

The polyomavirus major capsid protein VP1 interacts with the nuclear matrix regulatory protein YY1

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Abstract Polyomavirus reaches the nucleus in a still encapsidated form, and the viral genome is readily found in association with the nuclear matrix. This association is thought to be essential for viral replication. In order to identify the protein(s) involved in the virus-nuclear matrix interaction, we focused on the possible roles exerted by the multifunctional cellular nuclear matrix protein Yin Yang 1 (YY1) and by the viral major capsid protein VP1. In the present work we report on the *in vivo* association between YY1 and VP1. Using the yeast two-hybrid system we demonstrate that the VP1 and YY1 proteins physically interact through the D-E region of VP1 and the activation domain of YY1.

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Key words: Polyomavirus; VP1; Nuclear matrix; Yin Yang 1

1. Introduction

The mouse polyomavirus (Py) genome encodes three early tumor antigens (LT, MT and sT) and three late structural proteins VP1, VP2 and VP3 which make up the viral capsid. Polyoma late proteins, together with viral DNA and cellular histones (except H1) are assembled in the host cell nuclei into virions [1]. The icosahedral capsid is organized by 72 externally exposed VP1 pentamers with one VP2 or VP3 minor capsid proteins associated internally with each pentamer [2]. Py VP1 mutants have been isolated and characterized for modified host range or tumorigenesis. All of these mutations are located in the out-facing loops of VP1. The major out-facing protein of the capsid (VP1) has been characterized with regard to conformational organization and interaction with the cell membrane receptor [3–5]. According to X-ray diffraction studies of its tertiary structure [3], VP1 can be divided into three modules: an N-terminal arm, an antiparallel β -sandwich core and a long C-terminal arm. Four loops emanate from the β -sheet framework; three of them (B-C, D-E and H-I) form closely interacting structures at the out-facing side of the β -sandwich that are also exposed at the surface of the capsomeric structure. The long E-F loop is exposed on the side of the β -sandwich core (and on the side of capsomeres).

The external loops are likely to be the structures involved in cell receptor recognition by virions [4] and, therefore, to be the main antigenic determinants. The flexible C-terminal arms form interpentameric contacts and the basic amino acids of the N terminal arm are responsible for the non-specific DNA-binding activity of VP1 [6,7].

The necessity for viral genomes to associate to the nuclear matrix (NM) in order to be expressed, has been demonstrated in some DNA tumor viruses such as papilloma and adenoviruses; this association has been shown to be mediated by proteins encoded by the viral genome [8,9]. For the small polyomaviruses no such function has been found, although it has been shown that infecting polyomavirus reaches the nucleus encapsidated and its genomic DNA readily associates to the NM [10,11], and this association is thought to determine the ability of the incoming viral minichromosome to be transcribed and replicated in the permissive host. Furthermore, it has been shown that *in vivo* expressed VP1 associates to the NM [12].

Yin Yang 1 (YY1), a multifunctional cellular transcriptional regulatory phosphoprotein with predicted molecular weight 44 kDa, migrating on SDS gels as 68 kDa protein, contains four C₂H₂-type zinc fingers at the C-terminus responsible for the sequence-specific DNA binding activity, a glycine-rich repression domain and a N-terminal activation domain. Depending on the context, YY1 can activate or repress the transcription of number a of both cellular and viral genes (reviewed in [13]). Moreover, YY1 has been found to bind DNA in specific sites, also in the enhancer region of Py [14]. The protein-protein interaction has an important function in controlling the activity of YY1 and a number of proteins have been identified which form complexes with it. Another aspect of YY1 function has been unravelled by demonstration that YY1 is identical to the NM binding protein NMP-1 [15]. These data imply that YY1 may also be involved in the association of specific DNA sequences of cellular and viral genes to the NM.

Results from our and other laboratories [16–21] had suggested the possibility that VP1, the major capsid protein, could determine alterations in the host specificity of growth and tumorigenicity. In order to determine the possible mechanisms that allow the encapsidated polyomaviruses that reach the cell nucleus to associate with the NM, we investigated the possible role of YY1 in this necessary step of viral function. In this study we report that YY1 binds *in vivo* to the capsid protein VP1. This interaction involves a specific site in the D-E loop of VP1 and the activation domain of YY1. The

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possible relevance of VP1-YY1 interaction for the biology of the viral life cycle is discussed.

