ANALYSIS OF REGULATION OF POLYOMAVIRUS PROMOTERS BY A LUCIFERASE REPORTER SYSTEM

H. ŠPANIELOVÁ

Department of Genetics and Microbiology, Charles University in Prague, Viničná 5, 128 44 Prague 2, Czech Republic

Received November 4, 2002; accepted December 10, 2002

Summary. – The transcription factor Ying-Yang 1 (YY1), which has been shown to activate basal transcription from mouse polyomavirus (Py) late promoter, does not interact with the main viral regulatory protein, the large tumor antigen (LT) *in vivo* directly but via DNA (Španielová and Velková, 2001). In this report, the role of association of YY1 with LT in regulation of the Py late promoter was studied by a luciferase reporter system. The distance between YY1 and LT binding sites in the viral regulatory region was extended by insertion of a transcriptionally inert sequence 166 nucleotides (nt) long thus making physical contact between LT and YY1 bound to their sites impossible or difficult. Analysis of viral early and late promoter activities was performed in the presence or absence of viral early tumor antigens and in the presence or absence of an inhibitor of DNA replication. The study indicated that regulation of the Py late promoter is DNA replication-linked and that association of both proteins may be involved in this type of regulation. Moreover, the plasmid DNA replication assay showed that insertion of the inert sequence between the ori-core and enhancer restricted replication of the reporter plasmid.

Key words: polyomavirus; late promoter; YY1 protein, large T antigen

Introduction

Polyomavirus (Py) is a small non-enveloped double-stranded DNA virus. The viral capsid is composed of three viral proteins VP1, VP2 and VP3 and surrounds the viral minichromosome. The Py circular 5.3 kb long genome can be divided into an early and a late region. The early region is expressed prior to the onset of viral DNA replication and encodes three viral regulatory proteins – tumor antigens, which are designated according to their size large T (LT), middle T (MT) and small T (ST) antigens. The late region encodes three viral capsid proteins (for a review see Cole, 1996). The Py genome contains a single origin of DNA replication (ori). The core sequence of ori (ori-core) is 68 bp long and serves as a binding site for two hexamers of LT which bind ori (Cowie and Kamen, 1984) in an ATP-dependent manner (Lorimer *et al.*, 1991). LT then recruits the cellular DNA polymerase α-primase and replication protein A to the ori region (Collins and Kelly, 1991; Kautz *et al.*, 2001), thus forming an initiation complex. There are two auxiliary sequences (aux-1 and aux-2), located adjacent to the ori-core (Fig. 1), which synergistically activate initiation of DNA replication (Herbomel *et al.*, 1984). Aux-1 is located at the early site of ori-core and contains other LT-binding sites. Aux-2 is at the late side of ori-core and contains a transcription enhancer which is made up of various transcription factors binding sites determining the tissue specificity of virus replication. In the absence of the enhancer, Py replicates very poorly (de Villiers *et al.*, 1984).

The promoters of early and late transcription units are also located close to the ori and therefore this portion of the

E-mail: hs@natur.cuni.cz; fax: +4202 21951729.

Abbreviations: AraC = cytosine-ß-arabinofuranoside; aux-1, 2 = auxiliary sequences 1,2; CMV = cytomegalovirus; DME medium = Dulbecco's modified Eagle's medium; LT = large tumor antigen; MT = middle tumor antigen; ori = origin of DNA replication; ori-core = core of ori; Py = polyo-mavirus; ST = small tumor antigen; SV40 = Simian virus 40; VP1 = major capsid protein; wt = wild type; YY1 = Ying-Yang 1 protein

viral genome, containing promoters, enhancers and ori is referred to as the viral regulatory region. The early Py promoter contains typical eukaryotic promoter elements, such as TATA and CAAT box sequences upstream of the transcription initiation site and is regulated by cellular factors having their binding sites in the viral enhancer. The Py late promoter lacks the consensus TATA box and the late mRNAs are initiated at multiple sites (Cole, 1996) indicating that regulation of both promoters is different, although certain sequences reported to influence early transcription have been found to affect also the late promoter. There is strong evidence that PEA1 and PEA3 binding sites can function as initiator elements for late transcription (Yoo *et al.*, 1991). Moreover, two additional elements have been identified which positively contribute to the activity of the late Py promoter (Bourachot *et al.*, 1989; Cahill and Carmichael, 1989; Martelli *et al.*, 1996). These elements are binding sites for a multifunctional protein YY1 (Martelli *et al.*, 1996) which is known to act as an activator, repressor or initiator binding protein in a number of cellular and viral promoters (for a review see Shi *et al.*, 1997). YY1 is known to mediate basal transcription from the TATA-less promoters (Usheva and Shenk, 1994) and therefore the Py late promoter is an ideal target for YY1 action. In our previous work we have found a DNA-mediated association between YY1 and LT (Španielová and Velková, 2001). The role of YY1 and LT in activation of the Py late promoter has been studied separately in great detail in transient and stable expression assays with the late Py promoter only or with the whole Py regulatory region cassettes inserted into promoter-less expression vectors containing a reporter gene (Kern *et al.*, 1986; Bourachot *et al.*, 1989; Cahill and Carmichael, 1989; Cahill *et al.*, 1990; Martelli *et al.*, 1996). However, these assays have never been performed in the presence of both LT and YY1 and their binding sites.

