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Effect of Mutations and Deletions in a Bicistronic mRNA on the Synthesis of Influenza B Virus NB and NA Glycoproteins

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The mRNA derived from influenza B virus RNA segment 6 is functionally bicistronic and encodes the NB and NA glycoproteins in different, overlapping reading frames. NB protein synthesis is initiated at the 5′-proximal AUG codon, and 4 nucleotides downstream there is a second AUG codon which is used to initiate NA protein synthesis. The nucleotide sequence context of the first AUG codon conforms closely with the established 5′-CC(A/G)CCAUGG-3′ consensus sequence (M. Kozak, Nucleic Acids Res. 15:8125–8148, 1987), which should favor initiation of NB protein synthesis at this site, yet NB and NA are found to accumulate in approximately equal amounts in infected cells. To determine the features important for allowing initiation at the second 5′-proximal AUG codon, we made changes in the 5′-terminal region of the mRNA, including deletions, insertions, and site-specific mutations. The recombinant DNA molecules were expressed in eucaryotic cells, and the accumulation of NB and NA was quantitated. The data indicate that changes in the immediate sequence around the first AUG codon do not make a large difference in the amounts of NB and NA that accumulate, but that when the first AUG codon is displaced from its normal position it is now quite efficient at preventing downstream initiation events. In addition, the data indicate that an element of the B/NB/NA mRNA 5′ untranslated leader region acts in *cis* to enhance the expression of NB and NA.

It has been determined from surveys of the nucleotide sequences of eucaryotic cellular and viral mRNAs that protein synthesis usually initiates at the AUG codon nearest the 5′ end of the mRNA (20–22, 24, 32). However, approximately 10% of mRNAs do not fit this pattern, since protein synthesis begins in these mRNAs at initiation codons that are downstream of the first AUG codon (24, 32). The consensus nucleotide sequence containing the AUG codon for the initiation of protein synthesis is 5′-CC(A/G)C CAUGG-3′ (29), although less than 5% of natural eucaryotic mRNAs have the ideal consensus. Nonetheless, these sequences are thought to be an important determinant for translation initiation (21, 22). These conclusions are drawn from experiments involving site-specific mutagenesis of cloned cDNAs and expression of the mutants in eucaryotic cells, and they indicate that a large variability in protein production occurs depending on the nucleotide sequence context of the AUG initiation codon (13, 23, 25, 26, 28, 38, 39, 53). Both the A or G residue at position −3 (the A of the initiation codon is designated as position +1) and the G at position +4 have been found to be the nucleotides of greatest importance in determining initiation, but their contributions are not simply additive. For example, when an A is at −3, the presence of a G rather than a U in the +4 position makes less than a twofold difference, but the positive effect of a G at +4 is more significant when an A residue is not at position −3 (27).

A scanning model has been introduced to describe the mechanism by which eucaryotic mRNAs initiate translation (22). Ribosomes and initiation factors are proposed to bind at or near the 5′ end of the mRNA in a process facilitated by the 5′ cap structure and migrate in a linear manner on the mRNA, scanning until they reach the first AUG initiation codon. Two modifications were later made to the model, because several eucaryotic viral mRNAs have been reported which are polycistronic (26, 27). It is now thought likely that ribosomes can terminate translation of an upstream open reading frame and then resume scanning and initiate translation at the AUG codon of the downstream reading frame (27, 38). In addition, some viral mRNAs are functionally bicistronic, with protein synthesis occurring at two different AUG codons that are not separated by a termination codon; either the same open reading frame or two different overlapping reading frames are used (reviewed in reference 29). In these mRNAs, the 5′-proximal initiation codon is found to be in a suboptimal nucleotide sequence context as compared with the consensus sequence. It is presumed that initiation occurs at the second AUG in addition to the first AUG, because some ribosome preinitiation complexes bypass the 5′-proximal AUG (leaky scanning) and initiate protein synthesis at the second AUG codon (reviewed in references 29 and 30). The only reported exception to this hypothesis is the mRNA produced from influenza B virus RNA segment 6 (30).

