THE CELLULAR NUCLEAR MATRIX REGULATORY PROTEIN YY1 DOES NOT DIRECTLY INTERACT WITH THE MOUSE POLYOMAVIRUS LARGE TUMOR ANTIGEN

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Summary. – The transcription factor Yin-Yang 1 (YY1) is a multifunctional protein involved in repressing and activating many promoters of cellular and viral genes. YY1 functions via protein-DNA but also protein-protein interactions. The latter has been documented between YY1 and early gene products of adenoviruses (Lewis *et al.*, *J. Virol.* **69**, 1628–1636 (1995)) and papillomaviruses (Lee *et al.*, *J Virol* **72**, 4911–4917 (1998)). In this study, first of this kind on mouse polyomavirus (Py), we report that YY1 and the main viral regulatory protein, large tumor antigen (LT), do not interact directly *in vivo*. This evidence was obtained by use of two separate methods, immunoprecipitation (IP) and a yeast two-hybrid system

Key words: YY1, polyomavirus, large T antigen

The mouse Py is a small non-eveloped tumorigenic DNA virus. Its genome encodes three early regulatory proteins designated as tumor (T) antigens and three late structural (capsid) proteins, VP1, VP2 and VP3. Viral double stranded circular DNA is assembled with histones of cellular origin (except H1) in the form of a minichromosome encapsidated in an icosahedral capsid. The capsid is composed of major protein VP1 and minor proteins VP2 and VP3.

In the early stages of infection, the early region of viral DNA is transcribed by the host cell RNA-polymerase II and large tumor (LT), middle tumor (MT) and small tumor (ST) antigens are produced. LT initiates viral DNA replication and possesses a DNA helicase activity. It also autoregulates its own expression and by a complex series of events transactivates Py late promoter. These events include binding of LT to the Py DNA regulatory region, a possible interaction with enhancer-dependent transcription factors, and a down-regulation of early transcription. In addition, LT exerts an enhancer-mediated indirect effect on late genes expression that does not involve a specific interaction of LT with the viral DNA (Kern *et al.*, 1986). LT also directly binds many cellular factors, which together with MT and ST alter cell cycle signaling.

After the onset of viral DNA replication, the genes encoding capsid proteins are extensively transcribed from the late region of viral DNA. In contrast to the early genes promoter, the late genes promoter lacks a consensus TATA box and late mRNAs are initiated at multiple sites (for a review see Cole, 1996). Early and late regions of the Py genome are separated by the regulatory region consisting of an origin of DNA replication (ori) and enhancer of

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Abbreviations: α = anti; ECL = enhanced chemiluminiscence; IP = immunoprecipitation, h.p.i. = hours post infection, LT = large tumor antigen; MT = middle tumor antigen; NSA = non-specific antibodies; ori = origin of DNA replication; Py = mouse polyomavirus; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; ST = small tumor antigen; SV40 = simian virus 40, VP1 = viral protein 1 (major Py capsid protein); WB = Western blot analysis; YY1 = Yin-Yang 1 protein

