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Polyoma virus early–late switch: Regulation of late RNA accumulation by DNA replication

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ABSTRACT **Early in infection of permissive mouse cells, messages from the early region of the polyoma virus genome accumulate preferentially over those from the late region. After initiation of DNA replication, the balance between early and late gene expression is reversed in favor of the late products. In previous work from our laboratory, we showed that viral early proteins do not activate the polyoma late promoter in the absence of DNA replication. Here we show that activation of the late genes in replication-incompetent viral genomes can occur if actively replicating genomes are present in the same cell. A low level of DNA replication, however, is insufficient to induce the early–late switch. Furthermore, replication-competent genomes that fail to accumulate late RNA molecules are defective in the transactivation of replication-incompetent genomes. We suggest that titration of an unknown diffusible factor(s) after DNA replication relieves the block to late RNA accumulation seen in the early phase, with most of this titration being attributable to late-strand RNA molecules themselves.**

The productive life cycle of polyoma virus in permissive mouse cells involves a transition from early to late phase that is characterized by the expression of different groups of genes (1–5). Early in infection, gene products encoded by the early region of the viral genome [mRNAs encoding large, middle, and small tumor antigens (TAgs)] accumulate preferentially over those from the late region. After the onset of DNA replication, the balance between early and late gene expression is quickly reversed in favor of late products (mRNAs encoding the virion structural proteins VP1, VP2, and VP3); by late in infection, late messages exceed the early ones by a factor of 20–80 (3).

The molecular details underlying the early–late switch are poorly understood, although large TAg is known to play a pivotal role. Large TAg binds to its target DNA sequences within and adjacent to the replication origin (6–8) and is required for initiation and maintenance of polyoma virus replication. Previous experiments by others suggest that large TAg also transactivates the late promoter and represses transcriptional activity of early genes (2, 9–11). More recent data have suggested an additional involvement of middle TAg in regulating the early–late switch by interacting with host signal transduction pathways, which in turn modify transcription factors (10, 12). However, posttranscriptional regulation may play a more important role than large TAg in accumulation of late messages after DNA replication is initiated (3, 13–16).

Activation of polyoma virus late genes is replication-dependent because (*i*) polyoma mutants carrying temperature-sensitive large TAg mutations cannot proceed into the late phase at a nonpermissive temperature (2); (*ii*) cytosine arabinoside (araC), a DNA replication inhibitor, prevents the polyoma virus early–late switch (3, 14); and (*iii*) polyoma virus late genes are generally not expressed in significant levels in transformed nonpermissive cells, in which polyoma fails to replicate (15). Regulation of gene expression by DNA replication could be achieved via trans- or cis-acting mechanisms. Trans-acting factors may be modified, induced, or titrated as a result of DNA replication. Alternatively, changes in chromatin structure induced by replication may exert a direct cis effect on late gene expression (17, 18). In simian virus 40 (SV40), late genes can be activated in trans by large TAg in the absence of DNA replication (19–22). Recently, a trans effect involving depletion of an important transcriptional factor has been suggested for polyoma virus late gene activation (12). In contrast, previous reports presented evidence for a direct effect of the SV40 replication origin in activating the SV40 late promoter (23). Results from adenoviruses (24, 25) indicated cis-acting template effects on transcriptional control and posttranscriptional processing. A systematic study of a cis effect of DNA replication on polyoma late gene activation has not yet been reported.

We show here that TAg expression in the absence of DNA replication is insufficient to induce the polyoma virus early–late switch. Replication-defective constructs (i.e., those with deletions in the origin, deletion of large TAg binding sequences, or frame-shift mutations in the early region) failed to activate late genes in transient transfection assays. Cotransfection of a construct carrying a mutation in the replication origin with a distinguishable but replication-competent viral genome revealed that a replication-incompetent viral genome can be activated in trans by a replicating viral genome in the same cell. These results suggest that polyoma virus late gene expression is under negative regulation before the initiation of viral DNA replication. We propose that titration of an unknown diffusible factor(s) after DNA replication relieves the block to late RNA accumulation seen in the early phase. Surprisingly, most of this titration may result from late-strand RNA molecules.

MATERIALS AND METHODS

Plasmid Construction. Constructs TbsΔ (formerly E89) and oriΔ (formerly ΔT) were constructed as described (17). To create construct Tfs, 2 bp were added at each *Ava* I site in the early coding region (positions 673 and 1032 of strain 59RA, refs. 26 and 27). The "marked" Tfs (Tfs-M) was constructed by removing a *Bbs* I fragment (nt 978–1073) from the early region and by replacing a *Hinc*II fragment from the late region (nt 2985–3489) with a *Hinc*II fragment from the late region of SV40 (nt 2057–2297). Construction of oriΔ-M followed the same strategy.