2. Materials and methods

2.1. Antibodies

Rabbit polyclonal anti-VP1 (I58) and anti-D-E loop (against VP1-Leu¹³⁷-Glu¹⁵⁹ peptide) antibodies were kindly provided by R. Garcea (University of Colorado, CO, USA); mouse monoclonal anti-VP1 and anti-VP2/3B have been already described [22]; anti-YY1 (C-20), anti-Gal4-TA (768), anti-p27 (C-19) and anti-Grb-2 (C-23) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology, CA, USA. Secondary, Px-conjugated anti-mouse and anti-rabbit IgG antibodies were from Bio-Rad Laboratories, Hercules, CA, USA.

2.2. Py infection and immunoprecipitation

Mouse 3T6 fibroblast cells were infected with wild type (wt) Py [8] at a multiplicity of 50 pfu. At different times post-infection (p.i.) cells were lysed and lysates were subjected to ultrasonic treatment and subsequently clarified by centrifugation at 4°C following Pelicci et al. [23]. After classical immunoprecipitation, immunocomplexes were washed three times with cold lysis buffer and once with RIPA, eluted and denatured in Laemmli buffer (5 min). Proteins were resolved in 10% SDS-PAGE and transferred onto a nitrocellulose filter. Immunocomplexes were detected using either anti-VP1 or anti-YY1 antibodies and Px-conjugated secondary antibodies [24]. In some experiments secondary non-specific anti-rabbit antibodies (NSA) from SEVA, a.f. (Prague, Czech Republic) in control immunoprecipitations were used.

2.3. VP1, VP3 and YY1 over-production in mammalian and insect cells, and immunoprecipitation

COS cells were co-transfected using the calcium-phosphate technique [25] with pSVL-VP1 containing the VP1 gene under the SV40 late promoter and with pEB-DELTA:s (kindly provided by A. Felsani, Istituto Tecnologie Biomediche, Roma, Italy) expressing YY1 from the MSV-LTR promoter. 24 h post-transfection, cells were pre-incubated for 3 h in methionine-free medium, then labelled 12 h with ³⁵S-methionine (100 µCi/9 cm dish). SF9 insect cell cultivation, infection and transfection were performed according to Summers and Smith [26]. Baculovirus VL-YY1 expressing mouse YY1 from polyhedrin promoter was prepared using the pVL1393-YY1 recombinant transfer plasmid. Insect cells co-infected with baculovirus VL-YY1 and VL-VP1 or with VL-YY1 and Ac-VP1-VP3 (baculovirus prepared using pAcAB3 transfer vector [27] containing Py VP1 and VP3 genes; Fostová et al., manuscript in preparation) or with VL-YY1 and VL-VP3 were labelled 48 h p.i. by ³⁵S-methionine for 4 h. Lysates of cells producing YY1, VP1 and/or VP3 were immunoprecipitated with anti-YY1, anti-VP1 or with anti-VP2/3B antibody. Immunoprecipitates were resolved on 10% SDS-PAGE and detected by autoradiography [24].

2.4. In situ fractionation of 3T6 cells

Briefly, cells attached on the cultivation dish were washed with KM buffer (10 mM MES, pH 6.2, 10 mM NaCl, 1.5 mM MgCl₂, 10% glycerol, 30 µg aprotinin) and extracted sequentially with buffers I (KM, 1% NP40, 0.1 mM EGTA, 1 mM DTT), II (KM, 50 µg/ml DNase I) and III. (KM, 2 M NaCl, 0.1 mM EGTA, 1 mM DTT). Washing with KM buffer was performed after each extraction [28]. Proteins of extract III were precipitated with methanol-chloroform [25] and dissolved in Laemmli buffer.

