JOURNAL OF VIROLOGY, Oct. 1992, p. 6143–6154
0022-538X/92/106143-12$02.00/0

Vol. 66, No. 10

Internal Entry of Ribosomes on a Tricistronic mRNA Encoded by Infectious Bronchitis Virus

D. X. LIU[1] AND S. C. INGLIS[1,2]*

Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP,[1] and Cantab Pharmaceuticals Research, Ltd., 184 Cambridge Science Park, Milton Road, Cambridge CB4 4GN,[2] United Kingdom*

Received 9 April 1992/Accepted 20 July 1992

mRNA3 specified by the coronavirus infectious bronchitis virus appears to be functionally tricistronic, having the capacity to encode three small proteins (3a, 3b, and 3c) from separate open reading frames (ORFs). The mechanism by which this can occur was investigated through in vitro translation studies using synthetic mRNAs containing the 3a, 3b, and 3c ORFs, and the results suggest that translation of the most distal of the three ORFs, that for 3c, is mediated by an unconventional, cap-independent mechanism involving internal initiation. This conclusion is based on several observations. A synthetic mRNA whose peculiar 5′ end structure prevents translation of the 5′-proximal ORFs (3a and 3b) directs the synthesis of 3c normally. Translation of 3c, unlike that of 3a and 3b, was insensitive to the presence of the 5′ cap analog 7-methyl-GTP, and it was unaffected by alteration of the sequence contexts for initiation on the 3a and 3b ORFs. Finally, an mRNA in which the 3a/b/c infectious bronchitis virus coding region was placed downstream of the influenza A virus nucleocapsid protein gene directed the efficient synthesis of 3c as well as nucleocapsid protein, whereas initiation at 3a and 3b could not be detected. Expression of the 3c ORF from this mRNA, however, was abolished when the 3a and 3b coding region was deleted, indicating that 3c initiation is dependent on upstream sequence elements which together may serve as a ribosomal internal entry site similar to those described for picornaviruses.

Coronavirus gene expression occurs through the production of a set of 3′-coterminally capped, polyadenylated subgenomic mRNAs in infected cells (30). For the prototype coronavirus, infectious bronchitis virus (IBV), the third-largest of this nested set, mRNA3, appears to be functionally tricistronic (32). The mRNA contains at its 5′ end three open reading frames (ORFs) (5) now designated 3a, 3b, and 3c (8), which are all expressed in infected cells (32) but which are not found on the next-smallest mRNA (46). The implication that all three can be translated in the infected cell from a single mRNA species is further supported by the observation that a single synthetic mRNA can successfully direct synthesis of each of the three gene products in an in vitro translation system (32).

The precise mechanism by which mRNA3 may function polycistronically is of considerable interest. One possibility is through leaky scanning, as proposed by Kozak (24). In such a model, ribosomes would bind at the 5′ end of the mRNA and scan downstream to the initiation codon for the first ORF (3a). An occasional failure to initiate at this point would result in continued scanning downstream, introducing the possibility of initiation at the next available AUG (the start codon for 3b); failure once again would allow recognition of a third AUG, the initiation codon for 3c. One prediction from such a model would be that the efficiencies of initiation at the first and second ORFs (3a and 3b) would have to be relatively low if a reasonable balance of expression of the three gene products were to be maintained. Furthermore, the efficiencies of synthesis of the downstream ORFs should be directly dependent on the extent of ribosome loading at the 5′ end of the mRNA. A number of experimental observations suggested to us, however, that these predictions might not be fulfilled for translation of the 3c ORF. First, a synthetic mRNA bearing all three ORFs directed in wheat germ extracts the synthesis of much greater amounts of 3a than of 3b or 3c (32), suggesting that the 3a initiation codon can function efficiently, albeit in an in vitro system. Similarly, the 3b polypeptide was the most abundant product from a synthetic mRNA containing only the 3b and 3c ORFs, indicating again that the 3b initiation codon can be recognized efficiently. Thus, if a leaky scanning mechanism were operative, it might be expected that production of the 3c protein from mRNA3 would be very low in comparison to that of 3a and 3b. In infected cells, however, it appears that the 3c product, a 12,400-molecular-weight membrane-associated protein (12.4K protein) (33, 43), is the most abundant of the three (32). Further doubts about the leaky-scanning model arose from our analysis of the in vitro translation of a certain synthetic mRNA carrying all three ORFs as described above but with a different noncoding sequence at its extreme 5′ end. Here, synthesis of both the 3a and the 3b products was drastically reduced, suggesting that ribosome entry at the 5′ end had been inhibited, but the production of 3c appeared to be unaffected (32). This result therefore supported the idea that translation of the 3c ORF might be controlled independently of the upstream ORFs. We show here that this is indeed the case and that 3c translation operates by a cap-independent mechanism most likely involving internal ribosomal entry just upstream of the ORF, as has been described previously for initiation on a number of picornavirus mRNAs (for a review, see reference 17).

MATERIALS AND METHODS

Construction of plasmids. The plasmids pIBS1 and pIBS2, which contain the coding sequences for the IBV 3c protein

* Corresponding author.

alone and for the 3b and 3c proteins under control of the SP6 phage RNA polymerase promoter, were constructed as described by Smith et al. (44). Plasmids pIBT1 and IBT2 are equivalent to pIBS1 and pIBS2, except that they contain a T7 phage RNA polymerase, and their construction has been described by Liu et al. (32). Plasmid pIBS3.1 contains the 3a, 3b, and 3c ORFs adjacent to the SP6 promoter and was made by cloning a 910-bp *Bgl*II-*Hin*dIII restriction fragment containing the three ORFs from the plasmid pIBT4 (32) into *Bam*HI-*Hin*dIII-digested pSP65 (27). Plasmid pIBT3.1, like pIBS3.1, contains the 3a, 3b, and 3c ORFS, but in this case they are adjacent to the T7 RNA polymerase promoter. This plasmid (formerly called pIBT3) was constructed as previously described (32). Plasmid pIBS3.2 is identical to pIBS3.1 except that the 3a, 3b, and 3c ORFs have been fused together to create a single ORF (3abc). This plasmid was constructed by cloning a 913-bp *Bgl*II-*Hin*dIII restriction fragment containing the fused ORFs (3abc) from pIBM6 (32) into *Bam*HI-*Hin*dIII-digested pSP65. Plasmid pIBT3.2 was constructed by cloning the same fragment from pIBM6 into *Bam*HI-*Hin*dIII-digested pT7-1 and is thus similar to pIBS3.2 except that the IBV ORFs are adjacent to the phage T7 RNA polymerase and the mRNA transcribed from the plasmid has a slightly different 5′ untranslated region (5′ UTR).

Plasmids pIBM12 and pIBM13 were made from pIBT4 and pIBM6, respectively, through site-directed mutagenesis of the region lying between the T7 promoter and the IBV-coding sequence (corresponding to the 5′ UTR of *Xenopus* globin mRNA) with the synthetic oligonucleotide 5′ CAC TATAGGGAGACCGGAATTCGACCTCGCCCGGGGAT CTAAAAAGTC 3′. The effect of this alteration was to replace this region of the plasmid with the equivalent 5′ UTR from pIBT3.1.

