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The Structure and The Reason for Nuclear Accumulation of PolyA(-) Spliced SV40 RNA

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Poly A tail이 없는 SV 40 spliced RNA의 구조 및 핵내 축적의 원인

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ABSTRACT: The locations of 5' ends as well as the splicing pattern of viral polyA(-) 19S RNA from monkey cells infected with SV40 were determined by a modification of primer extension method. The 5' end of this RNA mapped at the major cap site at nucleotide residue 325, used most frequently by SV40 late RNAs. The intron from nt.373 to nt.558 was removed as the ordinary cytoplasmic polyA(+) 19S RNA. The 3' end of this RNA was very heterogeneous and distributed over 1 kb upstream of polyadenylation site, as determined by S1 nuclease mapping. The reason for this normally initiated and spliced RNA to accumulate in the nucleus was investigated. In order to test whether the presence of unused 3' splice region on this RNA caused such subcellular distribution, cells were transfected with SV40 mutant DNA containing deletion around 3' splice site. The RNA deleted of 3' splice region accumulated mainly in the cytoplasm. This accumulation did not result from the increased stability of the RNA due to the deletion, since the wild type and mutant RNAs exhibited similar half lives after chase with actinomycin D. Therefore it is likely that the 19S spliced RNA is hindered from being transported into the cytoplasm due to some pre-splicing complexes formed at the unused 3' splice site.

KEY WORDS□SV40 late RNAs; polyA(-) spliced RNAs; spliceosome complex; mRNA transport; primer extension; S1 nuclease mapping.

During the late phase of lytic infection, simian virus 40 (SV40) produces 2 classes of mRNAs, 16S and 19S in size, which encode different proteins. There are at least 3 kinds of RNAs among 16S RNAs and 4 kinds among 19S RNAs, which are all made by differential splicing of the primary transcripts (Ghosh *et al.*, 1978; Reddy *et al.*, 1978). There are 3 splice donor sites (5' splice sites; at nucleotide residue (nt.) 294, 373, and 526) and 3 splice acceptor sites (3' splice sites; at nt. 435, 558, and 1463) available on the late primary transcript. (See Tooze (1981) for nucleotide numbering.) Factors affecting the selection of splice sites are beginning to be understood, and include the locations of 5' ends of the primary transcript as well as the spatial arrangements and the length of introns (Somasekhar and Mertz, 1985a; Good and Mertz, 1988; Good *et al.*, 1988a, c; Aebi *et al.*, 1986). The amount of various species of spliced RNAs in the cytoplasm is regulated by the efficiency of splicing, the rate of nuclear transport, and the stability of the RNA. The relative abundances of the various species of spliced late RNAs present in the cytoplasm of SV40-infected monkey cells were determined (Ghosh *et al.*, 1978; Reddy *et al.*, 1978; Ghosh *et al.*, 1982; Barkan and

Mertz, 1984; Somasekhar and Mertz, 1985a), and it was reported that the ratio of 16S-to-19S cytoplasmic polyadenylated RNA was approximately four (Somasekhar and Mertz, 1985b).

Roe and Mertz(1988) have shown that the steady state level of the SV40 late spliced 19S RNAs, most of which are spliced from nt. 373, is similar in amount to that of the late 16S RNAs, most of which are spliced from nt. 526, in the infected whole cell. However, whereas most of the 526-spliced 16S RNA(16S 526-RNA) is polyadenylated and located in the cytoplasm, much of the 373-spliced 19S RNA(19S 373-RNA) lacks poly A and remains in the nucleus. This is why the majority of the 19S 373-RNA was overlooked in previous analyses. In order to find out the reason for this differential subcellular distribution of alternatively spliced RNAs, we needed to verify the structure of this abundant 373-RNA. Whether the 5' end and/or the splicing pattern of this RNA are different from the cytoplasmic 19S mRNA was investigated. In this work, we found that the structure of the abundant nuclear 373-RNA was the same as the cytoplasmic mRNA except the 3'half.