Cell Culture, Transfection, and RNA Isolation. Mouse NIH 3T3 cells were maintained and propagated as described (17). Transfection procedures followed a DEAE-dextran method (28) with 4 μg of DNA per 100-mm plate. For cotransfection experiments, equal amounts of plasmids were always used;

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Abbreviations: TAg, tumor antigen; araC, cytosine arabinoside; SV40, simian virus 40.

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controls for such experiments used pUC18 DNA. Total RNA isolation was as described (28). The RNA samples were further treated to remove the trace amount of DNA contamination by a modified acid-phenol method (29). Cytoplasmic RNA was isolated by a guanidinium isothiocyanate method as described (3).

RNase Protection. Clones for generating RNA probes VP1 and E(A) were described elsewhere (13). The L2 probe consists of the *Rsa* I–*Dde* I (nt 2043–2387) fragment of polyoma inserted at the *Sma* I site of pBS$^+$. Probe L4 consisted of an *Apa* I–*Hin*dIII fragment of Tfs-M into the corresponding sites of pBlueScript SKII. This probe spans the junction of polyoma and SV40 sequences in Tfs-M and oriΔ-M. After *in vitro* transcription by T3 or T7 RNA polymerase using [α-^{32}P]UTP, DNA templates were removed by RQ1 DNase digestion in combination with acid-phenol extraction. The labeled RNA probes were hybridized in excess to RNA samples at 57–60°C overnight (13). The resulting hybrids were digested with T1/T2 mixture (30) at 37°C for 2 h. Samples were resolved on 6% denaturing polyacrylamide gels. Quantitation of results was performed using a Betagen Betascope blot analyzer.

DNA Replication Assays. Episomal DNA was isolated as described (17, 31) and then digested with an excess of *Dpn* I, *Eco*RI, and *Bam*HI. Replication assays were as described (17), with the probe being either the ^{32}P-labeled viral *Ava* I fragment (nt 673–1032) or *Eco*RV–*Bam*HI fragment (nt 4132–4658). Bands were quantitated using a Betagen Betascope blot analyzer.

RESULTS

Replication-Defective Polyoma Viruses Fail to Display a Late Pattern of Gene Expression. We constructed three replication-defective polyoma virus mutants (Fig. 1*A*) to examine whether the polyoma large TAg plays a direct role in early–late switch or an indirect role by activating DNA replication. Tfs (*T*Ag *f*rame *s*hift) carries two translational frame-shift mutations in the early region so that functional large, middle, and small TAgs cannot be produced. Construct oriΔ lacks a stretch of 8 consecutive thymidine residues at the late border of the minimal replication origin. These thymidines are known to be required for DNA replication, both for polyoma and for SV40 (17, 32, 33). TbsΔ (*T*Ag *b*inding *s*ite deletion) lacks two high-affinity binding sites for large TAg on the early side of the origin palindrome; at least one of these two sites is required for DNA replication (6–8).

RNase protection assays of early-strand and late-strand gene expression from cells 48 h after transfection with these mutants are shown in Fig. 1*B*. Late cytoplasmic messages from a wild-type transfection were predominant over the early ones by a factor of 10 (lanes 1 and 5), mimicking a typical late phase of infection. In contrast, the late signals from all three replication-defective mutants were very low (lanes 6–8). The ratios of late to early signals for all three constructs were ≈1:5, resembling an early pattern of infection before the initiation of DNA replication (3). Results were the same when total RNA from transfected cells was examined (data not shown). Replication assays (Fig. 1*C*) confirmed that these mutants are replication-defective. The relatively low levels of early transcripts detected in these mutants likely result from low numbers of DNA templates due to the absence of DNA replication.

Trans-Activation of Polyoma Late Genes from a Nonreplicating Genome by a Wild-Type Virus Present in the Same Cell. The experiments described above suggest a central role for DNA replication in the polyoma early–late switch. However, they cannot distinguish between cis-effects of DNA replication, such as chromatin conformational changes on replicating templates, or trans-effects, due to induction or titration of a factor(s) that affects late gene expression. In the former case,

