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A REGION IMMEDIATELY ADJACENT TO THE ORIGIN OF REPLICATION OF BOVINE PAPILLOMA VIRUS TYPE 1 INTERACTS *IN VITRO* WITH THE NUCLEAR MATRIX

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SUMMARY: We have investigated the interaction of the 69% transforming fragment of the Bovine Papilloma Virus type 1 (BPV1) with the nuclear matrix from 1361.5 cells (NIH-3T3 cells transformed by a BPV chimeric construct). *In vitro* studies performed with end-labelled DNA fragments and nuclear matrices prepared using a high-salt extraction procedure demonstrate the binding of a 672 bp fragment adjacent to the viral origin of replication and containing the plasmid maintenance sequence (PMS-1). This fragment can be cleaved into two pieces (393 and 279 bp), both interacting equally well with the nuclear matrix. This indicates that a least two regions of the 672 bp DNA fragment are involved in the interaction.

The bovine papilloma virus, type 1 (BPV1) has a 7946 bp circular double stranded DNA genome (1), and replicates as an autonomous plasmid in the nuclei of tumor and transformed cells (2). The copy number of BPV1 DNA during prolonged passages of transformed cells is constant. In a non-synchronous population of BPV1-transformed C127 cells, the BPV1 genome seems to replicate at the same rate as the cellular genome, suggesting that each BPV1 DNA molecule replicates only once per cell cycle (3). These findings suggest that BPV1 may serve as a valid model system for mammalian DNA replication, in which each DNA molecule is only replicated once each S phase.

The origin of replication of the virus has been localized in the upstream, non-coding, regulatory region (URR) (4), at position 7730 $\pm$ 100 bp (5). This URR region also contains a transcriptional enhancer, transactivated by the viral protein E2 (6) and a promoter (7). Two plasmid maintenance sequences (PMS) have been identified on the basis of their ability to allow a linked selectable marker to be maintained as a nuclear plasmid in the presence of the required viral transactivating factors. PMS-1 is located in the URR (between

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positions 6945 and 7476) and is adjacent to the replication origin; PMS-2 is located in ORF E1 (between positions 1515 and 1655) (8).

In eukaryotic cells, the preferential association of pulse-labelled DNA with high-salt-insoluble nuclear structures, termed "nuclear matrix" or "nuclear cage", has been demonstrated (9). The trapping of nascent DNA and active DNA polymerase in the "nuclear cage" suggests that replication takes place at the nuclear matrix (10). In yeast, all the investigated autonomous replication sequences (ARS), that are replication origins (11), bind to the nuclear matrix (12,13). In higher eukaryotes, it has also been proposed that origins of replication interact with the nuclear matrix (14). However, precise interpretation of the data is difficult since replication origins have not been mapped with the same accuracy as in yeast. In this regard BPV1, that replicates in a synchronous fashion with cellular DNA and for which the origin of replication has been precisely mapped is a model of choice to study the functional role of nuclear matrix in DNA replication.

We have investigated the interaction of the different regions of a chimeric DNA construct containing the 69% transforming fragment of the BPV (15), linked to a hormonally regulated transcription unit, with the nuclear matrix of the cell line in which it has been permanently established (16). Our goal was to investigate the putative involvement of the different regions of the chimeric construct in dynamic interactions with the nuclear matrix. Studies on the interaction of the construct with the nuclear matrix were performed in cell line 1361.5 (16), *in situ* using a nuclei low-salt extraction procedure (17), and *in vitro* with nuclear matrices obtained by high-salt extraction of nuclei (18).

In this paper we report the results of the *in vitro* studies on the interaction of the 69% transforming fragment of the BPV with nuclear matrices from 1361.5 cells, the other results being reported elsewhere (19). The data show the interaction with the nuclear matrix of a 672 bp DNA fragment, containing the the PMS-1 region, and adjacent to the viral origin of replication.