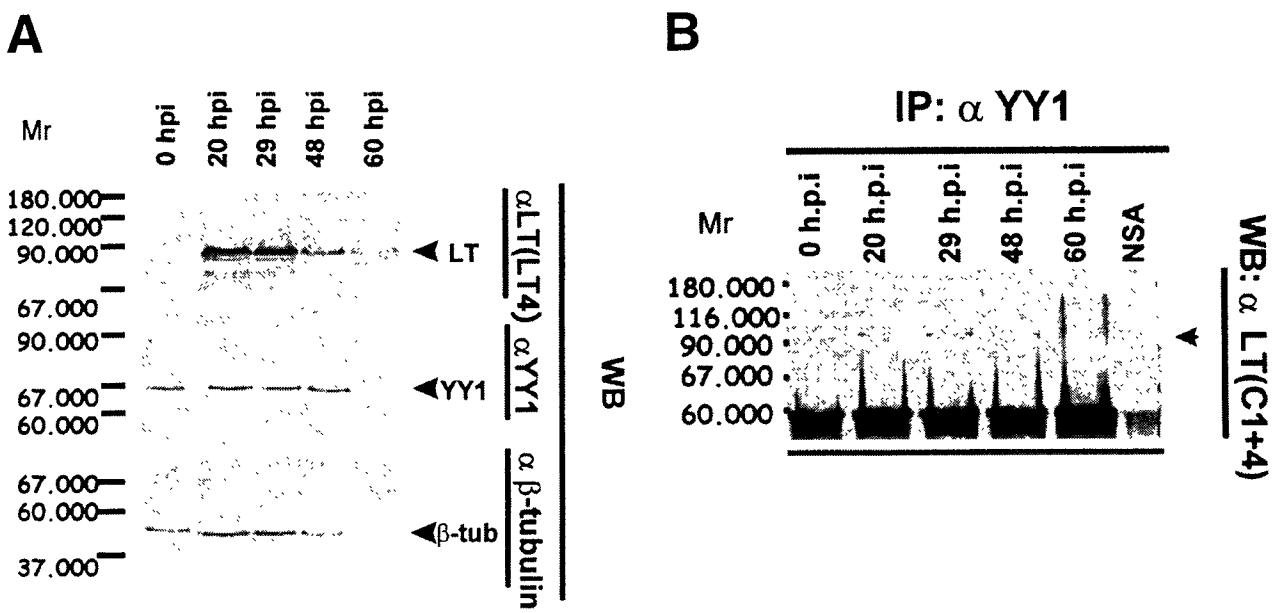

Fig. 1
Co-immunoprecipitation of Py LT with αYY1 antibody revealed by WB

A Cell extracts were prepared from Py-infected (50 PFU/ cell) 3T6 cells at different h p i Cell extracts, each sample corresponding to 0 2 mg of total protein, were electrophoresed and subjected to WB with αLT, αYY1 and αβ-tubulin antibodies.

B Cell extracts were prepared as above (A), and used for IP with αYY1 antibody Non-specific rabbit anti-mouse antibody (NSA) was used as control for IP with extracts from Py-infected cells harvested at 29 h p i The precipitates were electrophoresed and subjected to WB with the αLT (C1+C4) antibody LT is indicated by the arrow The strong bands at the bottom of the gel represent the immunoglobulin heavy chain

For the abbreviations see their list on the first page of the article.

replication. The ori has several binding sites for LT and the enhancer contains various transcription factor binding sites. Two specific binding sites for transcription factor YY1 have been found in Py enhancer (Martelli *et al.*, 1996). YY1 is known to be an important regulator of transcription, which can act as activator, repressor or initiator in a number of cellular and viral promoters (for a review see Shi *et al.*, 1997). YY1 is known to mediate basal transcription on TATA-less promoters (Usheva and Shenk, 1994), making the Py late promoter an ideal target for YY1 action. Indeed, YY1 has been found to stimulate the basal transcription from the Py late promoter, but the requirement of its function varied with the cell line used (Martelli *et al.*, 1996).

Protein-protein interactions with other transcription factors, co-activators and the nuclear matrix can modulate the YY1 activity and even extend the range of its functional potential in regulation of cellular and viral genes. The adenovirus early regulatory protein E1A interacts with YY1 and not only relieves the YY1-mediated repression but also activates transcription from YY1 binding sites (Lewis *et al.*, 1995). The papillomavirus early gene E2 product has also been shown to interact directly with YY1 *in vitro* (Lee *et al.*, 1998).

Taking into consideration multiple mechanisms by which Py LT activates Py late transcription (Cahill *et al.*, 1990; Kern *et al.*, 1986) and the extreme functional versatility of YY1 mediated by interacting proteins, we investigated the ability of Py LT to interact with YY1.