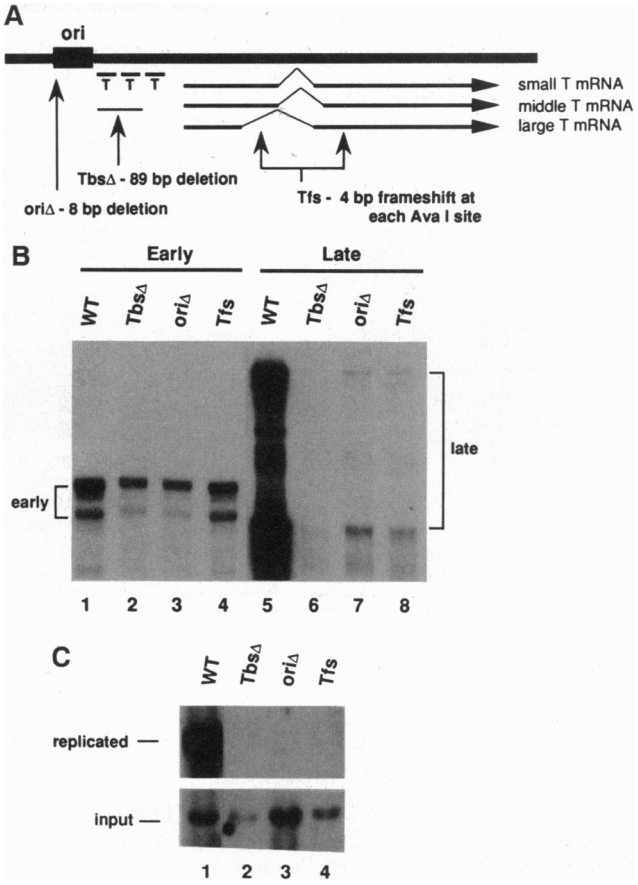

FIG. 1. Early and late gene expression in the absence of DNA replication. (*A*) Schematic diagram of polyoma mutants incapable of replication. Mutant oriΔ carries a deletion of 8 thymidine residues on the late border of the minimal replication origin. Mutant TbsΔ lacks 89 bp on the early side of the replication origin, including two high-affinity binding sites for large TAg. Mutant Tfs carries two frame-shift mutations, one in each of the viral *Ava* I sites in the early coding region. (*B*) Cytoplasmic RNA isolated from NIH 3T3 cells 48 h after transfection was analyzed for early and late transcripts by RNase protection assays with E(A) (early) and VP1 (late) RNA probes. Four-fold more RNAs were used to generate detectable signals in replication-defective mutants (lanes 2–4 and lanes 6–8). The ratio of late to early messages was calculated by correcting for probe lengths and uridine content. (*C*) DNA replication assays were performed using a probe for the early region. WT, wild type.

two coexisting independent viruses would display different patterns of gene expression if they were at different stages of infection. Those viruses that have undergone DNA replication would express predominantly late genes, whereas those in the early phase or replication-incompetent viruses would express predominantly early genes. In the case of a trans effect, however, both viruses would be phenotypically the same.

To distinguish between these possibilities, we marked both constructs Tfs and oriΔ by a small early region deletion and by replacing a portion of the late region with a corresponding fragment from SV40 (Fig. 2*D*). The early and late transcripts from these marked viruses could be easily discerned from those of wild type in our standard RNase protection assay. When transfected alone, both Tfs-M (marked Tfs) and oriΔ-M (marked oriΔ) failed to replicate (Fig. 2*C*, lanes 1 and 3). Analysis of total RNA showed that both Tfs-M and oriΔ-M failed to express late genes at a high level (Fig. 2*B*, lanes 1 and 3). However, the presence of large TAg supplied by cotransfected wild-type polyoma genomes corrected the replication defect of Tfs-M (Fig. 2*C*, lane 2). In these cotransfections, gene expression from Tfs-M shifted to a late-phase pattern