There are accumulating observations that presplicing complex is formed at the 3' splice region prior to the engagement of 5' splice sites in the formation of functional spliceosome complex (Frendewey and Keller, 1985; Krämer, 1987; Beyer and Osheim, 1988). We present evidence that the presence of 3' splice region without its 5' pair in the processed 373-RNA prevented it from being transported into the cytoplasm and thereby resulted in its accumulation in the nucleus.

MATERIALS AND METHODS

Virus infection and transfection

The CVIP cells (a transformed cell line of African green monkey kidney cells) were infected with wild type SV40(wt 830a) at the mutiplicity of infection of 20-30, as described by Mertz and Berg (1974). For transfection, cells were treated with DEAE-Dextran according to the modified method of McCutchan and Pagano (1968). The viral DNAs were obtained from cloning vectors by digesting with restriction endonuclease (EcoRI) and religating them into monomer circles at 2.5 μg/m*l*. DNAs in the ligation buffer were diluted fourfold with transfecting solution, containing 660 μg/m*l* of DEAE-Dextran (MW, 2×10^6; Pharmacia) and 20 mM Hepes (pH 7.1) in DMEM (Dulbecco's Modified Eagle's Medium). Nearly confluent cells in 100 mm diameter dish, which were plated one day before, were treated with transfecting solution containing DNA for 20 minutes in 37 °C, CO_2 incubator. Following removal of transfecting solution, the cells were washed with DMEM (20mM Hepes) and treated for 50 minutes with 100 μM chloroquine in 5 m*l* of DMEM (20 mM Hepes) to enhance the efficiency of transfection. Finally, the cells were washed, supplied with 2% fetal bovine serum in DMEM. At 40 to 48 hours after transfection or infection, the viral RNAs were purified from the cell as described by Roe and Mertz (1988).

Actinomycin D treatment of transfected cells

The transfected cells were further treated with actinomycin D for various lengths of time as follows. At 40 hours post-transfection, the medium was replaced with 4 m*l* of DMEM plus 2% fetal bovine serum containing 10 μg/m*l* of actinomycin D (Calbiochem) per 100 mm diameter dish. Following further incubation for 0.5, 1.5, 2.5, 7.5, and 13 hours, cells were harvested, and the RNAs were purified from the cytoplasm as described elsewhere (Roe and Mertz, 1988).

Primer extension method

Both conventional (Ghosh *et al.*, 1978) and modified primer extension methods (Somasekhar and Mertz, 1985b; Good *et al.*, 1988a) were employed. In the conventional method, a synthetic oligonucleotide complementary to the body of the 19S RNAs was 5' end-labeled at residue 604(604E; a 22-mer, a gift from Dr. J. Mertz, University of Wisconsin) and hybridized to the RNA samples (see Fig. 1). The cDNAs were synthesized with avian myeloblastosis virus reverse transcriptase (Life sciences, Inc.) at 40 °C for 2 hrs in 10 mM Tris(pH 8.4), 8 mM $MgCl_2$. 10 mM DTT, 50 μg/m*l* actinomycin D, 20u RNasin (Promega Biotech), and 1 mM each of dATP, dCTP, dGTP,

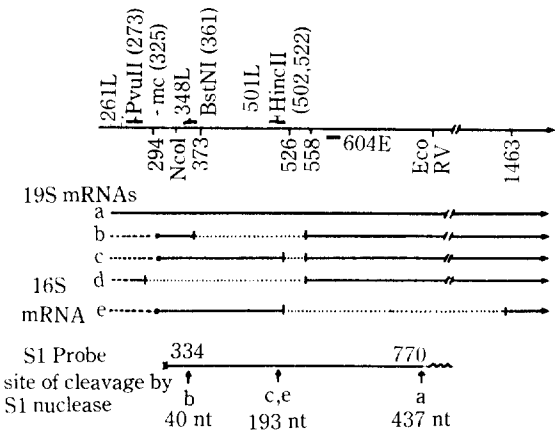

Fig. 1. *Structures of the late region of the SV40 genome, the abundant mRNAs it encodes, the primers used for conventional and modified primer extension, and the probe used for S1 mapping.* See text for more detailed descriptions.

