Regulation of viral and cellular promoter activity by polyomavirus early proteins

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Abstract. The chloramphenicol-acetyl-transferase (CAT) expression system has been utilized to study the ability of the polyomavirus (Py) early proteins, the 100K large T, the 55K middle T and 22K small T-antigens, to activate a variety of eukaryotic promoters (the SV40 early, the $\alpha 2(1)$ collagen, the rabbit β -globin, the polyomavirus early and the H-2 class I) in both transient and stable expression assays. We have found that either the complete polyomavirus early region or a plasmid expressing only the 55K middle T-antigen are capable of stimulating the expression of all the promoter-CAT plasmids in transient co-transfection experiments in both NIH-3T3 and Rat-2 cells. Conversely, the Py early proteins do not stimulate the transcription of most of the promoter-CAT genes stably introduced in the cell chromosomes, with the exception of H-2 class I promoter, when stimulation of transcription has been observed upon infection with recombinant retrovirus encoding the Py middle T-antigen.

INTRODUCTION

Regulation of gene transcription involves the interaction between specific DNA sequences and relevant cellular proteins. Recently, attention has focused on the ability of certain proteins to regulate transcription of a promoter in-trans, and this phenomenon has been termed trans-activation (1).

Trans-activation of transcriptional control elements has been described in a number of tumor virus systems. The proteins encoded by Adenovirus E1A region can stimulate a large number of viral and cellular promoters (2, 3, 4), although transcription from some promoters containing a strong enhancer is repressed in the presence of E1A proteins (5, 6). Moreover, the E1A products can increase the expression of RNA polymerase

III transcribed genes (7, 8). The SV40 and polyomavirus large T-antigens activate expression from their respectively viral late promoters (9, 10, 11).

In the present study, we have sought to define the ability of the polyomavirus early region to stimulate transcription of heterologous promoters. The polyomavirus (Py) early region encodes for three distinct proteins: the 100K large T (LT), 55K middle T (MT) and the 22K small T-antigen (ST) (12).

The LT protein is required for viral DNA replication (12), it is capable of binding specific DNA sequences (13) and it is an "immortalizing" gene, i.e. it confers on rat embryo fibroblasts the ability to grow indefinitely in culture (14). The Py MT is strictly required for viral transformation of cultured cells and for tumorigenesis in animals (12). It is bound to the membrane and has an associated tyrosine kinase activity in vitro (15). Using the chloramphenicol-acetyl-transferase system (16) in both transient and stable expression assays, we have studied the ability of the polyoma virus early region proteins to trans-activate the transcription of related and unrelated promoters. We have found that a number of viral and cellular promoters are indeed stimulated when they are co-transfected in rodent cells along with the polyomavirus early region. Using recombinant plasmids that could only express a single early viral protein, we have determined that the Py MT antigen is largely responsible to bring about this effect, whereas the expression of large T-antigen has only a limited stimulatory effect on the transcription from heterologous promoters.

MATERIALS AND METHODS

Plasmid-CAT recombinants

The pSV2-CAT, pA10-CAT and pCol-CAT have already been described (16, 17, 18). The pPy-CAT-12 contains 827 bp

BamHI-BstXI polyoma DNA fragment inserted via HindIII linkers in the HindIII site of pSV0-CAT, this plasmid was a gift from S. Pellegrini. The rabbit β -globin promoter-CAT plasmid p β -CAT was constructed after isolation of 750 bp SalI-HindIII DNA fragment from the plasmid pDVU103 (19), the DNA fragment contains 475 bp of the regulatory 5'-flanking sequences of β -globin transcription unit, and it was cloned in the SalI-HindIII sites of pEMBL8-CAT. The pH-2^k-CAT was constructed after isolation of 1.9 kb HindIII-TaqI (partial digest at nt. 935) DNA fragment from the cloned H-2^k gene (20), the fragment was inserted via HindIII linkers in the HindIII site of pEMBL8-CAT. pEMBL8-CAT was constructed by G. Ciliberto and L. Dente (unpublished) and contains the promoterless CAT gene carried by the HindIII-BamHI fragment of pSV2-CAT (16) replacing the HindIII-ClaI fragment of pEMBL8 plasmid DNA (21). All the plasmids were constructed and isolated by standard recombinant DNA techniques (22). The plasmids pPy, pPy-LT, pPy-MT and pPy-ST have been described (23) and they were donated by R. Kamen. The plasmids were purified through two rounds of ethidium bromide-CsCl gradient centrifugation before they were used for transfection.

