A Reiterated Leader Sequence Is Present in Polyomavirus Late Transcripts Produced by a Transformed Rat Cell Line

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In cells transformed by polyomavirus, the viral genome is integrated into the host DNA, and in the absence of excision, viral gene expression is limited to the early region. We report here that the ability of a unique transformed rat cell line, designated SS1A, to produce readily detectable levels of late mRNAs is due to rearrangements of the integrated viral sequences. The structure of the SS1A insertion, resulting from amplification and deletion events, allows for the formation of a primary late transcript that can subsequently be spliced to generate a reiterated leader attached to the body of the late mRNA coding sequences. The presence of transcripts containing such a leader was confirmed by sequencing the 5' end of cDNA copies of late mRNAs isolated from a library constructed with SS1A mRNA. These results suggest that a reiterated leader sequence is necessary to stabilize late mRNA.

Polyomavirus (Py) late gene transcription follows the onset of viral DNA replication during the course of lytic infection and has several unusual features (9, 26). Transcription initiation has been shown to be extremely heterogeneous, and inefficient termination leads to the formation of giant nuclear transcripts that arise from multiple rounds of transcription of the circular viral genome (1). Mature late mRNAs contain, at their 5' ends, variable numbers of a noncoding 57-nucleotide (nt) leader sequence (nt 5022 to 5078) that are generated by splicing out genomic-length introns by using splice donor and acceptor sites which border both ends of the late leader unit (21, 26).

The exact function of this reiterated leader is presently unknown. It has been suggested that multiple copies of this sequence, which contains a 40S ribosome-binding site and a 10-nt sequence complementary to the 3' end of mouse 18S rRNA, increase the efficiency of translation (20, 24). It has also recently been demonstrated that a mutant lacking the 48 central bases of this sequence is nonviable but that this sequence can be functionally replaced by a variety of prokaryotic sequences, indicating that the late leader sequences are not essential for either late gene expression or translation but are required as a spacer at either the RNA or DNA level (3).

In cells that lack the necessary factors for viral multiplication, the virus can undergo an alternative interaction resulting from the integration of the viral genome into the cellular DNA, most often in a tandem head-to-tail arrangement (5-7). Subsequent expression of the viral early region results in the cells acquiring a transformed phenotype. In Py-transformed rat cells, excision or amplification of the integrated viral sequences frequently occurs. This process is dependent on a functional origin of replication as well as on the presence of a functional large T antigen (LT) and is facilitated by the regions of homology resulting from tandem integration (5-7, 22). In the absence of excision, virtually undetectable levels of transcripts hybridizing to a late-region probe are observed even when it can be demonstrated that late coding sequences are integrated intact (10). This is

illustrated in Fig. 1, which shows a Northern blot (RNA blot) hybridization with total cellular poly(A)⁺ RNA extracted from the H6A cell line. This cell line is transformed by the tsA strain of Py (producing a thermolabile LT) and contains a tandem repeat of the Py genome (5, 6, 10). While no late transcripts can be detected at 39.5°C, shifting these cells to the permissive temperature for LT results in excision and limited replication of viral genomes (5, 6), and late transcripts become readily detectable. Thus, rat cells do not lack any cellular factors necessary for late transcription.

The reason for the lack of detectable late transcripts from integrated viral DNA sequences is currently unknown. However, we have observed that replacement of late coding sequences and attachment to the late promoter of various

FIG. 1. Northern blot analysis of stable early and late transcripts present in the H6A parental and SS1A revertant cell lines. Total cellular RNA was extracted by the guanidinium-isothiocyanate procedure, and poly(A)⁺ RNA was selected by chromatography, using oligo(dT)-cellulose as previously described (15). Lanes: 1, 5 µg of poly(A)⁺ RNA from the tsA Py-transformed cell line H6A grown at 39°C; 2, 5 µg of poly(A)⁺ RNA from SS1A cultured at 39°C; 3, 2 µg of poly(A)⁺ RNA from H6A grown at 33°C for 96 h. RNA was size fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized to a ³²P-labeled 1.8-kb *Pst*I Py early-region fragment (A) or to a ³²P-labeled 2.4-kb *Hha*I late-region fragment (B) as previously described. Numbers refer to the size in kilobases of the main hybridizing bands.