and dTTP as described by McKnight *et al.* (1981). The RNA was then hydrolyzed by the addition of NaOH to 0.1N and incubation at 48 °C for 30 min. After neutralization of the reaction mixture with HCl, the resulting cDNAs were precipitated with ethanol and analyzed by electrophoresis in 10% polyacrylamide gels containing 7M urea.

In the modified method, 5' end-labeled cDNAs which had been synthesized as described above were annealed with various synthetic oligonucleotides harboring distinct restriction enzyme sites. The synthetic oligonucleotides used in this study, 17-25 nucleotides in length, extended from nucleotide residues 261(261L), 348(348L), and 501 (501L), and harbored the restriction enzyme sites for PvuII, BstNI, and HincII, respectively (see Fig. 1). They were also kindly provided by Dr. J. Mertz. A molar excess of each one was incubated separately with the cDNAs first at 80-85 °C for 5 min and then at 60 °C for 15 min in 10 mM Tris(pH 7.4), 0.2M NaCl, and 1 mM EDTA to allow hybridization to occur. The DNA was then digested with the appropriate restriction enzyme and analyzed in a 10% polyacrylamide gel containing 7M urea.

S1 Mapping Analysis

The probes used for S1 mapping were described in Fig. 1 and Fig. 3. Both probes were labeled with ^{32}P at their 3' ends in order to map the 3' ends of the RNA or the splice donor (5' splice) site. The probe used for analyzing 3' ends of the 19S RNA consists of SV40 DNA from nt. 770 (an EcoRV site) to nt. 2770 (a BclI site) linked to 430 bp of pSP65 DNA. This probe (2.43kb) was prepared by digesting a recombinant plasmid containing BclI cut SV40 DNA linked to EcoRI fragment of pSP65, with EcoRV and SphI. The probe used for analyzing RNAs with different splice sites are described in Fig. 1. The mapping procedure were as described in Roe and Mertz (1988).

RESULTS

The Determination of the structure of poly A(−) 19S RNA

To determine the 5' ends and the splicing pattern of the polyA(-) 373-RNA accumulating in the nucleus of infected monkey cells, we employed the primer extension technique. The primer used (604E, see Fig. 1) cannot hybridize with 16S RNS and is, therefore, specific for 19S RNAs. If the polyA(-) 373-RNA has its 5' end mapping to the major cap site at nucleotide residue (nt.) 325 and had been generated using the acceptor site at nt. 558, the primer would extend to produce a cDNA 96 nucleotides in length. On the other hand, unspliced and 526-558 spliced 19S RNAs would generate cDNAs 280 and 249 nucleotides in length, respectively, while 294-558 spliced 19S RNA would generate 222 nucleotide long cDNA if its 5' end maps to nt. 120. Fig. 2, lane c, showed that a cDNA 96 nucleotides in length was abundant among the polyA(-) RNAs, as well as 280 nucleotide long cDNA derived from unspliced RNA. This finding indicated that the non-polyadenylated 373-RNA that was abundant in the nucleus was processed in the same way as the cytoplasmic 19S mRNA. cDNAs from 526-spliced and 294-spliced RNAs were not abundant enough to be detected in this gel.