2.5. Plasmid constructions

The amino acid sequence of the VP1 used is identical to the wt-Py sequence of GenBank, accession number P03091 with the following modifications: G₁₅₂, W₂₈₈, V₂₉₅, S₂₉₆, S₂₉₇, L₂₉₈, E₂₉₉, K₃₀₀, F₃₀₂. pSVL-VP1: contains the VP1 coding sequence (*EcoRV*/*XbaI*-blunt ended fragment) inserted into pSVL (Pharmacia; *XbaI*-blunt ended). pVL1393-YY1: contains the YY1 gene (*NcoI*-blunt ended/*EcoRI*) from pGEM-4Z.DELTA (kindly provided by A. Felsani) inserted into pVL1393 (Pharmacia) (*SmaI*/*EcoRI*). pGBT9-VP1 is composed of three DNA fragments: (1) the fragment of the 5' part of the VP1 gene (1–761 bp) amplified using synthetic primers (*EcoRI*/*PstI*); (2)

the fragment of the 3' part of the VP1 coding sequence from pVL1393-VP1 (11) (*PstI*/*BglII*) and (3) the plasmid pGBT9 carrying the GAL4 DNA-binding domain (DNA-BD)) (Clontech; *EcoRI*/*BamHI*). Production of the fusion protein in yeast was confirmed using anti-VP1 antibody. pGBT9-VP1₁₄₄ carries the fragment *StuI*-*NdeI* in pGBT9-VP1 replaced with the corresponding fragment of mutated VP1-M17 gene containing a *SmaI* site in position 432 (144 aa) from pSVL-VP1-M17 (Amati et al., manuscript in preparation); resulting pGBT9-VP1-M17 was subsequently cleaved by *SmaI* and *SalI*, blunt-ended and religated. pGBT9-VP1₁₄₀ and pGBT9-VP1₁₃₆: pGBT9-VP1-M17 was linearized by *SmaI*, treated with BAL31 nuclease, cleaved by *SalI*, blunt-ended, religated and electroporated into *E. coli* DH5α [24]; the extent of deletions was analyzed by sequencing (kindly performed by CEINGE, Napoli, Italy). pGBT9-DE_{118–170}: the DNA fragment of VP1 (Leu¹¹⁸-Gly¹⁷⁰) containing the VP1-D-E loop (Leu¹³⁷-Glu¹⁵⁹) was amplified using synthetic primers (*EcoRI*/*PstI*), fused with sequences encoding Gal4bd (Gal4-DNA binding domain) in pGBT9 (*EcoRI*/*PstI*); production of fusion protein in yeast was confirmed using the anti-D-E loop antibody. pGBT9-EF_{172–248}: constructed as described for pGBT9-DE_{118–170}, using amplified fragment of VP1 encoding amino acids 172–248. pGAD424-YY1: YY1 gene from pGEM-4Z.DELTA (*EcoRI*) was fused with sequences encoding GAL4 AD (GAL4-activation domain) in pGAD424 (Clontech; *EcoRI*), and the production of the fusion protein in yeast was confirmed using anti-YY1 antibody. YY1 deletions were derived from pGAD424-YY1 by cleavage with *XmaI* (pGAD424-YY1₂₀₀), *PstI* (pGAD424-YY1₈₉) and *EagI* (pGAD424-YY1_{157–414}), respectively, and subsequent religation.

2.6. Yeast strains and methods

S. cerevisiae HF7c (*MATa*, *ura3–52*, *his3–200*, *ade2–101*, *lys2–801*, *trp1–901*, *leu2–3,112*, *gal4–542*, *gal80–538*, *LYS2::GAL1-HIS3*, *URA3::(GAL17-mers)₃-Cycl-lacZ* and *S. cerevisiae* SFY526 (*MATa*, *ura3–52*, *his3–200*, *ade2–101*, *lys2–801*, *trp1–901*, *leu2–3,112*, *can1*, *gal4–542*, *gal80–538*, *URA::GAL1-lacZ*) strains were previously described by Bartel et al. [29]. Yeast transformations with appropriate plasmids were performed using the lithium acetate technique [30]. Yeast cell cultivation, media, selection of transformants, filter assay of β-galactosidase activity and detection of HIS3 expression were performed according to the Clontech ‘Two-Hybrid system manual’.