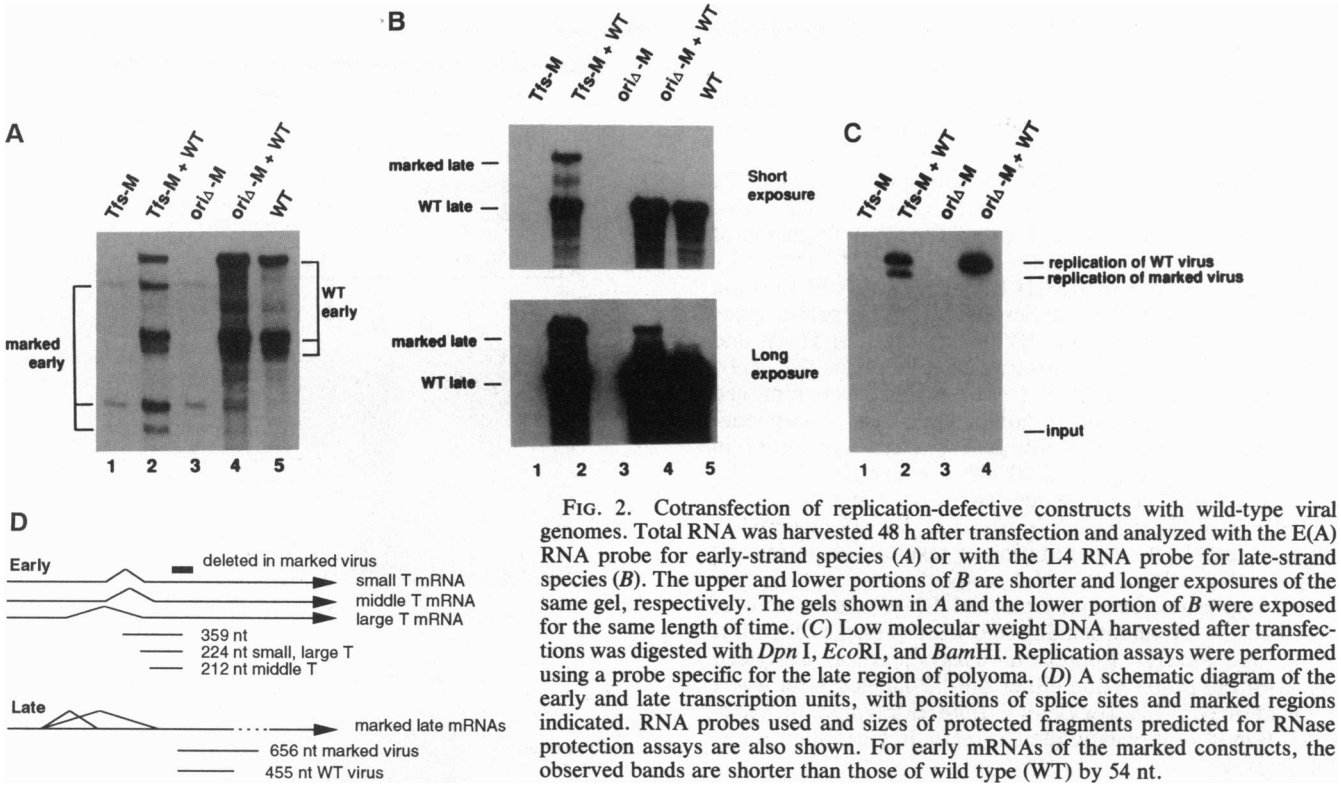

FIG. 2. Cotransfection of replication-defective constructs with wild-type viral genomes. Total RNA was harvested 48 h after transfection and analyzed with the E(A) RNA probe for early-strand species (*A*) or with the L4 RNA probe for late-strand species (*B*). The upper and lower portions of *B* are shorter and longer exposures of the same gel, respectively. The gels shown in *A* and the lower portion of *B* were exposed for the same length of time. (*C*) Low molecular weight DNA harvested after transfections was digested with *Dpn* I, *Eco*RI, and *Bam*HI. Replication assays were performed using a probe specific for the late region of polyoma. (*D*) A schematic diagram of the early and late transcription units, with positions of splice sites and marked regions indicated. RNA probes used and sizes of protected fragments predicted for RNase protection assays are also shown. For early mRNAs of the marked constructs, the observed bands are shorter than those of wild type (WT) by 54 nt.

(compare Fig. 2 *A*, lanes 1 and 2, and *B*, lanes 1 and 2), indicating that viral sequences missing in the marked regions are not essential for DNA replication or for the early–late switch. As expected, we could not detect any replication of oriΔ-M, even when cotransfected with wild type (Fig. 2*C*, lane 4). Surprisingly, however, late gene expression from oriΔ-M was stimulated >20-fold by wild type in trans (Fig. 2*B*, compare lanes 3 and 4). This experiment revealed that late gene activation can occur from a nonreplicating template.

Viral DNA Replication Induces the Early–Late Switch. The experiments described above indicated a DNA replication-dependent transactivation of late gene expression in transient transfection assays. We next used two different inhibitors to block the replication of wild-type virus in infected cells. araC and aphidicolin have different modes of action (34, 35), but each blocks both viral DNA replication (data not shown) and the early–late switch (Fig. 3). In the presence of replication inhibitors, even though there is no template amplification, early RNAs are nevertheless expressed at levels equal to or greater than those seen in parallel infections lacking the inhibitors (compare Fig. 3, lanes 2 and 3 with lane 1). This makes it unlikely that early proteins alone are sufficient to induce the switch. This result also suggests that the concentration of viral DNA (i.e., template number) might be important in regulating the switch. Additional experiments using constructs oriΔ and Tfs-M (Fig. 4*A*) provided evidence that support this hypothesis. Construct oriΔ generates 10–20% wild-type levels of early messages. Tfs-M cannot produce any functional TAgs but can replicate when cotransfected with wild type (Fig. 2*C*). When oriΔ and Tfs-M were cotransfected, the early–late ratio of RNA remained at 5:1 (Fig. 4*A*, lanes 1 and 3), a typical early pattern. Analysis of DNA replication (Fig. 4*B*) revealed that Tfs-M replicated to only