To verify the structure of 373-RNA, the modified primer extension technique was used in which appropriate synthetic oligonucleotides were hybridized to the 3' ends of the cDNAs and the cDNAs

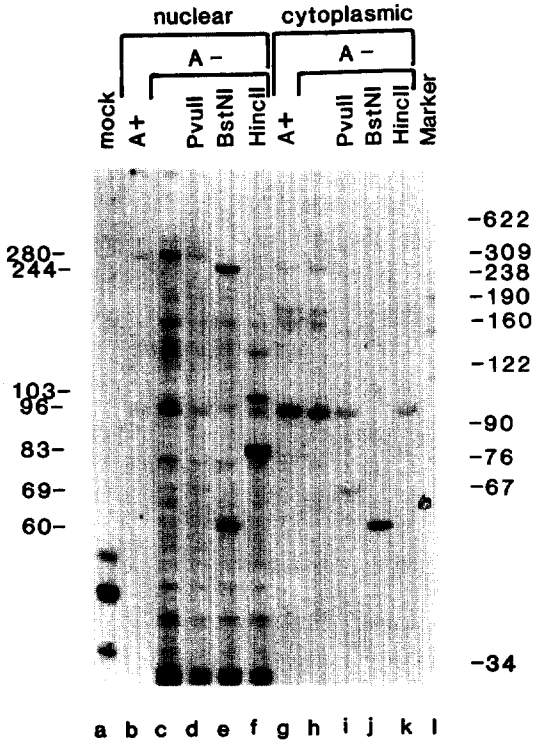

Fig. 2. *Structural analysis of the viral late 19S RNAs by the conventional and modified primer extension methods.*

Radiolabeled 604E primer (see Fig. 1) was extended by reverse transcription of RNA samples obtained from uninfected cells (lane a), nuclei (lanes b-f) and cytoplasm (g-k) of infected cells as described in the Materials and Methods. The RNA samples in lanes b and g had bound to oligo(dT)-cellulose, while those in lanes c-f, and h-k had not. The cDNAs made from RNA samples that failed to bind to oligo(dT)-cellulose were subsequently hybridized with synthetic oligonucleotides (261L, 348L, 501L) and cut with the appropriate restriction enzymes (PvuII, BstNI, HincII; see Fig. 1). The numbers on the left indicate the lengths of the cDNAs generated from the various 19S RNA species before and after digestion with restriction enzyme (see text for details). The numbers on the right indicate the lengths of molecular weight size markers (MspI-digested pBR322).

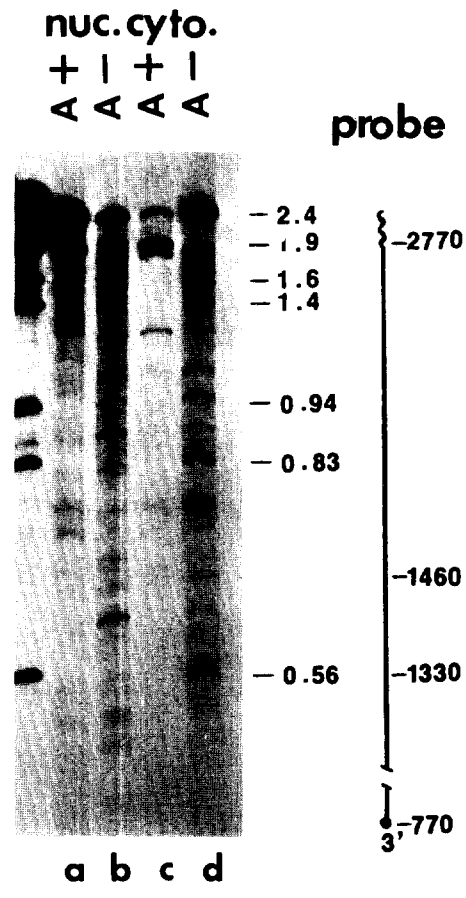

Fig. 3. *S1 mapping analysis of 3' ends of 19S RNAs.*

RNAs were isolated from nuclei (nuc.) and cytoplasm (cyto.) of cells infected with SV40, and were further fractionated through oligo(dT)-cellulose chromatography. The probe, depicted as a vertical line, contains SV40 sequences from nt.770 to 2770 linked to 430 bps of pSP65 DNA. The 3' end of the probe at nt.770 was labeled with ^{32}P. The numbers on the right of the autoradiogram are the lengths of molecular weight size markers (λDNA digested with HindIII and EcoRI).