We performed several IP tests with whole cell extracts from Py-infected mouse 3T6 cells (3T6 cells were obtained from DSMZ, Germany) soon after the onset of viral DNA replication. All IP tests were done exactly as described by Forstova *et al.* (1993). Cells were lysed in the RIPA buffer (150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l Tris-HCl pH 7.4, 0.05% NP-40, 1% deoxycholic acid, and 1% Triton X-100), centrifuged at 10,000 x g for 3 mins, and the supernatant was used for IP. Cell extracts were incubated for 1 hr on ice in an IP buffer (150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l Tris-HCl pH 7.4, 0.05% NP-40, and 0.2% gelatin) with an antibody. Then protein A-Sepharose 4B Fast Flow (Sigma) beads were added and the incubation was continued with end-over-end mixing for 30 mins at 4°C. The immunoprecipitates were washed 4 times and eluted with the Laemmli sample buffer. At 20-29 hours post infection (h.p.i) there was a high level of LT in infected 3T6 cells (Fig. 1A).

We carried out IP experiments with an anti-YY1 (αYY1) antibody (C-20; Santa Cruz Biotechnology). The eluted proteins were subjected to SDS-PAGE and identified by

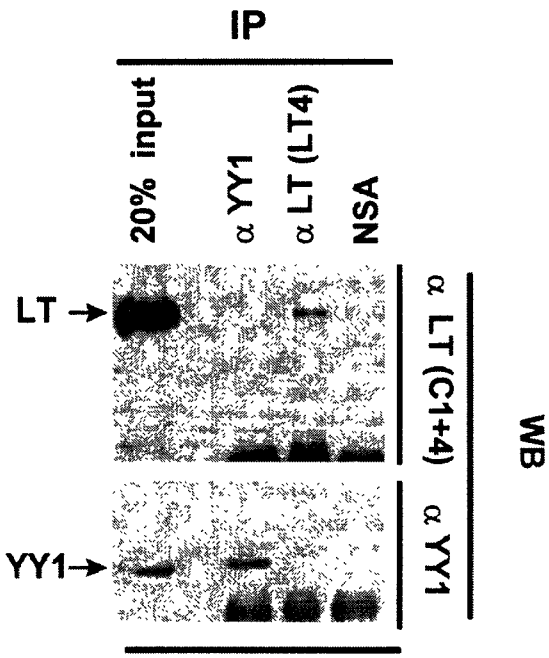

Fig. 2
Immunoprecipitation of LT and YY1 in an extract from insect SF9 cells overexpressing LT and YY1
An extract from SF9 cells was immunoprecipitated with αYY1 or αLT (C1+C4) antibodies. NSA was used as control as in Fig. 1B. The precipitates were electrophoresed and subjected to WB with αLT or αYY1 antibodies. LT and YY1 are indicated by the arrows. The experiments were repeated three times with identical results. For the abbreviations see their list on the first page of the article

Western blot analysis (WB) with an antibody against LT (αLT antibody). In all experiments we used either a LT4 antibody specifically recognizing LT or a mixture of C1 and C4 (1:1) antibodies directed against the common region of all T antigens (αLT antibodies were kindly provided by Dr. B. Griffin, St. Mary's Hospital, London, UK). Repeatedly, we detected a weak interaction. Fig.1B illustrates the results of the IP tests with the αYY1 antibody in cell extracts from Py-infected 3T6 cells prepared at different hrs p.i. ECL Western blotting analysis system (Amersham) was used for detection of LT with specific αLT antibody and only very faint bands of LT were seen.

To test for an interaction, we overexpressed LT and YY1 in a recombinant baculovirus system. SF9 insect cells (DSMZ, Germany) were co-infected with a recombinant baculovirus carrying YY1 gene (Palkova *et al.*, 2000) and a recombinant baculovirus carrying LT gene (M.Towey, B. Griffin and J. Forstova, unpublished data). Cells were harvested at 48 h.p.i., lysed in RIPA buffer and the IP was done with αYY1 and αLT antibodies as described by Forstova *et al.* (1993). We did not detect either LT in αYY1 immunoprecipitates or YY1 in αLT immunoprecipitates even though the crude lysates contained high levels of both proteins (Fig. 2).

