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Regulation of the Extent of Splicing of Influenza Virus NS1 mRNA: Role of the Rates of Splicing and of the Nucleocytoplasmic Transport of NS1 mRNA

FIRELLI V. ALONSO-CAPLEN† AND ROBERT M. KRUG†*

Graduate Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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Influenza virus NS1 mRNA is spliced by host nuclear enzymes to form NS2 mRNA, and this splicing is regulated in infected cells such that the steady-state amount of spliced NS2 mRNA is only about 10% of that of unspliced NS1 mRNA. This regulation would be expected to result from a suppression in the rate of splicing coupled with the efficient transport of unspliced NS1 mRNA from the nucleus. To determine whether the rate of splicing of NS1 mRNA was controlled by *trans* factors in influenza virus-infected cells, the NS1 gene was inserted into an adenovirus vector. The rates of splicing of NS1 mRNA in cells infected with this vector and in influenza virus-infected cells were measured by pulse-labeling with [^{3}H]uridine. The rates of splicing of NS1 mRNA in the two systems were not significantly different, strongly suggesting that the rate of splicing of NS1 mRNA in influenza virus-infected cells is controlled solely by *cis*-acting sequences in NS1 mRNA itself. In contrast to the rate of splicing, the extent of splicing of NS1 mRNA in the cells infected by the adenovirus recombinant was dramatically increased relative to that occurring in influenza virus-infected cells. This could be attributed largely, if not totally, to a block in the nucleocytoplasmic transport of unspliced NS1 mRNA in the recombinant-infected cells. Most of the unspliced NS1 mRNA was in the nuclear fraction, and no detectable NS1 protein was synthesized. When the 3′ splice site of NS1 mRNA was inactivated by mutation, NS1 mRNA was transported and translated, indicating that the transport block occurred because NS1 mRNA was committed to the splicing pathway. This transport block is apparently obviated in influenza virus-infected cells. These experiments demonstrate the important role of the nucleocytoplasmic transport of unspliced NS1 mRNA in regulating the extent of splicing of NS1 mRNA.

Splicing of eucaryotic pre-mRNAs occurs in the nucleus, and in most cases only the spliced mRNA products are detected in the cytoplasm. Retrovirus and influenza virus splicing deviates from this pattern (3, 14, 16, 17, 20). In these cases, both the spliced mRNA product(s) and the unspliced pre-mRNA(s) code for proteins, and with retroviruses, the completely unspliced pre-mRNA also serves as genomic RNA for progeny virus. Consequently, only a portion rather than all of the pre-mRNA(s) is spliced, and the unspliced pre-mRNA(s) is transported from the nucleus.

Two influenza virus mRNAs, the M1 (matrix) and NS1 (nonstructural protein 1) mRNAs, are spliced by host nuclear enzymes to form smaller mRNAs, M2 mRNA and $mRNA_3$ from M1 mRNA and NS2 mRNA from NS1 mRNA (11, 12, 15–17, 20). In infected cells, the extent of splicing is regulated such that the steady-state amounts of the spliced mRNAs are only about 10% of those of the unspliced mRNAs (16, 20). This regulation could occur at various levels. However, if the unspliced and spliced viral mRNAs are relatively stable, then the steady-state kinetic model for the generation of spliced mRNA would predict that the 1:10 ratio of spliced NS2 mRNA to unspliced NS1 mRNA in the cytoplasm of influenza virus-infected cells would result from a suppression in the rate of splicing coupled with the efficient transport of unspliced NS1 mRNA from the nucleus (see the equations for the two-compartment kinetic model in Materials and Methods). The 1:10 ratio in the whole cell would also result from these two types of regulation.

Studies have been carried out previously to analyze NS1 and M1 mRNA splicing in vivo in the absence of other influenza virus gene products by transferring the NS1 or M1 gene to a DNA vector (18, 19). However, the extent of splicing observed was different from that found in influenza virus-infected cells. Thus, in cells infected with a simian virus (SV40) recombinant containing the NS1 gene under the control of the SV40 late promoter, the cytoplasmic level of spliced NS2 mRNA was slightly greater, rather than much less, than that of unspliced NS1 mRNA (19). This was attributed either to instability of the unspliced NS1 mRNA in SV40-infected cells or to some effect on the rate of NS1 mRNA splicing (i.e., an increase in the rate) due to the SV40 sequences at the 5′ and 3′ ends of the NS1 mRNA in the construct. Other explanations are possible. For example, some evidence has been presented that one or more influenza virus-specific proteins are involved in the regulation of viral mRNA splicing (13, 27). It is conceivable that this putative protein, a protein other than the NS1 and NS2 proteins synthesized in the cells infected by the SV40 recombinant, might act by suppressing the rate of splicing. In addition, the efficiency of nucleocytoplasmic transport of unspliced NS1 mRNA could be different for influenza virus-infected cells and cells infected by the SV40 recombinant.

The present study was undertaken to sort out these possibilities. We inserted the NS1 gene into an adenovirus vector because the high level of mRNA synthesis with this vector allowed the determination of the rate as well as of the extent of splicing. It was found that the rate of splicing of NS1 mRNA was not significantly different from that in

* Corresponding author.
† Present address: Department of Molecular Biology and Biochemistry, Center for Advanced Biotechnology and Medicine, Rutgers University, 679 Hoes Lane, Piscataway, NJ 08854-5638.

