Induction of Polyomavirus DNA Replication by Carcinogens in Polyomavirus-Transformed Rat Cells: Evidence that the Viral Enhancer Is Not the Primary Target in the Induction Pathway

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In the polyomavirus (Py)-transformed rat cell line designated LPT, replication of the integrated Py DNA can be induced by exposure of the cells to carcinogens. In view of the observation that enhancer elements are essential components of the Py origin of replication, it appeared plausible that the induction is triggered by synthesis or modification of an enhancer-binding protein which is required for activation of the viral origin. To test this hypothesis, we have used a plasmid containing a modified Py origin (test plasmid), in which the Py enhancer has been replaced with five repeats of the yeast *GAL4* upstream activating sequence, and a plasmid encoding the *GAL4* transcriptional activator protein. Previous studies in which these two plasmids were cotransfected into mouse cells that are permissive for Py showed that the *GAL4* protein can transactivate the modified Py origin and cause replication of the test plasmid. When similar cotransfection assays were performed in LPT cells, no replication of the test plasmid was observed unless the cells were exposed to the carcinogen mitomycin C subsequent to the transfection, in which case replication of the test plasmid was induced. Control experiments showed that even though the *GAL4* protein was required for the induction, its concentration was not affected by the exposure to mitomycin C. These results indicate that the primary target in the induction pathway is not an enhancer-binding protein; instead, the induction appears to be triggered by changes in other components of the replication initiation complex which may be associated with the origin core.

Cells of the polyomavirus (Py)-transformed rat line designated LPT (10) contain multiple Py genomes integrated in tandem into a single chromosomal site (26). A small fraction of LPT cells (<0.20%) are spontaneously induced to synthesize free Py DNA molecules and infectious Py (10, 20, 29). The induction rate can be increased up to 300-fold by treatment of LPT cultures with physical and chemical carcinogens (10, 11, 29), with inhibitors of protein synthesis (22), or with agents that stimulate the intracellular level of cyclic AMP (3) or by fusion of LPT cells with mouse cells (10). Py induction in LPT cells is also manifested by amplification of the integrated Py DNA and flanking cell sequences (1, 2).

Previous studies of the induction process in LPT cells have indicated that the primary event in this process is activation of the Py origin of replication (ori) in the chromosomally associated viral DNA (23). It has been also demonstrated that continuous protein synthesis is required for induction of Py DNA replication by carcinogens in these cells (22). These studies, and the observation that replication of the virus was also induced by fusion of LPT cells with mouse cells (10), have indicated that protein factors that act in *trans* are involved in the induction process. Studies performed in other lines of Py- and simian virus 40 (SV40)-transformed cells have also provided evidence for involvement of *trans*-acting factors in induction of the integrated viruses (6, 14, 16, 18, 25, 32, 33).

Following the discovery that enhancer elements are indispensable components of the Py ori in permissive mouse cells, which allow Py DNA replication without being treated with inducing agents (9), it became apparent that the enhancer might be the primary viral target for induction of Py

DNA replication in LPT cells. A plausible hypothesis would be that exposure of LPT cells to virus-inducing agents causes enhancement of synthesis or modification of an enhancer-binding protein which is required for initiation of replication in addition to the Py large T antigen (T-ag) and the cell-encoded replication enzymes (7, 8). It is further assumed that the change in the enhancer-binding protein leads to activation of the Py ori. According to this hypothesis, the same protein is produced constitutively in permissive mouse cells. Alternatively, the primary target for induction of Py DNA replication in LPT cells is another component of the replication initiation complex, e.g., a protein that binds the viral origin core. In this case, the roles of the enhancer and of the enhancer-binding proteins in activation of the Py ori would be similar in induced LPT cells and in permissive mouse cells; in other words, the enhancer complex would not be the primary target in the induced transition of LPT cells from the nonpermissive to the permissive state for Py DNA replication.