were then cut in these regions with the corresponding restriction enzymes (see Fig. 1 and Materials and Methods). cDNA made from 373-558 spliced 19S RNA with a 5' end at nt. 325 should bind neither the PvuII-nor the HincII-containing synthetic oligonucleotides and, hence, not be cleaved by these enzymes. However, it should bind the BstNI-containing synthetic oligonucleotide and, therefore, be cleaved by BstNI enzyme to produce a DNA 60 nucleotides in length. This was exactly what was observed (Fig. 2, lanes d-f, and i-k). Similarly, cDNA from unspliced RNA with a 5' end at nt. 325 (280 nucleotides long) was not cleaved by PvuII but was cleaved by both BstNI and Hin-

cII to produce 244, and 83 plus 103 nucleotide long DNAs, respectively (lanes d-f). These latter bands, as expected, were not detected in the cDNAs generated from the cytoplasmic RNA samples because unspliced RNA was restricted to the nucleus. Lastly, the band 69 nucleotide in length, present only after cleavage with PvuII (lanes d and i), arose from 294-558 spliced 19S RNAs that had 5' ends mapping upstream of nt. 270. We therefore concluded that most of the abundant nonpolyadenylated 373-RNA had a 5' end at the major cap site and was spliced from 373 to 558 as is known to be true for the majority of polyadenylated cytoplasmic 19S RNA (Ghosh *et al.*, 1978).

In order to map the 3' ends of 19S RNA precisely, we performed S1 nuclease mapping using DNA probe 3' end labeled at EcoRV (nt.770) site (Fig. 3). Since this probe contains SV40 late region DNA up to BclI site (nt.2770) including polyA site at nt.2667, the polyadenylated RNA would protect 1.9kb of probe DNA. This was well represented in Fig. 3, lanes a and c. The pattern of probe protection by polyA(-) RNAs was quite different from that by polyA(+) RNAs(lanes b and d). There were multiple bands, the ends of which were spread over 1 kb upstream from the polyadenylation site. There was also a discrete band in nuclear polyA(-) sample (lane b), whose end was located downstream of polyA (nt. 2667) and BclI site. This band possibly represents a non-processed primary late transcript. The lack of this band in the cytoplasmic sample (lane d) is consistent with the idea that only the processed viral message is present in the cytoplasm. The multiple band patterns of nuclear polyA(-) RNA are similar to those of cytoplasmic sample, even though not identical. These bands could not be the product of random degradation during the RNA purification step nor the multiple breathings in RNA-DNA hybrids, since there were no such bands in polyA(+) samples. Therefore, the result demonstrated unambiguously that the 3' ends of the 373-RNA were heterogeneous and spread over a large distance (over 1 kb) upstream from the polyadenylation site.

The Effect of Unused 3' Splice Region on Nuclear Transport of Spliced RNA

The abundant presence of spliced 19S RNA in the nucleus of infected monkey cell evoked questions regarding its biogenesis. Why was 19S spliced RNA hindered from being transported into cytoplasm and accumulated in the nucleus in contrast to the 16S spliced RNA? One prominent feature in the 19S spliced RNA which is distinguished from 16S RNA is the presence of 3' splice region around nt. 1463 necessary for producing 16S RNA. We postulated that this region could form presplicing complex and thus caused the 373-RNA to remain in the nucleus longer in the absence of proper splicing donor site. This postulation was based on several observations from different laboratories that 3' splice region is important in initiating spliceosome assembly and forms RNA complexes prior to forming functional spliceosome (Frendewey and Keller, 1985; Krämer, 1987; Beyer and Osheim, 1988). In order to test this possibility, we tried experiments to see whether the deletion of 3' splice region for 16S RNA has any effect on the distribution of 373-RNA in the cell. For this, we analyzed SV40 late RNAs from cells transfected with either wild type (SVS) or mutant SV40 DNA deleted of 16S 3'-splice site from nt.1059 to 1463 (SV1777; see Good *et al.*, 1988b). Since SV1777 DNA does not produce VP1 protein due to the lack of 16S RNA, we tested another DNA (SV1773) which contains 7 bp deletion at AccI site (nt.1628) and lacks VP1 reading frame without impairing 16S 3' splice region, as a control against the effect of VP1.