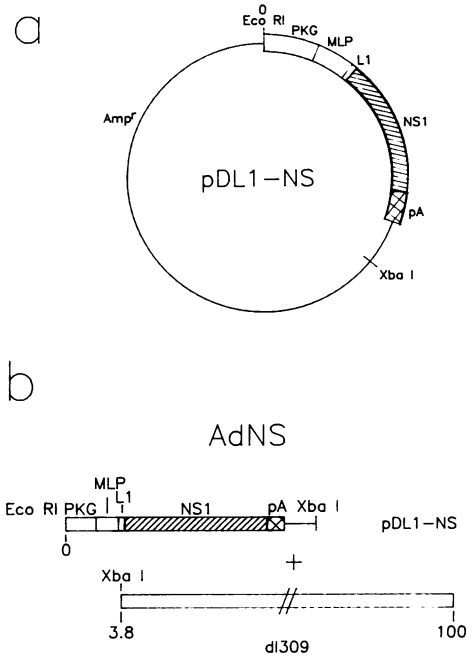

FIG. 1. Construction of the recombinant adenovirus AdNS. (a) Structure of the recombinant plasmid pDL1-NS. PKG, Adenovirus type 5 packaging sequences extending from 0 to 1 map unit; MLP, adenovirus MLP; L1, first 33 nucleotides of the first leader segment of the tripartite leader; NS1, the influenza virus NS1 gene; pA, SV40 early polyadenylation signal; Amp^r, ampicillin resistance gene. pBR322 sequences are indicated (——). (b) Generation of the recombinant adenovirus AdNS by in vitro ligation of the *Eco*RI-*Xba*I fragment of the plasmid (containing the modular transcription unit) with *Xba*I-cleaved *dl*309 DNA extending from 3.8 to 100 map units.

influenza virus-infected cells. In contrast, nucleocytoplasmic transport of unspliced NS1 mRNA was blocked in cells infected with the adenovirus vector, resulting in a much greater extent of splicing. The results provide strong evidence for two conclusions about general aspects of the regulation of the extent of NS1 mRNA splicing: (i) the rate of splicing of NS1 mRNA is most likely controlled solely by *cis*-acting sequences in NS1 mRNA itself, and (ii) the extent of splicing of NS1 mRNA can be regulated by the efficiency of nucleocytoplasmic transport of unspliced NS1 mRNA.

MATERIALS AND METHODS

Construction of recombinant adenoviruses containing the NS1 gene. The NS1 gene of influenza virus (A/Udorn/72) was excised by *Bam*HI digestion from plasmid pSP64-NS1 (25) and ligated to the *Bam*HI cloning site in the plasmid pDL1 (2) (Fig. 1a). This placed the NS1 gene under the control of the adenovirus major late promoter (MLP), with transcription terminating at the SV40 early-polyadenylation site. The recombinant plasmid pDL1-NS was digested with *Eco*RI and *Xba*I to remove the hybrid transcription unit and ligated to a sucrose gradient-purified large fragment of *Xba*I-cleaved *dl*309 DNA (Fig. 1b). The ligation mixture was transfected onto 293 cells and a recombinant adenovirus (AdNS) was obtained as previously described (2). In the construction of AdNS3ss, point mutations were first introduced into the NS1 gene at the positions shown in Fig. 5a by site-directed mutagenesis (Boehringer Mannheim Biochemicals). The mutated gene was then inserted into a recombinant adenovirus as described above.

Measurement of NS1 and NS2 mRNAs in infected cells. 293 cells infected with either AdNS for 20 h or with influenza virus (A/Udorn/72) for 4 h were labeled with 5 mCi of [^{3}H]uridine per ml for the times indicated below. The total poly(A)-containing RNA from the cells was annealed to an excess of an M13 single-stranded DNA containing sequences complementary to the entirety of spliced NS2 mRNA. After digestion with RNase T_2, the RNA-DNA hybrids were collected on nitrocellulose filters, and the RNA eluted from these filters by heating at 90°C was analyzed by electrophoresis on 5% denaturing polyacrylamide gels (26). The gels were fluorographed and exposed onto Kodak X-Omat AR films for 2 and 8 months. The relative amounts of label in the bands were determined by densitometry. Unlabeled 293 cells (at 20 h after infection with AdNS) were used for fractionation of cells into nuclei and cytoplasm. The cells were disrupted by Dounce homogenization in 0.5% Nonidet P-40-containing hypotonic buffer (9), and the nuclei were collected by centrifugation at 500 × *g* for 5 min. The total RNA from the nuclear and cytoplasmic fractions was assayed by S1 nuclease assay, using the 5′-end-labeled probe indicated in Fig. 3b.

Detection of NS1 and NS2 protein synthesis. 293 cells infected with AdNS or AdNS3ss for 20 h or with influenza virus for 4 h were labeled for 1 h with [^{35}S]methionine in methionine-free media. After a washing with ice-cold Hanks balanced salt solution, the cells were disrupted on ice with lysis buffer (2). One aliquot was analyzed directly on urea-containing sodium dodecyl sulfate (SDS)-polyacrylamide gels. Another aliquot was incubated with protein A-Sepharose beads precoated with monospecific antibodies directed against the NS1 or NS2 protein (obtained from M. Krystal and P. Palese [10, 28]), and the proteins which bound to the beads were eluted by boiling in gel-loading buffer (2) followed by SDS-polyacrylamide gel electrophoresis.

Two-compartment steady-state kinetic model for the generation of spliced mRNA. Pikielny and Rosbash (24) considered a two-compartment (nucleus and cytoplasm) kinetic model for splicing but did not present the equations for this model. The following symbols will be used: U_n and S_n, steady-state concentrations of pre-mRNA and mRNA, respectively, in the nucleus; k_{und} and k_{snd}, degradation rate constants of U_n and S_n, respectively; k_{sp}, rate constant of splicing; k_{ut} and k_{st}, rate constants for the nucleocytoplasmic transport of U_n and S_n, respectively; U_c and S_c, steady-state concentrations of pre-mRNA and mRNA, respectively, in the cytoplasm; and k_{ucd} and k_{scd}, degradation rate constants of U_c and S_c, respectively.