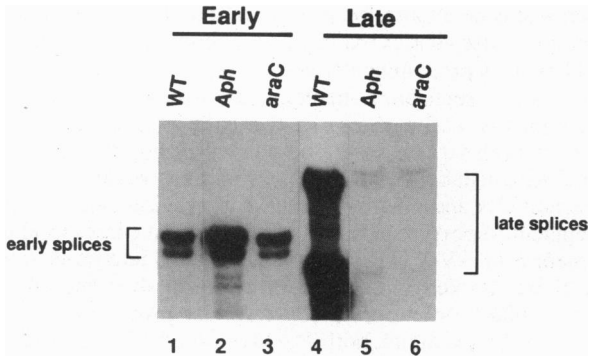

FIG. 3. Polyoma virus gene expression in the presence of replication blockers. Replication inhibitors, aphidicolin (2 μg/ml) or araC (20 μg/ml), were added to cells 1 h after infection with polyoma virus, and for araC experiments, the medium was replaced every 6 h. Cytoplasmic RNA isolated 24 h later was subjected to RNase protection assays with E(A) (lanes 1–3) and VP1 (lanes 4–6) RNA probes, respectively. WT, wild type.

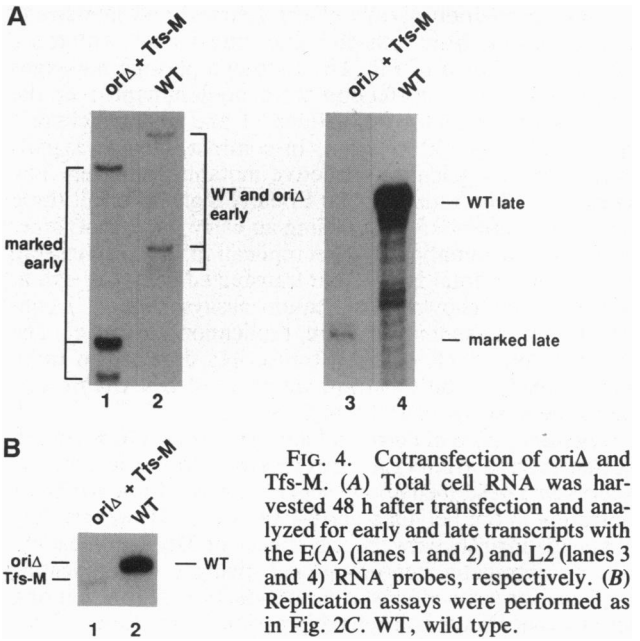

FIG. 4. Cotransfection of oriΔ and Tfs-M. (*A*) Total cell RNA was harvested 48 h after transfection and analyzed for early and late transcripts with the E(A) (lanes 1 and 2) and L2 (lanes 3 and 4) RNA probes, respectively. (*B*) Replication assays were performed as in Fig. 2*C*. WT, wild type.