Plasmids pIBM18 and pIBM24 were made from pIBM12 and pIBM13, respectively, through site-directed mutagenesis of the 5′ UTR lying between the T7 promoter and the IBV-coding sequence with the synthetic oligonucleotide 5′ CGACTCACTATAGAATACACGGAATTCGAC 3′. The effect of this alteration was to make the 5′ UTRs of these plasmids equivalent to that of pIBS3.1. Thus, in terms of their 5′ UTRs, pIBM12, pIBM13, pIBT3.1, and pIBT3.2 are equivalent and pIBM18, pIBM24, pIBS3.1, and pIBS3.2 are equivalent. Plasmids pIBM12, pIBM13, pIBM18, and pIBM24 all contain the T7 promoter and the intergenic region of bacteriophage f1 (12) and thus can be converted to a single-stranded form for mutagenesis by superinfection of plasmid-bearing bacteria with the bacteriophage R408 (42).

Plasmids pIBM48 and pIBM40 were made by altering, through site-directed mutagenesis of pIBT12 (32), the sequence contexts (surrounding sequences) around the initiation codons for 3a and 3b to those proposed to be optimal for eukaryotic translation (25) (e.g., A/GCCAUGG). Specifically, plasmid pIBM48 was made by changing the sequence around the 3a start codon from UUGAUGA to ACCAUGG with the synthetic oligonucleotide 5′ AAAAAGTCTGTAC CATGGTCCAAAGTCCC 3′. Plasmid pIBM40 was constructed by changing the sequence around the 3b start codon from CUAAUGU to ACCAUGG with the synthetic oligonucleotide 5′ CAGTCTAGAACCATGGTAAACTTAG 3′.

Plasmid pDC1 was constructed by cloning a 910-bp *Bgl*II-*Hin*dIII restriction fragment containing the 3a, 3b, and 3c ORFs (together with the beginning of the downstream membrane protein [M]-coding sequence) from pIBT4 into *Bgl*II-*Hin*dIII-digested plasmid pVB5+ (Fig. 1). Plasmid pVB5+ consists of a cDNA copy of the nucleocapsid (NP) protein gene or influenza A virus Puerto Rico/8/34 (3) cloned into the *Eco*RI site of the plasmid pSP65 immediately downstream of the SP6 promoter, and so SP6-driven transcripts of pDC1 contain a truncated form of the influenza virus NP gene, followed by the 3a, 3b, and 3c coding sequences as separate ORFs. A similar plasmid, pDC2, was made by cloning a *Bgl*II-*Hin*dIII fragment from pIBM6 corresponding to the fused 3abc ORF (together with the beginning of the downstream membrane protein [M]-coding sequence) into *Bgl*II-*Hin*dIII-digested plasmid pVB5+ (Fig. 1). This plasmid was further digested with *Eco*RI, end repaired with DNA polymerase I, and redigested with *Hin*dIII, generating a 1,750-bp fragment containing the truncated NP ORF together with the downstream 3abc fused ORF and the beginning of the M ORF. This fragment was then cloned into plasmid pIBT4, which had been digested with *Bgl*II, end repaired with DNA polymerase I, and redigested with *Hin*dIII, to give plasmid pDC3 (Fig. 1). Transcripts of the influenza virus-IBV-coding sequences may be produced from pDC3 with the T7 RNA polymerase, and further, since it contains the origin of replication for the single-stranded phage f1, the plasmid may be converted to single-stranded DNA for mutagenesis. Plasmid pDC4 was produced from pDC3 by fusing, through site-directed mutagenesis, the already fused 3abc ORF with the downstream truncated M ORF with the synthetic oligonucleotide 5′ CGAGACAAAATGATGTCTATGACTATG GAACAGTCAG 3′. At the same time, 4 additional methionine residues were created at the junction between the 3c and M ORFs (Fig. 1) in order to boost the incorporation of radioactive methionine during in vitro translation of proteins, including this region. Finally, pDC5, a derivative of pDC3 in which the 3a and 3b ORFs have been deleted, was constructed by digestion of pDC3 with *Bgl*II and *Pvu*II, repair of the overhanging end with DNA polymerase I, and religation.

Site-directed mutagenesis. Site-directed mutagenesis of single-stranded plasmid DNA was performed as described by Brierley et al. (7). The appropriate mutants were identified on the basis of colony hybridization and nucleotide sequencing.

In vitro transcription of plasmid DNA. Plasmid DNAs were prepared by a conventional alkaline-sodium dodecyl sulfate (SDS) miniprep method (2) and were linearized prior to being used by digestion with *Hin*dIII. In vitro transcription with SP6 and T7 phage RNA polymerase was carried out essentially as described by Melton et al. (38). Unless otherwise indicated, transcription was carried out in the presence of the synthetic cap structure ^{7Me}GpppG (New England BioLabs) to generate 5′ capped mRNA (9). Following transcription, product RNA was purified by extraction with phenol-chloroform, precipitation with ethanol, and, finally, gel filtration on a centrifuged 1-ml-volume Sephadex G50 column.

Translation of mRNA in wheat germ cell-free system. Wheat germ cell extracts were prepared as previously described (16). mRNA was translated in the extracts in the presence of 0.75 μCi of [^{35}S]methionine per μl. Translation products were separated by SDS-polyacrylamide gel electrophoresis on 17.5 or 20% polyacrylamide gels (29) and were detected by autoradiography or fluorography.

RESULTS

Influence of the 5′ UTR on expression of 3a, 3b, and 3c from a tricistronic mRNA. During the course of our studies on the

