Replication dependent and cell specific activation of the polyomavirus early promoter

Kenneth K. Yoshimoto and Luis P. Villarreal*

Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717, USA

ABSTRACT

The relationship of viral DNA replication to the activation of viral gene expression is usually considered with respect to late genes. In this report we examine the replication activation of the polyomavirus early promoter. Using origin active and inactive mutants to drive luciferase gene expression from the polyomavirus early promoter, we show that the early promoter is also subjected to a replication dependent activation. The degree of activation can be up to a hundred fold greater than that seen without replication and is about 13 fold on a per template basis. This replication based activation is, however, cell type dependent and was seen in FOP cells but not in 3T6 cells. Analysis of the requirements of cis acting DNA show that these enhancer elements affect early transcription predominantly through the activation of replication, although some replication independent stimulation can also be seen. The implications of this result for the regulation of polyomavirus early gene regulation are considered.

INTRODUCTION

It is generally observed that the activation of the late genes of eukaryotic DNA viruses follows the replication of viral DNA. The reasons for this relationship, however, remain in dispute. Early experiments with Adenovirus (1) and SV40 virus (2) infections indicated that the act of DNA replication per se was needed for late gene activation. Other results appear consistent with this view (3–5). Superinfection of cells in the late phases of viral gene expression with a second virus did not express late genes of the second virus (1). This failure to rapidly activate late gene expression appeared to indicate a need for viral replication to assemble stable active late transcription complexes and that the simple presence of appropriate transactivators for late transcription was insufficient (4,5). Such putative stable transcription complexes, however, would have numerous theoretical implications for the relationship of DNA replication to gene activation (6).

Other results with SV40 and polyomavirus, using DNA transfection methods, however, appear to clearly show the activation of late genes in the absence of DNA replication (7–12). This led to the view that the increased number of templates following DNA replication along with a trans-acting stimulation

of late promoter by T-antigen was mainly responsible for the increase in late gene activation. The role of T-antigen in activation of late genes appears to be indirect as SV40 T-antigen is not apparently bound to the majority of late transcription complexes (5). Also, early results using temperature sensitive T-antigen of polyomavirus (13) or SV40 appeared to indicate only a transient need for functional T-antigen to activate late genes (14,15).

Early SV40 gene expression, however, does not usually appear to be stimulated by viral DNA replication (see (16) for review). Early SV40 transcription appears to be under negative control of viral T-antigen (15,17–19). This negative regulation reportedly involves a T-antigen induced shift in transcriptional start sites from early-early to late-early positions (16). With polyomavirus, similar observations of shifting mRNA start sites were also made (20), but direct analysis of mRNA cap sites implied that the apparent late-early start site may be an artifact of the nuclease S1 assay and read-through transcription from the circular viral genome (21).

Even with SV40 early promoter, there appears to be a variable relationship to DNA replication. SV40 early gene expression can be repressed by the replication of DNA (22,23) in HeLa and CV-1 but not in adenovirus transformed 293. Also, Wildeman has reported that SV40 T-antigen will activate the SV40 late-early promoter in a replication dependent manner (24). In this report, we examine the replication dependence of the polyomavirus early promoter and unexpectedly show a clear replication dependence of early promoter in FOP cells. Permissive 3T6 cells, however, show much less of a replication dependence. Some implications of this result and earlier results are considered.

METHODS

Cells and reagents

FOP cells (provided by J. Hassell, McGill University, Montreal) are derived from FM3A mouse mammary tumor cells and constitutively express Py T antigen. FOP and 3T6 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum.

Plasmids

The Cytomegalovirus (CMV) early promoter was used as positive control for transcription. The plasmid with the CMV promoter (constructed by J. Guzowski, Department of Molecular Biology

* To whom correspondence should be addressed

Figure 1. The Polyomavirus noncoding region consists of the A and B enhancers and the origin of replication. The A enhancer contains the domain, with the PEA3, PEA1 and PEA2 binding sites. dl 5132–5269, which retains replication competence in FOP cells, is a deletion of the B enhancer. dl 5098–5269, which lacks replication competence in FOP cells, is a deletion of the B domain as well as the α domain. Sequences shown between dl 5132–5269 and dl 5098–5269 indicate nucleotides inserted into dl 5098–5269. Boxes indicate binding sites within inserted sequences. Replication levels observed previously are shown in the right column. The numbering system follows that of Soeda.

was stored at -80 degrees. Mobility shift assays were performed according to the Stratagene Gel Shift Kit. The PEA3/PEA1 combination site was excised to give a 23 bp XhoI-PvuII fragment. The XhoI site was a result of the oligonucleotide-directed mutagenesis used to insert the sites. The XhoI end was filled-in with radioactively labeled nucleotide. Non-specific competitor consisted of plasmid DNA.