To examine the role of enhancer elements in the induction process, we have developed a short-term assay for induction of replication of plasmids that contain a Py ori. In a typical assay, plasmids were transfected into LPT cells by the calcium phosphate technique (4, 12), and after 24 h the cells were exposed for 1 h to the carcinogen mitomycin C (MMC) (1 μ g/ml), which is a most effective inducer of the endogenous virus (11, 22, 29). These cells, and similarly transfected cells that had not been exposed to MMC, were harvested 24 h later. Small-molecular-weight DNA extracted from the cells was digested with the restriction enzyme *DpnI* and with another restriction enzyme that cuts each of the plasmids once. The digests were analyzed by agarose gel electrophoresis, Southern blotting, and hybridization with a plasmid probe. The amount of *DpnI*-resistant plasmid DNA determined by blot hybridization was a measure of the extent of

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replication of the transfected plasmids (4, 30). For quantitative assessments of this parameter, the cells were also cotransfected with a reference plasmid not containing a Py origin, which had been propagated in an *Escherichia coli dam* mutant. This plasmid was *DpnI* resistant even though it did not replicate in the LPT cells (4).

The initial replication induction assays were performed with plasmid pPy Δ cat26.2⁺, which contains a fragment of Py DNA that includes the viral ori and the early promoter (Fig. 1) (36). In this plasmid, the Py enhancer has been replaced with two tandem repeats of a 26-bp sequence that includes a segment of the Py enhancer designated α (nucleotides 5108 to 5130 in the A2 strain numbering system) (13). These sequences, and three cell-encoded transcriptional activator proteins which they presumably bind (24), have been previously found to provide the enhancer function needed for replication of this plasmid in permissive mouse cells (4, 36). Figure 1 (leftmost two lanes) presents an autoradiogram in which the results of these assays are shown. The position of the *DpnI*-resistant linear molecules of plasmid pPy Δ cat26.2⁺ is marked "test." The *DpnI*-resistant linear form of the reference plasmid pUC119 is also indicated. This autoradiogram does not show the *DpnI* digestion products of the plasmids. It can be seen that whereas barely detectable replication of plasmid pPy Δ cat26.2⁺ occurred in cells that had not been exposed to MMC, substantial replication of this plasmid was induced by exposure of the cells to MMC. It should be noted that plasmid pPy Δ cat26.2⁺ does not encode the Py large T-ag, and hence it must utilize for its replication the large T-ag encoded by the endogenous virus (see below).

To address the question of whether an enhancer-binding protein is or is not the primary target for induction of Py DNA replication by MMC, we carried out similar replication induction assays of plasmid pM101 (Fig. 1), which includes a modified Py ori in which the Py enhancer has been replaced with five repeats of the yeast *GAL4* upstream activating DNA sequences (5 $\times$ GAL4 sites) (4). We also used for these assays plasmids designated in Fig. 1 as activators; these plasmids encode either the whole yeast *GAL4* protein [(GAL4 (1-881))] or a truncated polypeptide constituting the N-terminal 147 amino acids of the *GAL4* protein [GAL4 (1-147)] which contains the DNA-binding domain of this transactivator (15, 19). Previous studies in which plasmid pM101 and activator plasmids were cotransfected into mouse cells showed that the whole *GAL4* protein, but not the truncated *GAL4* (1-147) protein, can transactivate the modified Py ori in plasmid pM101 (4). In the present experiments, plasmid pM101 was transfected into LPT cells with or without one of the two activator plasmids shown in Fig. 1. The transfected cultures were either subsequently exposed to MMC or incubated with no further treatment. The extent of replication of plasmid pM101 was determined in each of these cultures by the *DpnI* assay described above, and the results are displayed in the autoradiogram shown in Fig. 1. It can be seen that no detectable replication of plasmid pM101 occurred in any of the transfected LPT cultures that had not been subsequently exposed to MMC. Also, MMC did not induce replication of plasmid pM101 when this test plasmid was transfected into the cells alone or when it was cotransfected into the cells with the plasmid encoding the truncated protein *GAL4* (1-147). Substantial replication of plasmid pM101 was induced by MMC when this test plasmid was cotransfected into the cells with the activator plasmid encoding the whole *GAL4* protein; however, the extent of replication of plasmid pM101 in the induced cells was one-seventh that of plasmid pPy Δ cat26.2⁺.