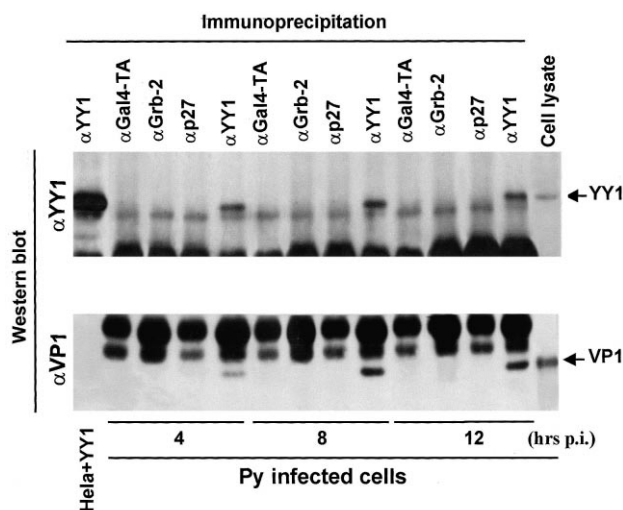


Fig. 1. In vivo co-immunoprecipitation of VP1 and YY1. 3T6 fibroblast cells at different times p.i. were lysed and immunoprecipitations (from 500 µg of total cell lysate) were performed with either anti-YY1 antibody or control antibodies specific for exogenous (αGal4-TA), cytoplasmic (αGrb-2) and nuclear (αp27) antigens. After SDS-PAGE separation, proteins were transferred onto nitrocellulose membrane and detected using antibodies specific to YY1 and VP1.

3. Results

3.1. VP1-YY1 association

Our starting point was the detection of VP1 in Py infected 3T6 fibroblast cell extracts immunoprecipitated with YY1 specific antibodies, a few hours p.i. and before the onset of viral replication. Fig. 1 shows the specific coimmunoprecipitation of VP1 obtained with anti-YY1 antibodies and the lack of coimmunoprecipitation with antibodies directed against exogenous, cytoplasmic or nuclear antigens. In order to determine if the interaction between VP1 and YY1 is direct, we overexpressed both VP1 and YY1 proteins in either mammalian cells, or recombinant baculovirus systems (Fig. 2A,B). Once again, the results showed that VP1 and YY1 efficiently coimmunoprecipitated with YY1-antibodies, thus confirming the interaction. Unexpectedly, under identical conditions, YY1 was not found to coimmunoprecipitate when immunoprecipitates were obtained using anti-VP1 antibodies. This result may reflect interference or competition between VP1 antibodies