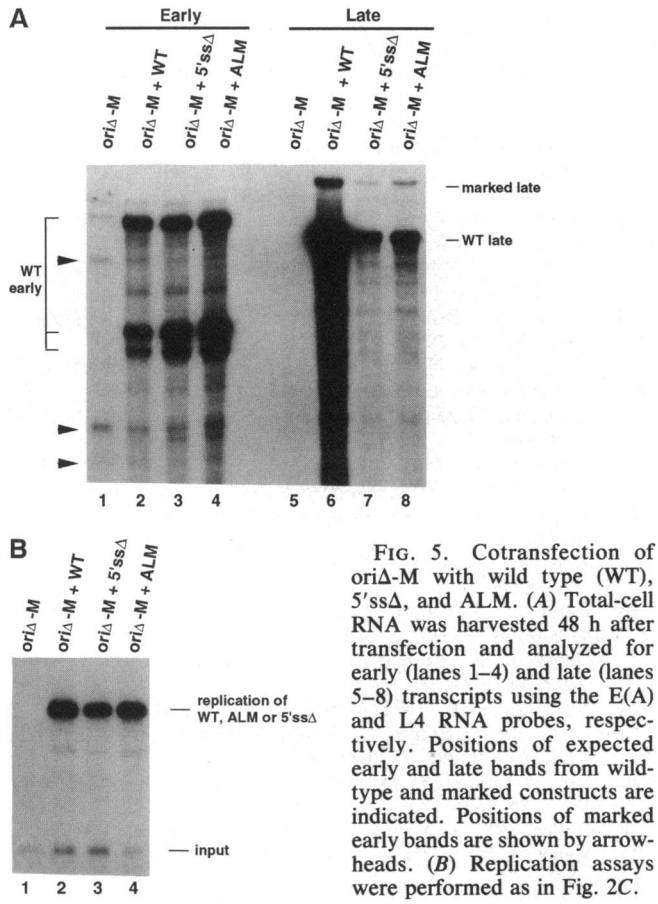

FIG. 5. Cotransfection of oriΔ-M with wild type (WT), 5'ssΔ, and ALM. (*A*) Total-cell RNA was harvested 48 h after transfection and analyzed for early (lanes 1–4) and late (lanes 5–8) transcripts using the E(A) and L4 RNA probes, respectively. Positions of expected early and late bands from wild-type and marked constructs are indicated. Positions of marked early bands are shown by arrowheads. (*B*) Replication assays were performed as in Fig. 2*C*.

5–10% the level of wild type. We conclude that the early–late switch requires a threshold level of viral DNA. Accumulating viral DNA, or some product of it, might induce or titrate a factor required for the switch.

Late-Strand RNA Molecules Themselves Contribute to the Early–Late Switch. Fig. 5*A* shows the results of a cotransfection of oriΔ-M with construct 5'ssΔ or with mutant ALM. Construct 5'ssΔ differs from wild type in having a 6-bp deletion of the 5' splice site used in all late pre-mRNA splicing (36). Mutant ALM contains an internal deletion of sequences within the late leader exon and lacks no splicing signals (13). The deletion in this mutant does not overlap that of mutant 5'ssΔ. Both mutants, however, express very low levels of late primary transcripts, whereas early messages are expressed at at least wild-type levels (refs. 13 and 36; and Fig. 5*A*, compare lanes 3 and 4 with lane 2). Fig. 5*A*, lanes 7 and 8, confirms the defect in RNA stability as reported (13, 36). Interestingly, however, these mutants can transactivate oriΔ-M late gene expression to <20% of the level seen with wild type (Fig. 5*A*, compare lanes 7 and 8 to lane 6). These experiments have been repeated five times, with transactivation levels always falling between 10 and 20% of those seen with wild type. Fig. 5*B* shows that constructs 5'ssΔ and ALM replicate as well as wild type in these cotransfections, indicating that neither DNA accumulation nor early proteins can fully account for the early–late switch observed for construct oriΔ-M when cotransfected with wild type. Late proteins are not involved, since frame-shift mutations in the late region did not affect the early–late switch (data not shown). We conclude that late-strand RNA molecules themselves must contribute to late gene activation and in some way positively regulate further late mRNA accumulation.

DISCUSSION

Large TAg and viral DNA replication are essential for the polyoma early–late switch (2, 3). Large TAg has also been proposed to be involved in the repression of early gene expression at late times and in transactivating the late promoter (2, 9, 10). Because mutations in large TAg or its binding sites always abolish DNA replication, it was unclear whether large TAg, DNA replication, or both are needed for the transactivation of polyoma virus late genes. To characterize the molecular event(s) needed for the switch, we set out to identify the cis and trans elements involved. To block viral replication, we separately mutated three targets: TAgs, high-affinity TAg binding sites, and the viral replication origin. None of these replication-defective mutants displayed high levels of late gene expression in transient expression assays (Fig. 1*B*). Results using araC and aphidicolin to block replication in infected cells also indicated that the polyoma early–late switch is dependent on replication of the virus, and not on early proteins; overexpression of early messages in the presence of these inhibitors did not activate late gene expression (Fig. 3). Further support for this conclusion comes from the cotransfection of constructs oriΔ-M and mutants 5'ssΔ and ALM (Fig. 5*A*), where a replicating viral genome that expressed levels of early messages equal to or higher than wild type, but low levels of late transcripts, was incapable of transactivating a replication-defective genome.

As DNA replication has been suggested to influence the gene expression of several animal viruses in cis (17, 23–25), we carried out cotransfection experiments with wild-type virus and a marked replication-incompetent genome, oriΔ-M (Fig. 2). When oriΔ-M was transfected alone into mouse cells it expressed predominantly early-strand RNAs; however, when it was cotransfected with wild-type virus, its late gene expression was activated (Fig. 2*B*). We conclude that late gene expression is activated in trans by replicating genomes. The fact that no replication was seen for oriΔ-M when cotransfected with wild type, even at long exposures of the film shown in Fig. 2*C*, argues that recombination between cotransfecting plasmids cannot account for the apparent transactivation of the late genes of oriΔ-M.