At steady state:

$$d(U_c)/dt = 0 = k_{ut}(U_n) - k_{ucd}(U_c)$$
$$d(S_c)/dt = 0 = k_{st}(S_n) - k_{scd}(S_c)$$

When these equations are solved for the ratio of S_c to U_c,

$$(S_c)/(U_c) = \frac{k_{sp}}{k_{st}+k_{snd}} \cdot \frac{k_{ucd}}{k_{ut}} \cdot \frac{k_{st}}{k_{scd}}$$

k_{snd} is usually $<<< k_{st}$, so that

$$(S_c)/(U_c) = \frac{k_{sp}}{k_{ut}} \cdot \frac{k_{ucd}}{k_{scd}}$$

If $k_{ucd} = k_{scd}$, i.e., the pre-mRNA and mRNA have similar stabilities in the cytoplasm, then

$$(S_c)/(U_c) = k_{sp}/k_{ut}$$

In other words, the ratio of spliced mRNA (e.g., NS2 mRNA) to unspliced pre-mRNA (e.g., NS1) in the cytoplasm is equal to the ratio of the rate of splicing to the rate of transport of unspliced pre-mRNA (NS1 mRNA). The same relationship holds for the ratio of spliced to unspliced mRNA in the whole cell if these two mRNAs have similar stabilities in the cytoplasm. Hence, if the transport rate (k_{ut}) is much greater than the splicing rate (k_{sp}), then most of the NS1 mRNA will not be spliced. On the other hand, if the splicing rate is greater than the transport rate, then most of the NS1 mRNA will be spliced. In the situation where the rate of splicing of NS1 mRNA in two different cell systems is the same, then the relative rates of nucleocytoplasmic transport of NS1 mRNA will determine the relative extent of splicing in the two systems.

RESULTS

Rate of splicing of NS1 mRNA was not significantly affected by transfer of the NS1 gene to an adenovirus DNA vector. To compare the rate of splicing of NS1 mRNA expressed with a DNA vector with the splicing rate in influenza virus-infected cells, it was necessary to use a recombinant adenovirus (AdNS) expressing NS1 mRNA under the control of the MLP (Fig. 1). With this expression system, the rate of NS1 mRNA synthesis was sufficient for determining the rate of splicing (see below). The MLP controlling NS1 mRNA synthesis replaced part of the E1A region of the adenovirus genome, which also contained its normal MLP. The expressed NS1 mRNA contained a minimal amount of non-influenza virus sequences: a small portion of the tripartite leader (33 nucleotides of the first leader, or L1) at the 5′ end and 200 nucleotides at the 3′ end. The small portion of the tripartite leader was present to enhance transcription from the MLP (2). The recombinant was grown in 293 cells, which should complement the E1A defect of the recombinant (8).

The splicing rate was measured directly by labeling cells with [^{3}H]uridine for short periods of time and determining the amount of labeled NS2 mRNA initially generated from newly synthesized NS1 mRNA. At 20 h postinfection, 293 cells infected with AdNS were labeled with [^{3}H]uridine for 5, 10, 15, 30, and 60 min. For comparison, 293 cells infected with influenza virus for 4 h were labeled with [^{3}H]uridine for the same lengths of time. The total poly(A)-containing RNA was annealed to an excess of an M13 single-stranded DNA containing sequences complementary to the entirety of spliced NS2 mRNA. After RNase T_2 digestion, the RNA-DNA hybrids were collected on nitrocellulose filters, and the RNA eluted from these filters by heating was analyzed by gel electrophoresis (26) (Fig. 2). The more slowly migrating band was intact spliced NS2 mRNA, and the faster-migrating band was the 3′ exon of unspliced NS1 mRNA. The small 5′ exon of NS1 mRNA migrated off the gel.

With the 5-min pulse, newly synthesized NS1 mRNA was detectable in influenza virus-infected cells but not in AdNS-infected cells. In the latter cells, an additional 5 min of labeling (i.e., a 10-min pulse) was needed for the detection of newly synthesized NS1 mRNA. This indicated that either the rate of transcription of NS1 sequences or the number of templates for transcription in influenza virus-infected cells was about twice that in AdNS-infected cells. Little or no spliced NS2 mRNA was detected after the 5-min pulse in influenza virus-infected cells or after the 10-min pulse in AdNS-infected cells. Hence, the ratio of spliced NS2 mRNA to unspliced NS1 mRNA that was found after an additional 5-min labeling period should provide an estimate of the rate of splicing. The ratio of these two mRNAs in influenza virus-infected cells (10-min pulse) was 0.2 and in AdNS-infected cells (15-min pulse) was 0.5. This suggested that the rate of splicing of NS1 mRNA in AdNS-infected cells could be about two and a half times that in influenza virus-infected cells. However, the results obtained with longer labeling periods indicated that the rate of splicing of NS1 mRNA in these two systems probably did not differ even by 2.5-fold.

With longer labeling periods (15, 30, and 60 min) in influenza virus-infected cells, little or no increase in the ratio of NS2 to NS1 mRNA occurred, indicating that the initial rate of splicing was similar or identical to the eventual extent of splicing. In other words, splicing occurred only for a brief period of time. In marked contrast, splicing of NS1 mRNA in AdNS-infected cells continued for a much longer time, most likely throughout the 60-min labeling period. The ratio of NS2 to NS1 mRNA increased dramatically from the initial value of 0.5 (15-min labeling period) to a value of 7 after 30 and 60 min of labeling. The decrease in NS1 mRNA labeling was more than accounted for by a larger increase in NS2 mRNA labeling. This strongly suggested that the NS1 mRNA was largely, if not totally, converted to NS2 mRNA rather than undergoing any significant breakdown. Thus, the most significant difference between AdNS- and influenza virus-infected cells was the length of time in which splicing of NS1 mRNA occurred and not the rate of splicing of NS1 mRNA. In fact, it is quite likely that the rate of splicing of NS1 mRNA in influenza virus-infected cells did not differ significantly from that in AdNS-infected cells. If splicing of NS1 mRNA in influenza virus-infected cells occurred for less than 5 min, the period during which the rate was measured, then the rate of splicing of NS1 mRNA in these cells would have been underestimated.