To investigate the interaction in mammalian cells with stabile expression of LT we used WOP cells (ICLC, Genova) for IP experiments. WOP cells are mouse 3T3 cells transformed by ori-defective Py. We found that WOP cells express high levels of LT and do not produce MT or any of the Py late gene products. We were not able to detect by IP any LT with the αYY1 antibody (Fig. 3A) and the αLT antibody was not able to bring down any YY1 from WOP cell extracts (Fig. 3B).

To prove the putative direct *in vivo* interaction between the two proteins we also employed a yeast two-hybrid system (Matchmaker Two-Hybrid System, Clontech). The LT gene was fused with the DNA binding domain of GAL4 of pGBT9 plasmid and the recombinant was designated as pGBT9-LT. The expression of LT from pGBT9-LT was confirmed by WB. The plasmid pGAD424-YY1 carrying YY1 sequence fused with GAL4 AD domain was described previously (Palkova *et al.*, 2000). Both plasmids were

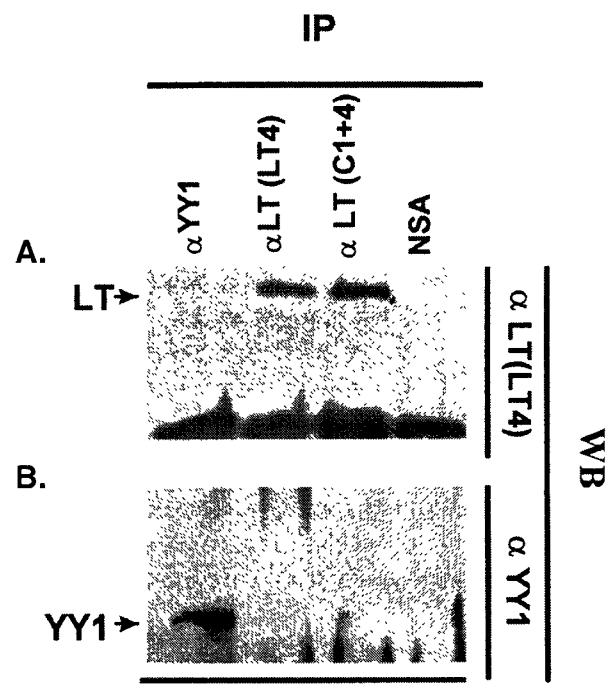

Fig. 3
Immunoprecipitation of LT and YY1 in an extract from WOP cells
An extract from WOP cells was immunoprecipitated with αYY1 or αLT (LT4 or C1+C4) antibody. NSA was used as control as in Fig. 1B. The precipitates were electrophoresed and subjected to WB with αLT (A) or αYY1 antibody (B). LT and YY1 are indicated by the arrows. The experiment was repeated three times with identical results For the abbreviations see their list on the first page.