Further studies were conducted to determine whether late gene expression is transactivated by an induced positive regulator or derepressed by removal of a negative one. As the results above indicate, the induction of a positive regulator could not be a direct one due to a viral early protein but must be an indirect consequence of DNA replication. Results of the Tfs-M and oriΔ cotransfection indicate that a low level of DNA replication does not appear to be sufficient to induce the transition from early to late phase (Fig. 4*A*). In this cotransfection, construct Tfs-M replicated to only 5–10% the level of wild-type polyoma virus (Fig. 4*B*) and neither Tfs-M nor oriΔ displayed a late pattern of gene expression, even though both the early-strand and the basal late-strand signals from the replicating Tfs-M genome were substantially enhanced due to a moderate increase in template number. As high-level expression of early genes is insufficient to induce the switch (Fig. 3), we suggest that the early–late switch involves the removal of a negative regulator, perhaps as a result of a titration event, rather than as the result of an induction by early proteins. The switch cannot take place until DNA templates have accumulated to a critical level, regardless of the levels of early proteins. Above that level, late RNAs accumulate rapidly. Consistent with this model, we have observed (unpublished data) that the onset of the polyoma late phase occurs sooner in high multiplicity infections than in low multiplicity infections.

Evidence for removal of a negative regulator of gene expression has been seen in another system. In SV40, models have been proposed for negative regulation of late gene expression by removal or depletion of a diffusible DNA-binding factor during infection (37, 38). For polyoma, Yoo *et al.* (12) suggested that a key transcription factor, possibly TFIID, is depleted by the accumulation of viral templates

during lytic growth. This could result in a failure of assembly of a TFIID-dependent transcription complex at the early promoter, leading to the activation of late genes, whose promoter may function well at lower TFIID levels. Consistent with this notion, TFIID has been shown to be maintained at a lower effective concentration relative to the other essential transcription factors in cells (39). Although our previous work has shown that late gene expression is not primarily regulated at the level of transcription initiation (3, 17), further work is required to determine whether the depletion of some type of DNA-binding factor contributes to the polyoma early–late switch.

The most obvious interpretation of the above results is that replicating viral DNA titrates out a limiting cell factor that negatively regulates late RNA synthesis or stability, such as a repressor of initiation from the late promoter. DNA titration, however, is not sufficient for the switch, because 5′ssΔ and ALM, mutants that express early proteins and replicate like wild type but fail to accumulate late RNAs (Fig. 5 and refs. 13 and 36), are defective in the titration. Even at early times in infection, and in the absence of replication, a small but measurable amount of late-strand RNA is produced (results presented here and refs. 1–5). One result of replication would be to amplify the basal level of late-strand transcripts. As we have ruled out the possibility that late proteins are involved, it is likely that the accumulation of late-strand transcripts themselves during infection in some way positively regulates further late mRNA accumulation.

How might late-strand RNA molecules autoregulate their own levels? We suggested previously that early in infection late-strand transcription occurs, but the primary transcripts are inefficiently spliced and fail to accumulate (3). At late times the efficiency of late-strand transcription termination may be reduced, resulting in giant multigenomic transcripts. These long transcripts are spliced more readily and give rise to late messages that accumulate rapidly (3, 14). Thus, the accumulation of late transcripts is regulated by RNA stability, which itself may be related to transcription termination and/or RNA splicing. One explanation for the data presented here is that a trans-acting RNA-binding factor(s) that regulates the elongation of the transcription complex is titrated out as the lytic cycle proceeds. The target of this titration is not yet known. Another explanation for our results would be that splicing is required for late RNA accumulation and that it becomes more efficient at late times. Although less is known about the trans-acting regulators than cis-acting elements for alternative splicing (for a review, see ref. 40), it has been postulated in adenovirus (41) that some splicing factors might become limiting after the accumulation of viral transcripts, resulting in a change in alternative splicing. Therefore, the increasing demand for splicing factors late in polyoma infection may titrate a trans-acting splicing regulatory factor(s).