In this study, the whole Py regulatory region, modified by insertion of a defined sequence that made the physical contact between LT and YY1 bound to their sites impossible or difficult, was used in a transient expression assay and in analysis of early and late promoter activities by luciferase reporter gene expression.

Materials and Methods

Cell cultures and virus. Swiss albino mouse fibroblasts (NIH 3T6, ATCC), normal murine mammary gland epithelial cells (NMuMG, ATCC) and WOP cells (ICLC, Genova) were grown at 37°C in a 5% CO_2-air humidified atmosphere using the Dulbecco's modified Eagle's (DME) medium supplemented with 2 mmol/l glutamine and 10% of fetal calf serum. WOP cells are mouse 3T3 cells transformed with the ori-defective Py which produce high levels of LT.

Py DNA extraction. Infection of 3T6 cells with Py was performed at a MOI of 5 PFU/cell. The cells were harvested 40 hrs post infection and DNA extraction was done according to Arad (1998).

Luciferase reporter gene constructs and expression plasmids. Viral DNA was obtained from the recombinant plasmid pMJG (Krauzewicz *et al.*, 1990), which contained a complete Py genome of the wt Py A3 strain, interrupted at the *Eco*RI site (nt 1560) and inserted into the *Eco*RI site of the pAT153 plasmid. Reporter gene plasmids were constructed by inserting the *Sac*I restriction fragment, excised from the Py A3 genome (GenBank Acc. No. J02288), spanning the Py regulatory region (nts 569-0/5292-4341) (Fig. 1) into the *Sac*I site of the promoter-less plasmid pGL3-BASIC (Promega). The luciferase reporter gene was put under control of either early or late promoters (the constructs pGL3-EARLY or pGL3-LATE, respectively), depending on the orientation of the regulatory region. The pGL3-LATE plasmid was further modified by insertion of a spacer sequence 166 nt long to obtain the construct pGL3-LATE+ with binding sites for LT and YY1 separated. For spacer sequence insertion, pGL3-LATE was digested with *Stu*I (nt 5226 in the Py numbering system) and the 166 bp long fragment, obtained from PCR amplification of the multiple cloning site from pBluescript KS+ (Stratagene), was blunt-end ligated into this site. The PCR was done by using a high fidelity DNA polymerase (the Vent-DNA-polymerase, New England Biolabs) and T3 and T7 primers.

The pGL3-BASIC plasmid (Promega) containing the luciferase gene but lacking an eukaryotic promoter and enhancer was used as a parental vector for reporter gene constructs and as a negative control to monitor a background activity of luciferase.

The pGL3-CONTROL plasmid (Promega) containing luciferase gene under the control of the SV40 promoter-enhancer. It displayed a strong expression of the luciferase reporter gene and was used as a positive control.

The pcDNA3 plasmid (Invitrogen) containing enhancer-promoter sequences for the immediate early genes of human CMV and SV40 ori but no gene to be expressed (therefore designated "empty") was used as a parental vector for cloning Py LT and MT, and a means to equalise the total amount of transfected DNA.

pCMVß plasmid (Clontech) expressing high levels of ß-galactosidase under the control of the human CMV immediate early promoter; was used as a reference plasmid when transfecting luciferase reporter gene constructs to monitor transfection efficiency.

The pCMV-LT plasmid was prepared by inserting the intron-less LT gene from plasmid pPyLT1 (Strauss *et al.*, 1986) into the *Bam*HI site of the plasmid pcDNA3.

The plasmid pCMV-MT, derived from the plasmid pcDNA3, was used for expression of Py MT (H. Lu and B. Griffin, personal communication).

The plasmid pCMV-YY1, derived from the plasmid pCB6+ was used for expression of YY1 (Austen *et al.*, 1997).