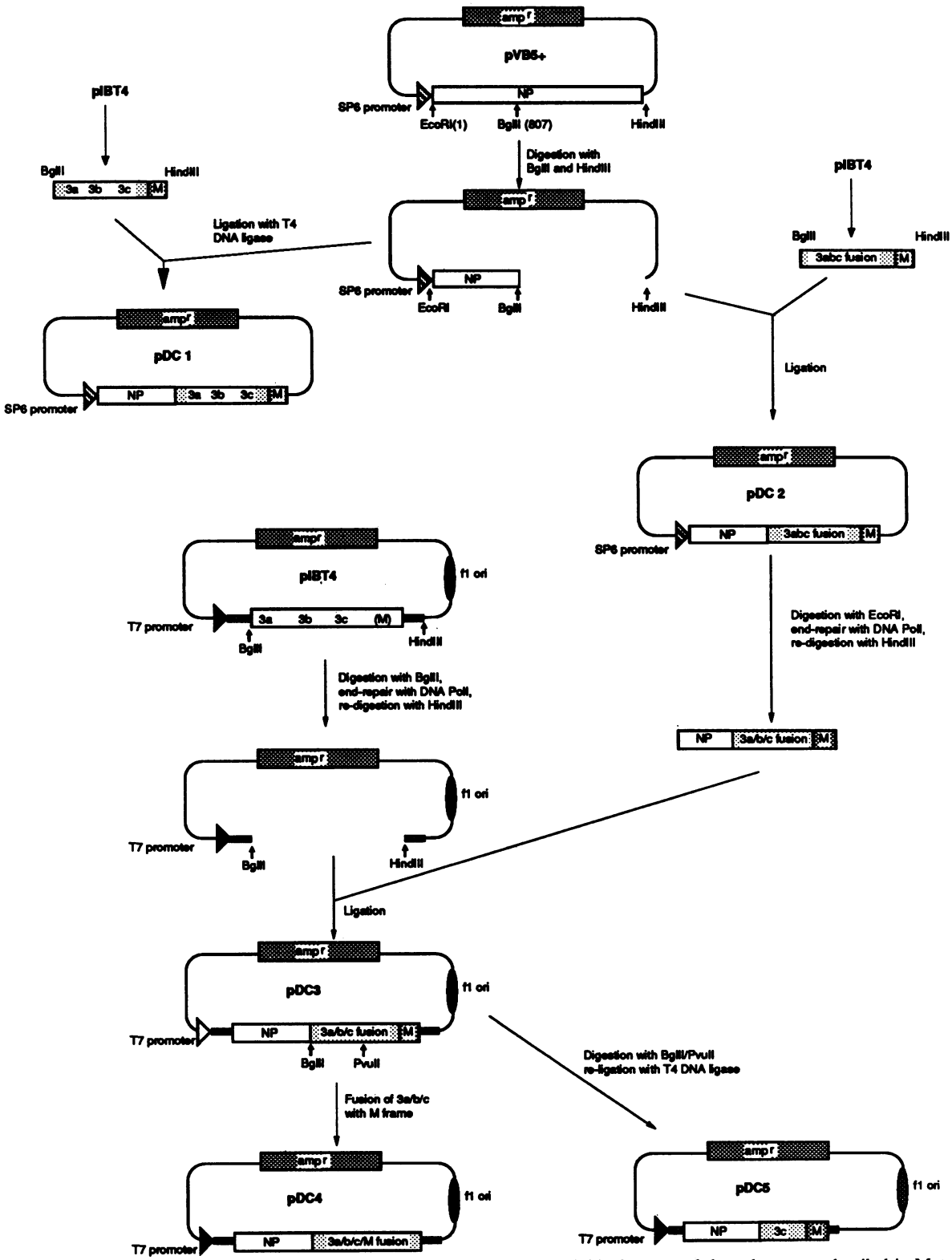

FIG. 1. Construction of plasmids pDC1, pDC2, pDC3, pDC4, and pDC5. Individual stages of the scheme are detailed in Materials and Methods.

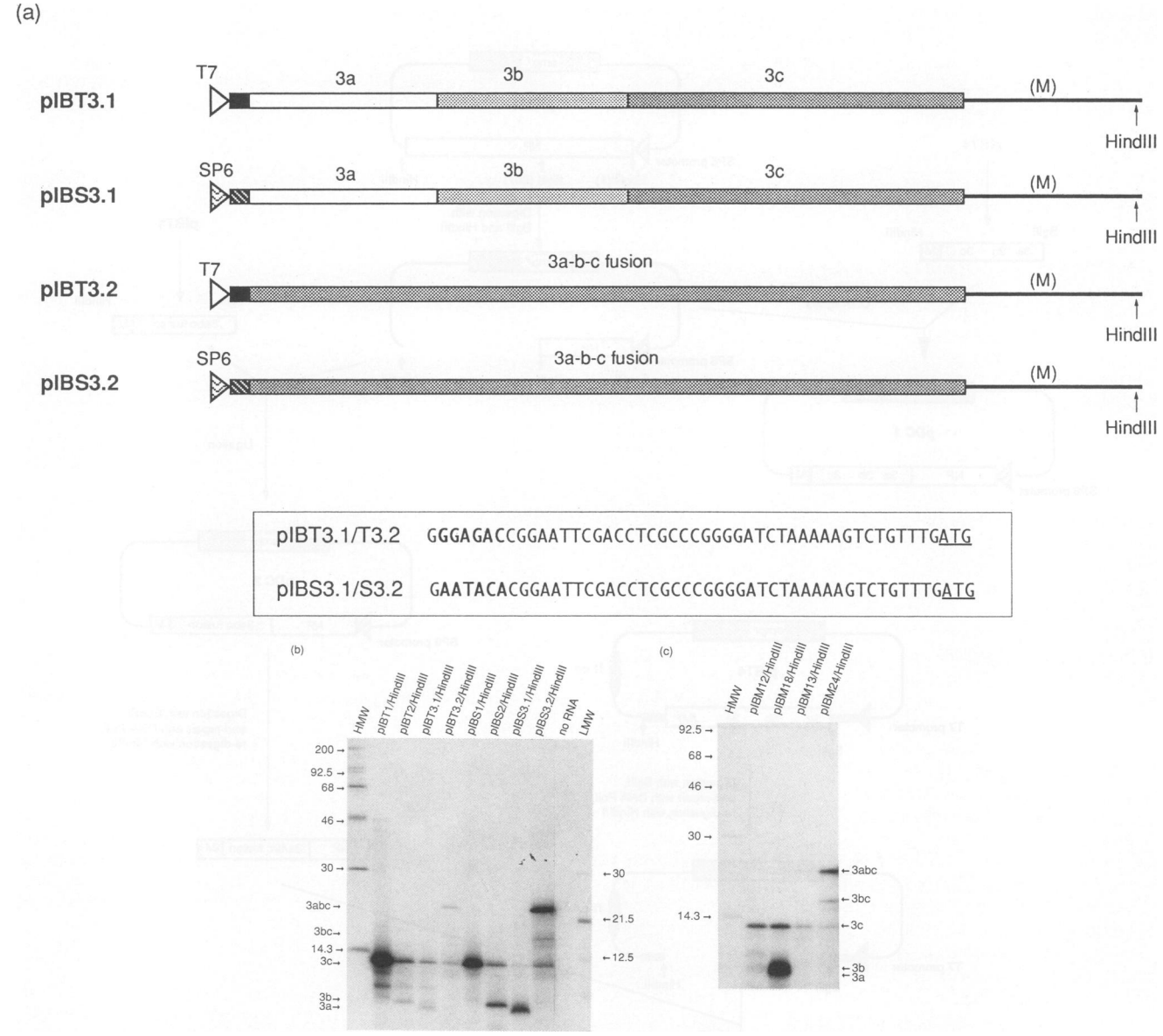

FIG. 2. (a) Diagram of plasmids pIBT3.1, pIBT3.2, pIBS3.1, and pIBS3.2 showing the SP6 and T7 RNA polymerase promoters and the *Hin*dIII restriction enzyme sites used to linearize the plasmids for in vitro transcription. The boxed panel shows a comparison of the 5′ UTRs of transcripts from these plasmids, with differences in nucleotide sequences in bold type and the initiator AUG of the 3a ORF underlined. The plasmids pIBM12, pIBM13, pIBM18, and pIBM24 are identical, with respect to the displayed regions, to pIBT3.1, pIBT3.2, pIBS3.1, and pIBS3.2, respectively. (b) Cell-free translation products of mRNA derived by in vitro transcription from *Hin*dIII-digested pIBT1, pIBT2, pIBT3.1, pIBT3.2, pIBS1, pIBS2, pIBS3.1, and pIBS3.2 with T7 or SP6 RNA polymerase. RNA was added to the wheat germ cell-free system, as indicated above each lane, at approximately 100 μg/ml. Translation products were labelled with [^{35}S]methionine, separated on an SDS–20% polyacrylamide gel, and detected by fluorography. (c) Cell-free translation products of mRNA derived by in vitro transcription from *Hin*dIII-digested pIBM12, pIBM13, pIBM18, and pIBM24 with T7 RNA polymerase. RNA was added as indicated above each lane. Translation products were prepared and analyzed as described in the legend to Fig. 1. Lanes HMW and LMW, ^{14}C-labelled high- and low-molecular-weight markers, respectively.

expression of mRNA3, we constructed a number of plasmids containing the 3a, 3b, and 3c ORFs under the control of promoters which allow their transcription in vitro into mRNA (Fig. 2a). One of these, pIBS3.1, contains the 3a, 3b, and 3c ORFs adjacent to the SP6 RNA polymerase promoter and was based on the vector pSP65. Another, pIBT3.1, contains the same set of ORFs, but they are adjacent to the T7 phage RNA polymerase promoter; this plasmid was based on the commercially available cloning vector pT7-1. mRNA transcripts produced from these two plasmids are thus identical except for some slight differences in their 5′ UTRs which result from differences in the vector sequences between the start site for transcription and the beginning of the IBV-specific insert; both 5′ UTRs are the same length, but they differ at 6 nucleotide positions (Fig. 2a).