FIG. 1. Replication induction assays of plasmids transfected into LPT cells. Test plasmid pPy Δ cat26.2⁺ (36) contains a fragment of Py DNA (nucleotides 5265 to 1560) that includes the Py origin core (ori), the early promoter, and a part of the early region. The Py enhancer (nucleotides 5021 to 5264) has been replaced in this plasmid with two tandem repeats of a 26-bp sequence that includes the Py α enhancer element (nucleotides 5108 to 5130). Test plasmid pM101 (4) contains a fragment of Py DNA (nucleotides 4632 to 152). The Py enhancer has been replaced in this plasmid with five repeats of a 17-bp inverted repeat sequence that includes the *GAL4* upstream activating sequence. The activator plasmid designated GAL4 encodes the whole *GAL4* protein, which includes 881 amino acids, under the control of the adenovirus major late promoter (15). The activator plasmid designated GAL4 (1-147) encodes an amino-terminal fragment of the *GAL4* protein (147 amino acids) under the control of the adenovirus major late promoter. This protein contains the DNA-binding domain of the *GAL4* transactivator (15, 19). These plasmids were transfected into LPT cells by the calcium phosphate technique (4, 12). Each transfection mixture contained 1 μ g of a test plasmid and 0.5 μ g of a pUC119 reference plasmid which was propagated in an *E. coli dam* mutant. Some of the mixtures also contained 3 μ g of an activator plasmid, as specified. Plasmid pUC119 was also added, such that the total amount of DNA in each transfection mixture was 10 μ g. Transfected cultures were subsequently exposed to 1 μ g of MMC per ml for 1 h and harvested after 24 h as previously described (22) or maintained under normal growth conditions until their harvest after 24 h. Small-molecular-weight DNA was selectively extracted from these cultures and was digested with *DpnI* and *Bam*HI. The DNA was analyzed by gel electrophoresis and Southern blotting (4, 26). ref. indicates the band including the pUC119 reference plasmid, which was used for quantitative assessments of the extents of replication of the test plasmids; these estimations gave the numbers displayed at the bottom of the autoradiogram, which represent the normalized test plasmid/reference plasmid ratios.

TABLE 1. Transactivation of the adenovirus E4 promoter by the yeast GAL4 protein in untreated and MMC-treated LPT cells

Transfected DNA ^a	Transactivator protein ^b	Exposure to MMC ^c	CAT activity (radioactivity extracted into organic solvent) ^d (avg cpm $\pm$ SD)
Herring	—	—	1,251 $\pm$ 81
	—	+	1,417 $\pm$ 28
pG5E472cat	—	—	1,740 $\pm$ 60
	—	+	1,549 $\pm$ 96
pG5E472cat + pAG147	GAL4 (1–147)	—	1,847 $\pm$ 60
	GAL4 (1–147)	+	1,616 $\pm$ 77
pG5E472cat + pAG4	GAL4	—	4,587 $\pm$ 148
	GAL4	+	4,839 $\pm$ 748

^a LPT cells were transfected with herring DNA or plasmid DNAs as described in the legend to Fig. 1. In the reporter plasmid pG5E472cat, the CAT gene was placed under the control of a modified adenovirus E4 promoter containing 5 $\times$ GAL4 sites (17). Each transfection mixture contained 2.0 μ g of the reporter plasmid and, where indicated, also 0.50 μ g of an activator plasmid. The control mixtures contained 10 μ g of herring DNA; herring DNA was also added to the other mixtures such that the total amount of DNA used to transfect each 60 mm dish was 10 μ g.

^b Proteins encoded by the transfected activator plasmids pAG147 and pAG4 (15).