MATERIALS AND METHODS

Cell line and tissue culture. Cells 1361.5 (NIH-3T3 cells transformed by transfection of a chimeric construct containing the 69% transforming fragment of the BPV1 genome (15) and the mouse mammary virus long terminal repeat (MMTV LTR) driving the v-Ha-*ras* gene) (16), were grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Nuclei preparation and nuclear matrix isolation. Unless otherwise stated, all steps used for nuclei and nuclear matrix isolation (18) were performed at 4°C. Cells were scraped in RBS (10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.5 mM PMSF), 0.25 M sucrose, collected by centrifugation, resuspended in the same buffer (2 x10^8 cells/10 ml), lysed in a Dounce homogenizer (20 strokes, pestle B), and the homogenate was centrifuged for 10 min. at 900 xg. The pellet was rinsed 3 times with RBS, 0.25 M sucrose,

resuspended in 18 ml of RBS buffer, containing 1.8 M sucrose, and the nuclear suspension was centrifuged for 30 min. at 24000 rpm, in a SW28 rotor (Beckman), through a layer of RBS, 1.8 M sucrose. Isolated nuclei were rinsed with RBS, 0.25 M sucrose containing a protease inhibitor mix (5 μl/ml antipain, 5 μg/ml leupeptine, 5 μg/ml chymostatine and 5 μg/ml pepstatine) and resuspended in 5 ml of the same buffer. Nuclei were either used immediately, or stored at -20°C after addition of an equal volume of glycerol. Nuclei ($\approx 2.5 \times 10^8$) were pelleted, resuspended in 4 ml of digestion buffer (RBS, 0.25 M sucrose, containing the above-mentioned protease inhibitor mix, 100 KIU aprotinin and 2 mM $CaCl_2$), and digested for 2 h. at 37°C with 4000 U DNaseI (Worthington). The digested nuclear suspension was centrifuged for 15 min. at 900 xg, the pellet resuspended in 4 ml of RBS, 0.25 M sucrose containing the protease inhibitor mix and 4 ml of extraction buffer (20 mM Tris-HCl pH 7.4, 20 mM EDTA, 4 M NaCl) were added. The samples were incubated on ice for 10 min. and centrifuged for 15 min. at 1500 xg. The pellets were resuspended in 8ml of purification buffer (10 mM Tris-HCl pH 7.4, 2 M NaCl, 10 mM EDTA, 0.5 mM PMSF, 0.25 mg/ml BSA) and rinsed 3 times in the same buffer, in order to eliminate the DNaseI. The nuclear matrices were resuspended in 8ml of RBS, 0.25 M sucrose containing the protease inhibitor mix and 0.25 mg/ml BSA (matrices from $\approx 5 \times 10^7$ nuclei/ml). The amount of residual DNA contained in the nuclear matrix preparation was determined using the mithramycine assay (20) and was always $< 0.3\%$ of the initial nuclear DNA. The nuclear matrices were either used immediately or stored at -20°C, after addition of an equal volume of glycerol.

Assay of DNA binding to nuclear matrix. Nuclear matrices were rinsed 3 times in 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 0.25 mg/ml BSA and 0.25 M sucrose. The indicated amounts of nuclear matrices were resuspended in 100 μl of binding buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.25 M sucrose, 0.25 mg/ml BSA), incubated at 23°C for 30 min. in the presence of various amounts of sonicated *E. coli* DNA (non-specific competitor) and for 2 h. at 23°C with the indicated amounts of each of the end-labelled DNA fragments. At the end of the incubation, 400 μl of the binding buffer were added to each tube, the samples were centrifuged for 1 min. at 10000 xg, the pellets were rinsed with 1 ml of the same buffer and the two supernatants were pooled. The DNAs purified from the pellet (associated to the nuclear matrix) and the supernatant were fractionated on 1% agarose gels. The gels were dried on DEAE paper, and autoradiographed.

RESULTS AND DISCUSSION

We have investigated the interaction of DNA fragments spanning all of the 69% transforming region of BPV1 (15) with nuclear matrices from cells 1361.5. The BPV1 genome is expressed in this type of cell line (16) thus making such cells susceptible to provide nuclear matrices containing viral components possibly involved in specific attachment of the BPV1 DNA. Figure 1 depicts the BPV1 DNA fragments used in the experiments presented here.