A representative S1 nuclease mapping analysis with DNA probe labeled at nt. 334 (an NcoI site) was shown in Fig. 4. The absolute amount of 373-RNA was not as dramatic as in the infected cell. However, the relative nuclear accumulation of 19S 373-RNA as compared with 16S 526-RNA was still observed in cells transfected with wild type (SVS) and VP1-less pseudo-wild type (SV 1773) DNA. The distribution of 373-RNA between the nucleus and the cytoplasm of cells transfected with SV1773 was about equal, and similar results were observed for cells transfected with

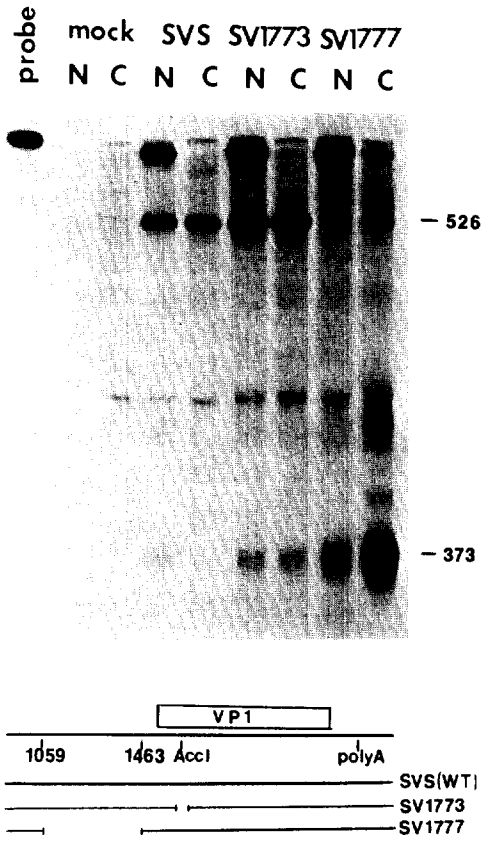

Fig. 4. *S1 mapping analysis of viral RNA from cells transfected with various SV40 DNAs.*
RNAs were prepared at 48 hrs after transfection from nuclei (N) and cytoplasm (C) of CV1P cells. The probe labeled at the 3' end of nt.334 was used for mapping (see Fig. 1). The SV40 DNAs used for transfection were described below the autoradiogram. The top line depicts a part of the late region of SV40 genome and the open reading frame for VP1. SV1773 contains 7 bp deletion at AccI site, whereas SV1777 contains deletion of 3' splice region from residue 1059 to 1463.

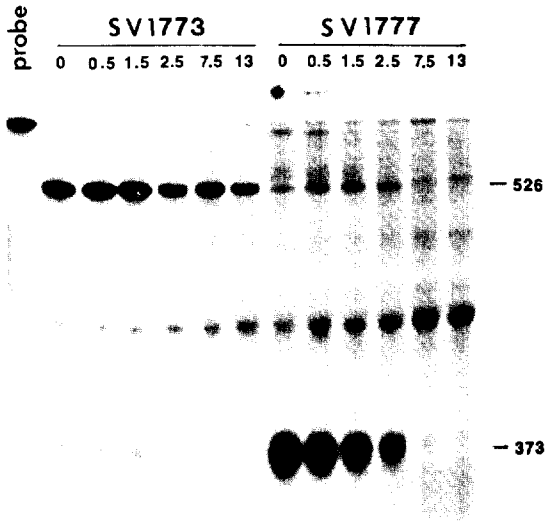

Fig. 5. *S1 mapping of cytoplasmic viral RNAs from cells treated with actinomycin D.*
RNAs were prepared from the cytoplasm of CV1P cells transfected with either SV1773 or SV1777 DNA and were treated wth actinomycin D (10 $\mu g/ml$) for 0.5, 1.5, 2.5, 7.5, and 13 hrs, at 40 hrs after transfection. The same probe as in Fig. 4 was used for analysis.