Determination of the nucleotide sequence of RNA segment 6 of influenza B virus from cloned cDNA (B/NB/NA DNA) indicated the presence of two overlapping open reading frames with the AUG initiation codons separated by 4 nucleotides (52). The first open reading frame of 100 amino acids encodes NB, and the second open reading frame encodes NA (51). Both NB and NA have been expressed from cloned cDNA, providing further evidence that the mRNA is functionally bicistronic (55). The rules of the scanning hypothesis suggest that the NB AUG initiation codon is in a good context for translation initiation, and it would be expected that this is where the majority of initiation events should occur (30) (Fig. 1). However, it has been found that in influenza B virus-infected cells, NB and NA

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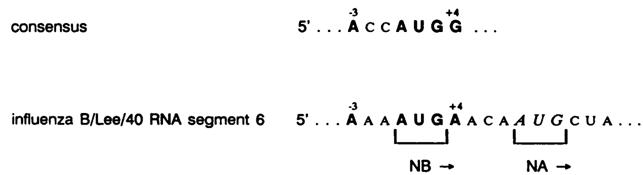

FIG. 1. Comparison of the nucleotide sequences surrounding the B/NB/NA initiation region with the consensus strong initiation context. The nucleotide sequences are shown for the region surrounding the translation initiation sites of B/NB/NA (data from references 52) and for a consensus strong initiation context (data from reference 29). The −3 and +4 nucleotides of the consensus sequence and the first B/NB/NA initiation codons are indicated in boldface type, and the initiation codons for NB and NA are shown in boldface and italic type, respectively.

accumulate in roughly equimolar amounts (51), suggesting that the ribosome preinitiation complex bypasses the first AUG codon more often than expected. The B/NB/NA mRNA system has been described as an exception to the scanning hypothesis (30), and thus this naturally occurring B/NB/NA initiation region is a unique model system.

To investigate the role of the nucleotide residues surrounding and including the B/NB/NA AUG initiation codons, we altered the nucleotide sequence of a cDNA clone of B/NB/NA by site-specific mutagenesis and expressed the resulting mutants in eucaryotic cells. The relative amounts of NB and NA protein that accumulated were used as a measure of initiation at each of the AUG codons. Data presented here indicate that changes in the immediate sequence around the first AUG codon do not make large differences in the amounts of NB and NA that accumulate, but that when the first AUG codon is displaced from the normal NB/NA initiation region, it is now more efficient at preventing downstream initiation events. In addition, the presence of the B/NB/NA-specific 5′ untranslated leader was found to enhance the expression of NB and NA.

MATERIALS AND METHODS

Viruses and cells. Influenza virus B/Lee/40 was grown in embryonated eggs as described previously (33, 35). Recombinant simian virus 40 (SV40) strains (SV40-BNA) that have been engineered to express the B/NB/NA DNA were grown as described previously (37, 55). CV1 cells were maintained and infected with viruses as described previously (33, 34, 37).

Isotopic labeling of infected cells, immunoprecipitation of infected cell lysates, and polyacrylamide gel electrophoresis. Cells were labeled with [^{35}S]cysteine from 7 to 9 h postinfection (p.i.) for influenza B virus infections and at 48 to 51 h p.i. for recombinant SV40 infections as described previously (55). [^{35}S]cysteine-labeled infected-cell lysates were immunoprecipitated as described previously (36). Antibodies used were as follows: a rabbit antiserum prepared against gel-purified NB (DNB) (55); rabbit anti-NA sera (kindly provided by R. G. Webster); and a tissue culture supernatant of SV40 T-antigen monoclonal antibody (MAb402) kindly made available by J. W. Yewdell. Immunoprecipitated samples were analyzed on 17.5% polyacrylamide gels containing 4 M urea as described previously (36).

Construction and expression of SV40-BNA recombinant viruses. The construction of the two parental recombinant viruses pSV93-BNAΔ4 and pSV93-BNAΔ39 that express NB and NA but differ in the length of influenza virus-specific 5′ untranslated region have been described previously (55). Transfection of DNA into CV1 cells and production of recombinant virus stocks were done as described previously (37).

Mutagenesis of B/NB/NA initiation region. Site-specific mutagenesis was done by using the method of Zoller and Smith (58) and mismatch oligonucleotides as described previously (55). Oligonucleotides were synthesized on a 380B synthesizer (Applied Biosystems, Inc., Foster City, Calif.) at the Northwestern University Biotechnology Facility. To reiterate upstream AUG codons, such that the NB and NA AUG initiation codons are effectively separated, two complementary oligonucleotides corresponding to B/NB/NA cDNA nucleotides 35 to 82 were annealed and ligated into the *Hae*III site at nucleotide 39 of B/NB/NA DNA. The insertion of these sequences extends the NB open reading frame at its 5′ end, adding an N-terminal extension of 14 amino acids to the NB protein and reiterating the NB N terminus except for a lysine residue at the fusion site. The smaller *Bam*HI fragment that contained the 5′-end extension and B/NB/NA sequences was ligated into an SV40 expression vector plasmid (pSV93) that contained the larger 3′-end *Bam*HI fragment of B/NB/NA cDNA, and molecules were screened for the appropriate orientation for the production of B/NB/NA mRNA by the SV40 late-region promoter: this construction was designated NBX. The DNA sequence of the extension and fusion regions was confirmed by chemical sequencing of 5′-end-labeled DNA fragments (41).