Cell lines, DNA transfection and CAT assay

NIH-3T3, Rat-2 (TK⁻) were maintained in DMEM (Gibco) supplemented with 5% of fetal calf serum. The ψ 2 producer cell lines: SV-X(1), LT-4.5 and MT-5 were kindly provided by P. Jat and used as described (24). In transient expression experiments 5×10^5 cells/100 mm. dish were transfected using the calcium phosphate co-precipitation technique (25). Briefly, 4 μ g of promoter-CAT plasmid DNA were transfected alone or with Py-plasmid DNAs in 1 to 1 molar ratio, and the total amount of DNA transfected was adjusted to 20 μ g by the addition of pEMBL8 DNA. The precipitate was removed from the cells after 16 hrs and fresh medium was added. The cells were harvested for CAT

assay 48 hrs later. CAT assays were performed as described (16). The protein of the cell extracts were quantitated using a protein assay kit from BioRad, routinely 200 μ g of total protein extract were incubated with 14 C- chloramphenicol (NEN) and 4 mM acetyl-CoA (Sigma) in 250 mM Tris-HCl (pH 7.8) at 37° for various times. The products of reaction were chromatographed by ascending TLC and quantitated by scintillation counting.

The transfection efficiency was routinely monitored by Southern blot analysis of Hirt supernatant DNAs prepared at the time the cells were harvested for CAT assays and RNA analysis.

Isolation of stably transfected cell lines and recombinant retrovirus infection.

The promoter-CAT plasmids were stably introduced in RAT-2 (TK⁻) cells by co-transfection technique (25). 10 μ g of each promoter-CAT plasmid DNA were co-precipitated with 500 ng of pTK plasmid DNA, the precipitate was added to 10⁶ Rat-2 (TK⁻) cells, and after 36 hrs the cells were split 1:6 and plated in HAT medium. Resistant colonies (~100 per 100 mm dish) appeared after 2 weeks. Both single colonies or pools of about 100 colonies (all the colonies in one dish) were isolated, grown in culture and analyzed for their endogenous CAT level. The cells of each pool (and in parallel single colonies) were then infected with the recombinant retrovirus SV-X(1) (vector), LT-4.5 (encoding for Py LT protein) and MT-5 (encoding for Py MT protein) in the presence of 8 μ g/ml of polybrene (24). At 48 hrs after infection the cells were split 1:6 and plated in G-418 (Geneticin, Gibco) containing medium (500 μ g/ml). Pools of about 80-100 colonies were trypsinized and grown in culture.

RNAase mapping and Northern blot analysis.

Total cellular RNA was prepared (26) from three to four plates of transfected NIH-3T3 cells at 48 hrs after transfection. Quantitative RNAase mapping (27) was performed using uniformly labeled RNA probe transcribed in vitro by T7

polymerase from linear DNA template. The XbaI-EcoRI fragment from pH-2K^k-CAT, containing 258 bp of CAT DNA sequence and 374 bp of flanking H-2-K^k promoter was cloned in pGEM-3 vector (Promega-Biotec). The linearized DNA template was transcribed in vitro using the reagents and conditions supplied by the Promega-Biotec. RNA-RNA hybridization was performed at 45°C overnight. Digestion with RNase A (40 µg/ml) and T₁ (2 µg/ml) was at room temperature for 30 min. (high temperature and longer incubation times resulted in signals of heterogeneous size). The double-stranded RNA products were analyzed by electrophoresis through 8% polyacrilamide denaturing gels. For Northern blot analysis, the poly(A⁺) fraction was isolated using the Hybound-mAP (Amersham). 4 µg of polyA⁺ RNAs were analyzed by Northern blot, using standard protocols (22). The DNA probes were labelled by nick-translation using a kit of Amersham.