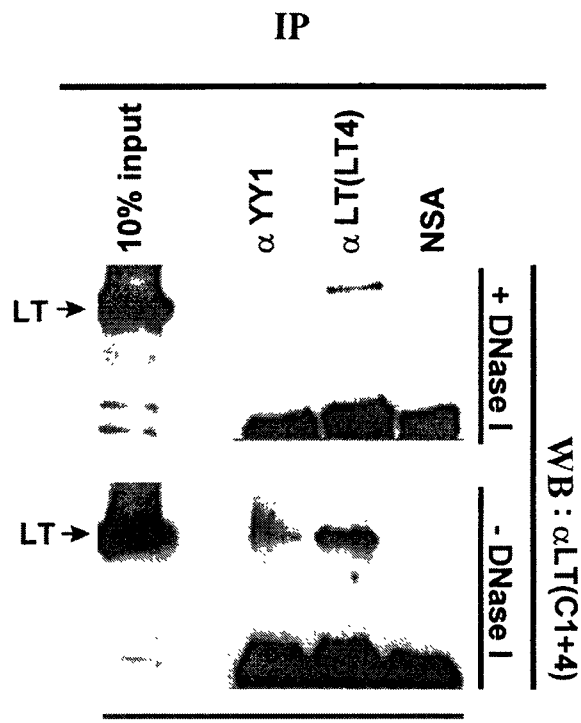

Fig. 4
Immunoprecipitation of LT and YY1 after DNase I treatment
Extracts were prepared from Py-infected 3T6 cells at 24 h p i. as above (Fig 1A) The extracts were immunop recipitated after 15 mins of treatment with DNase I (final concentration 50 μg ml) at 37°C αYY1 or αLT (LT4) antibody as well as NSA were used for IP The precipitates were electrophoresed and subjected to WB with αLT (C1+C4) antibody LT is indicated by the arrow For the abbreviations see their list on the first page

introduced either together or separately with an empty partner plasmid into *Saccharomyces cerevisiae* HF7-c strain This strain allows detection of interacting proteins by activation of HIS3 as well as lacZ gene promoter. These two-hybrid experiments were performed by following the manufacturer's instructions. The proteins did not activate the lacZ promoter when produced either separately or together as detected by filter assay of b-galactosidase activity. Expression from pGBT9-LT caused minimal growth of the yeast on the medium without histidine when co-transformed with empty pGAD424 plasmid, but this growth did not increase after co-transformation with pGAD424-YY1 plasmid.

LT from the closely related simian virus 40 (SV40) is known to exist in various biochemically defined forms, which differ in their functions and distribution. The distribution of LT within the nucleus follows a defined pattern during the course of infection (Schirmbeck and Deppert, 1989). Py LT is similar to SV40 LT in many respects. A significant portion of LT is strongly bound to the nuclear matrix (Buckler-White *et al.*, 1980). This makes YY1 an ideal interacting protein candidate as it is an intrinsic component of nuclear matrix. Nevertheless, the IP experiments with non-infected WOP and SF9 cells (where none of other Py proteins was present) showed absence of the presumed interaction.

Since these results could reflect the fact that proteins strongly bound to nuclear matrix could not be solubilized by the mild extraction buffer used, we employed a yeast two-hybrid system. We were once again not able to detect a direct *in vivo* interaction.

In our initial experiment we found that the YY1-LT association is detectable in Py-infected 3T6 cells. We believe that this association was mediated by DNA, since the DNase I treatment of cell lysates abolished the co-immunoprecipitation of both proteins (Fig. 4). This result could have been expected since LT as well as YY1 are known to be DNA binding proteins. Nevertheless, this fact does not exclude the possibility that Py LT can be physically and/or functionally connected with YY1 through specific DNA binding. The role of YY1 and LT in activation of Py late promoter has been studied in great detail in transient and stable expression assays with Py promoter or regulatory region cassettes inserted into promoterless expression vectors containing a reporter gene (Martelli *et al.*, 1996; Bourachot *et al.*,1989; Cahill *et al.*,1990; Kern *et al.*, 1986). However, these assays have never been performed in the presence of both proteins and their binding sites. We believe that this kind of assay could help to reveal the character of Py LT-YY1 association studied in this work. A transient expression study of the whole Py regulatory region, modified by the defined sequence insertion that prevents a direct contact between LT and YY1, is currently in progress.

It is worth recalling that all the results presented here were obtained from *in vivo* experiments, which means that the Py LT-YY1 association can also be mediated by other cellular or viral DNA binding proteins. For example, Py LT as well as YY1 are known to interact directly with histone acetylase p300 (Lee *et al.*,1995; Nemethova and Wintersberger, 1999), an important cellular regulator of transcription. Another interesting interaction between the activation domain of YY1 and Py VP1 has also been demonstrated recently (Palkova *et al.*, 2000), suggesting that Py late gene products may also be involved in regulation of the Py life cycle.

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