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foreign genes results in the efficient expression of these genes when these constructs are integrated (15, 16). Moreover, mapping of the 5' ends of transcripts containing these genes showed initiation from within the Py late promoter and the same heterogeneous mixture of start sites used during the late phase of lytic infection (15). This indicates that the late promoter is functional when integrated and suggests that the absence of stable late transcripts in transformed cells is due to regulation at a posttranscriptional level.

Our laboratory has previously reported the isolation of a unique transformed rat cell line in which late transcripts could be detected at appreciable levels by S1 nuclease analysis (4). This cell line, designated SS1A, was isolated as a cured revertant of the H6A cell line (7). It contains a single insertion of Py DNA constituting an intact late region and a number of amplified repeats of a viral DNA segment which includes the origin of replication, early and late promoters, and the enhancer region (4, 7). The insert no longer codes for a functional LT, and thus excision does not occur in this cell line. Figure 1 shows the results of a Northern blot analysis using total poly(A)⁺ RNA extracted from the SS1A cell line and hybridized to either a Py early- or Py late-region probe. No full-length early-region transcripts (2.9 and 2.5 kilobases [kb]) were detected due to the loss of most of the early coding sequences. However, partial early transcripts of ~1.3 and 1.0 kb that arise from a 3'-truncated early region and

utilize the alternative polyadenylation signal at 99 map units are present in both the parental H6A and the SS1A cell lines, in addition to other transcripts that probably represent readthroughs into the host DNA (10). Hybridization to the Py late-region probe showed the presence of the same species of 19S, 18S, and 16S late viral mRNAs (2.2, 1.8, and 1.2 kb) in the SS1A cell line that are detected in the H6A cell line upon shift to 33°C.

To understand whether the rearrangements of the viral genome which occurred in SS1A could be responsible for the expression of late transcripts in this cell line, we molecularly cloned a 17.6-kb *EcoRI* fragment from the integrated Py sequences by using the EMBL4 bacteriophage vector (11). This fragment contains most of the integrated viral sequences as well as a portion of the adjacent cellular DNA. The 17.6-kb insert and fragments subcloned in plasmid vectors were subjected to restriction enzyme mapping. The results of this analysis are shown in Fig. 2. The insert contains a single intact copy of the late coding region immediately adjacent to a single copy of the late promoter and Py-regulatory region. Fine-structure restriction enzyme mapping of the left-most 4.2-kb *EcoRI*-*AvaI* fragment that contained these sequences failed to reveal any gross rearrangement with respect to the A2 strain of Py when the enzymes *Bgl*II, *Sau*3AI, *Hph*I, *Pvu*II, and *Hin*FI were used (data not shown). Moreover, cotransfection of a plasmid

FIG. 2. Structure of the viral insertion present in the SS1A cell line and scheme for the generation of late mRNAs having a reiterated leader sequence. SS1A DNA was digested to completion with *EcoRI* and electrophoresed in a preparative agarose gel. DNA migrating with a size of 16 to 23 kb was eluted and ligated to dephosphorylated *EcoRI* arms of the EMBL4 phage vector (11) and packaged by using Gigapack packaging extracts. Recombinant phage were screened by using the same hybridization probes as described in the legend to Fig. 1. Shown are the locations of the cleavage sites for the enzymes *EcoRI* (E), *Bam*HI (B), *Bgl*II (Bg), and *Ava*I (A). Numbers in parentheses indicate Py nucleotide numbers for the A2 strain according to the modification of the numbering system of Soeda et al. (24) described by Tyndall et al. (28). The area with the diagonal stripes represents flanking rat cellular DNA. Triangles below the restriction map indicate areas of the Py genome that are deleted. The enlarged area contained within the box above the map shows the portion of the Py genome containing the late promoter, late leader, enhancer, and origin of replication that was found to be amplified. The black area within the box represents the late leader sequence. The line directly below the map represents a primary late transcript that could in theory extend from the first copy of the late promoter to the sole remaining late polyadenylation and mRNA cleavage signal at nt 2915. The direction of transcription is from right to left. This primary transcript could then be spliced to yield the mRNAs for VP1, VP2, and VP3. While the diagram indicates a leader structure containing nine copies of the 57-nt sequence, initiation within an internal copy of the late promoter or nonsequential splicing could yield mature mRNAs having the fewer copies.