^c The transfected cells were (or were not) exposed to MMC as described in the legend to Fig. 1.

^d CAT assays of the cell extracts were performed by the phase extraction method (34) as previously described (4). Each assay was carried out in duplicate. Numbers represent averages per 33 μ g of extract protein, which were recorded 6 h after the reactions were initiated.

One way in which exposure to MMC might lead to activation of the modified Py ori in plasmid pM101 is by enhancement of the synthesis or posttranslational modification of the GAL4 protein, such that the intracellular concentration of active GAL4 molecules increases. This possibility was examined in the following functional assays. LPT cultures were cotransfected with the activator plasmid encoding the GAL4 protein and with a reporter plasmid, designated pG5E472cat, expressing the enzyme chloramphenicol acetyltransferase (CAT) gene under the control of a modified adenovirus E4 promoter which includes the 5 $\times$ GAL4 sites (17). The extent of CAT synthesis was determined in these cultures and in similarly transfected cultures that were subsequently exposed to MMC as described above. Table 1 shows the results of these assays and of similar assays of LPT cells that were transfected with herring DNA, with the reporter plasmid alone, or with the reporter plasmid and the plasmid encoding the truncated protein GAL4 (1–147). It can be seen that a very low basal level of CAT expression was found in cells that were transfected with the reporter plasmid alone and that exposure of similarly transfected cells to MMC did not affect this level to a significant extent. The truncated GAL4 (1–147) protein did not stimulate expression of the CAT gene. In cells that were cotransfected with the reporter plasmid and with the plasmid encoding the complete GAL4 protein, CAT synthesis was stimulated manyfold. However, subsequent exposure of similarly transfected cells to MMC did not cause a substantial change in CAT expression. In these assays, the concentration of the GAL4 protein was limiting, as previously determined by similar assays in which the activator plasmid/reporter plasmid ratio was varied. Thus, any increase in the concentration of GAL4 protein caused by MMC would have led to an increase in CAT expression. It should be noted that GAL4 protein domains which activate transcription are also required for replication

transactivation, even though the two processes may not be causally related (4, 5). Therefore, these results indicate that exposure of the transfected LPT cells to MMC does not affect the concentration of GAL4 protein molecules which are capable of transactivating both transcription and replication. Consequently, it is unlikely that induction of replication of plasmid pM101 in these cells is caused by a change in the concentration of active GAL4 protein molecules. It should be noted that the use of the GAL4 plasmid constructs for these studies was advantageous because, in contrast to the cell-encoded Py enhancer-binding proteins, the concentration of the GAL4 protein could be controlled by varying the concentration of the plasmid encoding this protein in the transfection mixtures. Furthermore, the GAL4 protein was not expected to bind cellular promoter and enhancer elements, and therefore indirect effects resulting from enhancement or repression of synthesis of proteins controlled by these elements were avoided.