In vitro translation of RNA transcribed from these two

plasmids is shown in Fig. 2b. As expected from work described elsewhere (32), both mRNAs directed the synthesis of three separate polypeptides with molecular weights of 6,700, 7,400, and 12,400, corresponding to the 3a, 3b, and 3c gene products. The identities of these proteins were confirmed by comparison with the translation products of RNAs transcribed from related plasmids which contained either the 3c ORF alone (pIBT1 and pIBS1) or the 3b and 3c ORFs (pIBT2 and pIBS2). Surprisingly, however, in view of the similarity between the two mRNAs, the relative amounts of synthesis of the three proteins were markedly different. Whereas the 3a polypeptide was the most abundant product from pIBS3.1-derived transcripts, pIBT3.1-derived transcripts produced relatively little 3a, with 3c now the predominant product. This effect was reproduced with two further plasmids, pIBS3.2 and pIBT3.2, which are relatives of pIBS3.1 and pIBT3.1, respectively. In these plasmids, the junction regions between the 3a, 3b, and 3c ORFs were altered to create a single, continuous ORF comprising all three coding sequences (3abc); this was carried out originally to facilitate detection of the products of initiation at each of the three ORFs (32). In vitro translation of mRNA derived from these plasmids is expected to give rise to a 27K protein corresponding to the 3abc product, a 20K protein representing the 3bc product, and the 12.4K 3c protein. As shown in Fig. 2b, transcripts from both pIBS3.2 and pIBT3.2 do indeed produce polypeptides of the expected sizes but, once again, in very different proportions. As occurred before, initiation on the 3a ORF (giving rise to the 27K 3abc protein) was much more efficient on pIBS3.2-derived RNA, with the 3c protein being the most abundant product of pIBT3.2-derived RNA. Two additional products were observed migrating between the 3c protein and the 3a and 3b proteins. These were generally observed throughout the course of the experiments described here and probably represent premature termination products of translation from the 3c ORF.

In order to gather additional support for these findings and to provide a basis for further analysis of the phenomenon, we constructed another set of plasmids equivalent to those described above but which may be converted to single-stranded DNA in order to facilitate site-directed mutagenesis through the preparation of single-stranded DNA. These plasmids, pIBM12, pIBM13, pIBM18, and pIBM24, correspond with pIBT3.1, pIBT3.2, pIBS3.1, and pIBS3.2, respectively, in their coding sequences and 5′ UTRs. For each of these new plasmids, synthetic mRNA may be produced with the T7 RNA polymerase. Translation of RNA transcribed from these plasmids is shown in Fig. 2c, and once again the differences in the relative synthesis of the 3a, 3b, and 3c products described above were clearly visible.

Thus, it is evident that small changes in the vector-derived sequences within the 5′ UTRs of these mRNAs may have a dramatic effect on their translation. However, our own previous work and that of others indicate that the particular vector-derived sequence present at the 5′ ends of pIBT3.1, pIBT3.2, pIBM12, and pIBM13 transcripts (from which 3a is not translated efficiently) which corresponds to that present in the commercial transcription vector pT7-1 presents no general barrier to translation of downstream ORFs, since other mRNAs containing the same vector sequence at their 5′ ends function perfectly well (data not shown). We therefore considered the most likely explanation for the drastic reduction in translation of 3a and 3b from these transcripts to be that this vector sequence can, in conjunction with downstream IBV sequences, fold to produce an RNA structure which renders the 5′ end of the mRNA inaccessible to

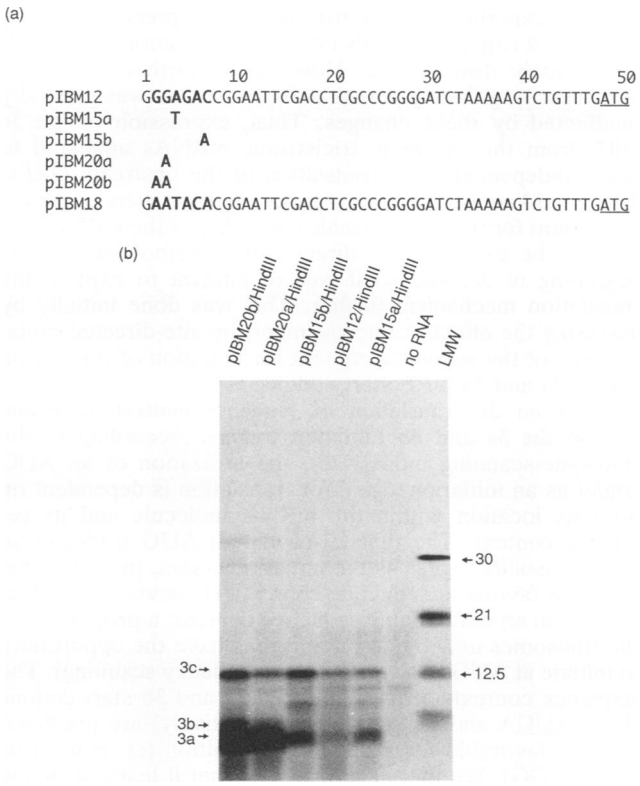

FIG. 3. (a) Primary structure of the 5′ UTR of transcripts from pIBM12 and mutated forms of the plasmid. Also shown, for comparison, is the 5′ UTR of pIBM18-derived transcripts. The nucleotide positions that differ between pIBM12 and pIBM18 are in bold type, and the initiation codon for the 3a ORF is underlined. (b) Cell-free translation products of mRNA obtained by in vitro transcription from *Hin*dIII-digested pIBM12, pIBM15a, pIBM15b, pIBM20a, and pIBM20b with T7 RNA polymerase. RNA was added to the wheat germ cell-free system as indicated above each lane. Translation products were prepared and analyzed as described in the legend to Fig. 1. LMW, ^{14}C-labelled low-molecular-weight markers.

ribosomal entry. The alternative possibility, i.e., that ribosomes can still bind at the 5′ end but for some reason do not recognize the 3a initiation codon, seems unlikely, since it might then be expected that initiation at 3b would be unaffected or perhaps even improved, which was not the case. We decided, therefore, to investigate the effect of specific nucleotide changes in the 5′ UTRs of pIBM12-derived transcripts on synthesis of the 3a, 3b, and 3c polypeptides. The results of this experiment are shown in Fig. 3. The 5′ UTRs of pIBM12- and pIBM18-derived transcripts differ at only 6 positions (Fig. 3a), and it can be seen that single-base changes at least 3 of these positions have a dramatic effect on the patterns of translation from the mRNAs (Fig. 3b), in each case enhancing initiation at 3a. The most striking example of this is mutant pIBM20a, in which a single G-to-A substitution restores 3a synthesis to a level equivalent to that observed with pIBM18-derived mRNA (Fig. 2c). These data are consistent with the idea that secondary or tertiary structures which can block ribosomal entry and which are exquisitely sensitive to alterations in their nucleotide constituents exist at the 5′ ends of pIBT3.1- and pIBM12-derived transcripts. However, as yet, the precise nature of these nucleotide structures remains unclear.

These experiments demonstrate that the precise nature of the 5′ UTR can dramatically influence translation of the ORF immediately downstream. However, a further interesting and surprising finding was that 3c translation was generally unaffected by these changes. Thus, expression of the 3c ORF from the synthetic tricistronic mRNAs appeared to occur independently of translation of the upstream ORFs. This would not be expected if leaky scanning were proposed to account for the polycistronic capability of the mRNA, but it could be explained by direct entry of ribosomes at the beginning of 3c, and so it was of interest to explore the translation mechanism further. This was done initially by assessing the effect of improvement, by site-directed mutagenesis, of the sequence context for initiation of translation on the 3a and 3b ORF start codons.

