The sequence and context of the 5' splice site govern the nuclear stability of polyoma virus late RNAs

Nancy L. Barrett⁺, Xiaotong Li and Gordon G. Carmichael*

Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030, USA

Received September 11, 1995; Revised and Accepted October 26, 1995

ABSTRACT

We have examined the influence of splicing signals on the stability of polyoma virus late RNAs in the nucleus. Late primary transcripts contain a single 5' splice site and three alternative 3' splice sites. In earlier work we showed that the presence of introns was not required for late RNA accumulation, however, the 5' splice site was essential, as removal of only the 5' splice site was sufficient to destabilize late RNAs up to 100-fold when compared with early RNAs. A complementary clone which retained the 5' splice site but which carried small deletions of all late region 3' splice sites produced wild-type levels of unspliced late RNA. In order to extend this work we have constructed additional types of mutants. Point mutations in the 5' splice site confirmed its importance for RNA stability. Other mutants included constructs in which the spacing between the 5' splice site and the late promoter was altered and 5' splice site insertion mutants where a 58 bp fragment containing the 5' splice site sequence was inserted separately at various restriction sites in the late region. Both types of mutants lacked all of the late 3' splice sites and had only a single 5' splice site. RNase protection analyses of late and early RNAs from these constructs revealed that moving the 5' splice site away from the late promoter (or from its normal context) destabilized late RNAs >10-fold relative to the wild-type. We conclude that both 5' splice site integrity and its proximity to the late promoter play important roles in the nuclear stability of polyoma virus late RNAs.

INTRODUCTION

In eukaryotic cells most pre-mRNAs contain introns which are removed by splicing to produce mature mRNA. The need for excisable introns for efficient mRNA accumulation has been demonstrated in a number of systems, such as SV40 late genes (1-4), mouse dihydrofolate reductase (5,6), rabbit β -globin (5), maize alcohol dehydrogenase-1 (7), rat growth hormone (8) and immunoglobulin heavy chain μ (9). However, splicing is not always essential for mRNA accumulation. There are numerous examples

of genes that contain no introns, but which can produce stable mRNA. These include cloned chicken thymidine kinase cDNA (10), histone genes (11), α -interferon (12) and *c-jun* (13).

The polyoma virus late region offers a convenient system for the study of the role splice sites play in the production of stable RNA. The late transcription unit consists of the late leader, a 57 nt non-coding exon flanked by near consensus 5' and 3' splice sites and two message body 3' splice sites (Fig. 1). Alternative choice of the message body 3' splice sites leads to formation of mRNAs for VP3 and VP1, which encode two of the viral capsid proteins. The third capsid protein, VP2 is encoded by an mRNA with no message body splice. Curiously, the most promoter proximal splicing signal in this system is a 3' splice site, rather than a 5' splice site. This is because at late times in infection a significant fraction of late-strand primary transcripts are multigenomic in length, due to inefficient transcription termination on the circular viral genome and must be processed by leader-leader splicing (14-19).

Extensive studies of the polyoma late leader have helped to define its role in late viral gene expression. Its position in late primary transcripts is unusual in that its presence generates molecules with 3' splice sites at or near their 5' ends. We have previously shown that the length, but not the sequence, of the late leader is important for virus viability (20,21). Mutants with leaders of substituted sequence are phenotypically wild-type in tissue culture, but those with leaders shorter than 33 nt are non-viable and produce reduced levels of late messages (22). This accumulation defect was associated with inefficient leader-body splicing in these mutants. We have hypothesized that the defect in short leader mutants is due to juxtaposition of the leader splice sites, one or both of which might be compromised as a result of steric hindrance (20).

Mutants with deletions or mutations in the late leader 3' splice site also exhibited a deficiency in leader-body splicing (22), as well as a defect in accumulation of late-strand mRNA in both the cytoplasm and nucleus. These results indicated that accumulation of polyoma late RNA in both the nucleus and the cytoplasm is dramatically affected by mutations that interfere with leader-leader splicing and led us to suggest that leader-leader splicing is required for efficient leader-body splicing (22).

More recently we constructed a number of mutants blocked in splicing and examined accumulation of both nuclear and cytoplasmic late-strand RNAs in transfected mouse cells (23).