RESULTS

Trans-activation by Polyomavirus T-antigens

We have studied the trans-activation function of the polyomavirus early proteins on CAT transient expression vectors in which the CAT gene was regulated by one of the following promoters: SV40 early, pSV2-CAT (16); the SV40 early promoter, deleted of its 72 base pair repeat enhancer element, pA10-CAT (17); the chicken $\alpha 2(1)$ collagen promoter, pCol-CAT (18); the polyomavirus early promoter, pPy-CAT-12; the rabbit β -globin promoter, p β -CAT, and the H-2 class I promoter pH-2K^k-CAT. The promoterless pEMBL8-CAT plasmid was utilized as an inactive control plasmid. The same 1630 bp HindIII-BamHI DNA fragment derived from pSV2-CAT (16), containing the bacterial CAT gene, the SV40 splice and polyadenylation signals is borne in all the CAT plasmids.

To assess the effect of the Py early proteins on gene expression from heterologous promoters, the promoter-CAT

Figure 1: CAT expression from pH-2K^k-CAT in the presence of Py early proteins. A typical autoradiograph from the thin layer chromatography of chloramphenicol acetylation catalyzed after 40' of reaction by extracts of NIH-3T3 cells transfected with the pH-2K^k-CAT plasmid DNA (4 μg) alone (T) or with equimolar amount of Py-plasmids expressing respectively the large T (pLT), the middle T (pMT), the small T (pST) or all the three Py early proteins (pPy).

plasmids described above were transfected into Rat-2 and NIH-3T3 fibroblasts, either alone or co-transfected with plasmids that could express the entire Py early region (pPy), or a single viral early protein; the LT (pPy-LT), MT (pPy-MT) and ST (pPy-ST), respectively. The Py-plasmids differ for the presence of deletions within the Py early region such that only a single Py early protein can be expressed (21). The transcriptional control both of 5' and 3' of the Py early regions are identical in all four Py-plasmids. Figure 1 shows the autoradiograph of the TLC plate from a CAT assay in which the pH-2K^k-CAT plasmid was transfected on NIH-3T3 cells alone or together with plasmids that could express the entire Py

Figure 2: Stimulation of CAT activity by Py early proteins on various promoter-CAT plasmids transfected in NIH-3T3. 4 μ g of each of the promoter-CAT plasmid DNAs were transfected alone or with equimolar amount of pPY-plasmids expressing all the three early Py proteins (pPy), the middle T-antigen (pMT), the large T (pLT) and small T (pST). The CAT activity is measured as the percent of chloramphenicol acetylated of the total input chloramphenicol, determined at various times of reaction. To simplify the graphic representation, the values of CAT activity for each of the promoter-CAT plasmids transfected alone are not reported since they are identical to those obtained in co-transfection with pST. The values reported are the average results of 3 to 4 different experiments. The variability from experiment to experiment was $\pm 12\%$ of the average values reported.

early region or only one of the viral early proteins. The measurement of the CAT expression indicates that the Py early proteins clearly stimulate CAT gene expression driven by the H-2 promoter. Moreover, a high degree of stimulation was observed in co-transfection with the pPy-MT plasmid, whereas the pPy-LT has only a limited effect. No stimulation was detected in co-transfection with the pPy-ST plasmid.

Figure 3: Stimulation of CAT activity by Py early proteins on promoter-CAT plasmids transfected in Rat-2 cells. The promoter-CAT plasmids were transfected alone or with a plasmid that could supply either all three Py early proteins or only one of them. The transfection procedure and the CAT assay was as described in the text. As in Fig.2, the CAT activities of each promoter-CAT alone are not reported since they are identical to those observed in co-transfection with pST plasmid. The values reported are the average of 3 to 5 different experiments, the variability from experiment to experiment was $\pm 9\%$ of the average values reported.

Experiments similar to the one shown in Fig.1 were performed with all the promoter-CAT plasmids, and the results of such extensive analysis are summarized in Fig.2 and 3. The stimulatory effect of Py early proteins was also seen using the SV40 and Py early promoters, both of which contain wherein a cis-acting enhancer element. We and others (F. G. Kern and C. Basilio, personal communications) found that CAT activity of any promoter-CAT plasmid was higher in rat cells than in mouse 3T3 cells. Consequently, the higher level of stimulation observed in NIH-3T3 cells was probably due to the different

baseline activity. The stimulation of CAT activity observed in co-transfection experiment of pPy-CAT-12 with pPy and pPy-LT plasmids was mainly due to replication of the test plasmid which contains a competent viral origin of DNA replication (data not shown). In fact, using an ori⁻ derivative of pPy-CAT-12, the large T protein represses the activity of the Py early promoter (F. G. kern and C. Basilico, personal communications).