Little or no transport of unspliced NS1 mRNA occurred in AdNS-infected cells. The difference in the duration of splicing of NS1 mRNA between AdNS- and influenza virus-infected cells could be due to different efficiencies of transport of unspliced NS1 mRNA from the nucleus to the cytoplasm. Previous experiments using nonaqueous fractionation showed that NS1 mRNA was transported efficiently in influenza virus-infected cells (26). It is not possible to use aqueous fractionation with influenza virus-infected cells because virus-specific RNAs leak out of the nuclei during fractionation (26). However, aqueous fractionation of AdNS-infected cells did yield relatively clear-cut results. These cells were fractionated into nuclear and cytoplasmic fractions in the presence of Nonidet P-40, and the amounts of NS1 and NS2 mRNAs in these fractions were determined by S1 nuclease analysis (Fig. 3). Most (more than 85%) of the spliced NS2 mRNA was in the cytoplasmic fraction, whereas most (more than 85%) of the unspliced NS1 mRNA was in the nuclear fraction.

To confirm this fractionation, we determined whether the NS1 protein was synthesized in AdNS-infected cells (Fig. 4). The cells were labeled with [^{35}S]methionine for 1 h at 20 h postinfection. Large amounts of the NS2 protein were synthesized. This could be seen both before (Fig. 4, lane 2) and after (Fig. 4, lane 8) immunoprecipitation. Consequently, NS2 mRNA containing 33 nucleotides of the first late adenovirus leader (L1) was efficiently translated in late adenovirus-infected cells, as was found previously for the influenza virus nucleocapsid protein mRNA (2). In contrast, no NS1 protein was detected, even after immunoprecipitation (Fig. 4, lane 5). It was therefore likely that little or no NS1 mRNA was transported to the cytoplasm and that the small