* To whom correspondence should be addressed

+Present address: Epidemiology Program, Department of Public Health, Hartford, CT 06106, USA

Figure 1. The early and late regions of polyoma virus. (A) The late region of the circular polyoma genome is displayed (not to scale) as a line with all known splicing signals indicated with arrows. The coding regions for the viral structural proteins VP1, VP2 and VP3 are marked. LL, the 57 nt non-coding late leader exon. Below the schematic are lines that indicate the sizes of protected bands seen in RNase protection experiments. (B) The early region of the genome is displayed (not to scale), along with the intron/exon structure for early messages. Splicing events involved in the production of messages for large T antigen, small T antigen and middle T antigen are indicated. Below the schematic are lines that indicate the sizes of protected bands seen in RNase protection experiments.

Mutants which lacked the leader 5' splice site had a profound defect, exhibiting virtually no late-strand cytoplasmic or nuclear RNA. A mutant with no intron, but having functional 3' and 5' splice sites bordering the leader exon, was capable of producing large amounts of unspliced late mRNA. This demonstrated that an excisable intron is not a requirement for late mRNA accumulation. Rather, accumulation of polyoma late mRNAs appeared to require the presence of leader exons bordered by functional 3' and 5' splice sites, whether or not these sites are used during pre-mRNA processing.

In this report we have further investigated the role of late splice sites in accumulation of late viral messages. Our approach has been to mutate specific late splice sites and then determine the effects on late viral RNA accumulation. After transfecting splice site mutants into NIH 3T3 cells the accumulation of both nuclear and cytoplasmic late RNAs was examined. Results confirm the importance of the 5' splice site for late RNA accumulation and further show that there is a positional requirement for its effect on nuclear RNA stability.

MATERIALS AND METHODS

Materials

T4 DNA ligase, T4 DNA polymerase and T4 polynucleotide kinase were obtained from New England Biolabs and were used as suggested by the supplier. *Escherichia coli* strains JM83, UT481, GM1634 (*dam*⁻) and CJ236 (*dut*⁻ *ung*⁻) were used to propagate plasmids by standard procedures (24,25). RNase T2

was from Bethesda Research Laboratories. [α -³²P]dATP and [α -³²P]UTP were from New England Nuclear. Oligonucleotides were synthesized using a Milligen/Bioscience Cyclone DNA synthesizer and phosphoramidite chemistry. Mutagenesis was performed using the BioRad Laboratories Muta-gene *in vitro* mutagenesis kit. Polymerase chain reaction analysis was performed using the GeneAmp kit from Perkin-Elmer Cetus. DNA sequencing was performed by the dideoxy chain termination method using the Sequenase kit from United States Biochemical Corp.

Cell culture and transfections

Mouse NIH 3T3 cells were obtained from the ATCC. The techniques for their propagation and transfection have been described elsewhere (26,27). Briefly, all transfections were performed using a modification (27) of the calcium phosphate transfection procedure of Chen and Okayama (28). Approximately 48 h prior to transfection cells were seeded at a concentration of 1×10^6 cells/150 mm plate in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum, L-glutamine, penicillin and streptomycin at 37°C in 5% CO₂. Sixteen hours prior to transfection the cells were serum stimulated by the addition of fresh medium. The total amount of DNA used per transfection was 40 μ g. Before transfection recombinant plasmids were digested with *Eco*RI and dilute ligated with T4 DNA ligase as described previously (21).

Plasmid constructs

The salient features of some of the constructs used in this study are illustrated in Figures 2 and 4. For each construct a polyoma virus genome was inserted into the unique *Eco*RI site of pBlueScribe (22). Before transfections genomes were released from plasmid backbones using *Eco*RI and re-circularized by dilute ligation.