Determination of the molar ratios of NB and NA present in infected-cell lysates. CV1 cells were infected with influenza B virus, labeled at 8 h p.i. with [^{35}S]cysteine for 20 min, and lysed. A sample of lysate was analyzed directly on gels, and a portion of the lysate was treated with 0.1 U of *N*-glycanase at 37°C for 1 h and then analyzed on gels. Autoradiographs were used as templates to excise the NB-specific and NA-specific bands. The gel pieces were rehydrated, and the radioactivity present in each band was quantitated by liquid scintillation counting (34). The background signal from gel slices containing no known protein band was subtracted, and then the molar ratio was determined by using the known number of cysteine residues present in each protein (7 in NB, 11 in NA) (52). The molar ratios of the amounts of unglycosylated NB (NB_0) and NA that accumulated in SV40-B/NB/NA recombinant virus (SV93-BNAΔ4)-infected cells were determined in a similar manner.

Quantitation of NB and NA expression levels in recombinant-virus-infected cells. CV1 cells were infected with recombinant SV40-B/NB/NA viruses and labeled at 48 h p.i. with 200 μCi of [^{35}S]cysteine for 3 h. Because the SV40 recombinant virus stocks differ in titer, it was necessary to devise a standardization scheme. A portion of the infected cell lysate was immunoprecipitated with monoclonal antibody to T antigen, and the amount of T antigen was quantitated from autoradiographs as described below. The relative expression level of T antigen by each recombinant virus was determined, and the immunoprecipitation was repeated by using a volume of infected-cell lysate adjusted for the relative expression level, such that on the final gel the relative levels of T antigen would be equivalent. Adjusted volumes of the same labeled cell lysates were also immunoprecipitated with rabbit anti-NB sera and goat anti-NA sera. To analyze NB as a single polypeptide species, we treated the immunoprecipitated NB with 0.1 U of *N*-glycanase for 3 h at 37°C. In all immunoprecipitation experiments the antibody concentration was in at least a twofold excess over the highest antigen concentration, and all antigen was quantitatively recovered.

Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gels were prepared for fluorography, and the X-ray films were scanned by using the LKB Ultroscan laser densitometer (Pharmacia, Inc., Piscataway, N.J.). The exposure times of the fluorographs were varied to allow each protein band to be quantitated in the linear range of the film response. Densitometer data were analyzed by using LKB Gelscan XL computer software. The amounts of NB and NA were normalized to the amount of T antigen found in the adjusted lysate volumes.

RESULTS

The molar ratio of NB to NA. It has been previously reported that NB and NA are synthesized in equimolar amounts in infected cells (51). However, it has recently been found that the carbohydrate chains on the high-mannose form (NB_{18}) are processed to a heterogeneous form of much higher molecular weight containing polylactosaminoglycan (NB_p) (56). Therefore, it was necessary to reexamine the relative molar ratio of NB to NA proteins that accumulate in influenza B virus-infected cells. In addition, the molar ratio of NB to NA expressed by the recombinant SV40 (SV93-BNAΔ4; see below) was determined. Infected cells were pulse-labeled with [^{35}S]cysteine for 20 min so that the majority of NB would be found in the NB_{18} form, and the molar ratio of NB to NA was determined as described in Materials and Methods. In influenza virus-infected cells the molar ratio of NB_{18} to NA accumulated was 0.9:1, while the ratio of NB_0 to NA, after *N*-glycanase treatment of the NB immunoprecipitated samples, was 0.6:1. The molar ratio of NB_0 to NA in the recombinant SV93-BNAΔ4-infected cells was 0.6:1. Thus, use of the SV40 expression vector to synthesize NB and NA does not change the level of accumulation of these proteins from that observed in influenza virus-infected cells. The accumulation of NB and NA is thought to reflect the number of initiation events, because both NB and NA are stable in CV1 cells with half-lives of more than 6 h (data not shown).