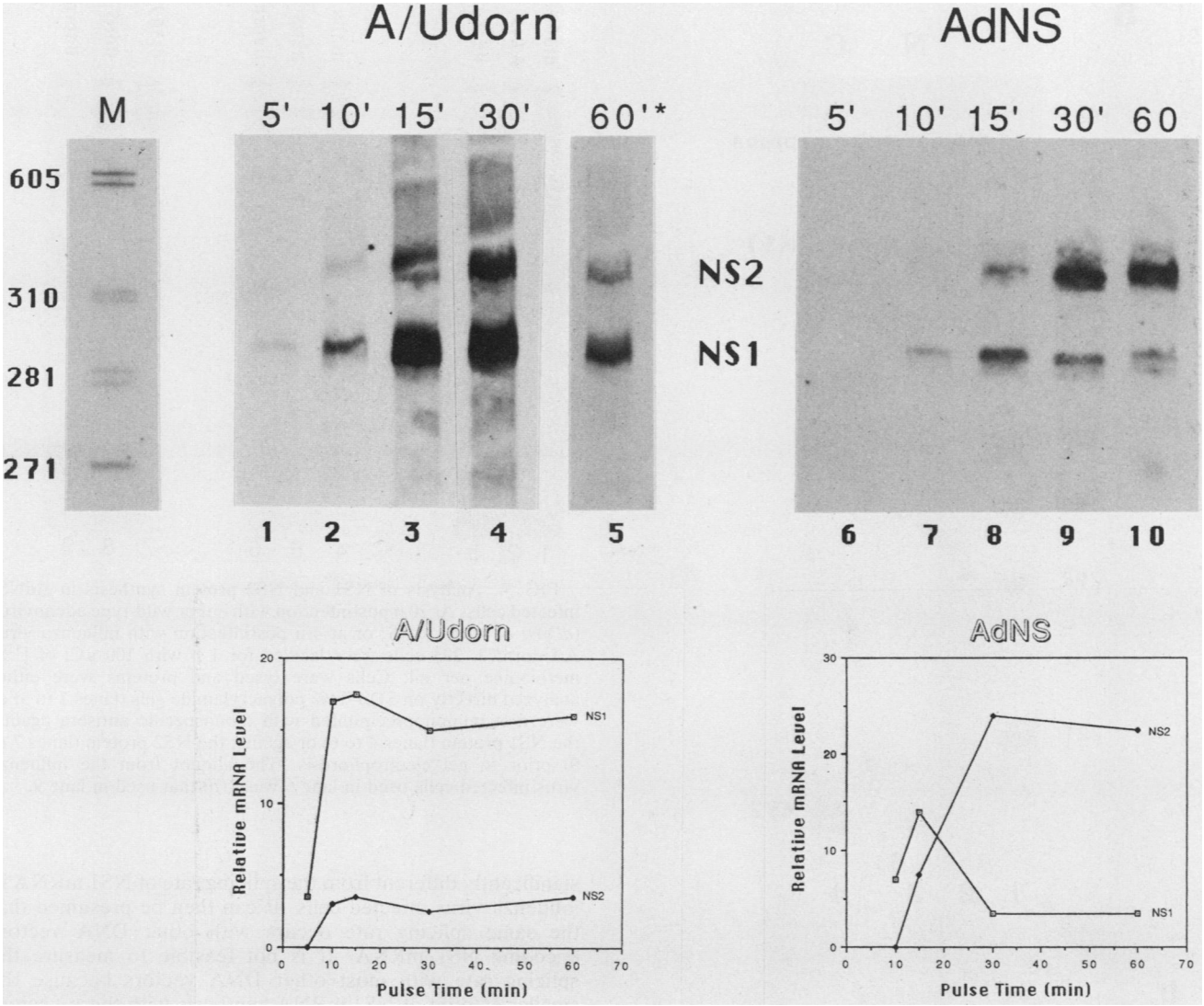

FIG. 2. Rate and extent of splicing of NS1 mRNA. At 4 h postinfection with influenza virus A/Udorn/72 (lanes 1 to 5) or at 20 h postinfection with AdNS (lanes 6 to 10), 293 cells were labeled with 5 mCi of [^{3}H]uridine per ml for 5, 10, 15, 30, and 60 min. Total poly(A)-containing RNA was annealed to an excess of M13 single-strand DNA complementary to NS2 mRNA sequences, and RNase T_2-resistant fragments were analyzed on a 5% denaturing polyacrylamide gel. The more slowly migrating band was intact spliced NS2 mRNA, and the faster-migrating band was the 3′ exon of the unspliced NS1 mRNA. The small 5′ exon of NS1 mRNA migrated off the gel. All lanes show an 8-month exposure of the gel except for lane 5, which is a 2-month exposure (*). Fragment sizes are indicated on the left. Appropriate exposures of the gels were quantitated by densitometry, and the resulting relative NS1 and NS2 mRNA levels were plotted against the duration of the labeling period.

amount of NS1 mRNA that was found in the cytoplasmic fraction after aqueous fractionation (Fig. 3) represented molecules that leaked out of the nucleus during fractionation.

Commitment of NS1 mRNA to the splicing pathway caused the block in its transport in AdNS-infected cells. According to the spliceosome retention hypothesis (21), pre-mRNA transport is normally prevented by the binding of splicing factors to the pre-mRNA, resulting in the formation of the spliceosome, a large complex containing multiple small nuclear ribonucleoproteins. If this were the case for NS1 mRNA in AdNS-infected cells, then inactivation of the 3′ and/or 5′ splice sites of NS1 mRNA would be expected to allow the transport of NS1 mRNA. We mutated the 3′ splice site AG to CG and also disrupted the polypyrimidine tract by mutating each of two T's to A's (Fig. 5a). None of these mutations caused a change in the amino acid sequence of the NS1 protein. This mutated NS1 gene was inserted into an adenovirus vector (AdNS3ss). In cells infected with AdNS3ss, NS1 mRNA was not spliced (data not shown). Transport of NS1 mRNA from the nucleus was assayed by translation (Fig. 5b). NS1 protein, but not NS2 protein, was synthesized in cells infected by AdNS3ss (Fig. 5, lanes 4 and 8), whereas the opposite was the case in cells infected with AdNS (Fig. 5, lanes 3 and 7). Consequently, it can be concluded that the transport of unspliced NS1 mRNA in AdNS-infected cells

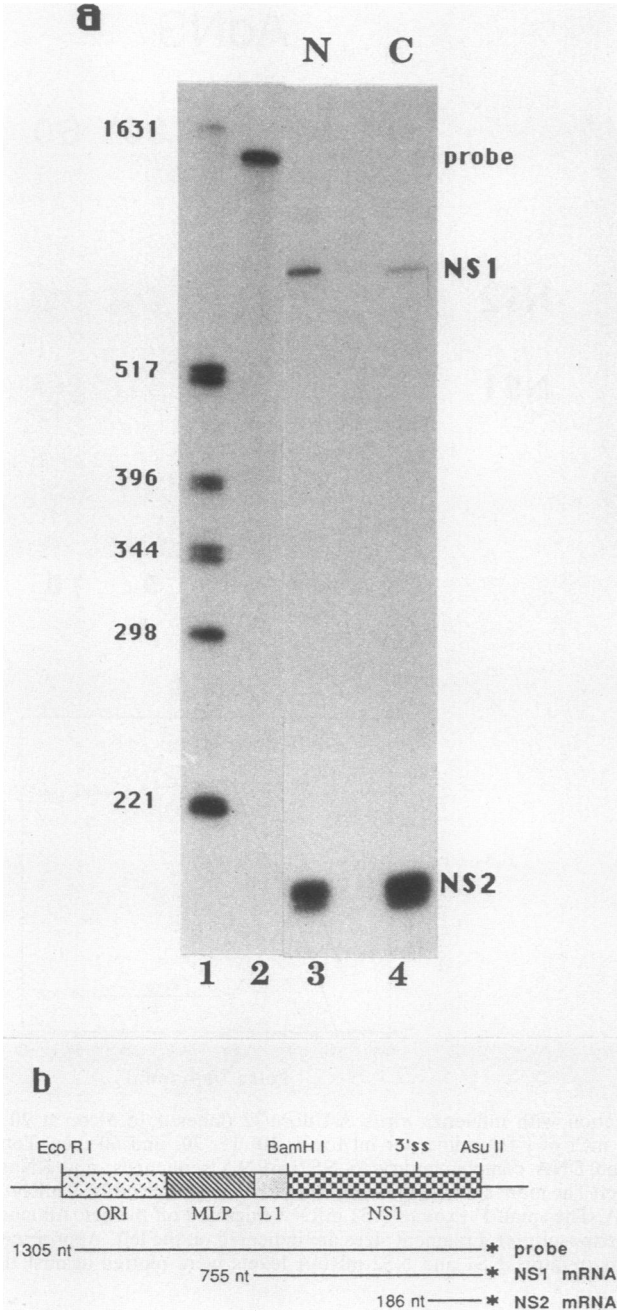

FIG. 3. Nucleocytoplasmic transport of NS1 and NS2 mRNAs in AdNS-infected cells. (a) 293 cells infected for 20 h with AdNS were fractionated into nuclei and cytoplasm, and the RNA in these two fractions was assayed by S1 nuclease analysis. Fragment sizes are indicated on the left. (b) Diagram of the probe obtained by digestion of pDL1-NS (Fig. 1a) with *Eco*RI and *Asu*II, followed by labeling at the 5′ end with [γ-^{32}P]ATP. The sizes of the probe and of the two protected fragments are indicated. nt, Nucleotides.