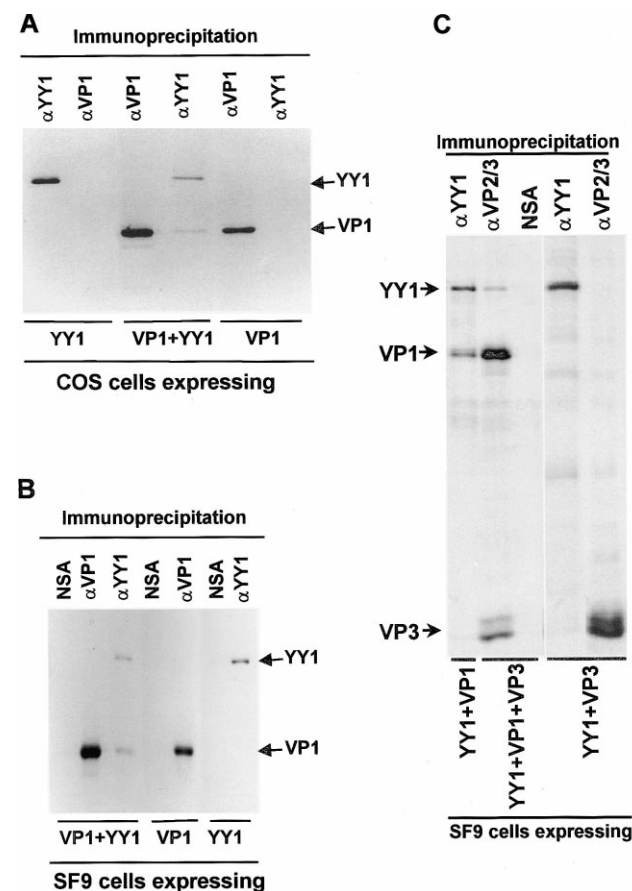


Fig. 2. Co-immunoprecipitation of VP1, VP3 and YY1 from cells overexpressing this proteins. A: COS cells transfected with pEB-delta:s expressing YY1, or with pSVL-VP1 expressing VP1, or co-transfected with both plasmids, respectively. Immunoprecipitation was performed with anti-YY1 polyclonal antibody or with anti-VP1 monoclonal antibody, as indicated. B: SF9 insect cells infected with either VL-YY1 or VL-VP1 baculoviruses alone or co-infected with both of them. Immunoprecipitations were performed with anti-VP1 monoclonal or with anti-YY1 polyclonal antibody. NSA were used in control immunoprecipitations. C: Insect cells co-infected with VL-YY1 and VL-VP1, or with VL-YY1 and Ac-VP1-VP3, or with VL-YY1 and VL-VP3. Immunoprecipitations were performed with anti-VP2/3B monoclonal or with anti-YY1 polyclonal antibody. NSA were used in control immunoprecipitations.

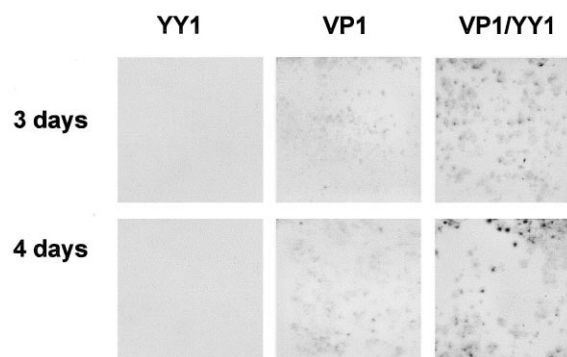


Fig. 3. Induction of *lacZ* expression in the yeast 2H system by interaction of VP1 and YY1. Colony-lift β -galactosidase filter assay of three and four day old colonies of *S. cerevisiae* SFY526 expressing YY1 (co-transformed with pGAD424-YY1 and pGBT9); VP1 (co-transformed with pGBT9-VP1 and pGAD424); or co-expressing VP1 and YY1 (co-transformed with pGBT9-VP1 and pGAD424-YY1).

and YY1 for the same binding site. If this were so, the interaction of VP1 with YY1 would be expected to involve regions of VP1 close to the D-E loop (see below) since the anti-VP1 monoclonal antibody used in this assay recognizes a site that maps in this region. Moreover, we were unable to coimmunoprecipitate YY1 even with a polyclonal antibody raised against intact virions, possibly reflecting the high immunogenicity of epitopes mapping in the D-E loop ([31], and unpublished results). The argument that the anti-VP1 antibody covers the YY1 binding site is supported by the results of immunoprecipitations of lysates of insect SF9 cells co-infected with baculoviruses expressing YY1 and VP1/VP3: the polyomavirus minor capsid protein previously shown to directly interact with VP1 (11). In fact, anti-VP2/3 specific antibody immunoprecipitated YY1 only in the presence of VP1 while no coimmunoprecipitation of VP3 by anti-YY1 antibody was observed (Fig. 2C).