containing this 4.2-kb *EcoRI*-*AvaI* fragment with a plasmid containing a dominant selectable marker for resistance to the drug G418 (8, 25) and subsequent Northern blot analysis of the RNA from cell lines derived from G418-resistant colonies failed to reveal detectable levels of late mRNAs. In contrast, late transcripts were easily observed by using RNA from G418-resistant cell lines obtained after cotransfection with a plasmid containing the complete 17.6-kb insert (data not shown). These results indicate that the phenotype was due to the arrangement of the viral sequences and not to a peculiarity of the SS1A cell line and that sequences upstream of the *AvaI* site are required for late transcripts to be detected.

Included in these upstream sequences are two *Bam*HI fragments that both contain a new region that through restriction enzyme analysis and dideoxy sequencing was shown to consist of 576-base-pair (bp) head-to-tail repeats of nt 4816 to 95. This amplified region contains the late promoter and the Py enhancer and origin of replication, as well as the splice donor and acceptor sites that could possibly be used to generate a reiterated late leader sequence (Fig. 2). Since this region is not amplified in the left-most 4.2-kb *AvaI*-*EcoRI* fragment shown in Fig. 2, transfection with this fragment would not generate a reiterated leader structure. Dideoxy sequencing and restriction enzyme mapping indicate that these 576-bp repeats are included in a viral DNA region from nt 4377 to 1119 that is itself repeated three times. The resulting DNA structure is deleted of the sequences from nt 1120 to 4376, a portion of the Py genome that in addition to the coding sequences for the carboxy-terminal portion of LT, also contains on the opposite strand the late polyadenylation addition and mRNA cleavage signal. Therefore, examination of the structure of the SS1A insert indicates that as a result of these rearrangements, it would be possible to generate a primary late transcript that could contain up to nine copies of the late leader sequence. Subsequent utilization of the splice donor and acceptor sites bordering the leader could then produce late mRNAs containing a reiterated leader sequence attached to the bodies of the late mRNAs (Fig. 2).

To determine whether such transcripts are indeed present in SS1A cells, a cDNA library was constructed with mRNA from the SS1A cell line and λ gt10 as a phage vector (12). A total of 200,000 plaques were screened by using the 2.4-kb Py *HhaI* fragment that spans the late coding region and the noncoding regulatory region as a hybridization probe. Of 28 plaques that hybridized, 9 plaques that were subsequently found to hybridize to a leader-containing 279-bp *ApaI*-*BclI* fragment were chosen for further analysis.

The *EcoRI* phage inserts were subcloned into the Bluescript KS plasmid vector (Stratagene Cloning Systems, San Diego, Calif.). Restriction enzyme analysis with the enzymes *Hind*III, which cleaves within the bodies of all three late mRNAs, *Bam*HI, which cleaves within the bodies for VP2 and VP3, and *ScaI*, which cleaves only within the body of VP2, indicates six clones that had a structure consistent with a cDNA for VP1, two clones consistent with that for VP2, and one clone consistent with that for VP3. For all nine clones, the size of the *Bam*HI or *Hind*III fragment containing the 5' end of the cDNA was larger than that expected of a cDNA that started within the late promoter region and contained a single copy of the late leader. To confirm that this increased size is due to the presence of multiple copies of the late leader sequences, the 5' ends of five clones were sequenced.