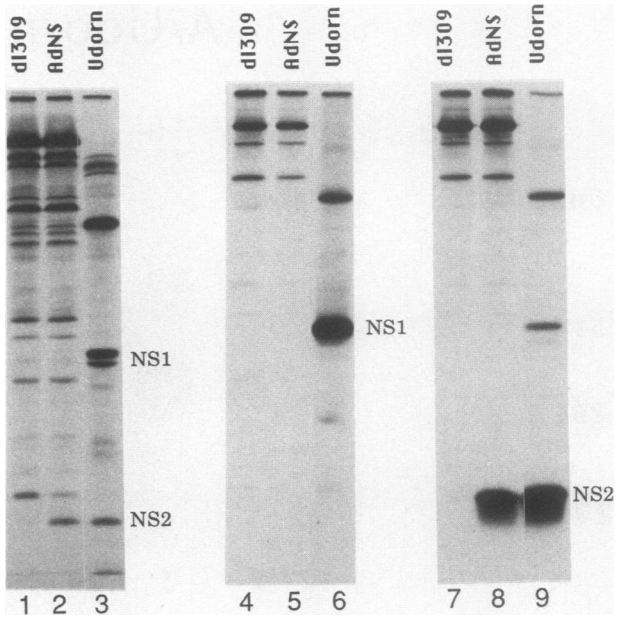

FIG. 4. Analysis of NS1 and NS2 protein synthesis in AdNS-infected cells. At 20 h postinfection with either wild-type adenovirus (*dl*309) or with AdNS, or at 4 h postinfection with influenza virus A/Udorn/72, 293 cells were labeled for 1 h with 100 μCi of [^{35}S] methionine per ml. Cells were lysed and proteins were either analyzed directly on SDS–14% polyacrylamide gels (lanes 1 to 3) or were first immunoprecipitated with monospecific antisera against the NS1 protein (lanes 4 to 6) or against the NS2 protein (lanes 7 to 9) prior to gel electrophoresis. The aliquot from the influenza virus-infected cells used in lane 6 was 1/10 that used in lane 9.

was blocked because of the commitment of the mRNA to the splicing pathway.

DISCUSSION

We have shown that the rate of splicing of NS1 mRNA encoded in an adenovirus recombinant (AdNS) was not significantly different from the splicing rate of NS1 mRNA in influenza virus-infected cells. It can then be presumed that the same splicing rate occurs with other DNA vectors encoding NS1 mRNA. It is not feasible to measure the splicing rate with most other DNA vectors because the smaller amount of NS1 mRNA synthesis with these vectors precludes the use of short pulses of [^{3}H]uridine. Even with the AdNS vector, it was necessary to expose the X-ray film of the gel analysis of the pulse-labeled RNA for 8 months (Fig. 2). It can be concluded that the rate of splicing of NS1 mRNA is independent of the following factors: (i) the genetic material, DNA or RNA, from which NS1 mRNA is transcribed; (ii) the enzyme system, host RNA polymerase II or the influenza virus transcriptase, that synthesizes NS1 mRNA; (iii) the presence of additional 5′ and 3′ sequences in the AdNS-encoded NS1 mRNA; and (iv) all virus-specific proteins, except possibly the NS2 protein, the only influenza virus-specific protein synthesized in AdNS-infected cells.

The most likely conclusion is that the rate of splicing of NS1 mRNA in influenza virus-infected cells is controlled solely by *cis*-acting sequences in NS1 mRNA itself. Candidates for such *cis*-acting sequences have been identified in in vitro splicing experiments. Though NS1 mRNA contains 5′ and 3′ splice sites that closely fit the consensus sequence (17), it was not detectably spliced in vitro with nuclear extracts from uninfected (or infected) HeLa cells (25). Only extremely small amounts of a splicing intermediate, intron lariat-3′ exon, were detected. Nonetheless, NS1 mRNA, like other splicing precursors, formed ATP-dependent 55S complexes containing the U1, U2, U4, U5, and U6 small nuclear