Effect on 3c translation of sequence context alteration around the 3a and 3b initiation codons. According to the ribosome-scanning model (26), the utilization of an AUG triplet as an initiation signal for translation is dependent on both its location within the mRNA molecule and its sequence context. The first (5′-proximal) AUG triplet in an mRNA usually serves as the initiation codon, provided that it is in a favorable sequence context. However, if the first AUG is in an unfavorable sequence context, a proportion of the ribosomes may bypass it and thus have the opportunity to initiate at AUG triplets downstream (leaky scanning). The sequence contexts around both the 3a and 3b start codons (UUGAUGA and CUAAUGU, respectively) are predicted to be unfavorable for ribosome recognition (cf. consensus ACCAUGG). We therefore reasoned that if leaky scanning is the mechanism by which the 3b and 3c ORFs are recognized during translation of mRNA3, improvement of the sequence contexts of the 3a and 3b initiation codons (i.e., changing them to the Kozak consensus sequence A/GC CAUGG) should decrease translation of the 3c ORF. As a basis for these experiments, we used the plasmid pIBT12 (32), which contains the 3a, 3b, and 3c ORFs under the control of a T7 promoter and which may be converted to single-stranded DNA for mutagenesis but which contains a 5′ UTR corresponding to the natural IBV leader sequence; this sequence of about 65 nucleotides is present at the 5′ ends of all of the IBV mRNAs and is thought to be acquired by a leader-primed transcription mechanism (30). This plasmid was chosen so that the synthetic mRNA analyzed in these experiments would be as close as possible to the naturally occurring counterpart. Two mutant derivatives of pIBT12 were constructed (Fig. 4a). The first (pIBM40) was made by mutating the sequence context around the 3b initiator from CUAAUGU to the Kozak consensus sequence ACCAUGG, and the second (pIBM48) was made by changing the sequence context around the 3a initiation codon from UUGAUGA to the same consensus sequence. As shown in Fig. 4b, the translation of RNA derived from these mutant plasmids produced patterns of translation similar to those resulting from pIBT12-derived mRNA, though the accumulation of the 3b polypeptide appeared to be slightly increased in pIBM40. No obvious reduction in the expression of the 3c product was observed with the mutants. The fact that the relative expression of 3c is not significantly affected by improvement of the sequence contexts of initiator codons of the upstream ORFs lends support to the idea that translation of 3c may not be linked to the translation of the 3a and 3b ORFs.

Effect of 7-methyl-GTP on translation of the 3c ORF from the tricistronic mRNA. The initiation of translation by eukaryotic ribosomes on capped mRNA is believed to begin

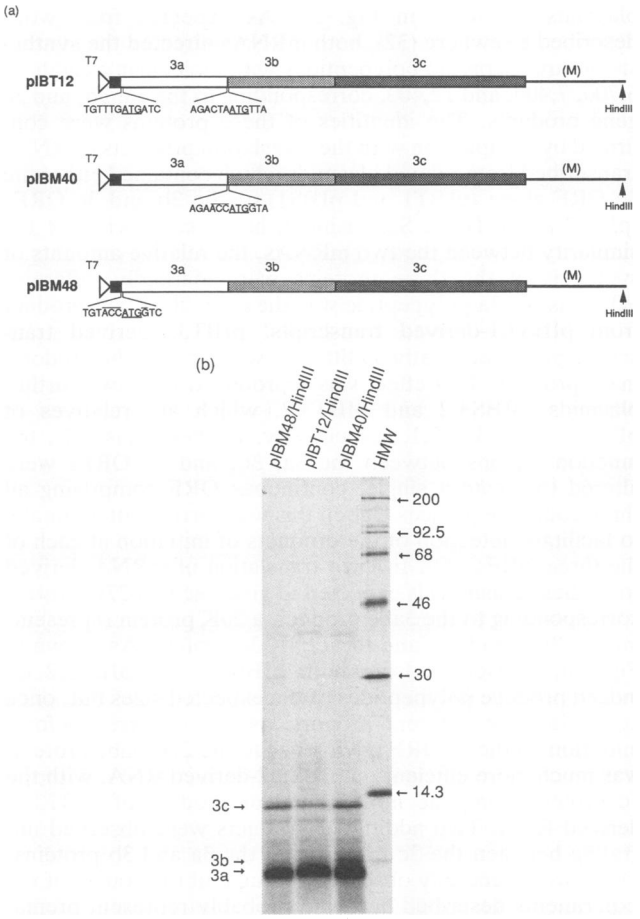

FIG. 4. (a) Diagram of plasmids pIBT12, pIBM40, and pIBM48 showing the T7 promoter sites and the restriction enzyme sites used to linearize the plasmids for in vitro transcription. Also shown are the initiator AUGs and the surrounding sequence contexts for the 3a and 3b ORFs. Nucleotides in pIBM40 and pIBM48 differing from those in the parental pIBT12 plasmid sequence are identified by a rule above them. (b) Cell-free translation products of mRNA obtained by in vitro transcription from *Hin*dIII-digested pIBT12, pIBM40, and pIBM48 with T7 RNA polymerase. RNA was added to the wheat germ cell-free system as indicated above each lane. Translation products were prepared and analyzed as described in the legend to Fig. 1. HMW, ^{14}C-labelled high-molecular-weight markers.

with the formation of a complex consisting of the mRNA, the 40S ribosome subunit, and several translational factors (45). This recognition between the mRNA and the 40S subunit appears to be mediated specifically by a cap-binding protein which can recognize the methylated cap structure at the 5′ end of the mRNA. Several analogs of the 5′ cap have been shown to be able to compete under certain conditions with capped mRNA for the limited supply of cap-binding protein and thereby inhibit cap-dependent initiation of translation (15). We therefore set out to test the effect of one such analog, 7-methyl-GTP, on translation of the synthetic tricistronic 3a/b/c mRNA, reasoning that if translation of 3c occurred by internal ribosomal entry, it should be insensitive to the addition of the compound. Accordingly, RNA transcripts derived from pIBM12, pIBM13, pIBM18, and pIBM24 were translated in wheat germ extracts in the presence or the absence of 0.2 mM 7-methyl-GTP, and the

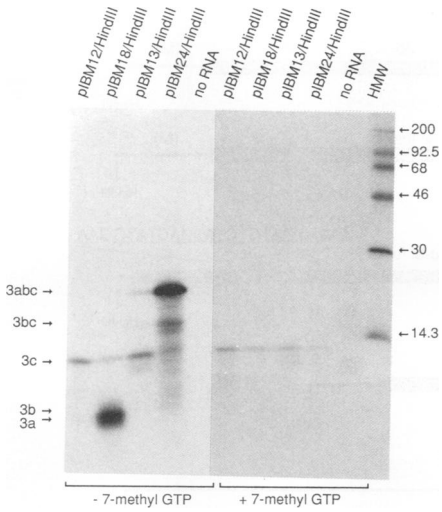

FIG. 5. Effect of 7-methyl-GTP on cell-free translation of mRNA derived by in vitro transcription from *Hin*dIII-digested pIBM12, pIBM18, pIBM13, and pIBM24 with T7 or SP6 RNA polymerase. RNAs were added to the wheat germ cell-free system in the presence or the absence of 0.2 mM 7-methyl-GTP as indicated. Translation products were prepared and analyzed as described in the legend to Fig. 1. HMW, ^{14}C-labelled high-molecular-weight markers.

results are shown in Fig. 5. It is clear that translation of the upstream ORFs (3a and 3b) was highly sensitive to the presence of 7-methyl-GTP. Translation of 3c, on the other hand, was relatively resistant, strongly suggesting that the initiation of the 3c ORF occurs independently of cap recognition and possibly by the direct internal entry of ribosomes into some specific region near the 3c start codon.