Transient transfections. 3T6 or NMuMG cells were transfected using the DEAE-dextran method. The cells were plated at a density of 2.5 x 10^5 per 60-mm-Petri dish and incubated overnight at 37°C in DME medium supplemented with 5% of fetal calf serum. In the standard procedure, the cells were transfected with 6 µg of DNA

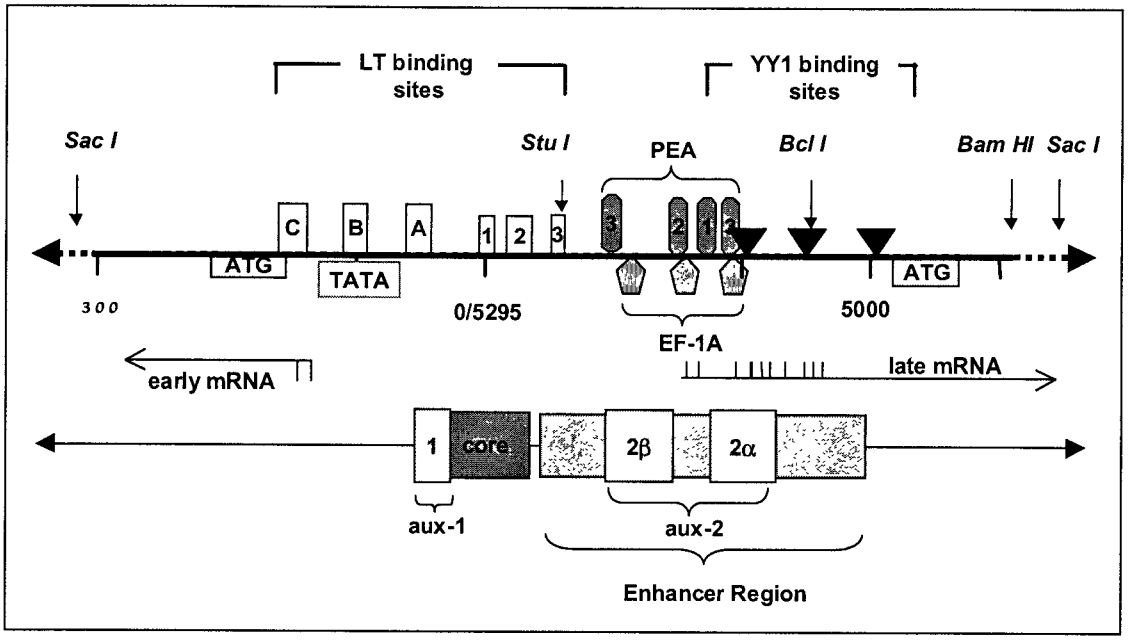

Fig. 1
Regulatory region of Py

The location of the ori-core of viral DNA replication and the location of auxiliary sequences (aux) that enhance viral DNA replication are shown. Replication requires either aux-2α or aux-2ß (Cole, 1996). The transcription factors binding sites in the regulatory region are indicated as follows: the three major and three minor LT binding sites are represented by white rectangles designated A, B, C and 1, 2, 3, respectively (Cowie and Kamen, 1984); the black triangles indicate YY1 binding sites (Martelli *et al.*, 1996); the black-dotted pentagons represent EF-1A-binding sites (Bolwig and Hearing, 1991); PEA1, PEA2 and PEA3 elements are illustrated by grey hexagons (Yoo *et al.*, 1991). The PEA1 motif is bound by the mouse homolog of transcription factor AP-1 (Piette and Yaniv, 1986; Martin *et al.*, 1988), the PEA3 motif is bound by members of Ets-encoding family of protooncogenes (Wasylyk *et al.*, 1990) and the PEA2 element is bound by the Py enhancer binding factor 2 (PEBP2), a homolog of the human AML1 gene product (Kamachi *et al.*, 1990; Ogawa *et al.*, 1993). The restriction enzyme recognition sites are indicated by arrows. In the *Stu*I site the 166 bp long sequence was inserted in this study and the aux-1 and ori-core became more distant from the enhancer region. The light grey boxes indicate the early TATA box and start codons for early and late genes; the nucleotide numbering system is as originally described (Soeda *et al.*, 1980).

which consisted of the following plasmids: 2 μg of a luciferase reporter construct, 2 μg of the reference plasmid pCMVß and 2 μg of a trans-activator plasmid (as indicated in figure legends). When no trans-activator DNA was needed for co-transfection, the total amount of DNA was equalized with the "empty" pcDNA3 plasmid. The plasmid DNA mixture was resuspended in 600 μl of phosphate-buffered saline (PBS) containing 250 μg/ml DEAE-dextran, added to the cells previously rinsed with PBS and incubated for 30 mins, followed by addition of 2 ml of DME medium containing 80 μmol/l chloroquine (Sigma) to each dish. After 3 hrs, the incubation medium was replaced by 1 ml of 10% DMSO (Sigma) in DME medium for 1.5 min and the latter was immediately removed. Fresh DME medium was added or the cells were further maintained in the fresh DME medium containing 20 μg/ml cytosine-ß-arabinofuranoside (AraC) to inhibit viral DNA replication.

WOP cells were transfected using a low voltage electroporation. A suspension of freshly grown WOP cells (5 x 10^5) was transfected with identical amounts of plasmid DNA (as described for 3T6 and NMuMG cells) in 220 μl of the OPTIMEM medium in a 0.4 cm cuvette using the Gene Pulser II Electroporation System (Bio-Rad) set at 960 μF and 0.2 kV with a time constant 60–70 secs.