Previous work has indicated that exposure of LPT cells to MMC enhances synthesis of the Py large T-ag encoded by the endogenous viral DNA (21, 34a). In view of these data, it appeared possible that the induction of replication of plasmids bearing a Py ori in LPT cells exposed to MMC was not caused by the primary event that causes induction of the endogenous virus; instead, enhancement of plasmid replication might be caused by the increase in the concentration of the large T-ag. To examine this possibility, we used a subclonal derivative of the LPT line designated subclone 4. This subclone was found by Southern mapping techniques to harbor two complete copies of the Py DNA tandemly integrated into the same chromosomal site at which the viral DNA of the parent LPT clone has integrated (23). However, spontaneous induction of replication of the viral DNA was not observed in these cells, nor could replication of the viral DNA be induced by exposure of the cells to carcinogens (23). This noninducible phenotype was due to absence of functional Py large T-ag molecules in these cells (see below). Figure 2 (leftmost four lanes) shows the results of replication induction assays in which subclone 4 cells were transfected with test plasmid pM101, which contains the Py origin core and the 5 $\times$ GAL4 sites, or with both this plasmid and the activator plasmid encoding the complete GAL4 protein. It can be seen that the test plasmid did not replicate irrespective of whether the cells had or had not been exposed to MMC subsequent to transfection. Replication induction of the test plasmid was, however, rescued by transfecting into the same subclone 4 cells a plasmid encoding a functional large T-ag under the control of the mouse ribosomal protein L7 promoter (27) (Fig. 2, lanes T-Ag +). In fact, some replication of test plasmid pM101 was observed even in the absence of the GAL4 activator protein. However, in the absence of the GAL4 transactivator, exposure to MMC did not affect the replication of the test plasmid. The truncated protein GAL4 (1–147) apparently inhibited both the spontaneous and the MMC-induced replication of the test plasmid. In cells that had been cotransfected with the test plasmid and with the activator plasmid encoding the whole GAL4 protein, as well as with the plasmid encoding the large T-ag, the basal level of replication of the test plasmid was slightly stimulated. However, subsequent exposure of similarly transfected cells to MMC caused a substantial enhancement of replication of the test plasmid, as was previously observed in the experiments performed with the parent LPT cells (Fig. 1). It should be noted that in MMC-treated subclone 4 cells, the extent of replication of plasmid pPy Δ cat26.2⁺ was three times higher than that of plasmid pM101 (not shown).

To determine whether the induction of plasmid replication in the MMC-treated subclone 4 cells was caused by an increase in the concentration of the large T-ag, we carried out Western immunoblot assays of the large T-ag, using polyclonal anti-T-ag antibodies. These assays did not reveal any differences in the concentration of the large T-ag in transfected cells that were subsequently exposed to MMC versus cells that were not treated with MMC (not shown). In fact, even in cells that were not transfected with the plasmid encoding the large T-ag, the endogenous virus encoded large T-ag molecules which were functionally inactive but immunologically reactive. We realized, however, that only a small proportion of the cells were transfected with the plasmid, and hence changes in the concentration of the large T-ag that might occur in the transfected cells may have not been detected by this type of measurement. Therefore, we used a different approach to examine this question. We made a plasmid construct in which the CAT gene was placed under the control of the mouse ribosomal protein L7 promoter instead of the gene encoding the large T-ag. The new plasmid, designated pM121, was transfected into subclone 4 cells, which were subsequently exposed to MMC. CAT expression was determined in these cells and in similarly transfected cells that had not been exposed to MMC. It can be seen in Table 2 that CAT expression in cells transfected with two different amounts of plasmid pM121 was not substantially affected by subsequent exposure of the cells to MMC. Thus, MMC does not appear to affect the activity of the L7 promoter. Since functional large T-ag molecules were

TABLE 2. Activity of the mouse ribosomal protein L7 promoter in untreated and MMC-treated cells of LPT subclone 4

Transfected DNA ^a	Exposure to MMC ^b	CAT activity (radioactivity extracted into organic solvent) ^c (avg cpm $\pm$ SD)
Herring	—	1,576 $\pm$ 72
	+	1,273 $\pm$ 110
pM121		
3 μ g	—	21,735 $\pm$ 3,793
	+	28,634 $\pm$ 2,858
10 μ g	—	64,020 $\pm$ 6,302
	+	71,083 $\pm$ 3,708

^a LPT subclone 4 cells were transfected with herring DNA or with the specified amounts of pM121 plasmid DNA as described in the legend to Fig. 1. In plasmid pM121, the CAT gene was placed under the control of the mouse ribosomal protein L7 promoter (27).

^b The transfected cells were (or were not) exposed to MMC as described in the legend to Fig. 1.

^c CAT assays were performed as described in Table 1, footnote d.

expressed only in the subclone 4 cells under the control of the L7 promoter, these results indicated that the synthesis of these large T-ag molecules was not affected by exposure of the cells to MMC. Hence, it appears unlikely that the induction of replication of the test plasmid in subclone 4 cells and in the parent LPT cells that had been exposed to MMC was caused by enhanced expression of the large T-ag molecules.