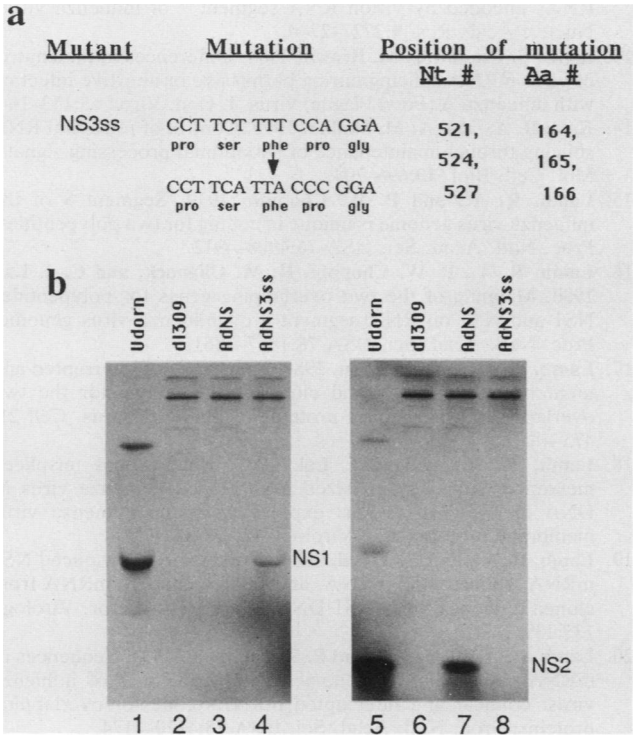

FIG. 5. Analysis of NS1 and NS2 protein synthesis in cells infected with AdNS3ss. (a) Prior to insertion into the adenovirus vector, pDL1, the three point mutations shown were introduced into the NS1 gene by site-directed mutagenesis. (b) After 293 cells were infected with wild-type adenovirus (*dl*309), AdNS, or AdNS3ss for 20 h, or at 4 h postinfection with influenza virus A/Udorn/72, the cells were labeled with 100 μCi of [^{35}S]methionine per ml for 1 h. Aliquots of the cell lysates were subjected to immunoprecipitation with anti-NS1 (lanes 1 to 4) or anti-NS2 (lanes 5 to 8) antibodies, followed by electrophoresis on SDS–20% polyacrylamide gels. The aliquot used in lane 1 was 1/10 that used in lane 5. Nt, Nucleotide; Aa, amino acid.

ribonucleoproteins (1). These 55S complexes had the properties of spliceosomes except that essentially no catalysis occurred, not even of the first step in splicing. We have recently identified two sequence elements in NS1 mRNA, one in the intron and one in the 3′ exon, that are responsible for the block in splicing after spliceosome formation (23a). It can be proposed that these sequences also operate in vivo, where they would decrease the rate of splicing rather than block splicing completely.

The dramatic increase in the extent of splicing of NS1 mRNA in AdNS-infected cells, relative to that occurring in influenza virus-infected cells, can be attributed largely, if not totally, to a block in the transport of unspliced NS1 mRNA. This block occurred because NS1 mRNA was committed to the splicing pathway. When the 3′ splice site of NS1 mRNA was inactivated by mutation, NS1 mRNA was transported. In contrast, Chang and Sharp (7), using a β-globin transcript as a precursor, found that mutations in either the 3′ splice site or the 5′ splice site did not lead to transport of the unspliced precursor from the nucleus. Mutations in both splice sites were needed. These authors argued that the splicing machinery recognizes either a wild-type 5′ splice site or a 3′ splice site even when the partner splice site is mutant and that this recognition leads to the retention of the pre-mRNA in the nucleus. Clearly, this cannot be a general rule because NS1 mRNA was transported from the nucleus when it had a wild-type 5′ splice site and a mutant 3′ splice site. It should be noted that in yeast cells, deletion of either the 5′ splice site or the branchpoint sequence from a pre-mRNA resulted in the transport of most of the pre-mRNA (21).

Most, if not all, of the NS1 mRNA was retained on nuclear spliceosomes in AdNS-infected cells. In contrast, with other DNA vectors encoding NS1 mRNA (19), transport of some NS1 mRNA must have occurred because some NS1 protein was synthesized, and, presumably as a consequence of this transport, the extent of splicing of NS1 mRNA was intermediate between that in AdNS-infected cells and that in influenza virus-infected cells. Recently, we have determined the extent of transport of NS1 mRNA expressed with a transient transfection vector; a small fraction (about 10%) of the NS1 mRNA was transported, resulting in a lesser extent of splicing than in AdNS-infected cells (2b). Hence, the complete retention of NS1 mRNA on nuclear spliceosomes occurred only in cells infected by the adenovirus recombinant. In cells in which NS1 mRNA was expressed with other DNA vectors, a significant, though small, fraction of NS1 mRNA escaped from nuclear spliceosomes and was transported. It is not likely that the retention of unspliced NS1 mRNA in the nuclei of AdNS-infected cells reflects the action of the adenovirus block in nucleocytoplasmic transport of cellular mRNAs (4) because mRNAs expressed from heterologous genes integrated into the adenovirus genome are not subject to this block (2, 5, 6, 23), and, in fact, spliced NS2 mRNA is efficiently transported. One possible explanation is that the nucleocytoplasmic transport of even virus-expressed mRNAs in adenovirus-infected cells is significantly slower than the transport of all mRNAs in uninfected cells. As a consequence, splicing rather than transport of NS1 mRNA would predominate.

In influenza virus-infected cells, most of the NS1 mRNA fails to be retained on nuclear spliceosomes and is transported. It is not known how unspliced NS1 mRNA is efficiently transported in influenza virus-infected cells. With human immunodeficiency virus type 1, a virus-encoded protein, the Rev protein, mediates the transport of unspliced and incompletely spliced pre-mRNAs from the nucleus (22). It is conceivable that there is an influenza virus counterpart to the Rev protein. As one approach to demonstrating the existence of such an influenza virus factor, AdNS-infected cells were superinfected with influenza virus. This did not lead to the transport of the NS1 mRNA that was encoded in the AdNS genome (2a). Thus, if there was in fact an influenza virus-specific factor required for the transport of NS1 mRNA, it did not act in *trans* on the adenovirus-encoded NS1 mRNA.

Our results indicate that the extent of splicing of NS1 mRNA is almost certainly determined by competition between the splicing rate and the rate of nucleocytoplasmic transport of NS1 mRNA, as predicted by the two-compartment steady-state kinetic model for the generation of spliced mRNA (see Materials and Methods). When NS1 mRNA is expressed with a DNA vector, its transport is either totally blocked (adenovirus vector) or inefficient (19), and transport is the rate-limiting factor that determines the extent of splicing. Even though *cis*-acting sequences probably suppress the rate of NS1 mRNA splicing, NS1 mRNA resides in the nucleus for relatively long periods of time and is extensively spliced. In contrast, in influenza virus-infected cells, transport of NS1 mRNA is efficient, and the rate of splicing

largely, if not totally, determines the extent of splicing. If an inhibition in the transport of NS1 mRNA occurred in influenza virus-infected cells, then different extents of splicing would be expected to result. This could account for the host-dependent effects on the extent of NS1 mRNA (and M1 mRNA) splicing that have been reported elsewhere (13, 27). Such host-dependent effects on the transport of NS1 mRNA (and M1 mRNA) would be expected to have profound effects on the course of influenza virus replication.