Structure of mutations in the 5′ region of B/NB/NA mRNA. To investigate a possible contribution of the 5′ untranslated region of B/NB/NA mRNA in the accumulation of NB and NA in cells, we used two recombinant viruses differing in the length of 5′ untranslated region derived from B/NB/NA mRNA. SV93-BNAΔ4 lacks only the 5′-proximal 4 nucleotides, whereas SV93-BNAΔ39 lacks all the influenza virus-specific 5′ untranslated region except for 6 nucleotides before the NB initiation codon (Fig. 2). Both recombinant viruses express NB and NA in infected cells as described previously (55).

To examine whether the hypothesized ribosome scanning occurs on the B/NB/NA mRNA, we separated the NB-specific and NA-specific initiation codons. DNA sequences containing the NB AUG codon in its 5′-proximal context and followed by the nucleotides encoding the first 14 amino acid residues of NB were inserted into a position 5′ to the NB protein synthesis initiation region. The mutant NBX was generated (Fig. 2 and 3A). In the 5′ extension, the NA-specific AUG codon following the first NB AUG codon was changed to an AAC, since the objective was to examine protein synthesis from the NB AUG codon in isolation from that of NA.

Nucleotide substitutions in the initiation codon region of B/NB/NA mRNA. To examine the relative importance of the nucleotide sequence context of the first initiation codon of B/NB/NA mRNA in permitting the synthesis of both NB and NA, we made site-specific mutations in the initiation region by oligonucleotide-directed mutagenesis (Fig. 3B). Three mutations were made which changed the nucleotide sequence of the first AUG from the wild-type 5′-AAAAUGA (A−3; A+4) to contexts predicted from data obtained with other systems to be more favorable (G+4) or less favorable (C−3; C+4) (27) for initiation of NB protein synthesis. Another mutant was made in which the A residue at postion −2 was changed to a C. Again, the B/NB/NA mutant C−2 could be predicted to slightly enhance the initiation of NB but to have less of an effect than that of the G+4 mutant (27). Two further mutations were made in which the initiation codons for NB and NA were changed to ACC (NB) or ACG (NA). These were synthesized to provide further evidence for the bifunctional nature of B/NB/NA mRNA and to investigate a possible synergistic effect of the two AUG codons on protein synthesis initiation.

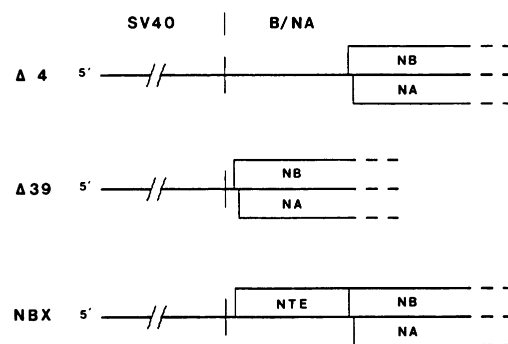

FIG. 2. Schematic diagram of the mRNA structure of the B/NB/NA initiation region mutants. The mRNA structure predicted for the initiation region mutants is shown. Insertion of a foreign gene in the SV40-based late-region replacement vector (pSV93) placed the insert cDNA between the unique *Hae*II and *Bam*HI sites of SV40 and under the control of the SV40 late promoter and polyadenylation signals. The vector also contains a 183-nucleotide deletion (SV nucleotides 336 to 519) spanning the agnoprotein initiation codon (19). The region in the diagram designated SV40 represents untranslated leader sequences derived from the vector, and the region designated B/NA is derived from the B/NB/NA cDNA insert. The 5′ untranslated leader regions are expected to contain approximately 324 nucleotides at the 5′ end transcribed from the SV40 late region, beginning at the major start site of SV40 late mRNA (3) (nucleotide 325), fused to the influenza B virus-derived nucleotide sequence at nucleotide 832. The coding regions for NB and NA are shown by boxes. The construction labeled Δ4 is considered wild type, and this mRNA contains an SV40-derived 5′ end and 43 of 46 influenza B virus-specific nucleotides in the 5′ untranslated leader region. The initiation region point mutants to be described below, C−3, C−2, C+4, G+4, NA−, and NB−, have the general mRNA structure designated Δ4 and are shown in more detail in Fig. 3. The construction labeled Δ39 contains the SV40-derived 5′ end and 6 influenza B virus-specific nucleotides in the 5′ untranslated leader region. The N-terminal extension of the NB coding regions in the mutant NBX (14 amino acid residues) is designated by the box labeled NTE. The mutant NBX is shown in further detail in Fig. 3.