The interaction of fragments A (containing PMS-1) and B (containing PMS-2) with the nuclear matrix was assayed in the presence of increasing amounts of *E. coli* non-specific DNA competitor. Results are presented in fig.2. The upper band is a 2967 bp PBR322 fragment, spanning from AvaI to HindIII, used as a negative control. The identical behavior of fragments B and PBR322 demonstrates that fragment B is not retained in the nuclear

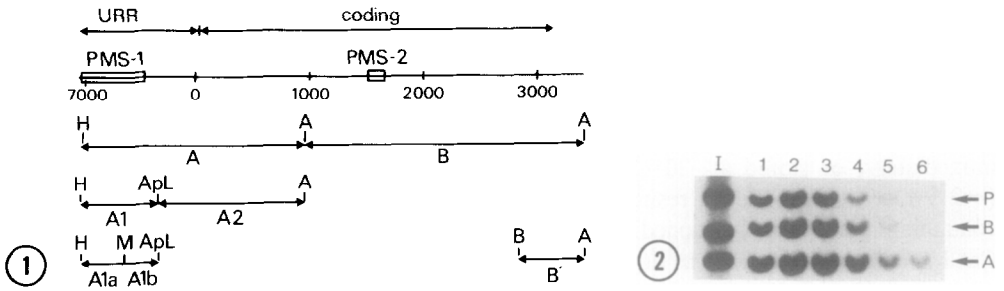

Figure 1. BPV1 genome fragments assayed for nuclear matrix binding. The numbers refer to the numbering of BPV1. PMS-1 and PMS-2 are the plasmid maintenance sequences. URR: upstream regulatory region. Letters on top of the fragments indicate the enzymes used to generate the fragments. H: HindIII, A: AvaI, ApL: ApaLI, M: MluI, B: BglI.

Figure 2. Interaction of BPV1 genome fragments A and B with the nuclear matrix from 1361.5 cells. Nuclear matrices (equivalent to 5.10^7 nuclei) were incubated with 2 ng of a mixture of end-labelled fragments (generated by the digestion of plasmid pM23 (13) with AvaI, BamHI, and HindIII) and increasing amounts of *E. coli* non-specific competitor (lane 1: 0, 2: 50, 3: 100, 4: 200, 5: 400, and 6: 600 μg/ml). On the figure are presented the top 3 bands of an autoradiogram showing the DNAs associated with the pellet fraction. P: PBR322 fragment, B: BPV1 fragment B , and A: BPV1 fragment A.

matrix pellet. In contrast, displacement of fragment A from the pellet is only achieved for higher amounts of non-specific *E. coli* competitor, suggesting a binding of fragment A to the nuclear matrix. A fragment, spanning from AvaI (3409) to BamHI (4191) and corresponding to the rest of the transforming region, was also assayed and found unable to bind the nuclear matrix (data not shown).

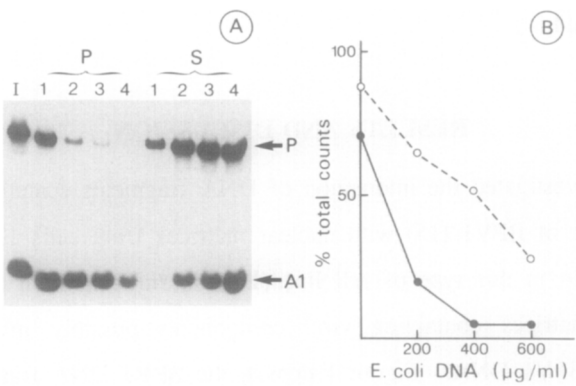

Figure 3. Interaction of BPV1 genome fragment A1 with the nuclear matrix from 1361.5 cells. Labelled fragments P (PBR322) and A1 (0.5 ng each) were mixed and incubated with nuclear matrices (equivalent to 5.10^6 nuclei) in the presence of increasing amounts of *E. coli* non-specific competitor (lane 1: 0, 2: 200, 3: 400, and 4: 600 μg/ml). Lane labelled I shows the input radioactive fragments. The arrows on the side of the gel mark the position of the fragments. The lanes labelled P and S are the pellet supernatant fractions, respectively. Panel A: autoradiogram of the dried gel, B: quantitation from a radioactivity scan of the dried gel (closed circles: PBR322 fragment, open circles: BPV1 fragment)