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1. Beard, P., Acheson, N. H. & Maxwell, I. H. (1976) *J. Virol.* **17**, 20–26.
2. Farmerie, W. G. & Folk, W. R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6919–6923.
3. Hyde-DeRuyscher, R. & Carmichael, G. G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8993–8997.
4. Kamen, R., Lindstrom, D. M., Shure, H. & Old, R. W. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 187–198.
5. Piper, P. (1979) *J. Mol. Biol.* **131**, 399–407.
6. Cowie, A. & Kamen, R. (1984) *J. Virol.* **52**, 750–760.
7. Pomerantz, B. J., Mueller, C. R. & Hassell, J. A. (1983) *J. Virol.* **47**, 600–610.
8. Gaudray, P., Tyndall, C., Kamen, R. & Cuzin, F. (1981) *Nucleic Acids Res.* **9**, 5697–5710.
9. Kern, F. G., Pellegrini, S. & Basilico, C. (1986) *Cancer Cells* **4**, 115–124.
10. Kern, F. G., Pellegrini, S., Cowie, A. & Basilico, C. (1986) *J. Virol.* **60**, 275–285.
11. Bergqvist, A., Nilsson, M., Bondeson, K. & Magnusson, G. (1990) *Nucleic Acids Res.* **18**, 2715–2720.
12. Yoo, W., Martin, M. E. & Folk, W. R. (1991) *J. Virol.* **65**, 5391–5400.
13. Adami, G. R., Marlor, C. W., Barrett, N. L. & Carmichael, G. G. (1989) *J. Virol.* **63**, 85–93.
14. Hyde-DeRuyscher, R. P. & Carmichael, G. G. (1990) *J. Virol.* **64**, 5823–5832.
15. Kern, F. G., Bovi, P. D. & Basilico, C. (1987) *J. Virol.* **61**, 4055–4059.
16. Lanoix, J., Tseng, R. W. & Acheson, N. H. (1991) *J. Virol.* **65**, 4728–4734.
17. Cahill, K. B., Roome, A. J. & Carmichael, G. G. (1990) *J. Virol.* **64**, 992–1001.
18. Villarreal, L. P. (1991) *Microbiol. Rev.* **55**, 512–542.
19. Coulombe, J., Berger, L., Smith, D. B., Hehl, R. K. & Wildeman, A. G. (1992) *J. Virol.* **66**, 4591–4596.
20. Ernoult-Lange, M., May, P., Moreau, P. & May, E. (1984) *J. Virol.* **50**, 163–173.
21. Keller, J. M. & Alwine, J. C. (1984) *Cell* **36**, 381–389.
22. Keller, J. M. & Alwine, J. C. (1985) *Mol. Cell. Biol.* **5**, 1859–1869.
23. Grass, D. S., Read, D., Lewis, E. D. & Manley, J. L. (1987) *Genes Dev.* **1**, 1065–1074.
24. Thomas, G. P. & Mathews, M. B. (1980) *Cell* **22**, 523–533.
25. Adami, G. & Babiss, L. E. (1991) *EMBO J.* **10**, 3457–3465.
26. Feunteun, J., Sompayrac, L., Fluck, M. & Benjamin, T. L. (1976) *Proc. Natl. Acad. Sci. USA* **68**, 283–288.
27. Freund, R., Mandel, G., Carmichael, G. G., Barncastle, J. P., Dawe, C. J. & Benjamin, T. L. (1987) *J. Virol.* **61**, 2232–2239.
28. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
29. Kedzierski, W. & Porter, J. C. (1991) *BioTechniques* **10**, 210–214.
30. Lichtler, A., Barrett, N. L. & Carmichael, G. G. (1992) *BioTechniques* **12**, 231–232.
31. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
32. Deb, S., DeLucia, A., Koff, A., Tsui, S. & Tegtmeyer, P. (1986) *Mol. Cell. Biol.* **6**, 4578–4584.
33. Luthman, H., Nilsson, M. G. & Magnusson, G. (1982) *J. Mol. Biol.* **161**, 533–550.
34. Catapano, C. V., Perrino, F. W. & Fernandes, D. J. (1993) *J. Biol. Chem.* **268**, 7179–7185.
35. Decker, R. S., Yamaguchi, M., Possenti, R., Bradley, M. K. & DePamphilis, M. L. (1987) *J. Biol. Chem.* **262**, 10863–10872.
36. Barrett, N. L., Carmichael, G. G. & Luo, Y. (1991) *Nucleic Acids Res.* **19**, 3011–3017.
37. Brady, J. & Khoury, G. (1985) *Mol. Cell. Biol.* **5**, 1391–1399.
38. Alwine, J. C. & Picardi, J. (1986) *J. Virol.* **60**, 400–404.
39. Colgan, J. & Manley, J. L. (1992) *Genes Dev.* **6**, 304–315.
40. McKeown, M. (1992) *Annu. Rev. Cell Biol.* **8**, 133–155.
41. Gattoni, R., Chebli, K., Himmelspach, M. & Stevenin, J. (1991) *Genes Dev.* **5**, 1847–1858.