Quantitation of the in vivo expression of the B/NB/NA initiation region mutants. SV40 recombinant molecules containing wild-type Δ4 B/NB/NA DNA and DNA of each of the mutants shown in Fig. 3 were used to transfect CV1 cells together with DNA of SV40 early-region deletion mutant *dl*1055 (46), as a helper virus, and recombinant SV40 virus stocks were made. CV1 cells were infected with the recombinant viruses, labeled at 40 h p.i. with [^{35}S]cysteine for 3 h, and then lysed for immunoprecipitation. Because NB mi-

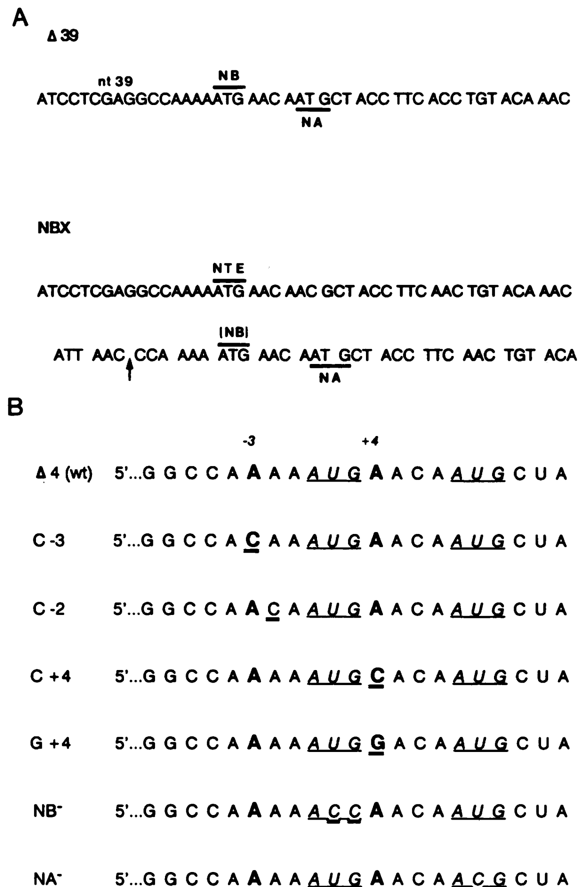

FIG. 3. Nucleotide sequences of the mutated regions of B/NB/NA. (A) The nucleotide sequences of the mutants Δ39 and NBX are shown. B/NB/NA nucleotide 39 is indicated, and an arrow denotes the point of fusion of the extension oligonucleotide to B/NB/NA cDNA to form mutant NBX. The initiation codons for the N-terminal extension (NTE), NB, and NA proteins are indicated, and the second, in-frame initiation codon in the N-terminal extension mutants is indicated by brackets. (B) The nucleotide sequences of the initiation region mutants are shown for the regions surrounding the initiation codons of NB and NA. The −3 and +4 sites relative to the NB AUG codon are indicated in boldface type, and the NB and NA initiation codons are italicized. The mutants are named after the nucleotide(s) altered, except NB− and NA−, which indicate the elimination of the initiation codon. The nucleotides changed by mutagenesis are doubly underlined.

grates on gels in more than one form (NB_{18} [high-mannose sugar form] and NB_p [polylactosaminoglycan-containing form]) (56), samples were treated with *N*-glycanase to remove all N-linked carbohydrate from NB such that it would migrate on gels as unglycosylated NB_0 (M_r ca. 10,500 [55, 56]).

To compare the relative expression levels of NB and NA synthesized by the different recombinant viruses, we determined the amount of SV40 T antigen synthesized so that the effect of different titers of the virus stocks could be normalized. With the vector system used, full-length SV40 T antigen is the only protein uniquely produced in *cis* with the NB and NA proteins (40, 46). Even though SV40 T antigen negatively regulates SV40 early transcription (1, 48, 49, 54), the relative accumulation of T antigen reflects the number of input genomes, since all the recombinant-virus-infected cells were examined at the same time p.i. (49). Thus, each