Luciferase activity assay. If not indicated otherwise, forty hrs post transfection, cells were washed twice with PBS, scraped into an Eppendorf tube, resuspended in 100 μl of 250 mmol/l Tris-HCl pH 7.4 and lysed by three freeze-thaw cycles. Cell debris was removed by centrifugation at 10 000 x g for 1 min and the supernatant ("the extract") was directly used for measuring the luciferase and ß-galactosidase activity. Assay of luciferase activity was done in a Microlite TLX2 luminometer (Dynatech Laboratories, Inc.). Ten μl of the extract was mixed with 50 μl of the Luciferase Assay Substrate Solution (Promega) and integrated values of luminescence intensity for 10 secs were recorded. Another 10 μl of the extract was used for the ß-galactosidase activity assay (Sambrook *et al.*, 1989). The protein concentration in cell extracts was measured according to Bredford (1976). The luciferase activity was normalized for ß-galactosidase activity and protein content. All transfections were done at least three times and all assays were done in duplicate.

Plasmid replication assay. Forty hrs post transfection total DNA from transfected WOP cells was extracted (Park and Hoffmayer, 1997). Samples were subjected to *Hind*III and *Dpn*I digestion and resolved by 1% agarose gel electrophoresis. The resolved DNA was blotted onto a nylon membrane (Zeta-Probe, Bio-Rad) by

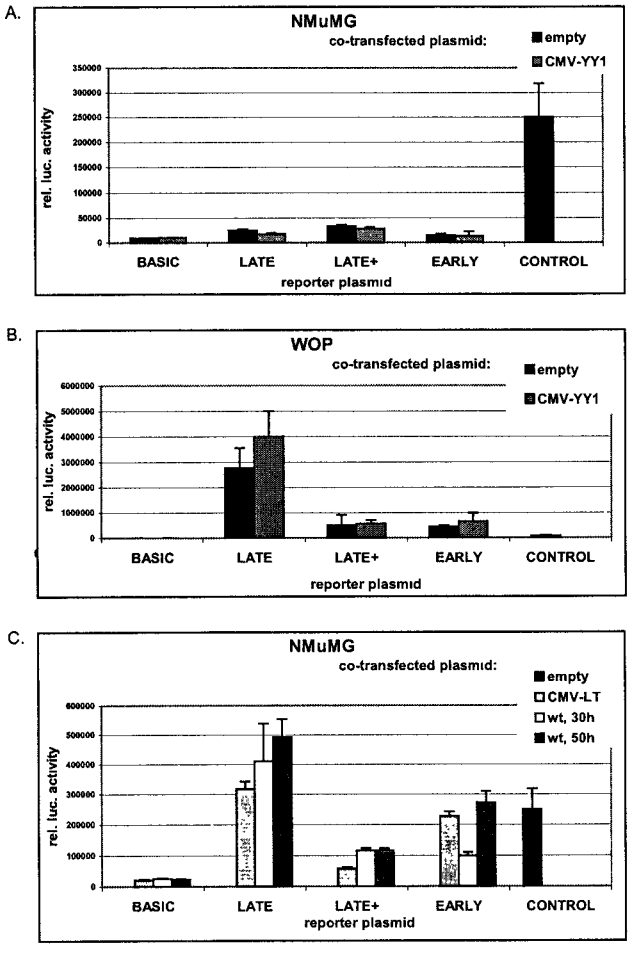

Fig. 2
Analysis of Py promoter activities by reporter gene expression and effect of co-expression of YY1, LT or viral antigens on promoter activity

Cells were co-transfected with individual reporter plasmid pGL3-BASIC (as a negative control), pGL3-LATE (Py late promoter), pGL3-LATE+ (Py late promoter with the 166bp insertion), pGL3-EARLY (Py early promoter) or pGL3-CONTROL (positive control) together with reference plasmid pCMVß and trans-activator DNA. (A) NMuMG cells were co-transfected with a reporter plasmid, reference plasmid and trans-activator plasmid, which was either pcDNA3 without the insert ("empty") or pBC6^{+}-YY1 (CMV-YY1) expressing YY1. (B) WOP cells were co-transfected with plasmids as above. (C) NMuMG cells were co-transfected with a reporter plasmid, reference plasmid and trans-activator plasmid pcDNA3-LT (CMV-LT) expressing LT or wtPy DNA was used as a trans-activator for co-expression of all viral antigens. In the latter cases, the luciferase activity was assayed 30 or 56 hrs post transfection (as indicated) to monitor activity of promoters during the virus life cycle. Average values of normalized luciferase activity from three independent experiments are shown. Bars indicate the standard deviation of the mean. Note the differences in scale on (A), (B) and (C).

capillary transfer in 0.4 mol/l NaOH and 0.6 mol/l NaCl and hybridized with the ^{32}P-labeled luciferase gene probe (Sambrook *et al.*, 1989) at 68°C for 20 hrs. Washing (4 x 30 mins) was performed with 0.1 x SSC and 1% SDS at 68°C and the blots were then exposed to X-ray films or analyzed by PhosphoImager.

