topoisomerase II-treated DNA with 10 μM teniposide. The bracketed region shown on the left encompasses the 306-bp MAR located at position 4071 to 4377 (17).

necessary for the terminal stages of SV40 DNA replication, primarily at the decatenation step (43, 44, 54). Therefore, since topoisomerase II in the nuclear matrices that we used could be responsible for binding the SV40 MAR, we decided to directly map the organization of topoisomerase II sites on SV40 DNA. Although Liu and co-workers have found that multiple topoisomerase II cleavage sites exist on SV40 DNA (26, 52), the map positions and relative strengths of these sites with respect to the MAR remained to be determined.

We located the positions of topoisomerase II-mediated double-stranded DNA cleavage in the absence or presence of the antitumor drugs 4′-(9-acridinylamino)methanesulfon-m-anisidide and teniposide (14, 26, 37, 49, 53). Topoisomerase II, purified from mouse L1210 cells (29), was incubated with ^{32}P-end-labeled SV40 DNA as described previously, and the resulting DNA samples were separated by gel electrophoresis (24, 26, 37). As shown in Fig. 3, DNA cleavage was greatly stimulated by the drugs and occurred within the MAR (arrowed bracket) as well as elsewhere. However, because the enzyme cleavage specificity was influenced somewhat by the drugs, we decided to analyze only the cutting pattern obtained in the absence of these agents. As summarized in Fig. 4, the highest density of topoisomerase II cleavage sites within SV40 occurred within the MAR between nucleotides 4000 to 4400. About 20% of the topoisomerase II cutting within the entire SV40 genome occurred within this region.

While the MAR is located in the region of the SV40 genome that contains the highest density of topoisomerase II double-stranded cutting sites, several other segments also exhibit prominent enzyme cutting (Fig. 4). However, these other segments do not bind significantly to nuclear matrices (Fig. 1). Thus, while major sites of interaction of topoisomerase II with SV40 DNA colocalize with the MAR, it is clear that interaction with this protein is not sufficient to specify matrix attachment, since other fragments bearing topoisomerase II sites are not recognized by the matrix. Furthermore, MARs have been observed to interact with matrices from nondividing cells (8, 10, 36) that appear to be deficient in topoisomerase II (16, 20, 21). These findings agree with the previous conclusion that topoisomerase II binding sites are neither necessary nor sufficient to specify cellular MARs (45).

Like all other MARs, the SV40 MAR is made up of several hundred base pairs of A+T-rich DNA. However, there are

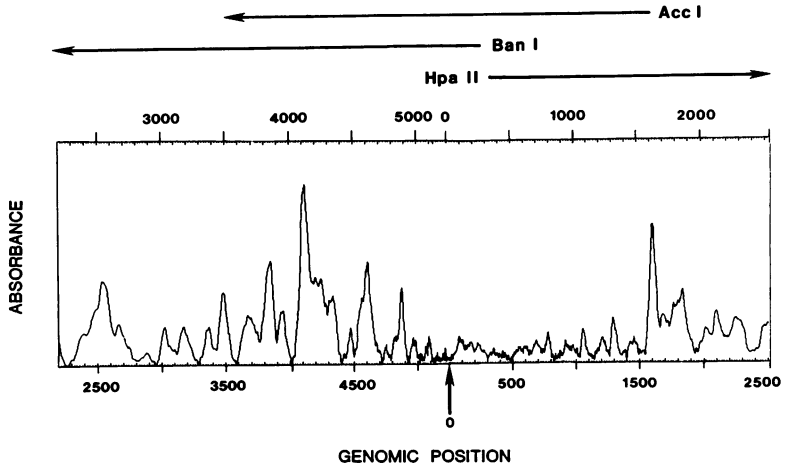


FIG. 4. Location of topoisomerase II-mediated DNA cleavage in SV40 DNA. Three independent uniquely ^{32}P-end-labeled SV40 DNA fragments were used. The labeling sites were *Ban*I (nucleotide 298 of the Crick strand), *Acc*I (nucleotide 1631 of the Crick strand), and *Hpa*II (nucleotide 347 of the Watson strand). The arrows indicate the direction away from the label. Topoisomerase II reactions were performed in the absence of drugs as described in the legend to Fig. 3. DNA samples were separated on 1% agarose gels, and autoradiograms were analyzed by computer densitometry (24, 37). The arrow indicates the origin of replication (position 0 [or 5243]) of SV40 (17). The densitometric profiles obtained with the three SV40 DNA fragments have been added together by computer to generate a profile for the whole genome.

six other regions in the SV40 genome that have at least 300 bp of 63 to 66% A+T-rich DNA that bind to the matrix poorly by comparison with the 306-bp, 68% A+T-rich MAR. We conclude, therefore, that A+T richness may be a necessary but not a sufficient condition to specify matrix association. Regions of sequence homology do indeed exist between the SV40 and cellular MARs. Both are enriched in the MAR consensus elements ATATTTTT and AATATT (8). Although a search against the topoisomerase II consensus cleavage sequence (40) revealed little homology within the SV40 MAR or elsewhere within the genome (17), this consensus does not always predict strong topoisomerase II cleavage sites (24, 45).

The region of the SV40 genome corresponding to the MAR has been shown previously to have several interesting properties, indicating that this segment may have an altered conformation or a partial single-stranded character when supercoiled. It has been demonstrated that this region is preferentially sensitive to S1 nuclease (2), prefers to bind gene 32 protein of bacteriophage T4 (33), reacts preferentially with carbodiimides (7), and is hypersensitive to micrococcal nuclease (Pommier et al., unpublished results). In addition, minor DNase I and DNase II hypersensitive sites reside at or near this region in SV40 minichromosomes (13, 42). Whether the SV40 MAR has a *cis*-acting effect on the biological functions of the virus is a key question remaining to be answered, particularly since in studies to be reported elsewhere, the κ gene MAR has been shown to exert a positive quantitative effect on gene expression (Blasquez et al. and Xu et al., in press). In addition, cellular MARs appear to be prone to illegitimate recombination events (45).

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