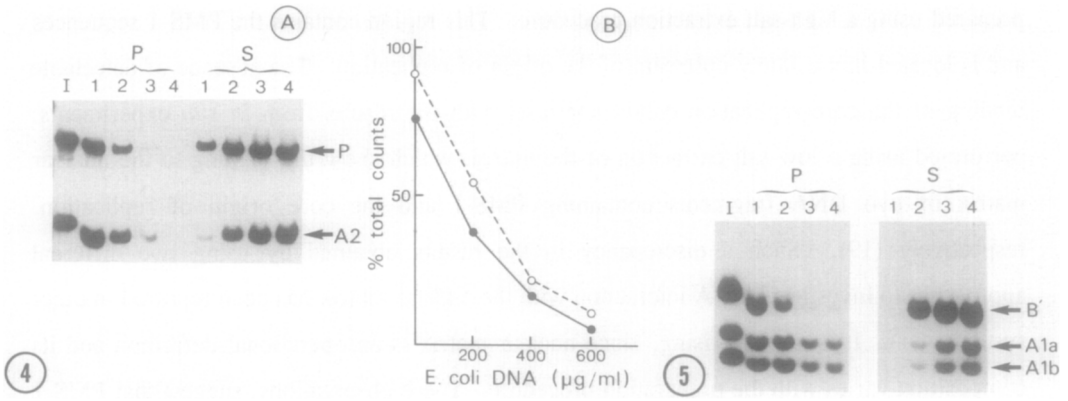

Figure 4. Interaction of BPV1 genome fragment A2 with the nuclear matrix from 1361.5 cells. Results are presented as in figure 3.

Figure 5. Interaction of BPV1 genome fragment B', A1a, and A1b with the nuclear matrix from 1361.5 cells. Results are presented as in figure 3.

In order to localize better the region involved in the nuclear matrix binding, fragment A was cut with ApaLI, generating fragments A1 and A2 (fig.1). Fragment A1 contains all PMS-1, with most of the URR region. Fragment A2 contain the origin of replication, the E2 transactivated enhancer, the open reading frames E6 and E7, and 139 bp of open reading frame E1. Binding of these fragments to the nuclear matrix was assayed along with the, above mentioned, PBR322 fragment. Results are presented in fig. 3 (fragment A1) and fig. 4 (fragment A2). Panels A show the distribution of the fragments between pellet and supernatant fractions, as a function of the amount of non-specific *E. coli* competitor in the assay. The upper band is the PBR322 fragment, used as a negative control, and the lower band, the BPV1 fragment assayed. Panels B present a quantitation of the fragments in the pellets. Fragment A2 (fig.4) behaves almost in the same way as the PBR322 fragment, allowing to conclude that it does not bind to the nuclear matrix. In contrast, fragment A1 (fig.3), is clearly retained more efficiently in the pellet fraction than the PBR322 fragment. Thus the region responsible for the binding of fragment A to the nuclear matrix is part of fragment A1.

Figure 5 shows the result of an experiment in which the two A1 subfragments A1a and A1b, mixed with fragment B' (part of fragment B that does not bind the nuclear matrix) were assayed. In this experiment, fragment B' was used as a negative control (instead of PBR322) because of its size comparable to that of A1a and A1b. The autoradiogram clearly shows that, as expected, B' is not retained in the pellet fraction. Fragments A1a and A1b display the same behavior, showing that they both interact with the nuclear matrix.

These results demonstrate that within the 69% transforming region of BPV1, only one region, located in the 672 bp A1 fragment, interacts *in vitro* with nuclear matrices

prepared using a high-salt extraction procedure. This region contains the PMS-1 sequences and is located immediately upstream of the origin of replication. The absence of detectable binding of the core replication origin contrasts with the results from *in situ* experiments, performed using a low-salt extraction of the nuclei, which show the binding to the nuclear matrix of two DNA fragments containing PMS-1 and the core origin of replication, respectively (19). Such a discrepancy in the results obtained by using two different approaches to investigate DNA interaction with the nuclear matrix has been reported in other systems (21). It is not surprising, since nuclear matrix is an operational definition and its composition varies with the preparation procedure. These observations, suggest that PMS-1 and core replication origin interactions with the nuclear matrix involve different sets of proteins. This is interesting, since the appurtenance of PMS-1 to a multipartite replication origin has been recently proposed (5). This region could provide information at a distance and be required for effective replication initiation. It would be important to determine if in higher eukaryotes replication origins display such a composite structure and if permanent nuclear matrix attachment sites (independent of the transcriptional activity) (14) coincide exactly with replication origins or map at their vicinity.

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