Results

Reporter plasmid construction and comparison of basal and LT-activated promoter activity

The Py regulatory region spanning the ori-core, enhancer and the first thirds of both transcription units was inserted in the early (the plasmid pGL3- EARLY) or late (the plasmid pGL3-LATE) orientation relative to the luciferase reporter gene. The choice of having two transcription units present in these plasmid constructs was made to approximate more closely the physiology of viral infection and to include all of the LT-binding as well as YY1-binding sites to the sequence under investigation. To extend the distance between these binding sites, a 166 bp long fragment was inserted in the *Stu*I site of the Py regulatory region (the plasmid pGL3-LATE+). Albeit the *Stu*I site is located at the minor LT-binding site 3 (Fig. 1), its modification should not interfere with the transcriptional regulation since it has been earlier shown that this site can be deleted without any effect on the ability of LT to stimulate expression from the late promoter (Kern *et al.*, 1986). The insertion should also leave the ori-core as well as the enhancer region intact. Individual reporter plasmids were transfected into the permissive mouse 3T6, NMuMG or WOP cells and luciferase activity was measured. Since the NMuMG cells were transfected with better transfection efficiencies than 3T6 cells, the data presented here for the cells non-expressing Py T antigens were obtained entirely from the NMuMG cell line. The results presented in Fig. 2A show that, in NMuMG cells, the basal level of luciferase gene expression was very low for all the reporter gene constructs compared to pGL3-CONTROL showing a strong luciferase expression. Luciferase activity measured for pGL3-LATE was reproducibly slightly lower than that for pGL3-LATE+. Interestingly, the early Py promoter (pGL3-EARLY) gave even a lower activity than the late Py promoter (pGL3-LATE) but just slightly higher than the background activity detected for the promoter-less pGL3-BASIC (Fig. 2A). When the same experiment was performed in WOP cells (mouse cells expressing high level of LT), the results were completely different (Fig. 2B). As expected, in the presence of LT antigen, the luciferase expression from the late Py promoter dramatically increased. The luciferase activity measured for pGL3-LATE was 40–80 times higher than that for pGL3-CONTROL. The results presented in Fig. 2B indicate that insertion of the 166 bp long sequence into the Py regulatory region led to a 5-fold decrease in the LT-stimulated late promoter activity. To confirm that the overall increase in luciferase activity was indeed due to the presence of the early T antigens and not to the different cell type, reporter gene plasmids, trans-activator pCMV-LT plasmid or Py wtDNA were co-transfected into NMuMG cells. Expression of LT was confirmed by immunostaining. Albeit

the overall luciferase activities for NMuMG cells were lower than those for WOP extracts, the data presented in Fig. 2C show a similar mode of stimulation in the reporter gene expression. It may be important to note, that under all described conditions the Py early promoter, although functional, was never activated to the same extent as the Py late promoter (see Fig. 2).

Effect of exogenous expression of YY1 on reporter gene transcription

In order to determine the significance of DNA-mediated association of LT and YY1 in regulation of the Py late promoter, reporter gene constructs were tested with increased intracellular level of YY1 ensured by its over-expression from pCMV-YY1. In mouse cells non-expressing Py early T antigens, the luciferase expression was not stimulated by higher levels of YY1 (Fig. 2A); even a slight repressive effect was observed. In WOP cells (Fig. 2B) or NMuMG cells co-transfected with pCMV-LT (data not shown), over-expression of YY1 led to an approximately 1.5 fold increase of luciferase expression regulated by early or late promotors (pGL3-EARLY or pGL3-LATE, respectively). However, no significant increase in luciferase activity was detected for pGL3-LATE+. Thus insertion of the 166 bp long sequence into the Py regulatory region resulted in loss of the ability of exogenously expressed YY1 to stimulate the luciferase expression under the control of the Py late promoter.

Effect of the sequence insertion on reporter plasmid replication

Since LT initiates Py DNA replication, the difference in its stimulatory effect on luciferase activity for pGL3-LATE and pGL3-LATE+ could result either from a difference in the reporter plasmid replication or transcription efficiency. To distinguish between these possibilities, the plasmid replication was analysed. pGL3-LATE, pGL3-LATE+, pGL3-EARLY and pGL3-BASIC (a negative control) were individually transfected into WOP cells. Forty hrs post transfection total DNA was extracted and digested with *Hind*III to linearise the plasmid DNA and with *Dpn*I to degrade the unreplicated DNA. After gel electrophoresis DNA samples were subjected to Southern blot analysis with a ^{32}P-labeled luciferase gene DNA probe. Fig. 3 shows that replication of pGL3-LATE+ was reduced to 10% as compared to pGL3-LATE or pGL3-EARLY plasmids.