Constructs shown in Figure 2. The wild-type and construct 5'ss Δ have been described previously (23). 5'ss Δ contains a 6 nt deletion of the late leader 5' splice site. Site-directed oligonucleotide mutagenesis (23) was used to alter bases 1 (mutant 5-1) and 5 (mutant 5-5) of the late leader 5' splice site to the indicated bases. Similarly, site-directed mutagenesis was used to remove 11 nt upstream of and including the leader 3' splice site, 6 nt upstream of and including the VP3 3' splice site and 9 nt upstream of and including the VP1 3' splice site, creating mutant 3 $\times$ 3'ss Δ . Mutant 26-6 lacks all polyoma late splicing sites and includes the mutations of mutants 5'ss Δ and 3 $\times$ 3'ss Δ . Mutants 5'ss-Bam and 5'ss-RV were made by inserting a 58 bp *Sau*3A-*Taq*I fragment spanning the late leader 5' splice site into the unique *Bam*HI site of mutant 26-6 (construct 5'ss-Bam) or into the unique *Eco*RV site of 26-6 (construct 5'ss-RV). This 58 bp fragment consists of 6 bp upstream of 5'ss and 52 bp downstream of the leader 5' splice site. Ins 350, ins 500 and ins 1700 were made by inserting phage M13mp18 *Sau*3AI fragments of 350, 500 and 1700 bp, respectively, into the unique *Bcl*II site of mutant 26-6.

Constructs shown in Figure 4. The wild-type, ALM, SLM, ALM-4, ALM-11 and ALM-15 constructs have been described before (21,22). The wild-type late leader exon is 57 nt in length. ALM contains an abbreviated late leader exon of only 9 nt. Constructs ALM-4, ALM-11 and ALM-15 lack 4, 11 and 15 nt,

Figure 2. Mutants with altered splicing signals. The top line depicts the wild-type polyoma virus late region, as in Figure 1. Mutants below were constructed as described in Materials and Methods. The inverted triangles beneath the lines denote splice site deletions of the indicated sizes. Point mutations in the 5' splice sites of constructs 5-1 and 5-5 are shown with small arrows and M13 spacer inserts in ins 350, ins 500 and ins 1700 are indicated below the late leader exon boxes. Inverted triangles above the lines in mutants 5'ss-Bam and 5'ss-RV denote 58 bp inserts of the late leader (LL) 5' splice site at downstream locations.

respectively, immediately upstream of the late leader 3' splice site. SLM contains a 51 nt leader exon of substituted sequences unrelated to polyoma virus, deriving from phage M13mp8 (21). Constructs ALM-4R, ALM-11R and ALM-15R were made by inserting the phage M13mp8 sequence from SLM into the unique *Bcl*I site within the leaders of ALM-4, ALM-11 and ALM-15, respectively. These mutants lack the leader 3' splice site, but restore the spacing of the leader 5' splice site to near its wild-type position downstream of the viral late promoter. Constructs ALM-4R', ALM-11R' and ALM-15R' are the same as ALM-4R, ALM-11R and ALM-15R, respectively, except that the M13mp8 spacer insert is in the opposite orientation to that shown in Figure 4.

Preparation of probes and RNA analysis

Total cellular RNAs were isolated and analyzed by quantitative RNase T2 protection experiments as previously described (22).

The riboprobes used are shown in Figure 1 and are as follows. Analysis of the VP1 3' splice site was done using a 306 bp *Bam*HI–*Hind*III late fragment cloned into pBS⁺ (20). RNAs containing the VP1 splice protected a band of 203 nt, while unspliced RNAs and VP3 messages protected a fragment of 306 nt. As an internal control early splicing was analyzed using a 357 bp *Ava*I early fragment cloned into pBS⁺ (20). Unspliced pre-mRNAs protect a band of 357 nt, while spliced early messages protect bands of 224 (small T and large T antigen mRNAs) and 212 nt (middle T antigen mRNA).

RESULTS

The polyoma virus late 5' splice site is required for late RNA accumulation, but a spliceable intron is not

We previously reported that deletion of the 5' splice site seriously destabilized polyoma virus late primary transcripts, while deletion of all of the 3' splice sites had no deleterious effects on RNA stability (23). To confirm and extend that work a number of mutants were examined (5'ssΔ, 5-1, 5-5, 3x3'ssΔ and 26-6, Fig. 2). Mutant 5'ssΔ lacks the first six intronic nucleotides (GUAAGU) of the 5' splice site. In mutant 5-1 the first base was changed from a G to a U and in mutant 5-5 position 5 was changed from a G to a C. Mutant 3x3'ssΔ lacks all three late region 3' splice sites but contains the 5' splice site. Mutant 26-6 lacks all late splicing signals. These mutant genomes were propagated as plasmids. Intact viral genomes were liberated from vector backbones with *Eco*RI and re-circularized with T4 DNA ligase. Ligated DNAs were then transfected into mouse NIH 3T3 cells, total cell RNA isolated 48 h later and RNase protection assays with uniformly labeled riboprobes used to quantify RNA levels. Relative steady-state levels of late RNAs were determined by calculating the ratio of total early signal (internal control; Fig. 3B) to total late signal (Fig. 3A).