All five cDNAs contained the same reiterated 57-bp leader

FIG. 3. Presence of a reiterated leader sequence in a cDNA copy of a VP1 mRNA from SS1A. Shown is a region of the dideoxy sequencing gel of the 5' portion of the *EcoRI* insertion of plasmid pSSVP6. Bracketed areas mark the locations of the sequences that indicate the splice of nt 5022 to 5078, which generates the repeated leader structure, and the leader-to-body splice of nt 5022 to 4124.

sequence that arises from the splicing of nt 5022 to 5078 found in the late mRNAs in lytically infected cells (Fig. 3 and Table 1). One cDNA was found to start at nt 5195 and to contain three complete copies of the leader followed by a splice of nt 5022 to 4124, which is the proper splice for a VP1 message (14). A second cDNA was found to start within the late leader sequence at nt 5069 and to contain three copies of the complete leader before proceeding directly into the VP2 coding sequences. A third clone started at nt 5076 and was found to contain four additional copies of the leader before it continued into the VP2 coding sequences, confirming the absence of a leader-to-body splice for VP2 mRNA. A fourth cDNA started at nt 5068 and was found to contain at least four leader copies. The fifth cDNA started at nt 5090 and contained at least five complete copies of the leader. We were unable to detect the leader-to-body splice in the latter two cDNAs due to the length of the leader sequence and the resulting distance of the leader-to-body splice from the primer-binding site used for dideoxy sequencing. Sequencing of the 3' end of one cDNA (pSSVP18C) indicated the proper utilization of the late polyadenylation signal, since a poly(A) tract was found added to nt 2893, 16 nt downstream of the end of the hexanucleotide signal AATAAA at nt 2915. This location is 7 nt further downstream than the previously assumed position of the 3' end of late mRNAs determined by S1 nuclease analysis (14). Together these results indicate that the structure of the late mRNA present in SS1A cells is the same as that observed in lytically infected mouse cells.

TABLE 1. Characterization of Py late cDNAs isolated from an SS1A library^a

Plasmid	Coding sequence contained	First nucleotide	No. of copies of complete leader sequence	Leader-to-body splice
pSSVP6	VP1	5195	3	5022-4124
pSSVP7A	VP2	5076	4	None
pSSVP15	VP2	5069	3	None
pSSVP17	VP1	5068	>4	ND
pSSVP18C	VP3	5090	>5	ND

^a A cDNA library was constructed by using the Amersham cDNA synthesis system, and poly(A)⁺ RNA was isolated from the SS1A cell line by the guanidinium-isothiocyanate procedure and oligo(dT) chromatography as previously described (15). After methylation with *Eco*RI methylase and the addition of *Eco*RI linkers, the digested linkers were removed and the cDNA was size fractionated by chromatography over an A50m column (Bio-Rad Laboratories, Richmond, Calif.). The cDNA was then ligated to *Eco*RI-digested and dephosphorylated λ gt10 arms (Promega Biotec, Madison, Wis.). The ligated cDNA was then packaged by using Gigapack extracts (Stratagene Cloning Systems, San Diego, Calif.), and plated by using C600 Hfl as a host strain. Plaques were screened as described in the text. *Eco*RI phage inserts were subcloned into an *Eco*RI-digested and dephosphorylated Bluescript KS plasmid vector, and the orientation and the coding sequence were determined by digestion with *Bam*HI, *Hind*III, or *Sca*I as described in the text. Dideoxy sequencing was performed by using the Gem Seq. K/RT system (Promega).

These results indicate that an integrated genomic structure that allows the formation of a reiterated leader sequence accounts for the detection of viral late transcripts in the SS1A cell line. As mentioned above, our previous work with chimeric plasmids containing foreign coding sequences linked to the late promoter suggested that the reason for the absence of late transcripts in Py-transformed cells is not due to the inefficient utilization of an integrated template for transcription (15, 16). The results presented here suggest that the absence may result from the inability to produce a primary transcript that can subsequently be processed to form a reiterated leader structure, implying that late transcripts lacking this structure would be inherently unstable. Transfection with the chimeric constructs results in transcripts that contain only a single copy of the leader sequence (15), and this implies that the instability is conferred by the late coding sequences. Several factors would militate against the formation of such a primary transcript even in the common situation of tandem head-to-tail insertions of the Py genome. In addition to the fact that the number of tandemly integrated viral genomes is generally limited to two or three, the presence of late polyadenylation and mRNA cleavage signals may preclude the possibility of transcribing a tandem copy of the insertion. Although this signal functions inefficiently during a lytic infection (2, 27), our previous results indicate that the signal is efficiently utilized when integrated (15). Another factor would be the likely selection against cells transcribing multimeric transcripts, since such transcripts would contain anti-early RNA, which in turn would be expected to reduce the level of expression of the middle T transforming gene (13).