Effect of sequence insertion on reporter gene transcription in absence of DNA replication

In order to minimize any variation in template concentration the experiments were repeated in the presence of a

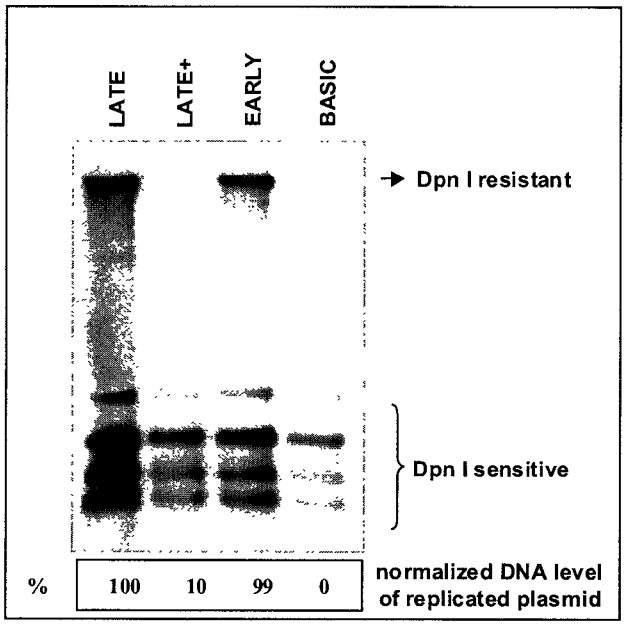

Fig. 3
Plasmid DNA replication analysis

WOP cells were transfected with 2 μg of each reporter plasmid (as designated) and 40 hrs post transfection total DNA was extracted. Plasmid DNA was linearized by digestion with *Hind*III and the unreplicated plasmid was digested with *Dpn*I. The analysis was performed with ^{32}P-labeled luciferase gene as a probe. Hybridization signals of *Dpn*I-resistant and *Dpn*I-susceptible DNA were quantified using the PhosphoImager, and relative signal intensities were calculated.

DNA replication inhibitor, AraC in the medium. Reporter plasmids pGL3-LATE and pGL3-LATE+ were transfected into NMuMG or WOP cells and the luciferase activity was measured (Fig. 4). The luciferase gene expression remained at basal level in WOP cells and it was higher for pGL3-LATE+ than for pGL3-LATE. Over-expression of YY1 had a slightly repressive effect on pGL3-LATE and pGL3-LATE+ driven luciferase gene expression. Since the stimulatory effect of MT on the activity of Py late promoter under similar conditions has been previously described (Yoo *et al.*, 1991), individual reporter plasmids were co-transfected with pCMV-MT into NMuMG cells. No stimulatory effect of MT on the luciferase expression driven by pGL3-LATE or pGL3-LATE+ was detected. Interestingly, the promoter-less plasmid pGL3-BASIC exhibited an increase in basal luciferase gene expression, thus showing that MT can stimulate transcription independently of promoter sequences.

The results show that extension of the distance between the LT-binding and YY1-binding sites (considering a linear form of the DNA region of concern) and consequently extension of the distance between LT and YY1 bound to

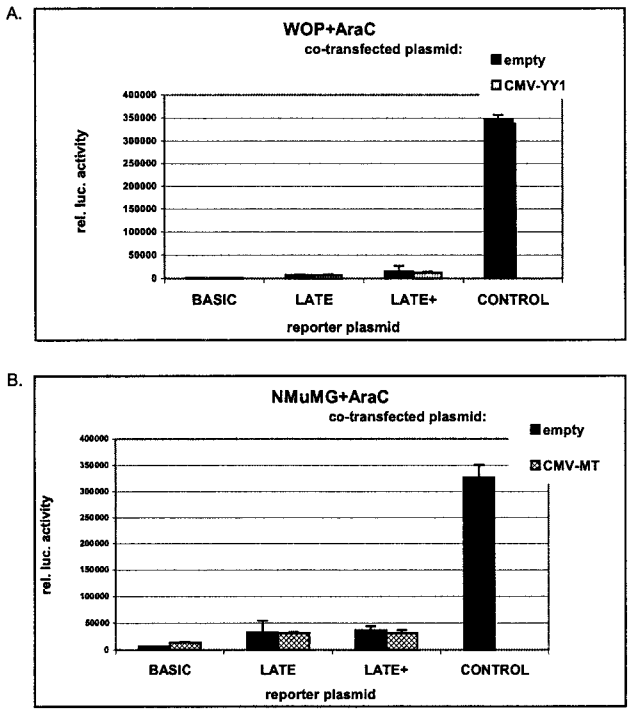

Fig. 4
Analysis of Py promoter activities by reporter gene expression in the presence of AraC

Cells were transfected as in Fig. 2 with a reporter plasmid, reference plasmid pCMVß and trans-activator plasmid. AraC was added to the medium immediately after transfection. (A) WOP cells were co-transfected as in Fig. 2B. (B) In NMuMG cells, each co-transfection was performed with the reporter plasmid, reference plasmid and trans-activator plasmid, which was either pcDNA3 without insert ("empty") or pcDNA3-MT (CMV-MT) expressing MT. The luciferase activity was assayed 40 hrs post transfection. Average values of normalized luciferase activity from three independent experiments are presented. Bars indicate the standard deviation of the mean.

their sites had almost no effect on the late promoter transcription *per se*.