Polyoma late transcripts can be made intronless and unspliceable in several different ways: by destroying all splice sites, by destroying all three 3' splice sites or by destroying the single 5' splice site. Mutant 26-6, which lacks all late splice sites, shows a severe defect in late RNA accumulation (Fig. 3 and Table 1). This would be consistent with an intron requirement for late RNA accumulation. In contrast, on comparing the ratios of early RNA with late RNA it is apparent that construct 3x3'ssΔ, which lacks all late 3' splice sites, produces even more late RNA than the wild-type (Fig. 3 and Table 1). This result was repeated in numerous experiments and use of other riboprobes failed to detect any cryptic late splicing for this mutant (data not shown). Further, the unspliced RNA of mutant 3x3'ssΔ was predominantly retained in the nuclear compartment of the cell (Y.Huang and G.Carmichael, unpublished results). Thus unspliceable (and intronless) polyoma late RNA can be stable within the nucleus. On the other hand, mutant 5'ssΔ, which lacks only the late 5' splice site, shows the same stability defect as mutant 26-6. This result demonstrates the importance of the 5' splice site in late RNA accumulation. Constructs 5-1 and 5-5 contain base changes that are known to interfere with splicing and the interaction of U1 small nuclear RNA with the 5' splice site (29). Interestingly, these mutants also showed severe defects in late RNA accumulation (Fig. 3 and Table 1). Taken together, these results demonstrate that the late 5' splice site itself is the most important determinant of late RNA stability and that a 5' splice site

Figure 3. RNase protection assays of total cell RNAs. NIH 3T3 cells were transfected, RNA isolated 48 h later as described in Materials and Methods and subjected to quantitative RNase protection analysis using either late-specific (A) or early-specific (B) probes (see Fig. 1). Early RNAs served as internal controls to allow accurate quantitation of late RNA levels, as discussed in the text.

and not an excisable intron is required for RNA accumulation in the polyoma system.

The distance of the 5' splice site from the late transcription start sites is important for late RNA accumulation

The 5'-end of polyoma virus late pre-mRNA molecules contains a 57 nt long non-coding exon, the late leader (Fig. 1). We

previously reported that there is a minimum late leader exon size of between 27 and 33 nt for both pre-mRNA splicing and stability in polyoma virus late RNA processing (21,22). These and other results were consistent with a model where the late leader 3' splice site is critical for late RNA splicing and accumulation, with short leader mutants indicating steric interference with proper splice site recognition across short exons (21). To further examine the function and spacing requirements of the late leader we constructed several new mutants with truncated leaders (Fig. 4).

Table 1.

Mutant	Relative RNA levels
Wild-type	100
5-1	1.1
5-5	0.6
5'ssΔ	1.8, 2.4
3x3'ssΔ	162
ins 350	1.8
ins 500	2.4
ins 1700	8.3
26-6	2.2
5'ss-Bam	3.4
5'ss-RV	1.9

Relative RNA levels were determined from the data presented in Figure 3. Band intensities were determined using a Packard Instant Imager. Late probe RNA levels were calculated by summing the signals of the 306 and 203 nt bands after correcting for relative uridine content. Values were then normalized to levels of early RNAs determined in the same manner. The values shown for mutant 5'ssΔ were from two independent experiments.

If most of the internal sequence of the leader exon is replaced with unrelated sequence RNA levels are unaffected (Fig. 5A, lane 3; 20,21). Mutants ALM-4, ALM-11 and ALM-15 carry a 48 bp internal deletion of the 57 bp leader exon. In addition they lack 4, 11 and 15 bp, respectively, from the 3' splice site region upstream

Figure 4. Mutants with truncated and substituted leaders. Mutants were made as described in Materials and Methods. The top line shows the sequence of the polyoma virus late region immediately surrounding the late leader exon (boxed). The splice sites flanking the leader are shown with arrows and the conserved AG at the 3' splice site and the conserved GT at the 5' splice site are underlined. Late transcription start sites are heterogeneous and lie upstream of the late leader. Hyphens denote wild-type sequences missing in the mutants. Lower case letters represent sequences deriving from phage M13mp8.