The results presented here also suggest that the formation of a reiterated leader structure may be involved in the temporal regulation of viral gene expression during lytic infection. Using transient assays with constructs containing the *cat* gene linked to the late promoter, we have demonstrated that the late promoter is functional even in the absence of early gene expression (17). This raises the possibility that early in the course of infection, late sequences are actually being transcribed but are properly terminated without traversing the circular genome and thus yield transcripts that lack a reiterated leader sequence and are then rapidly

degraded. Results from another laboratory (19) suggest that late in infection, cellular factors required for termination and polyadenylation become limiting and therefore multimeric transcripts are produced that are then spliced to yield stable late mRNAs containing the reiterated leader. This putative change in mRNA stability via the addition of a leader sequence could therefore act in concert with template amplification via DNA replication and transactivation of the late promoter by the viral early proteins (17) as another means of increasing the amount of late mRNA present in the infected cell.

We have attempted, by performing in vitro nuclear run-on assays of the rate of transcription of late DNA sequences in the SS1A and H6A cell lines, to provide conclusive evidence for the hypothesis that the presence of late transcripts in Py-transformed cells is regulated posttranscriptionally. However, in our hands, the sensitivity of this technique was insufficient to detect not only late but also early transcription from integrated viral templates in a variety of Py-transformed cell lines. It therefore remains to be determined if two other mechanisms suggested by the analysis of the structure of SS1A insertion are instead responsible for the phenotype observed. The same amplification event that gave rise to multiple copies of the reiterated leader sequence also resulted in an increase in the number of enhancer elements present in the insertion. It is therefore formally possible that the ability to detect late transcripts in the SS1A cell line is due to an increased transcription rate owing to an increased density of enhancer elements. The study of the effect of an increased number of copies of the simian virus 40 enhancer on the expression of stably integrated sequences revealed that while 4 copies of the enhancer result in a modest stimulation of gene expression, 10 copies of the same sequence result in a diminution of expression (18). The presence of minor enhancer elements within the reiterated late leader sequence of Py (29) makes the dissection of two functions provided by the same stretch of DNA a difficult question to approach experimentally. If increased enhancer density is responsible for the ability to detect late transcripts in the SS1A cell line, then it remains to be explained why there was no increase in the level of the truncated 1.3- and 1.0-kb early transcripts in the SS1A cell line with respect to the same transcripts produced in the parental H6A cell line. Enhancers by definition act bidirectionally, and the structure of integrated early-region viral DNA giving rise to these truncated transcripts, located at the far right end of viral insertion, appears to be identical in both cell lines (7; Fig. 2), with the exception of the 576-bp repeated structure, which is present only in the SS1A cell line. If enhancer density were responsible for the detection of late transcripts in the SS1A line, one would also expect to find an elevated level of truncated early-region transcripts, and this was not observed (Fig. 1).

A last explanation that remains to be formally excluded arises from the examination of the SS1A sequence in the region containing the Py enhancer. With respect to the Py A2 strain (24), a single base change of a T to a C on the L strand at nt 5122 was detected when the region spanning the *Bcl*II-to-*Apa*I site was sequenced. This change was confirmed by the observation of an A to G transition on the E strand when the same region derived from a different portion of the SS1A insertion was sequenced in the opposite direction. This mutation is present also in two naturally occurring Py strains, TOR and P16 (23), and therefore we tend to doubt its functional significance. Additional evidence against this hypothesis comes from the fact that the *Eco*RI-*Ava*I fragment

of the SS1A insertion does not transmit the late RNA phenotype, whereas the whole insertion does. We therefore think it likely that the same base change was present in the H6A parental cell line.

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