Discussion

A wide array of studies using the reporter gene system have been done in order to elucidate regulation of the Py late promoter. Albeit some data are contradictory, several interesting findings can be noted from these studies. The study performed with an ori-defective reporter plasmid containing the complete enhancer and half of the early region (nts 1587-0/5292-5022) (Kern *et al.*, 1986) indicated that (i) induction of the late transcription is dependent on some enhancer elements and is LT-mediated; (ii) removal of the early transcription unit highly increases late transcription which is otherwise very low. In contrast to (i), (iii) Yoo *et al.* (1991) have reported no effect of LT on the late transcription in a viral construct in the presence of the inhibitor of DNA replication while MT stimulated the late transcription under the same conditions. (iv) The Py late promoter itself (nts 5130–4639) appeared to be very efficient when analyzed without involvement of other regulatory sequences (Bourachot *et al.*, 1989). (v) In the experimental setting almost identical to that described previously, YY1 has been reported as an activator of the late transcription (Martelli *et al.*, 1996).

To gain further insight into the role of DNA-mediated interaction of LT with YY1 and taking into consideration all the abovementioned findings, the entire Py regulatory region (nts 569-0/5292-4341) was analyzed by a luciferase reporter system. An intact viral sequence comprising the first third of both transcription units was inserted in both orientations proximal to the luciferase gene. This approach facilitated measurement of luciferase activity under the control of the early as well as late promoter.

In permissive mouse cells, the activities were very low, supporting the finding that in the viral context both transcription units mutually suppress each other (Kern *et al.*, 1986). This could explain why insertion of the 166 bp inert sequence between the ori-core and enhancer resulted in a small increase in the basal activity of the late promoter.

Here it should be noted that the assumption implicated in our and other studies that insertion of a sequence between the two transcription factor binding sites may really cause extension of the distance between these factors is based on a linear model of the region of concern. However, linear arrangement of the DNA region is obviously different from real three-dimensional one, which is, strictly speaking, unknown. Therefore also the effects of the insertion on interactive capabilities of LT and YY1 bound to their specific sites on DNA should be interpreted very cautiously.

The exogenously expressed YY1 did not substantially affect the activity of the late promoter. Taking into consideration the work of Martelli *et al.* (1996), this finding is surprising but consistent with the data obtained earlier by point mutational analysis of the Py enhancer (Shivakumar and Das, 1998), which showed that inactivation of YY1 binding sites had no effect on late transcription under DNA non-replicating conditions.

When the activity of reporter gene constructs was assayed in the presence of LT, the luciferase gene expression dramatically increased thus showing that the system was functional in both orientations. The early promoter has never been activated to the same extent as the late one. Even when the wt Py DNA was used as a trans-activator to simply approximate viral infection, the early promoter exhibited a lower activity than the late promoter at all tested times, but the continuous increase of luciferase activity driven by the

early promoter was detected throughout the experiment. This is consistent with the recent findings of Chen and Fluck (2001a), which refute the deep-rooted idea that an extensive transcription of early genes in the early stages of infection is abolished after onset of the late transcription. Insertion of the 166 bp sequence between the LT and YY1 binding sites impaired the reporter gene expression from the late promoter in the presence of LT. The explanation of this finding came from DNA replication analysis, which proved that modification of the regulatory sequence led to the reduction of reporter plasmid replication. It is known that transcription factors, whose binding sites were found in the Py enhancer, can stimulate the Py DNA replication. This was shown for the transcription factor AP1 (Murakami *et al.*, 1991), which activated the Py DNA replication via the AP1 binding site in the enhancer. The increasing distance between the ori-core and AP1 binding sites resulted in a sharp decrease in the replication activity, but the AP1 ability to stimulate transcription had not been affected under these circumstances. This is probably not true for YY1. In our experiments, in the presence of LT, an exogenously expressed YY1 protein increased the luciferase activity for pGL3-LATE and pGL3-EARLY by a factor of 1.5, but no significant stimulatory effect of higher levels of YY1 on pGL3-LATE+ driven luciferase expression was observed. A straightforward interpretation of this finding could be that the 166 bp sequence insertion changed the ability of YY1 to activate the late promoter thus favoring the hypothesis that the physical contact of LT with YY1 is important for regulation of the late transcription. However, the results from the experiments performed in the presence of AraC, an inhibitor of DNA replication, offer another interpretation. These results showed that not LT but the DNA replication *per se* is important for further stimulation of the reporter gene expression by exogenous YY1. It is also possible that the endogenous intracellular level of YY1 is high enough for regulation of Py promoters until the template concentration reaches some threshold, when the exogenously added YY1 is needed. This threshold may never be achieved by the replication-defective pGL3-LATE+ and the effect of higher levels of intracellular YY1 will be apparent only for fully replication-competent plasmids pGL3-LATE and pGL3-EARLY.