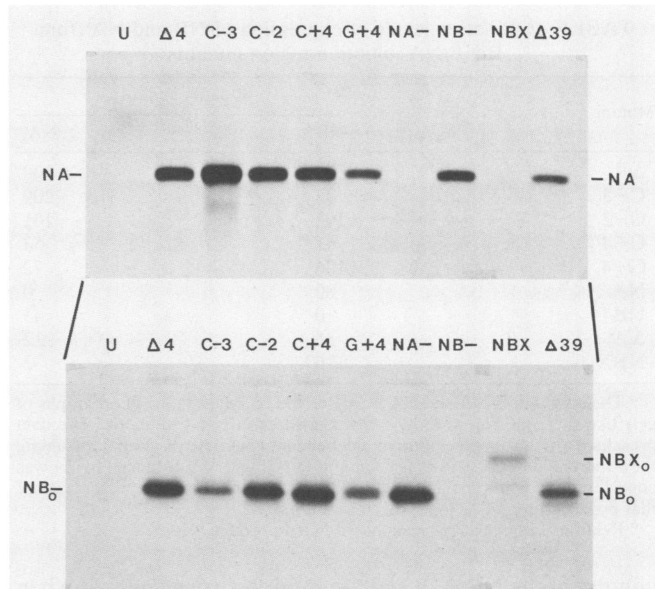

FIG. 4. Expression of B/NB/NA initiation mutants in an SV40 recombinant vector. CV1 cells were infected with recombinant SV40 containing the B/NB/NA initiation mutants and labeled for 3 h with [^{35}S]cysteine at 48 h p.i. Cells were lysed, and differences in infectivity of each virus stock were corrected as described in Materials and Methods. Adjusted amounts of each lysate were immunoprecipitated with antisera specific for NB or NA or T antigen. The NB-specific immunoprecipitates were treated with 0.1 U of *N*-glycanase at 37°C for 3 h, and the proteins were visualized by gel electrophoresis on separate gels. Lanes: U, uninfected cells; Δ4 to Δ39, expression of the mutants indicated. Only the relevant region of the gels showing the NA immunoprecipitation and the NB immunoprecipitation are shown.

[^{35}S]cysteine labeled, recombinant-virus-infected cell lysate was immunoprecipitated with a twofold excess of either T antigen-, NB-, or NA-specific antibody.

The amounts of NB, NA, and related hybrid proteins that accumulated in the labeling period in the mutant-infected cells are shown in Fig. 4. Although the relative amount of NB and NA for any given mutant can be directly compared, the amounts of NB and NA between mutants are not finally adjusted for an equal amount of T antigen. However, they are a close approximation of the corrected expression levels determined after laser densitometry of the autoradiograms (Table 1). The DNB antiserum (55) immunoprecipitated from uninfected cells a small amount of a protein of a similar (but different) mobility to that of NB_0 (Fig. 4). This cellular protein is an artifact of the immunoprecipitation or *N*-glycanase treatment and does not affect the data described here.

The 5′-terminal extension mutation. The protein product immunoprecipitated by NB-specific sera and derived from the 5′-terminal extension mutation NBX is designated NBX_0. It has a slower electrophoretic mobility than wild-type NB_0 does (Fig. 4), as would be expected from the addition of 14 N-terminal residues to NB. The synthesis of NB_0 and NA could barely be detected from the NBX DNA construction. Thus, these data for the NBX mutation can be interpreted to support the notion that ribosomes migrate in a linear manner from the 5′ end of the mRNA and that initiation of protein synthesis occurs at the 5′-proximal (extension) AUG codon. More importantly for the experiments described here, these data suggest that the sequence

TABLE 1. Relative levels of expression of NB and NA from B/NB/NA initiation region mutants

Mutant	Relative amt of:	
	NB[a]	NA[a]
Δ4	100	100
C−3	33	209
C−2	102	101
C+4	68	53
G+4	106	72
NA$^-$	80	0
NB$^-$	0	43
Δ39	35	12
NBX	16[b]	2

[a] The amounts of NB and NA were derived by quantitating fluorographs of gels like those in Fig. 4, as described in Materials and Methods. The data presented are the average of three independent experiments. Variation among the experiments was ±10%. The amount of NB and NA produced by Δ4 was considered the wild-type amount, and levels of expression are percentages of that produced by Δ4.

[b] Relative amount of N-terminal-extended product.

context of the NB AUG codon is fairly strong and that when it is separated from its natural position in the mRNA, initiation at this AUG codon severely limits initiation at downstream AUG codons.

Single-nucleotide changes in the B/NB/NA initiation region affect the level of NB and NA accumulation. The single-nucleotide changes around the NB AUG codon designated C−3 and C+4 (Fig. 3B) were examined to test whether they placed the first AUG codon in a less optimal context for initiation, thus causing reduced synthesis of NB and increased synthesis of NA. The C−3 mutation caused the accumulation of only one-third the normal amount of NB as did the wild type, whereas the amount of NA increased by more than twofold (Fig. 4; Table 1). However, the predicted result was not obtained with the C+4 mutation, since the amount of both NB and NA decreased relative to the wild type.