Figure 5. Quantitative RNase protection assays of total cell RNA isolated from cells transfected with the wild-type and mutants with truncated and substituted late leaders. The experiment was carried out as in Figure 3. (A) Analysis with late-specific riboprobe. (B) Analysis with early-specific riboprobe.

of the leader exon. Consistent with previous observations (22), these mutants did not produce measurable amounts of late RNA (Fig. 5A, lanes 4–6). The same results were seen with nuclear RNA (data not shown), indicating that the defect is one of nuclear RNA stability. Nuclear run-on assays confirmed that the defects were not due to any effects on late transcription rates (N.L. Barrett and G.G. Carmichael, unpublished results). Taken together these results are consistent with a model where recognition of the leader 3' splice site is the most important determinant of nuclear RNA stability. Alternatively, however, one could postulate that the defects result simply from moving the 5' splice site closer to the major start sites for late transcription, which lie between 10 and 100 nt upstream of the leader 3' splice site (30–34).

To discriminate between these two alternative models we restored the late leader exons of mutants ALM-4, ALM-11 and ALM-15 to near wild-type lengths (ALM-4R, ALM-4R', ALM-11R and ALM-15R; Fig. 4). As shown in Figure 5A (lanes 7–10), each of these constructs exhibits essentially wild-type levels of late RNA. These results argue strongly that the spacing of the 5' splice site from the late promoter is more important than the nucleotide sequence between the start sites and the 5' splice site.

The above results point to the critical role played by the 5' splice site in late RNA expression. Is this 5' splice site itself sufficient for stable RNA accumulation, is its sequence context important or must it lie within a critical distance of the 5'-ends of late primary transcripts? To address these questions we constructed mutants 5'ss-Bam, 5'ss-RV, ins 350, ins 500 and ins 1700 (Fig. 2). Mutants 5'ss-Bam and 5'ss-RV were made by inserting a 58 bp cassette containing 6 bp upstream and 52 bp downstream of the late leader 5' splice site into construct 26-6 at either the unique *Bam*HI or *Eco*RV site. Each of these new constructs contained the wild-type

Figure 6. Quantitative RNase protection assays of total cell RNA isolated from cells transfected with wild-type, 5'ss Δ and mutants with spacer inserts in the late leader exon. The experiment was carried out as in Figure 3. (A) Analysis with late-specific riboprobe. (B) Analysis with early-specific riboprobe.

polyoma late 5' splice signal, but positioned ~390 and 910 nt, respectively, downstream of its normal location. All 3' splice site were deleted. Mutant 5'ss-Bam exhibited only 3.4% wild-type levels of late RNA, while 5'ss-RV showed <2% wild-type levels (Fig. 3, lanes 4 and 5, and Table 1). These results indicated that the late 5' splice site itself cannot confer RNA stability when either taken out of its normal context or placed too far from the pre-mRNA 5'-end.

While constructs 5'ss-Bam and 5'ss-RV still contained the wild-type viral late 5' splice site, it is possible that late exon sequences flanking the repositioned splicing signal might adversely affect its function. Therefore, we constructed several mutants in which the 5' splice site position was changed, but only by inserting sequences upstream of the 5' splice site. Work shown here and published elsewhere previously (20–22) argues that sequences upstream of the 5' splice site are not important for late RNA accumulation. Mutants ins 350, ins 500 and ins 1700 contained ~350, 500 and 1700 nt, respectively, of phage M13 sequences as spacer elements to reposition the 5' splice site further from late 5'-ends. Results presented in Figure 6 and Table 1 show that these mutants also exhibit a striking defect in late RNA accumulation. The conclusion from these studies is that not only is the integrity of the late 5' splice site critical for RNA accumulation, but that it must be neither too close to late transcription start sites nor too far from them.

DISCUSSION

Analysis of the sequence requirements for polyoma virus late RNA accumulation have revealed an unexpected dependency on the integrity of a 5' splice site which is normally situated between ~70 and 170 nt downstream of the major late transcription start sites and which is the only 5' splice site in late primary transcripts. Deletion of this site typically leads to levels of late transcripts that are >50-fold lower than wild-type. Even point mutations in this signal that would be predicted to disrupt base pairing interactions with U1 snRNA have the same severe stability defect. On the other hand, mutations that eliminate 3' splice sites also lead to unspliced transcripts, but ones that show no defects in stability. These results are reminiscent of the situation for the Tat/Rev 5' splice site of HIV, which has been shown to be necessary for the stability of unspliced HIV pre-mRNAs in the nucleus (35).