The results presented in Fig. 1, 2 and 3 indicate that the Py early proteins stimulate gene expression in both permissive and semi-permissive cells, and such activation greatly depend on the expression of the 55K middle T protein, although the 100K large T itself and possibly in conjunction with the MT, can also exert a stimulatory effect on promoter function. We also note that the pPy-MT is less effective than wild type polyomavirus (pPy), this result is likely due to a synergistic effect of MT and LT proteins on heterologous promoters. No detectable effect was observed with the ST protein with any of the promoter-CAT tested.

Stimulation of gene expression by Py T-antigen occurs at the transcriptional level.

Using the promoterless pEMBL8-CAT plasmid in control experiments, we did not observe any CAT activity even in the presence of Py viral proteins (data not shown). This data implies that to detect CAT activity and any stimulation by Py early proteins, an eukaryotic promoter must be utilized in the promoter-CAT plasmids. Moreover, it has generally been found that the level of CAT activity in transient expression assays and the relative CAT mRNA levels varied in a proportional manner. Therefore, the CAT activity is usually presented as a measure of transcriptional promoter activity (9, 10, 11, 16, 17, 18).

We sought to determine the amount of CAT mRNA in NIH-3T3

Figure 4: Stimulation of transcription from chimeric H-2K^k promoter-CAT plasmid by Py early proteins. NIH-3T3 cells were transfected with 4 μ g of pH-2K^k-CAT DNA together with an equal amount of pPy (Lane 1), pMT (Lane 2) or pST (Lane 3) plasmid DNAs. Cytoplasmic RNA was isolated at 48 hrs after transfection, and quantitative RNAase mapping was performed on 40 μ g of RNA as described in the text. The expected correct size of CAT mRNA is 272 nt. (M) Py DNA restricted with HinfI and end-labelled.

cells after co-transfection of pH-2K^k-CAT with pPy, pPy-MT and pPy-ST plasmids, in order to test whether the increased CAT activity observed in co-transfection with both pPy and pPy-MT was a result of an enhanced transcription of the pH-2K^k-CAT plasmid. As control, the pH-2K^k-CAT was co-transfected with pPy-ST plasmid that, as reported in Fig.1, has no detectable

trans-activity on H-2 promoter. Total cellular RNA was isolated at 48 hrs after transfection and analyzed by quantitative RNAase mapping (27). Fig.4 shows the results of such analysis, indicating that the level of mRNA observed correlates with the level of CAT activity and the H-2K^k-CAT mRNA has the appropriate 5' end in both the presence and absence of trans-activation.

Stimulation of endogenous CAT genes by Py early proteins.

To determine whether the Py early T-antigens, the LT and particularly MT, could stimulate the expression of integrated genes, we have stably introduced the promoter-CAT plasmids, used in transient expression experiments, in Rat-2 (TK⁻) fibroblasts using the Herpes virus tk gene as selectable marker. The majority of stable transformants on each plate (over 100) was pooled to minimize the effect of the integration site, copy number and reorganization during the transfection. In addition, few colonies of each transfection were individually isolated and analyzed in parallel. The Southern blot analysis was used to identify both mass cultures and single clones that contained 1 to 3 copies of promoter-CAT gene per haploid Rat-2 genome (data not shown). Both mass cultures and single clones derived from each transfection were infected with recombinant retroviruses encoding the Py, LT or MT antigens (24). The retrovirus producing ψ 2 cell lines were: (1) SV-X(1), in which there is the vector retrovirus without the insert, (2) LT-4.5, producing a retrovirus with LT, and (3) the cell line MT-5, that produce a recombinant retrovirus with the MT protein. Virus supernatant from each of these cell lines was used to infect Rat-2 cells that stably contain the promoter-CAT genes, and at 48 hrs after infection the cells were splitted and plated in G-418 selective medium. The level of endogenous CAT activity was determined both at 48 hrs after retrovirus infection and from pools of G-418 resistant colonies (100 colonies/pool).