3.2. Mapping of the interacting domains of VP1 and YY1

To map the VP1 and YY1 domains involved in VP1-YY1 interaction, we resorted to the yeast two-hybrid system (2HS). Two plasmids, pGBT9-VP1, carrying VP1 fused with the GAL4 DNA-BD sequences, and pGAD424-YY1, carrying YY1 sequence fused with the GAL4 AD sequences, were introduced into *Saccharomyces cerevisiae* strains using the activation of *HIS3* or *lacZ* gene in order to detect interactions between the co-produced proteins. Fig. 3 shows the *lacZ* expression in yeast colonies producing both YY1 and VP1 proteins and in control yeast cells, transformed with only one plasmid and producing either the VP1 or the YY1 protein alone. This showed that YY1 does not activate the expression of *lacZ*, while the presence of pGBT9-VP1 alone allows some expression of the reporter gene. Similar results were obtained in the *S. cerevisiae* HF7c strain with the *HIS3* reporter gene (data not shown). However, co-expression of both VP1 and YY1 genes increased substantially the level of β -galactosidase production from the *lacZ* gene. These results showed a direct interaction between the two proteins despite the existence of a yeast factor(s) capable of interacting with VP1 and to switch on the expression of the reporter gene. To determine whether the VP1 domains involved in the interaction with YY1 and with the hypothetical yeast factor(s) coincide, we assayed dif-