wild type SVS DNA. This distribution changed dramatically, however, in cells transfected with SV1777 DNA, a mutant deleted of 16S 3' splice region. The level of 373-RNA in the cytoplasm was about 10 fold higher than in the nucleus as determined by densitometric tracing of linearly exposed film. This result unambiguously demonstrated that 373-RNA was held in the nucleus due to the presence of unused 16S 3' splice region. However, the result does not tell the mechanism by which such a deletion could have caused the accumulation of 373-RNA in the cytoplasm.

One can acribe the result from the deletion to the absence of splicing complex at this site and at the same time, to the possible increase in the stability of the 373-RNA in the cytoplasm. In order to compare the half lives of 373-RNAs in the cytoplasm of cells transfected with pseudo-wild type (SV1773) or deletion mutant (SV1777) DNA, we treated transfected cells at late times after transfection with actinomycin D for varying lengths of time and analyzed their RNAs by S1 nuclease mapping as described in Fig. 4. Fig. 5 demonstrated that the half lives of cytoplasmic 373-RNA were the same for cells transfected with either SV1773 or SV1777 DNA. This clearly excluded the possibility that the deletion of unused 16S 3' splice region increased the stability of the mRNA. From the above results, we concluded that the presence of the unused 16S 3' splice region in the 19S 373-RNA caused its accumulation in the nucleus via forming presplicing complex.

DISCUSSION

We have shown that the 19S 373-spliced RNA, present in the nucleus of monkey cells infected with SV40, contains the same 5' end and the exon structure as the cytoplasmic viral 19S mRNA. Since the amount of the spliced 19S RNA was similar to that of the spliced 16S RNA within the cell as a whole, we could argue that the two classes of SV40 late RNAs are produced equally efficiently within the nucleus. Why, then, is this normally spliced 19S RNA retained in the nucleus whereas spliced 16S RNAs are transported efficiently into the cytoplasm? What is the main determinant in this differential transport? Could it be the presence or the absence of polyA tail or something else?

An insight to solve these questions came from 2 lines of observations. One was from the studies using intronless mutant SV40 to produce pre-spliced RNA. When the cells were forced to produce the late primary transcript as pre-spliced 19S RNA following transfection with SV40 DNA deleted of 19S intron sequences from nt.373 to 558, it was observed that a large proportion of it remained in the ncleus (Wangshick Ryu, personal communications). This 373 pre-spliced RNA also produced low levels of 16S RNA by splicing out nucleotides from 294 to 1463, which is rarely used otherwise (Good *et al.*, 1988c). The other line of observations was those, which provided accumulating evidences that the assembly of splicing machinery gets initiated at the 3' splice region by forming stable ribonucleoprotein complexes (Frendewey and Keller, 1985; Krämer, 1987; Beyer and Osheim, 1988; Green, personal communications). Based on these observations we hypothesized that the presence of efficiently used 3' splice region in the 19S RNA could be the culprit for the current phenomena that we observed. Since the 5' ends of the abundant 373-RNA is on the major cap site at nt.325 and the spliced-out intron contains another 5' splice site at nt.435, splicing complex formed at 3' splice region around nt.1463 will not find its 5' pair. We, therefore, postulated that the unsuccessful formation of pre-splicing complex could have caused such an accumulation of 19S spliced RNAs. The SV40 mutant SV1777 that we used to test this hypothesis, contains deletion of not only the 16S 3' splice site but also a functional branch site as well as polypyrimidine tracts, which are thought to be required for the formation of pre-splicing complex (Kramer, 1987). The dramatic change in cellular distribution of 373-RNA shown for this mutant was demonstrated not to be due to the changed stability of this RNA in the cytoplasm (Fig. 5). Therefore, this study clearly shows that the presence of 16S 3' splice region caused the inefficient transport of the spliced RNA into the cytoplasm, possibly by forming a pre-splicing complex.