Initiation of 3c translation from a chimeric mRNA containing an additional upstream ORF. In order to test this possibility further, we adopted a strategy similar to that used by others to establish that translation of picornavirus RNA initiates independently of the 5′ end by direct ribosomal entry (19, 39). Thus, the 3a/b/c coding sequence was placed downstream of another efficiently translated ORF, encoding the influenza virus NP protein, and a synthetic mRNA transcript encompassing both the IBV and influenza virus sequences was produced for in vitro translation studies. If 3c is indeed recognized by internal initiation, it should still be translated, even when preceded by an additional ORF. This kind of experiment also provides a convenient internal control, in that any ribosomes entering at the 5′ end of the mRNA and simply bypassing the first ORF would be expected to initiate at 3a or 3b rather than at 3c. The absence of 3a and 3b translation may thus be taken as additional evidence for the specificity of the internal initiation process.

The results of this experiment are shown in Fig. 6. Plasmid pDC3 (Fig. 6a) consists of the fused 3abc ORF located downstream of a truncated form of the influenza virus NP gene. Transcripts derived from this plasmid should translate to give a major influenza virus-specific 32K product, and additional initiation at the 3a, 3b, or 3c start codon would give rise to easily recognizable products with molecular weights of 27,000 (3abc protein), 20,000 (3bc protein), and 12,400 (3c protein), respectively. In addition, these transcripts contain at their 3′ ends the initiation codon and the 5′ end of the IBV M protein-coding sequence, which begins just at the terminus of 3c. Translation of mRNA derived from this plasmid is shown in Fig. 6b (left panel). As expected, the major translation product was a protein with a molecular weight of approximately 32,000 which corresponds with the product of the 5′-proximal NP ORF (confirmed by immunoprecipitation with NP-specific antiserum [data not shown]), but, in addition, a clear band with the molecular weight expected for 3c was evident. The relative abundance of this protein, in molar terms, was high, given that 3c contains only 2 methionines, compared with the 19 predicted for the NP product. No other obvious translation products which were consistent with initiation at 3a or 3b were apparent. In a further experiment, we tested the effect of the cap analog 7-methyl-GTP on the translation of pDC3-derived transcripts (Fig. 6b, right panel). It was apparent that synthesis of 3c was much less sensitive to inhibition by the cap analog than was that of the influenza virus NP protein, suggesting that initiation at 3c, as expected, is occurring through a cap-independent mechanism.

In order to provide further support for the conclusion that the 3c initiation codon can function efficiently even when preceded by other functional initiation codons, we constructed a derivative of pDC3 in which the 3′ end of the 3c ORF was mutated to become contiguous with the downstream M ORF, which initiates close to the 3c stop codon (pDC4). Initiation at 3c on transcripts from this plasmid should now produce a 22K instead of a 12.4K protein. As can be seen clearly in Fig. 6c, pDC4 transcripts do indeed translate to give the predicted protein. As part of the same mutagenesis procedure, we also introduced an additional 4 new methionine codons at the junction between the 3abc ORF and the M ORF, in order to enhance the radioactivity incorporated into proteins, including this coding sequence, and to provide a more sensitive means of detecting initiation at 3a and 3b. In spite of this, we did not observe products of the sizes expected for initiation at 3a and 3b (36K and 29K, respectively), which once again emphasized the relative specificity of 3c initiation. These transcripts did, however, direct the synthesis of a new protein with a molecular weight of about 9,000. The identity of this protein is not certain, but from its size, it appears most likely to represent a product of initiation from the start of the M ORF, perhaps from one of the newly introduced Met codons (some of which have fortuitously favorable surrounding contexts).

In the above examples, 3c initiation was demonstrated on chimeric mRNAs in which the 3c start codon was located within a fused 3abc ORF positioned downstream of the influenza virus NP ORF. We also analyzed the translation products of transcripts from pDC1, a plasmid in which the unfused 3a, 3b, and 3c ORFs are placed downstream of the truncated influenza virus NP gene (Fig. 6c). Though the efficiency of translation was generally lower with this mRNA, synthesis of 3c protein was clearly evident, ruling out the possibility that internal initiation on 3c is an artifact arising from the creation of the fused 3abc ORF.

These results clearly indicate that ribosomal initiation on the 3c ORF can occur even when the entire 3a/b/c coding region is itself placed downstream of an efficiently expressed ORF and strongly support the idea that 3c may be translated through internal ribosome entry on the mRNA. By analogy with other systems in which internal ribosome entry is known to occur (19, 39), it might be expected that sequences upstream of the 3c ORF would contribute to this ribosomal entry site. In order to test this possibility, we deleted from pDC3 the coding region for 3a and 3b, leaving the intact 3c ORF downstream of the influenza virus NP ORF. The 3c protein was not synthesized in response to RNA derived