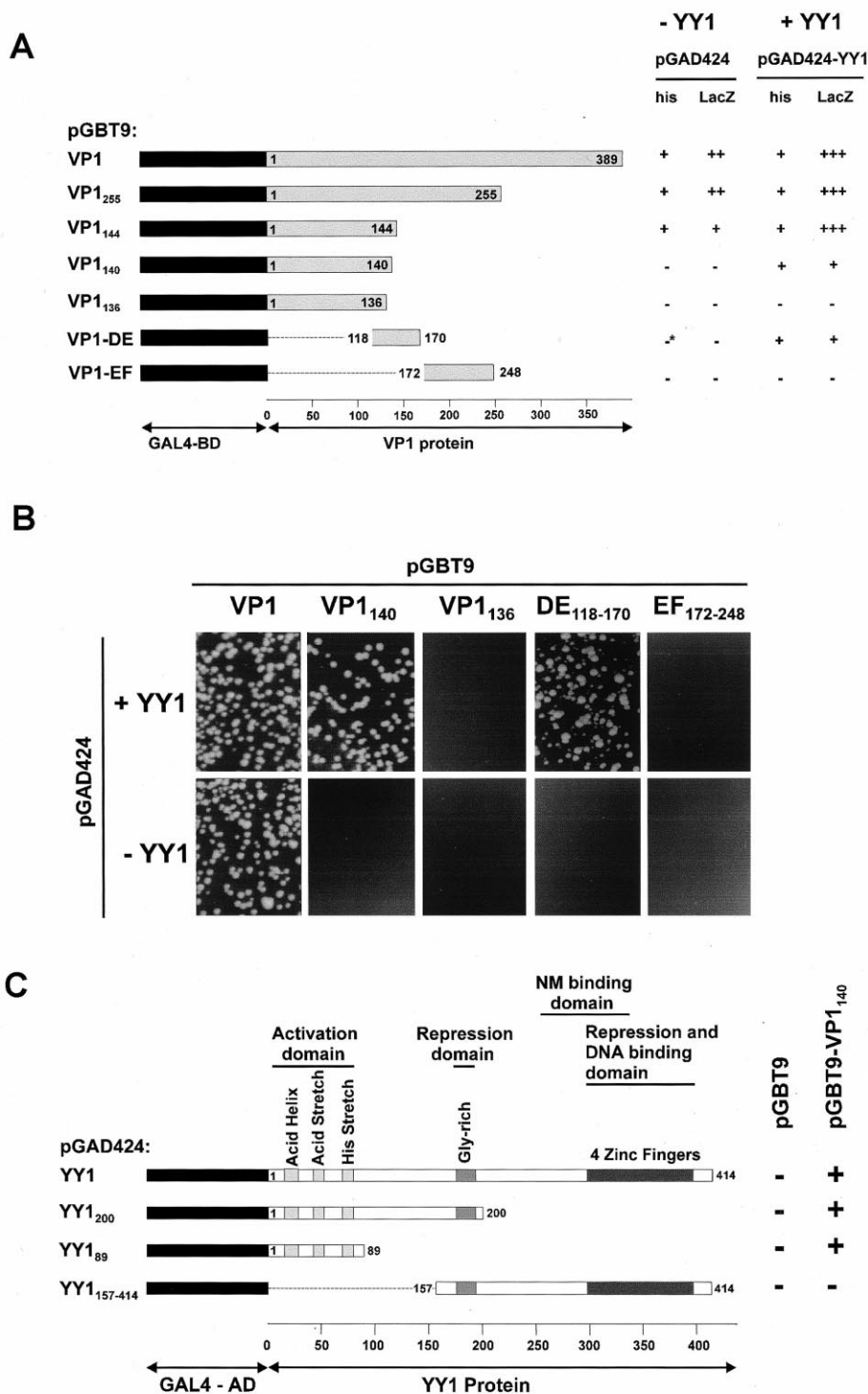


Fig. 4. Ability of VP1 and YY1 deletion mutants to induce *HIS3* or *lacZ* genes expression. A: Different VP1 fragments in pGBT9 tested in yeast 2HS for their ability to induce *HIS3* or *lacZ* gene expression in the absence or presence of YY1. (* very small colonies appeared after prolonged cultivation). B: Interaction of different fragments of VP1 protein with YY1 detected by colony-formation induced by *HIS3* expression in *S. cerevisiae* HF7c. HF7c cells transformed by the plasmids indicated were grown on minimal agar medium in the absence of histidine. C: Schematic representation of YY1 deletion mutants tested for their ability to induce the *HIS3* reporter gene expression in yeast 2HS either alone (pGBT9) or in the presence of VP1 (pGBT9-VP1₁₋₁₄₀) as in (A).

ferent deletion mutants of VP1 in the 2HS. To this end, two VP1 mutants carrying a C-terminal deletion obtained through cleavage of the gene with *Pst*I (VP1₁₋₂₅₅) or *Sma*I (VP1₁₋₁₄₄) were tested either alone, or in combination with YY1 for the

induction of the expression of *lacZ*. The results of this assay (reported in Fig. 4) showed that VP1₁₋₂₅₅, when tested alone, behaves like the full length protein, while VP1₁₋₁₄₄, cleaved within the D-E loop, reduced substantially the level of the

lacZ gene activation; by contrast, in the presence of YY1 the induction of the reporter gene was not diminished. To map more precisely the VP1 site that interacts with YY1, deletions starting from *Sma*I site (aa 144) of VP1 were generated by BAL-31 limited digestion and the mutants VP1_{1–140} and VP1_{1–136} were analyzed in the 2HS. Results reported in Fig. 4A,B show that VP1_{1–140} retains the ability to bind YY1, although completely loses its interaction with the yeast factor. VP1_{1–136} shows to be totally unable to activate the reporter genes even in presence of YY1, therefore, indicating that aa 137–140 are required for VP1-YY1 interaction in this experimental system and that the interacting sites with the yeast factor and YY1 are distinguishable. Since our previous results indicated that the D-E loop of VP1 could be responsible for the viral-host specificity [18,19] and may be involved in the formation of the complex of the viral genome with the NM (Amati et al., manuscript in preparation), we fused the sequences encoding the D-E_{118–170} or the E-F_{172–248} loops with the GAL4 DNA-BD. The results of the 2HS as in Fig. 4A,B, show that neither YY1 alone nor VP1 D-E_{118–170} alone allow the growth of yeast in the absence of histidine in the medium, while yeast colonies grow efficiently in the presence of both proteins. Essentially comparable results were obtained with the *lacZ* reporter gene induction although the expression level was lower than that observed with the VP1₁₄₄ mutant. By contrast, neither *HIS3* nor *lacZ* expression was observed when VP1 E-F_{172–248} was tested (Fig. 4 and data not shown).

Various characterized regions of YY1 protein comprise the domains involved in repression, activation, DNA binding and NM association [13,32,33]. To determine the YY1 region(s) involved in the binding to VP1, YY1 sequences carrying deletions extending from the C or the N terminal ends were examined for their ability to interact in 2HS with VP1_{1–140}, the minimal fragment that fully interacts with YY1 without being activated by the yeast factor(s). The YY1 deletion mutants used, and their respective ability to activate the expression of *HIS3* when co-expressed with VP1 are presented in Fig. 4C. The results show that the interacting sequence of YY1 encompasses aa 1–89, comprising the activation domain.