As discussed above with the mutants G+4 and C−2, it might be expected that they would have the phenotype of increased initiation at the first AUG codon and reduced initiation at the second AUG codon and that the G+4 mutation would have the greater effect (27). In fact, the amount of NB was virtually unchanged with the G+4 mutation, and the amount of NA was slightly reduced as compared with the wild type (Fig. 4; Table 1). The C−2 mutation had no effect on the accumulation of NB and NA as compared with the wild type.

If scanning of the mRNA by preinitiation complexes involves only strong-context AUG codons as defined by the consensus sequence described above, elimination of the first AUG codon (mutant NB$^-$) might be expected to cause an increase in initiation at the second AUG codon and hence increase the amount of NA. Correspondingly, elimination of the second AUG (mutant NA$^-$) should have little effect on NB protein production. Both the NB$^-$ and NA$^-$ mutations eliminated NB and NA synthesis, respectively, as expected, but the NA$^-$ mutation caused the accumulation of only 80% of NB as compared with the wild type, and the NB$^-$ mutation caused a twofold reduction in the amount of NA accumulated (Fig. 4; Table 1).

A role for the influenza virus-specific 5′ untranslated mRNA leader region in the efficient synthesis of NB and NA. Deletion of the influenza virus-specific 5′ untranslated leader region in mutant Δ39 greatly reduced the amount of NB and NA accumulation with respect to the wild type (Fig. 4; Table 1) and also altered the ratio of NB to NA, since approximately threefold more NB was found than NA (Table 1). The Δ39 mutant and the 5′-terminal extension mutant NBX have their 5′-proximal AUG codons in the same nucleotide sequence context as the first initiation codon in the mutant NA−, but the latter mutant contains the wild-type 5′ untranslated region, whereas NBX has the Δ39 deletion. It can be seen that with mutant NBX, smaller amounts of NB- and NA-related products accumulate than with mutant Δ39 (Table 1). Thus, these data suggest that the influenza virus-specific 5′ untranslated leader region of B/NB/NA mRNA has a positive effect on translation initiation, but they also suggest that the effects of the 5′ untranslated leader region and the initiation region may be cooperative.

DISCUSSION

The B/NB/NA mRNA is a naturally occurring mRNA, and from examination of the mRNA nucleotide sequence in conjunction with the rules of the modified scanning hypothesis (29), it would be expected that only NB and not NA would be synthesized in infected cells. However, NB and NA accumulate in a 0.6:1 ratio, suggesting that approximately 60% of ribosome preinitiation complexes on the B/NB/NA mRNA do not initiate protein synthesis at the first AUG codon, but use the second AUG codon 4 nucleotides downstream.

The data described here, as well as data from all previous studies on the effect of sequence context on protein synthesis initiation (e.g., references 13, 23, 26, 28, 31, 38, 39, and 53) have been obtained by using protein accumulation as a measure of in vivo protein synthesis initiation. Although not ideal, accumulation is the best available assay for proteins such as NB and NA which are stable, because determination of the rate of protein synthesis directly, using dipeptide synthesis rates (4), relies upon in vitro translation assays. However, such assays would be difficult to interpret, because the ratios of proteins produced from bicistronic mRNAs in vitro can vary from the ratio synthesized in vivo (5, 7, 8, 11, 44, 57).

A previous study of translation initiation on a synthetic mRNA containing two AUG codons separated by 5 nucleotides has been made on a mutated preproinsulin mRNA, and initiation at the second AUG was not found to occur (26). Thus, it was thought that the proximity of two AUG codons does not alter the rules of nucleotide sequence context for initiation of protein synthesis (26). The data presented here suggest that several features of the nucleotide sequence of the influenza B virus-specific NB/NA mRNA are involved in the control of the number of initiation events specifying the synthesis of NB and NA in infected cells. The data for the 5′-terminal extension mutant suggest that the 5′-proximal AUG codon is in a fairly strong context for initiation of translation when separated from the second AUG codon by 35 nucleotides, since initiation at downstream AUG codons was greatly reduced. The effects of some of the single-nucleotide changes on NB and NA synthesis were different from those that might be predicted on the basis of previous studies (24, 27), but our data do suggest that the A residue at position −3 is important for NB initiation. These data were obtained from three independent experiments with a variation of ±10% in protein levels, but previously it has been considered that in these types of experiments, differences in protein accumulation of twofold or less are insignificant (27). Thus, with the exception of the C−3 mutation it could be argued that none of the changes really affect the NB-to-NA