Nevertheless, these results question the actual role of YY1 in regulation of the Py late promoter. Albeit there is no doubt that YY1 functions as an activator of the basal transcription when the late promoter is analyzed out of the viral context (Martelli *et al.*, 1996), its role in the replication of the viral DNA cannot be excluded. The fact that luciferase gene expression from Py late and early promoters (which should differ in their regulation) was stimulated by exogenous YY1 to the same extent (1.5 fold; Fig. 2B) supports the latter notion and indicates a necessity of further investigation.

Since the reporter plasmid replication contributes to the expression of the reporter gene, experiments were also done in the presence of AraC, an inhibitor of DNA synthesis (elongation). Under these conditions no increase in the luciferase gene expression driven by the tested promoters was detected in the presence of the early gene products. In WOP cells a very low luciferase activity corresponding to basal levels, was detected for pGL3-LATE and pGL3-LATE+. The work, which has been earlier done under very similar conditions (Yoo et al., 1991), showed that LT/ST antigen is not required for the late transcription and MT mediates stimulation of the late transcription. In contrast to the latter notion, co-transfection of reporter constructs with pCMV-MT into NMuMG cells had no effect on the activity of both tested promoters. Nevertheless, the role of MT/ST in the early-to-late transcriptional switch has been also demonstrated by use of MT/ST-defective viral mutants (Chen and Fluck, 2001b), but AraC abolished the synthesis of late transcripts and proteins when added before the onset of viral DNA replication in the same study. Considering the high sensitivity of the luciferase assay in comparison to the Northern blot analysis in the work mentioned above, it is in agreement with the results presented here. In general, no significant difference between pGL3-LATE and pGL3-LATE+ driven luciferase gene expression was observed when assayed in the presence of early T antigens and AraC. The previously noted increase of basal transcription from the modified late promoter (pGL3-LATE+) is obviously LT-independent, since it was also observed in its absence.

The results reported in this study clearly show that not only the late but also the early Py promoter exhibited very low activity under conditions of no replication of viral DNA when assayed in the viral context. The increase in gene expression driven by Py promoters appeared to be highly dependent on the viral DNA replication and increased template copy. These data, consistent with those of others (Hyde-DeRuyscher and Carmichael, 1988; Liu and Carmichael, 1993; Cahill *et al.*, 1990; Chen and Fluck, 2001a), indicate that the Py early-to-late transcriptional switch is not controlled at the level of transcription initiation and can be viral DNA replication-linked (Cahill *et al.*, 1990; Chen and Fluck, 2001b). Extension of the distance between YY1 and LT binding sites slightly promoted the basal late transcription by the mechanisms, which is probably independent on the mutual interaction of both proteins. The extension mentioned above meant at the same time extension of the distance between the ori-core and enhancer; it dramatically lowered the replication of viral DNA and consequently decreased the gene expression from the modified late promoter. The results suggested that YY1 can be involved in the viral DNA replication-dependent regulation of Py promoter, but the role of its association with LT in this kind of regulation cannot be fully clarified from

the data presented here. It is worth recalling that YY1 is an intrinsic component of nuclear matrix (Guo *et al.*, 1995) and may mediate the Py DNA-matrix interaction important not only for proper viral DNA replication and gene expression, but also for the virus assembly. An association between YY1 and the major capsid protein VP1 has been demonstrated previously (Palkova *et al.*, 2000). The DNA-mediated association between Py DNA replication protein LT, nuclear matrix regulatory protein YY1 and the capsid protein VP1 may be important particularly in the process of virus assembly. This possibility is currently under investigation.

Acknowledgement. I am grateful to Dr. J. Forstová, Charles University Prague, Prague, Czech Republic, for helpful discussion and comments to the manuscript, Dr. A. Kalousová, Center for Integrated Genomics, Prague, Czech Republic, for experimental advice, Dr. M. Pospíšek. Charles University Prague, Prague, Czech Republic, for the Microlite TLX2 equipment, Dr. S. Cushing, Corvallis, Oregon, USA, for assistance in preparation of the manuscript, Dr. M. Austen, Medizinische Hochschule Hannover, Hannover, Germany, for the pCMV-YY1 plasmid, Dr B. Griffin for the pPyLT1 plasmid and Dr. H. Lu for the pCMV-MT plasmid, both from St. Mary's Hospital, London, UK. This work was supported by the grants No. 204/00/02071 from the Grant Agency of the Czech Republic and No. MSM 113100003 from the Ministry of Education of the Czech Republic.

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