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REFERENCES

1. **Agris, C. H., M. E. Nemeroff, and R. M. Krug.** 1989. A block in mammalian splicing occurring after formation of large complexes containing U1, U2, U4, U5, and U6 small nuclear ribonucleoproteins. Mol. Cell. Biol. **9**:259–267.
2. **Alonso-Caplen, F. V., M. G. Katze, and R. M. Krug.** 1988. Efficient transcription, not translation, is dependent on adenovirus tripartite leader sequences at late times of infection. J. Virol. **62**:1606–1616.

2a. **Alonso-Caplen, F. V., and R. M. Krug.** Unpublished data.

2b. **Alonso-Caplen, F. V., M. E. Nemeroff, and R. M. Krug.** Unpublished data.

3. **Arrigo, S., and K. Beemon.** 1988. Regulation of Rous sarcoma virus RNA splicing and stability. Mol. Cell. Biol. **8**:4858–4867.
4. **Babich, A., L. T. Feldman, J. P. Nevins, J. E. Darnell, Jr., and C. Weinberger.** 1983. Effect of adenovirus on metabolism of specific host mRNAs: transport control and specific translational discrimination. Mol. Cell. Biol. **3**:1212–1221.
5. **Berkner, K. L., and P. A. Sharp.** 1984. Expression of dihydrofolate reductase and of the adjacent E1b region in an Ad5-dihydrofolate reductase recombinant virus. Nucleic Acids Res. **12**:1925–1941.
6. **Berkner, K. L., and P. A. Sharp.** 1985. Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant. Nucleic Acids Res. **13**:841–857.
7. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59**:789–795.
8. **Graham, F., J. Smiley, W. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
9. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–438.
10. **Greenspan, D., M. Krystal, S. Nakada, H. Arnheiter, D. S. Lyles, and P. Palese.** 1985. Expression of influenza virus NS2 nonstructural protein in bacteria and localization of NS2 in infected eucaryotic cells. J. Virol. **54**:833–843.
11. **Inglis, S. C., T. Barrett, C. M. Brown, and J. W. Almond.** 1979. The smallest genome RNA segment of influenza virus contains two genes that may overlap. Proc. Natl. Acad. Sci. USA **76**:3790–3794.
12. **Inglis, S. C., and C. M. Brown.** 1981. Spliced and unspliced RNAs encoded by virion RNA segment 7 of influenza virus. Nucleic Acids Res. **9**:2727–2740.
13. **Inglis, S. C., and C. M. Brown.** 1984. Differences in the control of virus mRNA splicing during permissive or abortive infection with influenza A (Fowl Plague) virus. J. Gen. Virol. **65**:153–164.
14. **Katz, R. A., and A. M. Skalka.** 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. Mol. Cell. Biol. **10**:696–704.
15. **Lamb, R. A., and P. W. Choppin.** 1979. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc. Natl. Acad. Sci. USA **76**:4908–4912.
16. **Lamb, R. A., P. W. Choppin, R. M. Chanock, and C.-J. Lai.** 1980. Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. Proc. Natl. Acad. Sci. USA **78**:1857–1861.
17. **Lamb, R. A., and C.-J. Lai.** 1980. Sequences of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. Cell **21**: 475–485.
18. **Lamb, R. A., and C.-J. Lai.** 1982. Spliced and unspliced messenger RNAs synthesized from cloned influenza virus M DNA in an SV40 vector: expression of the influenza virus membrane protein (M1). Virology **123**:237–256.
19. **Lamb, R. A., and C.-J. Lai.** 1984. Expression of unspliced NS1 mRNA, spliced NS2 mRNA, and spliced chimera mRNA from cloned influenza virus NS1 DNA in an SV40 vector. Virology **135**:139–147.
20. **Lamb, R. A., C.-J. Lai, and P. W. Choppin.** 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc. Natl. Acad. Sci. USA **78**:4170–4174.
21. **Legrain, P., and M. Rosbash.** 1989. Some cis- and trans-acting mutants for splicing target pre-mRNA to the cytoplasm. Cell **57**:573–583.
22. **Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 Rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338**:254–257.
23. **Mansour, S. L., T. Grodzicker, and R. Tjian.** 1985. An adenovirus vector system used to express polyoma virus tumor antigens. Proc. Natl. Acad. Sci. USA **82**:1359–1363.

23a. **Nemeroff, M. E., and R. M. Krug.** Unpublished data.

24. **Pikielny, C. W., and M. Rosbash.** 1985. mRNA splicing efficiency in yeast and the contribution of nonconserved sequences. Cell **41**:119–126.
25. **Plotch, S. J., and R. M. Krug.** 1986. *In vitro* splicing of influenza viral NS1 mRNA and NS1-β-globin chimeras: possible mechanisms for the control of viral mRNA splicing. Proc. Natl. Acad. Sci. USA **83**:5444–5448.
26. **Shapiro, G. I., T. Gurney, Jr., and R. M. Krug.** 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J. Virol. **61**:764–773.
27. **Smith, D. B., and S. C. Inglis.** 1985. Regulated production of an influenza virus spliced mRNA mediated by virus-specific products. EMBO J. **4**:2313–2319.
28. **Young, J. F., U. Desselberger, P. Palese, B. Ferguson, A. R. Shatzman, and M. Rosenberg.** 1983. Efficient expression of influenza virus NS1 nonstructural proteins in *E. coli*. Proc. Natl. Acad. Sci. USA **80**:6105–6109.