RESULTS

Enhancer and origin requirements for early promoter stimulation

We have previously shown that FOP cells (Py early region expressing mouse FM3A cells) have simpler enhancer requirements for DNA replication than do 3T6 cells (27). FOP cells will efficiently replicate a Py origin containing plasmid if only the adjacent PEA3 and PEA1 binding sites remain of the enhancer DNA. Various other binding sites (including the PEA2 site) or combinations of binding sites show much less activity for driving Py DNA replication in FOP cells. To determine if these elements which were active for stimulating Py DNA replication were also active for stimulating the early promoter the chimeric plasmids which express the luciferase gene from the Py early promoter, plasmids which had various combinations of enhancer elements (see figure 1) were transfected into FOP cells and the level of luciferase expression measured as described previously. The results of this experiment are shown in figure 3. It was observed that the PEA3/PEA1 binding site combination was most active at stimulating the early promoter, almost as active as the full wild type polyomavirus enhancer. At roughly half the level of wildtype levels of early promoter activation were the PEA1/PEA2 and PEA3/PEA3. The single binding sites, as well as the enhancer deleted negative control dl5098-5269 were low, but detectable, for luciferase expression. Activation was less dramatic (2 or 3-fold) for the PEA3 and PEA1 single constructs. Interestingly, the PEA2/PEA1 and PEA1/PEA3 'reversed' combinations were lower than their 'natural' counterparts even though they contained the same individual binding sites. The

'reversed' constructs maintain the orientation of their binding sites with respect to the origin, but switch the binding site order as shown in Fig. 1. This effect was also seen in the replication study; the 'reversed' constructs were repressed for replication (27). In general, the stimulation of early promoter by these elements appeared to closely follow their ability to stimulate Py DNA replication as we had shown previously (27).

These results were obtained with plasmids having a functional Py origin of replication. When the Py origin was inactivated by a small deletion, all enhancer combinations were low in their ability to stimulate the early promoter and they were also low in comparison to a transcriptionally active nonreplicating control plasmid in which the CMV regulatory region drives luciferase gene expression (Fig. 3). The net effect on luciferase expression of an intact origin of replication was to increase expression from the constructs containing the wildtype or the PEA3/PEA1 enhancers by 13-fold and 12-fold, respectively. The intact origin did not activate transcription from the enhancerless dl5098-5269. Origin dependent activation was less dramatic (2 or 3-fold) for the PEA3 and PEA1 single constructs. The control CMV plasmid did establish that the transfection efficiencies and transient expression efficiencies for a nonreplicating plasmid can be almost as high as that seen with the replicating polyoma based plasmids. The low level of transcription which was observed in the Py replication deficient plasmids, however, still showed some differences with respect to the enhancer. The wildtype enhancer gave the highest expression, followed by the PEA3/PEA1 construct.

DNA levels following transfection

The quantity of plasmid DNA present following transfection was measured with a Southern transfer of plasmid extracted by the Hirt procedure (Fig. 4). Although there is some visible variation in the levels of transfected plasmid seen, this variation is rather small, about two fold. In general, most transfections show similar levels of plasmid DNA in cells 48 hours after transfection. That the PEA3/PEA1 plasmid was slightly higher than the other transfections is possibly due to some plasmid replication. The nonreplicating CMV control plasmid, which showed high levels of luciferase activity, gave levels of plasmid DNA in cells similar to that seen following transfection with the polyoma based

Figure 2. Luciferase expression plasmid with wildtype Polyomavirus enhancer. Variants replace the enhancer with the PEA binding sites indicated in Figure 1.

Figure 3. Luciferase expression from various constructs in FOP cells. Enhancer column specifies the enhancer activating transcription from the early promoter. A (-) in the Ori column indicates the origin of replication has been inactivated by Apa I digestion and blunt-ending. Expression is given in microvolts per microgram of protein in cell extract. Data in this figure are from one experiment and are qualitatively representative of other experiments.