Regarding the mechanism by which the 16S 3' splice region actually inhibited nuclear transport, we can only postulate. The formation of pre-splicing complex at this site might have hindered the release of the RNA from the nuclear matrix, and thereby impaired transport. Alternatively, the pre-splicing compelx could have caused the lack of polyA tail, either by inhibiting polyadenylation process or by inducing deadenylation of the mature RNA, and thereby repress transport (Schroeder *et al.*, 1987). There can be several pathways for the generation of nonpolyadenylated 373-RNA. First, it may have undergone splicing without ever having been polyadenylated. The heterogeneity in the 3' ends of this RNA may have been generated by partial degradation *in vivo* following transcription termination, prior to fractionation of the cells for the isolation of RNA. Second, the 373-RNA may have undergone deadenylation and partial degradation after having been spliced and polyadenylated. Many examples of shortening in the cytoplasm of the polyA segment, as well as the complete loss of it from the polyadenylated mRNA have been reported (Adams *et al.*, 1981; Dworkin *et al.*, 1977; Merkel *et al.*, 1976; Bergman and Brawerman, 1980). Some nonpolyadenylated mRNAs are even associated with polysomes and are translated efficiently (Dworkin *et al.*, 1977; Bergman and Brawerman,

1980). Whether the same process of deadenylation occurs naturally in the nucleus as in the cytoplasm is not known.

In order to elucidate the molecular mechanism for the hindrance in nuclear transport of 373-RNA by the remaining unused 3' splice region, one has to have information on the pathways outlined above. For this, one needs to perform pulse chase or actinomycin D chase experiment. From these experiments, we also expect to elucidate the interrelationships between splicing, polyadenylation and nuclear transport.

The current result, which demonstrated that the choice of splice sites affect nuclear transport, reveals yet another level of regulation that alternative splicing exerts on gene expression. Not only the alternative splicing produces RNAs of different reading frame and half life, it can also change the amount of mRNA in the cytoplasm via regulating the rate of transport.

적 요

SV 40 바이러스가 원숭이 세포(CVIP)에 감염한 후기에 생기는 poly A(−) 19S RNA의 5'끝과 splicing 유형을 알아보기 위하여 primer extension 및 그 변형방법을 행하였다. 이 RNA의 5' 끝은 SV 40 후기 RNA들이 가장 많이 사용하는 cap 자리인 잔기 325 위치임이 밝혀졌다. 또한 splicing 유형도 세포질의 poly A(+)인 19S RNA와 같은 잔기 373에서 잔기 558까지의 intron이 제거된 형태이었다. S1 뉴클리아제에 의한 분석결과 이 RNA의 3' 끝은 polyadenylation 위치로부터 상위로 약 1 1kb에 걸쳐 다양하게 존재함을 알았다. 정상적인 cap 자리와 splicing 형태를 지닌 이 RNA가 왜 핵에 축적되는지의 이유를 조사하였다. 이 RNA상에 사용되지 못한채로 남아있는 3' splice 부위가 핵내 편재를 유발했는지의 여부를 알아보기 위하여, 3' splice 부위를 결손시킨 돌연변이 SV 40 DNA를 세포에 도입시켰다. 그 결과 3' splice 자리가 없는 RNA는 세포질에 많이 축적됨을 관찰하였다. 이 결손 RNA의 세포질내 축적은 결손으로 인해 RNA의 안정성이 증가함으로써 비롯된 것이 아니라는 것을 actinomycin D 추적실험을 통해 밝혔다. 따라서 정상적인 19S spliced RNA가 세포질로 이동되는 과정을 방해하는 것은 사용되지 않은 3' splice 부위에 형성된 pre-splicing 복합체 때문인 것으로 여겨진다.

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