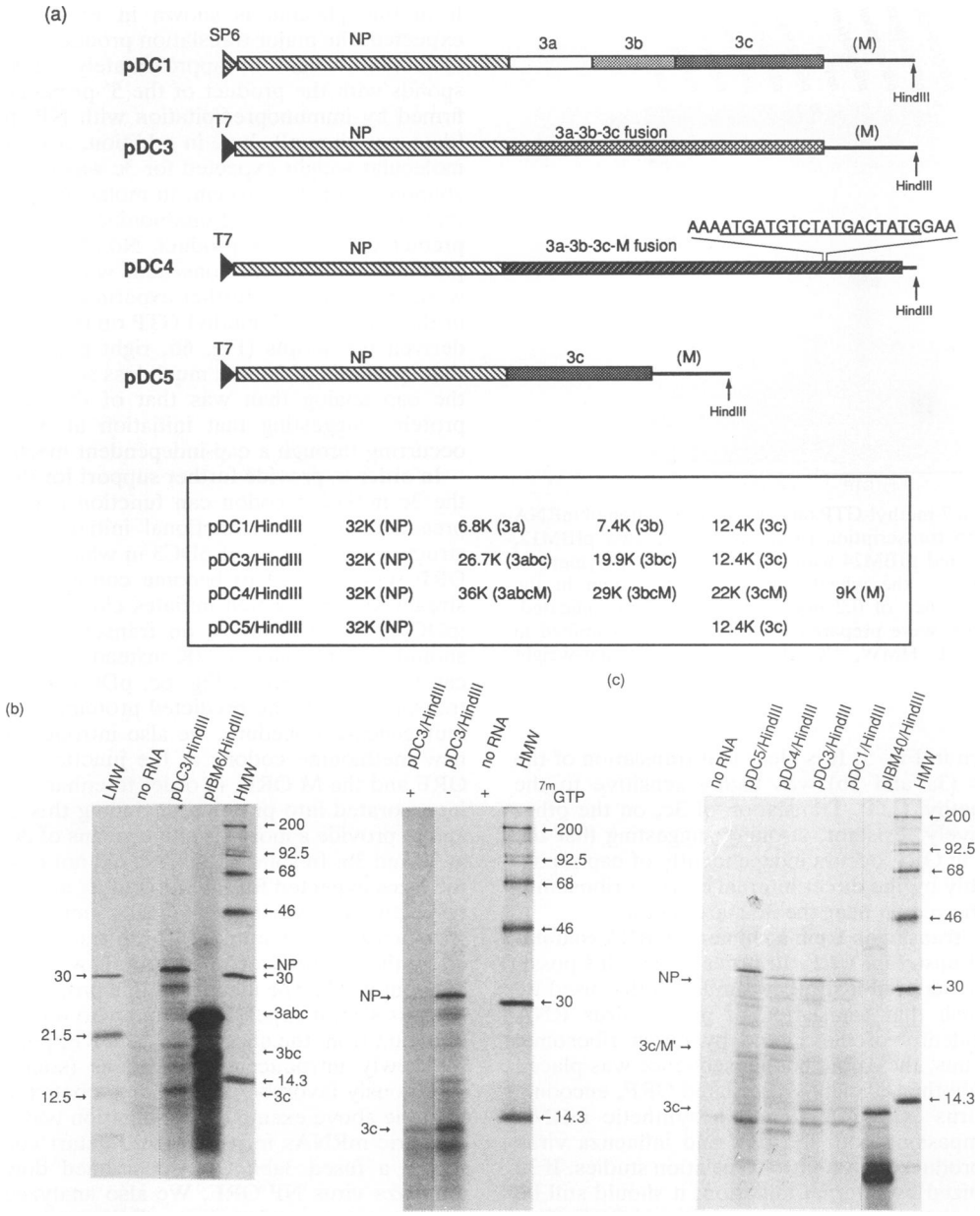

FIG. 6. (a) Diagram of plasmids pDC1, pDC3, pDC4, and pDC5 showing the T7 promoter sites and the restriction enzyme sites used to linearize the plasmids for in vitro transcription. Also shown is the nucleotide sequence from the end of the 3c coding region in pDC4 at the point where nucleotide changes were made to fuse the upstream ORF with the downstream M ORF and to introduce additional methionine codons (see Materials and Methods). Nucleotides which differ from those of the natural IBV sequence are underlined. The boxed panel shows the translation products that could be produced from transcripts of each plasmid by initiation at the start of different ORFs (indicated in parentheses). (b) Left: Cell-free translation products of mRNA obtained by in vitro transcription from *Hin*dIII-digested pDC3 with T7 RNA polymerase. pIBM6-derived transcripts were also translated to provide markers for the 3abc, 3bc, and 3c proteins. RNA was added to the wheat germ cell-free system as indicated above each lane. Translation products were prepared and analyzed as described in the legend to Fig. 1. Right: Translation of pDC3-derived RNA in the presence and the absence of 7-methyl-GTP (25 mM). Translation products were prepared and analyzed as described in the legend to Fig. 1. (c) Cell-free translation products of mRNA derived by in vitro transcription from *Hin*dIII-digested pDC1, pDC3, pDC4, and pDC5 with T7 RNA polymerase. pIBM40-derived transcripts were also translated to provide markers for the 3a, 3b, and 3c proteins. RNA was added to the wheat germ cell-free system as indicated above each lane. Translation products were prepared and analyzed as described in the legend to Fig. 1. HMW and LMW, ^{14}C-labelled high- and low-molecular-weight markers, respectively.

from this plasmid, pDC5 (Fig. 6c), suggesting that the capacity of ribosomes to recognize the 3c initiation codon is dependent on sequences from within the 3a-3b coding region.

DISCUSSION

We previously showed that synthetic mRNAs equivalent to IBV subgenomic mRNA3 can direct the synthesis in vitro

and in vivo of three different proteins (the products of the 3a, 3b, and 3c ORFs). Since IBV does not appear to specify subgenomic mRNAs intermediate in size between mRNA3 and mRNA4 (encoding the M protein), and since all three proteins are known to be produced in IBV-infected cells (32), our work indicates that naturally produced mRNA3 is functionally tricistronic. We are not aware of any other mRNAs which display this kind of versatility in eukaryotic cells, and so the mechanism which controls the initiation of protein synthesis on mRNA3 is of particular interest.

In most cases, translational initiation on eukaryotic mRNAs appears to be consistent with the scanning model (22, 26). This model originally proposed that the 40S ribosomal subunit binds initially to the 5′ end of the mRNA and migrates toward the 3′ end until it encounters the first AUG triplet, which will be used to initiate the protein synthesis. Such a model would predict, however, that all eukaryotic mRNAs should be functionally monocistronic, and although this is true to a large extent, an increasing number of exceptions have been previously described (for a review, see reference 23 and references therein). For a number of virus mRNAs which are known to encode more than one different polypeptide from separate ORFs, it has been proposed that expression of the distal regions of the ORFs is mediated by a leaky-scanning mechanism (26). This model is based on the idea that the efficiency with which an initiator AUG is recognized by the 40S ribosomal subunit is dependent on its location in the mRNA and its surrounding sequence (sequence context). If the first AUG is located in an optimal context [e.g., GCC(A/G)CCAUGG, proposed by Kozak (25) on the basis of a survey of 699 vertebrate mRNAs], it would act as an efficient initiator; if, on the other hand, its context is poor, a portion of the 40S subunit may bypass the first AUG and thus have a chance to initiate on a downstream ORF. The general arrangement of sequences on mRNA3 of IBV appeared to be consistent with this mechanism, in that aside from the initiation codons for 3a and 3b, no AUG triplets could be found in any reading frame upstream of 3c (5). Furthermore, the sequence contexts for initiation around the 3a and 3b start codons are poor, according to the Kozak "rule." In such a model for the expression of mRNA3, translation of all three ORFs on the mRNA should depend on the efficient entry of ribosomes at the 5′ end, and, since coronavirus mRNAs are known to contain a 5′ cap structure (31), it might be expected that the synthesis of each encoded protein should be effectively cap dependent. The data reported here, however, strongly suggest that translational initiation on the 3c ORF is not due to leaky scanning and that it occurs independently of conventional ribosomal entry at the 5′ end. This conclusion is based on the following observations: (i) translation of 3c was maintained from synthetic tricistronic mRNAs whose particular 5′ UTR structures resulted in drastically reduced synthesis of 3a and 3b; (ii) alterations of the sequences around the 3a and 3b start codons designed to improve the context for ribosomal initiation did not lead to a reduction in the synthesis of 3c; (iii) the inclusion of the synthetic cap analog, 7-methyl-GTP, during in vitro translation of the synthetic tricistronic mRNA affected synthesis of the 3a and 3b polypeptides much more dramatically than that of 3c; and (iv) the translation of a synthetic mRNA in which the 3a/b/c coding sequence was placed downstream of the influenza A virus NP gene led to efficient synthesis of 3c, as well as NP, whereas initiation at the 3a and 3b ORFs could not be detected. Synthesis of 3c, however, was abolished when the 3a and 3b ORFs were removed from this mRNA, suggesting that 3c initiation is dependent on upstream sequence elements. Taken together, these data imply that ribosomes can bind directly to a region within the 3a or 3b coding sequence and initiate synthesis of the 3c protein. Precisely how this interaction occurs is not clear. It could be argued that ribosomal entry at this point is the result of sequence-specific RNA cleavage somewhere within the 3a-3b region, providing a free 5′ end, and that our in vitro translation experiments do not accurately reflect events that occur in the infected cell. This possibility is difficult to eliminate completely, as has been pointed out previously (26), particularly as mRNA degradation does occur to some extent in the wheat germ system. However, our in vitro studies, together with those of IBV-infected cells, have indicated that translation of 3c is quite efficient in relation to that of upstream ORFs. Indeed, infected cells contain considerably more 3c than 3a protein (32). Thus, if specific RNA cleavage were required to generate a new mRNA for 3c, one might expect the cleaved product to be at least as abundant as the uncleaved precursor. However, analysis of IBV-infected cells has not revealed any major RNA species which could correspond to such a cleaved mRNA. The most likely explanation remains, therefore, that 3c translation from mRNA3 is initiated in infected cells by direct binding of the ribosome to an internal ribosome entry site somewhere within the 3a-3b coding region. Proof of this point, however, will require in vivo studies using the naturally occurring mRNA.