Figure 4. Quantitation of total episomal DNA. Hirt-extracted episomal DNA was linearized with XhoI and subjected to Southern blot analysis.

Figure 5. Luciferase expression normalized to the template level of the corresponding ori-construct. Total episomal DNA was extracted and quantified by Southern blot and densitometer reading. Ori + expression was normalized to template level by the following formula:

$$\text{Normalized ori + expression} = (\text{raw ori + expression}) \times (\text{ori - DNA}) / (\text{ori + DNA})$$

Figure 6. Luciferase expression from various constructs transfected into 3T6 cells. Ori column indicates whether an active origin of replication is present. Tag column indicates whether or not a Py T antigen expressing plasmid was cotransfected with the reporter plasmid. Expression is given in microvolts per microgram of protein in cell extract.

plasmids. It appears from this measurement that the amount DNA transfected into cells was substantial and that an increase in total template due to replication does not account for a large increase in total plasmid DNA. When the levels of luciferase expression were normalized to the level of DNA in the corresponding origin-

inactivated construct, the data as shown in Fig 5 resulted. Replicated DNA was again seen to be 13 fold more active for early transcription than nonreplicated DNA on a per template basis. Because the total DNA was roughly the same between origin-active and origin-inactive templates, and because the level of expression was 13 fold in replicating plasmids in this experiment (and up to 100 fold higher on an absolute scale in other experiments, not shown) a simple increase in numbers of templates would not appear to account for the large stimulation of early promoter seen, implying that it is replication per se that is needed for high activation of the polyomavirus early promoter. The possibility exists that a very small portion of the transfected DNA is in the correct state for both replication and transcription. In this case, a fractional increase this active template following replication might account for the increase in luciferase expression from ori (+) constructs. This possibility is further considered in the discussion section.

Replication dependent activation of early promoter is cell type dependent

We had previously shown that the enhancer requirements for Py DNA replication in FOP cells were not the same as that of the fully permissive 3T6 cells. FOP cells appear to have simpler enhancer requirements for full levels of DNA replication. Others have also reported cell type effects on the replication dependent repression of the SV40 early promoter which is seen in 293 but not HeLa cells (22,23). We therefore examined 3T6 cells to determine if the replication dependent activation of the early promoter we observed was cell type dependent. Because the above plasmids, which do not code for T-antigen, were used in this experiment, T-antigen was also supplied in trans by cotransfection with a second plasmid to allow viral DNA replication. Origin and T-antigen dependence of Py early promoter activity was examined as shown in Fig. 6. All the Py based plasmids examined gave low luciferase expression relative to the CMV promoter control. Cotransfection of a Py T antigen expressing plasmid (PiAN7 + A2 BamHI) increased the expression from the wildtype and the PEA3/PEA1 constructs somewhat (about 2 fold). This activation was much less than that observed in FOP cells. The enhancerless dl5098-5269 failed to activate expression. Somewhat surprising was the large increase in expression of the nonreplicating control CMV promoter plasmid seen in the presence of T-antigen (about 6 fold), probably indicating the trans-stimulatory effect of T-antigen on some promoters as described by others (11).

Gel retardation analysis

To investigate the possibility that FOP cells have trans-acting factors which bind specifically to the PEA3/PEA1 binding sites, a mobility shift assay was performed on FOP cell extract (Fig. 7). A broad band of radioactivity was retarded but disappears on addition of non-specific competitor. A smaller, sharper band remains even at high concentrations of non-specific competitor. (This band was not significantly competed off by the specific competitor plasmid used here. The fact that a plasmid containing a single PEA3/PEA1 site was used, rather than a double-stranded oligonucleotide, made it difficult to attain a high molar ration of specific competitor.) This retarded band appears similar to that described by Bolwig and Hearing, due to binding of the PEA3/PEA1 sites (31) and appears to establish that FOP cells have factors which bind to these sites in vitro.