accumulation ratio. Although the data derived from the 5′-terminal extension mutation indicate that ribosome scanning can occur on the hybrid mRNA, the data obtained from the point mutations are consistent with the notion that with the naturally occurring NB/NA mRNA, once ribosomes arrive in the region of the two initiation codons, it is essentially a random choice as to which AUG is used. Thus, this implies that scanning does not occur over short distances on this mRNA. However, it is possible that other untested nucleotides outside the −3 to +4 region with respect to the NB AUG codon are important in determining the ratio of initiation events for NB and NA. Data supporting this idea were obtained in another study, in which it was found that the difference between the defective avian provirus *ev*-1 and a nondefective provirus was in nucleotides −4 and −7 of the AUG codon used for initiation of the proviral *gag* and *env* genes (14).

The deletion of 40 of the 46 influenza virus-specific 5′ untranslated-region nucleotides in mutants Δ39 and NBX has the largest effect on the accumulation of NB and NA. These data suggest that an unrecognized *cis*-acting element of the entire region, e.g., a specific secondary structure, also has a major role in the initiation of synthesis of NB and NA. With the expression vector used, the mRNAs contain 5′ additional SV40 late-region-specific sequences before the influenza virus B/NB/NA sequences, and initially it was of concern that they might alter the NB-to-NA ratio from that observed in influenza B virus-infected cells. However, as described above, the additional SV40-specific 5′ untranslated leader sequences on the mRNAs did not change the ratio of NB to NA synthesized in wild-type Δ4 recombinant-virus-infected cells.

The 5′ untranslated leader region of several other naturally occurring capped mRNAs has been shown to be important in the regulation of initiation of protein synthesis, including positive regulation of the heat shock protein HSP70 (6, 42) and trypsinogen (45) or negative regulation of type I collagen (50). Other data indicating that the 5′ untranslated region of mRNAs can alter the pattern of initiation of protein synthesis come from studies with an SV40 mutant (9). The 5′-proximal region of the untranslated leader region of the 16S late-region mRNA was replaced with the first 33 nucleotides of the adenovirus type 2 late-region 5′ untranslated leader, and it was found that only the upstream AUG codon initiating the agnoprotein was used: VP1, which is initiated by a downstream AUG codon, was not synthesized (9). Elimination of the 5′-proximal AUG codon from this mutant allowed the production of VP1 from the downstream reading frame, suggesting that the adenovirus 5′ untranslated leader region caused the loss of the normal bicistronic function (10). The importance of the intrinsic structure of individual mRNAs was also emphasized in a study in which the differential translation of the reovirus S_1 and S_4 mRNAs was examined (2). Recently, it has been shown that the 5′ untranslated region of the naturally uncapped picornavirus RNA contains a sequence element that allows translation initiation on the RNA via the binding of ribosomes to an internal entry site (12, 43). Thus, all these studies indicate a role of the 5′ untranslated region in initiation of protein synthesis.

The 5′ untranslated leader region nucleotides, perhaps specified by secondary structure elements, interact with specific cellular factors to mediate the initiation of protein synthesis. Initiation factor eIF-4A has been postulated to function as a discriminatory factor in the selection of reovirus mRNAs over cellular mRNAs for translation initiation by binding to a reovirus-specific nucleotide sequence or secondary structure and enhancing the initiation of synthesis of reovirus proteins (47). It is possible that an analogous process could occur in influenza B virus-infected cells. It has been suggested that influenza virus mRNAs have, as part of their linear mRNA sequence or secondary structure, signals that allow more efficient translation initiation with respect to cellular mRNAs (15, 16, 18). These signals could interact with cellular components that are modified by virus infection (17) or perhaps could interact with translation factors more efficiently than the signals of cellular mRNAs could, thereby limiting the factors to viral mRNAs. The 5′ untranslated region of the B/NB/NA mRNA may contain a signal that mediates the interaction of cellular factors to cause the formation of a specific RNA-protein structure which allows the synthesis of both NB and NA. Determination of the factors involved in the formation of this putative structure and the interaction of the ribosome preinitiation complex with the B/NB/NA mRNA is necessary to further understand the control of synthesis of NB and NA.

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