TABLE 1: CAT activity in stable promoter-CAT Rat-2 cell transformants after infection with recombinant retroviruses

Plasmids	Cells	Endogenous CAT activity	Fold CAT increase at 48 hr p.i.			Fold CAT increase of G-418 ^R cells		
			SV-X(1)	LT-4,5	MT-5	SV-X(1)	LT-4,5	MT-5
pH-2K ^k -CAT	P. #1	10	1	2.5	4.5	1	3	7
	P. #2	9	1	1.5	4	1	4	9
	P. #3	13	1	1.9	3.5	1	2.5	12
	Cl. #1	8	1	3	6	1	2.5	10
	Cl. #2	14	1	1.8	4	1	2.5	14
pSV ₂ -CAT	Cl. #1	20	1	1	1	1	1	1
pβ-CAT	Cl. #1	0.8	1	1	1	1	1	1
pCol-CAT	Cl. #1	1	1	1	1	1	1	1
pPy-CAT	Cl. #1	18	1	1	1	1	1	1

The endogenous CAT activity was assayed on 100μg of cell extract. The symbol P. indicates a pool of >80 single clones. The symbol Cl. indicates a individually isolated cell colony.

The results of such analysis are reported in Table 1. We found that only the CAT gene under the transcriptional control of H-2K^k promoter was stimulated upon infection with MT-5 virus, and in less extent with LT-4.5 virus, both 48 hrs after infection and after G-418 selection. Conversely, Rat-2 cells expressing the CAT gene under the control of other promoters did not change the level of endogenous CAT expression after infection with any of the recombinant retroviruses. In Table 1 it is reported the results obtained with a representative cell clone of each of the non-stimulated promoter-CAT genes. Absence of stimulation on endogenous promoter-CAT was also observed using pooled clones (data not shown).

To determine whether the increase in CAT activity reported in Table 1 for the H-2K promoter was related to increase in

Figure 5: Analysis of endogenous CAT mRNA level in cells infected with recombinant retroviruses. **A.** 4 μ g of polyA⁺ mRNAs isolated from G-418 resistant cells derived after infection with LT4.5 (Lane 1), MT-5 (Lane 2) and SV-X(1) (Lane 3) recombinant retroviruses of Rat cells containing the pH-2K^k-CAT plasmid were analyzed by Northern blot hybridization using a specific CAT probe (the HindIII-BamHI fragment from pSV2-CAT) labeled by nick-translation. After removal of the first probe, the filter was then rehybridized to a chicken β -actin probe (41) for normalization. **B.** Northern blot hybridization of 4 μ g of polyA⁺ mRNAs from cells containing the pH-2K^k-CAT gene (Lane 1) and two different pools (Lane 2 and 3) of G-418 resistant cells derived after infection with the vector recombinant retrovirus SV-X(1). The filter was hybridized with the HindIII-BamHI CAT specific probe. The filters were processed as described in the Materials and Methods section. The arrows indicate the positions of 28S and 18S rRNA.

the steady-state level of CAT mRNA, Northern blot hybridization was performed. Cytoplasmic mRNA was isolated from pools of G-418 resistant colonies isolated after infection with MT-5, LT-4.5 and SV-X(1) viruses respectively. Hybridization with a CAT specific probe revealed a MT dependent increase in the

level of CAT mRNA (Fig. 5A). A slight but significant increase of CAT mRNA was also found in G-418^R cells expressing the Py 100K large T-antigen. No difference in CAT mRNA level was found in cells infected with the SV-X(1) vector retrovirus (Fig. 5B).

DISCUSSION

The polyomavirus early proteins are capable to stimulate the transcription of viral and cellular promoter in transient expression experiments. We found that the Py 55K middle T-antigen is the viral gene protein required to bring about this effect, whereas the large T-antigen and the small T-antigens show little or no effect in stimulation of heterologous gene expression, respectively. In addition, the Py early proteins stimulate transcription from the SV40 and Py early promoters, both of which contain a cis-acting element. This indicates that trans-activating proteins can stimulate the expression of genes already activated by enhancer elements. From the results reported in Fig. 4, the stimulation of gene expression appears to be due to elevated levels of CAT-transcripts, however we cannot formally exclude the possibility of a post-transcription regulation by PY early proteins. Direct experimental proofs, in the form of run-on experiments are required to establish this point.