The data presented in this report indicate that whereas enhancer elements and enhancer-binding proteins are required for initiation of DNA replication at the Py ori in rat LPT cells as well as in mouse cells, these elements are not primary targets for induction of Py DNA replication in LPT cells by MMC. In particular, we have shown that replication of test plasmid pM101, which includes a Py origin core and the 5 $\times$ GAL4 sites but lacks the Py enhancer sequences (Fig. 1), was induced by exposure to MMC of LPT cells that had been cotransfected with this plasmid and with the plasmid encoding the whole GAL4 activator protein. We have also shown that MMC treatment of LPT cells did not affect the concentration of active GAL4 protein molecules in these cells. Hence, this treatment must have affected another component of the replication initiation complex which is not bound to the enhancer and thereby induced replication of the test plasmid. In line with this conclusion, we failed to detect changes in the three proteins that bind the α enhancer element in LPT cells that have been exposed to MMC (15a), even though replication of plasmid pPy Δ cat26.2⁺, which contains this enhancer element, was induced by MMC (Fig. 1).

What is the primary target in the induction process? One possible candidate is a protein that specifically binds a DNA sequence in the Py origin core and is required for formation of the replication initiation complex. It is interesting to note in this connection that a protein that binds a sequence included in the early palindrome of the SV40 origin core (35) has been reported to be a target for induction of amplification of SV40 DNA by UV light (18). However, while the sequence binding this protein has been conserved in several primate polyomaviruses, it does not occur in the Py origin core (35). It has been also reported that exposure of Py-transformed rat cells to UV light induces synthesis of proteins that bind an 8-bp sequence designated URE (nucleotides 5256 to 5263), which maps at the end of the Py

enhancer (33). It has been suggested that these proteins might be involved in triggering Py DNA amplification in UV-treated cells (33). However, since neither plasmid pPy Δ cat26.2⁺ nor plasmid pM101, whose amplification could be induced by MMC, contains this sequence (Fig. 1), it is clear that in LPT cells, induction by MMC does not depend on the presence of the URE sequence. It would be interesting to determine whether, in analogy to the SV40 origin-binding protein mentioned above, there are proteins other than the Py large T-ag that specifically bind sequences within the Py origin core and whether such proteins might be involved in the induction process.

Another possible primary target in the induction process is the large T-ag; for even though we have presented evidence indicating that the induction of plasmid amplification by MMC cannot be accounted for by an increase in the concentration of large T-ag molecules, the possibility that a modification induced in these molecules is the event that triggers the amplification process has not been ruled out. A third possible primary target for the induction of replication by MMC could be a protein intermediary that neither directly binds the enhancer elements nor binds the origin core. Such an intermediary might, however, interact with enhancer-binding or core-binding proteins and would be analogous to postulated protein intermediaries that might activate transcription (31). Finally, it is possible that the target for the induction is a replication enzyme. It is interesting to recall in this connection the observation that addition of a polymerase α -primase complex prepared from human cells that are permissive for SV40 to an extract prepared from mouse cells that are nonpermissive for SV40 allows in vitro replication of SV40 DNA in this extract, and that addition of a polymerase α -primase complex prepared from mouse cells that are permissive for Py to an extract prepared from human cells that are nonpermissive for Py allows in vitro replication of Py DNA in this extract (28). In view of these data, it is possible that the target for induction of Py DNA replication by carcinogens in LPT cells, which is a transition from a nonpermissive to a permissive state for Py DNA replication, is the LPT polymerase α -primase complex. These various possibilities may be tested in an in vitro replication system, using extracts prepared from uninduced and induced LPT cells.

In conclusion, we have initiated a detailed study of the mechanism of induction of Py DNA replication by carcinogens. The data presented in this report indicate that although the induction is dependent on the presence of an enhancer sequence next to a Py origin core and of a transactivator protein that binds this sequence, these elements do not appear to be the primary targets in the induction pathway.

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