Interestingly, however, the effects of the polyoma late 5' splice site on RNA stability appear to be manifested only when this site is positioned within a rather narrow range of distance from the late promoter. If it is too close to or too far away from the late start sites then RNA stability is drastically reduced. These defects are manifested in nuclear RNA, since in every case where a defect was noted for total cell RNA it was also observed for nuclear RNA.

Our results might help to explain why some genes lack introns and yet can still express stable RNAs. Introns might serve to protect pre-mRNAs from degradation within the nucleus (4,5,36). It is possible that some intronless genes are stabilized in a similar fashion by encoding unused or unpaired 5' splice sites. Such 5' splice sites might lead to nuclear transcript stability by the same mechanism that we have revealed for polyoma virus late RNAs. We predict that molecular analysis of signals that contribute to RNA stability in intronless messages will reveal some elements that resemble 5' splice sites. Likewise, cDNA constructs that can be expressed as stable messages without added introns might themselves encode cryptic 5' splice sites near their 5'-ends.

What is the nature of the stabilization by the polyoma late 5' splice site signal? 5' Splice sites can be recognized by multiple components of the splicing machinery, including U1 snRNA (29,37) and SR proteins (38,39). We do not know whether any of these or other protein-RNA interactions are important for the phenotype we have described here, but all attempts to suppress the stability defects of mutants 5-1 and 5-5 with altered mouse U1 genes that could restore complementary base pairing interactions have been unsuccessful (N.Barrett and G.Carmichael, unpublished results). This suggests that the stability effects we have observed are not the direct result of interactions between the polyoma late 5' splice site and U1 snRNA.

Why is there a spacing requirement for nuclear RNA stabilization by a 5' splice site? Although the reason for the apparent requirement in the polyoma system is still obscure, we speculate that stabilization of intronless RNAs is in some way related to the stabilization by intron-containing RNAs and as such requires proper recognition by the splicing machinery. It is conceivable that exon recognition is a universal requirement for mRNA expression in eukaryotes. The exon definition model (40) suggests that the exon is often the initial event in committing a pre-mRNA molecule to the splicing pathway. In this model pairs of exons must be recognized across exons in order for either site to be used. As proposed, this model also invoked a rather strict exon length requirement; if exons are too long then they are skipped in RNA splicing. In fact, most eukaryotic exons are shorter than 300 nt (41). Internal exons would be defined by a 3' splice site paired with a downstream 5' splice site. Terminal exons would be defined by a 3' splice site paired with a polyadenylation signal or the message cap site paired with a 5' splice site. We speculate that for many intronless RNAs some sort of exon recognition must occur for proper RNA accumulation. In the case of polyoma late transcripts the late leader 5' splice site is positioned appropriately downstream of the cap sites to ensure efficient 5' terminal exon recognition. If this site was moved too close to the cap sites or too far away then exon recognition would be less efficient and RNA stability would decrease.

Finally, Liu and Mertz (42) have recently identified a *cis*-acting element within the intronless herpes simplex virus thymidine kinase gene that allows intron-independent gene expression. Interestingly, this element was shown to interact with the nuclear

protein hnRNP L and did not appear to contain a 5' splicing signal of the type needed in the polyoma system.

ACKNOWLEDGEMENTS

We thank the other members of the laboratory for helpful comments throughout this work and D. Batt, M. Kumar, Y. Huang, P. Leahy, L. Rapp, M. Szlachetka and K. Wimler for helpful comments on the manuscript. This work was supported by grant CA45382 from the National Cancer Institute.