Our results indicate that Py middle T-antigen increases the expression of transfected genes, yet it does not stimulate transcription from most of the same promoter-CAT genes when they are stably introduced in the cell chromosomes. A similar result has been reported for other trans activating gene products, as Adenovirus E1A and Herpes 1E proteins. These proteins stimulate transcription from a number of viral and cellular promoters when these genes are newly introduced into cells (2, 3, 4), but with few exceptions the expression of

Adenovirus E1A proteins do not significantly alter the transcription of endogenous genes (2, 3, 4, 28, 29, 30, 31). The most likely explanation of the failure of Py early proteins to stimulate transcription of endogenous genes may be because they are already assembled into a transcriptional context (i.e. chromatin structures, association to nuclear cage), which can determine their relative rates of transcription. Overall our results suggest a general lack of specificity of the Py middle T-antigen in activation of the transfected genes and a lack of stimulation of endogenous chromosomal genes. The exception is in the case of H-2K^k promoter where a significant level of stimulation was observed. It may be noted that activation by MT protein of H-2K^k promoter-CAT plasmid stably introduced in rat fibroblasts is consistent with previous reports showing that activation of MHC Class I gene expression occurs upon polyoma virus induced transformation (32) and lytic infection (33). However, since we found that interferon treatment of cells containing the H-2K^k-CAT gene results in a 5 fold increase of the endogenous CAT activity (data not shown), we cannot exclude that the gene activation may be mediated by a general anti-viral state induced by cellular proteins in response to polyoma virus middle T-antigen.

Recent data concerning the subcellular localization of the MT antigen (34) indicate that the bulk of this protein resides in intracellular membranes, most particularly associated with the endoplasmic reticulum, rather than at the plasma membrane as suggested by earlier experiments (35, 36). No staining in nucleoplasm has been observed. Conversely, the viral trans-activator proteins described so far are localized into the nucleus (1). In other experiments, we have used an SV40 L-T mutant unable to be transported into the nucleus, and we found that the cytoplasmic located SV40 L-T mutant still retains the

ability to trans-activate transfected genes in simian cells (37). This result substantiates our data on MT protein, indicating that viral transforming proteins located outside the nucleus may have a direct effect on transcription.

Although the molecular mechanisms which are responsible for the trans-activation of transfected genes by Py MT-antigen are completely unknown, one can formally come to some hypotheses based on the MT properties. It has been recently reported that a subclass of MT is able to bind to DNA cellulose (38). This result could suggest that the trans-activation on transfected genes observed in our experiments could be directly correlated to the MT DNA-binding. However, our result indicate that MT is able to enhance expression from a variety of promoters, suggesting that it can act as general non specific stimulator of transcription. This is also supported by our failure to identify common DNA sequences among the different promoters tested. Thus, it seems more likely that MT acts indirectly, either through protein-protein interaction or through the activation of cellular transcription factors.

The middle T-antigen has been previously identified in plasma membrane and it has an associated tyrosine kinase activity in vitro due to the interaction with the pp60 c-src protein (15). As a result of the association of MT, the specific activity of the pp60 c-src tyrosine phosphotransferase is enhanced (39), as well as increased the level of pp60 c-src-associated phosphatidylinositol kinase activity (40). It has been suggested that the 55K middle T-antigen induced transformation may be connected to the complex network of second messengers generated from the phosphatidylinositol kinase. In recent work (42) Imbra and Karin have reported that a phorbol ester (TPA) has a stimulatory activity on SV40 enhancer in transient expression experiments. Since the TPA is thought to exert this action by a mechanism involving the

activation of protein kinase C (43), it has been suggested that the possible mechanism of induction of the SV40 enhancer by TPA could involve a specific phosphorylation event. On the basis of these observations, we suggest that the possible mechanism of transcription induction mediated by polyomavirus 55K middle T-antigen in transient expression experiments, could be exerted through a specific protein phosphorylation event leading to a post-translation modification of one or more of the factors involved in the RNA polII transcription process. However, determination of the exact mechanism of activation will require the identification and isolation of trans-acting factor(s) induced by the polyomavirus 55K middle T-antigen.

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