4. Discussion

In this report we show that the major capsid protein VP1 interacts directly with the host NM protein YY1. The in vivo detection of this interaction shows to be specific and occurs at early times after infection in mouse cells, as well as in insect cells when both proteins are over-expressed. The fine mapping of VP1 site interacting with YY1, by means of the yeast 2HS, allows the conclusion that the site on VP1 molecule interacting with YY1 is located into the D-E region (aa 118–170). In the yeast 2HS we have found that there is a host activator factor(s) that is able to interact with VP1 fused with GAL4 DNA-BD and to activate *HIS3* or *lacZ* genes. However, no protein homologous to YY1 has been found by screening of yeast protein sequences. The activation by the yeast protein was significantly lower than that observed when VP1 gene was co-expressed with YY1 gene fused to GAL4 AD. We also showed, by the analysis of deletion mutants of VP1 that the interaction sites of the putative yeast activator factor is located in close proximity of the YY1 binding site but can, nevertheless, be distinguished from the latter. Mapping of

the VP1-BAL31 mutants localized the YY1 binding site onto the left arm of the D-E loop (aa 118–140), upstream from that of the yeast factor(s). The fact that D-E_{118–170} alone did not exhibit detectable interaction with a yeast factor, while the VP1_{1–144} mutant retains a reduced ability to switch on the reporter gene expression, may reflect a steric interference resulting from the direct interaction between the sequences of the GAL4 DNA-BD and the D-E_{118–170} fragment or, alternatively, the binding site for the yeast factor includes also amino acids from the deleted part of VP1 (aa 1–118). We have demonstrated, in the yeast 2HS, that the interaction of YY1 with VP1 can occur when VP1 is in monomeric form, since VP1 fused with the GAL4 DNA-BD does not form pentamers (our unpublished results).

By deletion mutants of YY1 we have determined that the interacting region with VP1 is the N terminal encompassing the first 89 aa; this region comprises the three activation domains and does not involve the repression domains, the DNA binding domain nor the sequences devoted to the NM binding. This finding can be interpreted in different ways. A possibility could be that YY1 is involved in targeting the virions or the viral nucleocore (consisting of viral genome, histones and capsid proteins [11]) to the appropriate compartment immediately after the virions have reached the cell nucleus. For example, the YY1-VP1 interactions could be involved in the viral genome association to the NM, a localization shown to be essential for expression and replication of some DNA tumorigenic viruses, such as polyoma, papillomavirus and adenovirus [8,9,34]. In this context, the role of VP1-YY1 complex resembles that of the adenovirus terminal protein [35]. This possibility is supported by the recent finding that the amino acids of YY1 responsible for NM interaction are located in the C-terminal domain (aa 257–341) [33], i.e. far from the activation domains we have shown to bind VP1 (aa 1–89); therefore, a single molecule of YY1 can concomitantly interact with both VP1 and NM.

Another interesting possibility is that the properties of VP1 may also be influenced by its interaction with YY1. In this context, it is worth recalling that VP1 has already been shown to be a multifunctional protein with several interesting properties, including binding to the cellular membrane receptor [16], self-assembling into capsid like structures [36] capable of encapsidating heterologous DNA [37,38] and, intriguingly, binding DNA non-specifically [6,7]. Since YY1 binds DNA in a specific manner, an additional biological role of VP1-YY1 interaction could be that of conferring DNA binding specificity to VP1. In this regard we should recall that two YY1 consensus sequences are present in the polyoma enhancer [14], therefore, this could be a mechanism by which the enhancer region is found in the virions in a nucleosome-free configuration [1]. These alternatives deserve further investigation.

The role of YY1 in forming different complexes with other cellular proteins so as to regulate gene expression in different ways [13], and its possible involvement in viral DNA recombination [39], is subject to extensive research. Our finding that a viral structural protein interacts with a protein as YY1, that appears to be a pivotal component of NM regulatory mechanisms, support the notion that viral gene expression could be conditioned not only by its early functions but also by late structural proteins. To define the functional role of YY1-VP1 association in the viral life cycle, the biological properties of

Py-VP1 mutants in the amino acids involved in this interaction are under investigation.

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