REFERENCES

- Gruss,P., Lai,C.-J., Dhar,R. and Khoury,G. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4317-4321.
- Gruss,P. and Khoury,G. (1980) *Nature*, **286**, 634-637.
- Hamer,D. and Leder,P. (1979) *Cell*, **18**, 1299-1302.
- Ryu,W.S. and Mertz,J.E. (1989) *J. Virol.*, **63**, 4386-94.
- Buchman,A.R. and Berg,P. (1988) *Mol. Cell. Biol.*, **8**, 4395-405.
- Gasser,C.S., Simonsen,C.C., Schilling,J.W. and Schimke,R.T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6522-6526.
- Callis,J., Fromm,M. and Walbot,V. (1987) *Genes Dev.*, **1**, 1183-1200.
- Brinster,R.L., Allen,J.M., Behringer,R.R., Gelinis,R.E. and Palmiter,R.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 836-840.
- Neuberger,M.S. and Williams,G.T. (1988) *Nucleic Acids Res.*, **16**, 6713-6724.
- Gross,M.K., Kainz,M.S. and Merrill,G.F. (1987) *Mol. Cell. Biol.*, **7**, 4576-4581.
- Kedes,L.H. (1978) *Annu. Rev. Biochem.*, **48**, 837-870.
- Nagata,S., Mantei,N. and Weissmann,C. (1980) *Nature*, **287**, 401-408.
- Hattori,K., Angel,P., Beau,M.M.L. and Karin,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9148-9152.
- Acheson,N.H. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4754-4758.
- Acheson,N. (1981) *J. Virol.*, **37**, 628-635.
- Hyde-DeRuyscher,R. and Carmichael,G.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8993-8997.
- Hyde-DeRuyscher,R.P. and Carmichael,G.G. (1990) *J. Virol.*, **64**, 5823-5832.
- Treisman,R. (1980) *Nucleic Acids Res.*, **8**, 4867-4888.
- Treisman,R. and Kamen,R. (1981) *J. Mol. Biol.*, **148**, 273-301.
- Adami,G.R. and Carmichael,G.G. (1986) *J. Virol.*, **58**, 417-425.
- Adami,G.R. and Carmichael,G.G. (1987) *Nucleic Acids Res.*, **15**, 2593-2610.
- Adami,G.R., Marlor,C.W., Barrett,N.L. and Carmichael,G.G. (1989) *J. Virol.*, **63**, 85-93.
- Barrett,N.L., Carmichael,G.G. and Luo,Y. (1991) *Nucleic Acids Res.*, **19**, 3011-3017.
- Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G. and Struhl,K. (1989) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York, NY.
- Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1-545.
- Cahill,K.B. and Carmichael,G.G. (1989) *J. Virol.*, **63**, 3634-3642.
- Cahill,K.B., Roome,A.J. and Carmichael,G.G. (1990) *J. Virol.*, **64**, 992-1001.
- Chen,C. and Okayama,H. (1987) *Mol. Cell. Biol.*, **7**, 2745-2752.
- Zhuang,Y. and Weiner,A.M. (1986) *Cell*, **46**, 827-835.
- Cowie,A., Tyndall,C. and Kamen,R. (1981) *Nucleic Acids Res.*, **9**, 6305-6322.
- Fenton,R.G. and Basilico,C. (1981) *J. Virol.*, **40**, 150-163.
- Flavell,A.J., Cowie,A., Legon,S. and Kamen,R. (1979) *Cell*, **16**, 357-372.
- Flavell,A., Cowie,A., Arrand,J.R. and Kamen,R. (1980) *J. Virol.*, **33**, 902-908.
- Treisman,R. and Kamen,R. (1981) *J. Mol. Biol.*, **148**, 273-301.
- Lu,X., Heimer,J., Rekosh,D. and Hammarskjöld,M.-L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7598-7602.
- Hamer,D., Smith,K., Boyer,S. and Leder,P. (1979) *Cell*, **17**, 725-735.
- Zhuang,Y., Leung,H. and Weiner,A.M. (1987) *Mol. Cell. Biol.*, **7**, 3018-3020.
- Tarn,W.-Y. and Steitz,J.A. (1994) *Genes Dev.*, **8**, 2704-2717.
- Staknis,D. and Reed,R. (1994) *Mol. Cell. Biol.*, **14**, 7670-7682.
- Robberson,B.L., Cote,G.J. and Berget,S.M. (1990) *Mol. Cell. Biol.*, **10**, 84-94.
- Hawkins,J.D. (1988) *Nucleic Acids Res.*, **16**, 9893-9908.
- Liu,X. and Mertz,J. (1995) *Genes Dev.*, **9**, 1766-1780.