Figure 7. Mobility shift assay of FOP cell extract. A radioactively-labeled double-stranded 23 bp fragment comprising the PEA3/PEA1 sites was incubated with FOP cell extract. The specific competitor used was the PEA3/PEA1 replication assay plasmid. 1) probe + extract; 2) probe alone; 3)–6) probe + extract + 0.6, 6, 60 and 600 ng, respectively, specific competitor; 7)–10) probe + extract + 0.6, 6, 60 and 600 ng, respectively, non-specific competitor. Sequence of 23mer: TCGAGCAGGAAGTGACTAACCAG.

DISCUSSION

In this report we have demonstrated that FOP cells show a significant replication dependent stimulation of the polyomavirus early promoter. The highest absolute levels of polyomavirus early promoter activity were seen with replicated DNA in FOP cells. On a per template basis, replicated DNA in FOP cells was about 13 fold more active for early promoter activity than unreplicated DNA in either FOP or 3T6 cells. The nonreplicating CMV promoter plasmid also achieved similar high level of luciferase expression. This indicates that our transfection procedure can generate a sufficient quantity of biologically active template, without replication, for efficient transcription and implies that an increase in template number need not be required to account for the observed stimulation of luciferase expression from replicating polyoma plasmids. Instead, replication may allow the assembly of transcriptionally active templates. Also, aphidicolin prevents replication dependent stimulation of the early promoter (unpublished observation), supporting the view that replication

itself is required for the observed activation of transcription. A genetic analysis of the requirements of cis-acting elements show that those elements which stimulate Py DNA replication (PEA3/PEA1) also stimulate the early promoter, predominantly through DNA replication as origin inactive DNA's showed low activity. Yet in some cases the enhancer requirements for transcription are distinguishable from those for replication. For example, the highly synergistic behavior of the PEA3 and PEA1 seen previously for replication (27) was not seen here for transcription from origin-inactivated constructs. This indicates that there are cell specific differences in the way cis-acting DNA binding sites activate replication and transcription as has previously been reported by us *in vivo* (32).

We have also shown that FOP cells appear to have factors which specifically bind to the PEA3/PEA1 binding sites *in vitro*. This raises the interesting question of why there should be a replication dependent stimulation of early promoter in these and not other (3T6) cells, which also have factors which bind this DNA (33). Because naked DNA is being transfected it is expected that it will assemble with the prevailing chromatin proteins and trans-acting factors into mini-chromosome like structures (34). As both FOP and 3T6 cell lines are permissive for wild type Py replication, why should Py DNA in FOP cells require replication for active early transcription? Others have proposed models in which cell specific negative factors assemble onto transfected DNA and that DNA replication increases template levels to the point where such negative factors are titrated out, allowing increased transcription (8,35). If such a model is generally applicable, it would not, however, explain the early mixed infection results of Thomas and Mathews (1). Nor does this model appear to readily account for our results as the levels of DNA replication which were occurring in our experiments do not increase the total plasmid DNA to significant degrees (less than two fold). Also, because the CMV promoter was highly active under our transfection conditions, it appears that newly transfected DNA can efficiently assemble into active transcription complexes. It appears, therefore, that any putative negative acting factor would have to specifically bind to transfected and not replicated polyomavirus DNA sequences. A possible explanation of this, which we favor, is inferred from the results of Cereghini and Yaniv (34) and others (36–39). They show that replicated DNA has a distinguishable chromatin structure from non-replicated DNA *in vivo* and *in vitro* (36,39). Along these lines, Thomas and Mathews (1) and others (4) have proposed that the replication dependent activation of the Adenovirus, SV40 and polyomavirus (40) late promoters may occur during replication by allowing assembly of stable and active late transcription complexes. The observations that in some conditions, late promoter activity is seen without replication might indicate cell specific differences in the assembly of chromatin competent for transcription.

Previously, it appeared that replication based transcriptional stimulation was specific to the late, not early promoters. Our results, that polyomavirus early promoter can also show a replication dependent stimulation are unexpected and appear similar to those of Wildeman (24) who also observed a cell specific replication based stimulation of the SV40 early (and late) promoter. Although such an observation may appear to be at odds with prevailing views of an autoregulated early promoter, the characterization of early promoter activity by Kamen and colleagues showed a large increase in activity following DNA replication and is not inconsistent with our results (20,21). It is

also possible that replication stimulation of the Py early promoter is made more apparent in FOP cells due to the relatively low levels of T-antigen which may be insufficient for repression, but not replication.

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