There are now several reports of internal initiation occurring on eukaryotic mRNAs. A prominent example is translational initiation on picornavirus RNAs. These RNAs are unlike most eukaryotic mRNAs in that their 5′ ends are not capped but instead have a small covalently linked viral polypeptide (Vpg). They also have unusually long 5′ UTRs, portions of which can apparently direct internal ribosomal entry to initiate protein synthesis (1, 18, 19, 28, 39). Internal translation has also been previously reported to occur on several capped viral mRNAs, such as the vesicular stomatitis virus phosphoprotein mRNA (14, 15), the cowpea mosaic virus M RNA (47), and the mRNA encoding the adenovirus DNA polymerase (13). Furthermore, Sarnow (43) reported that translation of the cellular glucose-regulated protein 78/immunoglobulin heavy-chain-binding protein (GRP78/BiP) is enhanced by poliovirus infection. Since poliovirus infection can inhibit host cell mRNA translation by inactivating the cellular cap-binding protein (45), this observation suggests that synthesis of GRP78/BiP may be initiated by a cap-independent mechanism. Indeed, the 5′ UTR of GRP78/BiP mRNA has been recently shown to be able to mediate the translation of a reporter gene through internal ribosome binding in mammalian cells (35).

The precise mechanism by which ribosomes can bind and initiate translation directly at certain internal sites on mRNA remains unclear. It has been proposed, however, that part of this process may be the recognition of specific RNA structures within these internal ribosome entry sites by particular cellular proteins. For example, Meerovitch et al. (37) identified a cellular protein (p52) that specifically binds to a stem-loop structure within the poliovirus 5′ UTR (nucleotides 567 to 627); Del Angel et al. (10) also reported that certain cellular proteins, among them the eukaryotic initiation factor eIF-2a, can form a complex with the poliovirus 5′ UTR. Using encephalomyocarditis virus (EMCV) RNA, Borovjagin et al. (4) found a protein factor (p58) in extracts of Krebs-2 cells which could bind specifically to the 5′ UTR. Evidence as to the nature of the RNA structure involved is also beginning to emerge. Jang and Wimmer (20) reported

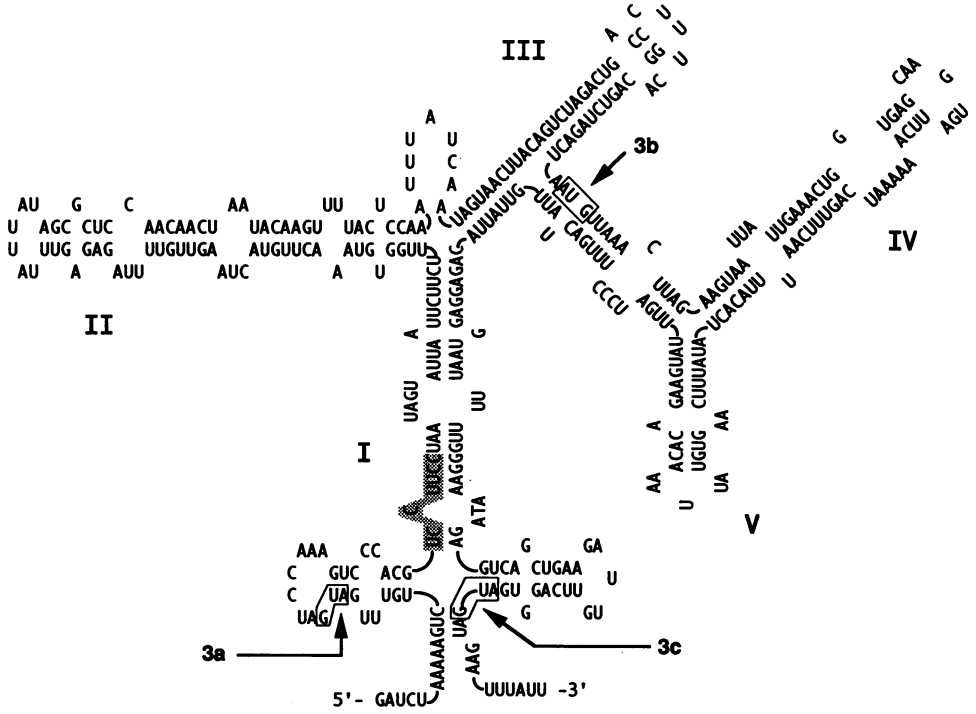

FIG. 7. Computer-predicted secondary structure of the IBV 3a and 3b coding region (11). The region chosen for analysis maps from nucleotides 23836 to 24216 on the IBV genome, according to the numbering scheme of Boursnell et al. (6). Initiator codons for 3a, 3b, and 3c are boxed and indicated with arrows. The shaded region indicates homology with 18S rRNA.

recently that a stem-loop structure, stem-loop E, located 400 nucleotides upstream from the initiation codon is crucial for translation of EMCV RNA. This structure, which contains a pyrimidine-rich sequence (UCUUU) in its loop region, is apparently recognized by a cellular protein, p57. Together with another pyrimidine-rich sequence (UUUUCCUUU) which lies near the initiator AUG triplet and which is also thought to be critical for the initiation of translation (20), stem-loop E may play an essential role in the formation of the initiation complex. Luz and Beck (34) have recently shown that the cellular p57 protein binds specifically to the equivalent two, pyrimidine-rich sequences of foot-and-mouth disease virus RNA. Similarly, a pyrimidine-rich region located between nucleotides 556 and 585 has been identified as the core ribosome internal binding site in poliovirus RNA (36, 41); this region is highly conserved across all enteroviruses and rhinoviruses (40). These results suggest that oligopyrimidine tracts in the 5′ UTR may play an essential role in the internal initiation of translation on picornavirus RNA.

Our data suggest that nucleotide sequences from within the 3b ORF and at least part of the 3a ORF are important for cap-independent initiation of the 3c ORF. We therefore analyzed the potential secondary structure formed by this region using a computer-based RNA-folding program (11). Comparison of this predicted structure with that published for the EMCV UTR (Fig. 7) suggests some similarity between stem-loop III and stem-loop E of the EMCV UTR, and so it may be that the two stem-loop structures serve a similar function. We also note that there is a 7-nucleotide sequence (shaded region of Fig. 7) near the beginning of 3a, which is complementary to a sequence beginning 6 nucleotides from the 3′ end of human 18S rRNA; this region of 18S rRNA has been implicated recently in the internal initiation of translation on picornavirus RNAs (41), and so it is possible that base pairing involving these sequences might also play some role in internal initiation on IBV mRNA.

A further interesting question relating to internal initiation of translation concerns the precise mechanism for selection of the initiator AUG following binding of the ribosome internally on an mRNA. Pelletier and Sonenberg (39), on the basis of experiments with the internal ribosome entry of poliovirus, reported that upon binding internally, the ribosome may scan downstream to find the proper initiator AUG. However, the work of Kaminski et al. (21) on translation of EMCV RNA suggested that the ribosome may move directly to the initiator AUG, ignoring potential start signals lying between this and the initial entry points. Translation of transcripts from pDC5, in which the 3c ORF was fused with the downstream M ORF (Fig. 5c), led to significant synthesis of not only 3c, but also of a small polypeptide probably corresponding to initiation at the beginning of M. This would imply that ribosomes binding to the mRNA in the 3a-3b coding region can occasionally scan past the 3c initiation site and initiate in some way downstream. Confirmation of this finding will, however, require further work.

Finally, it is interesting that the putative internal ribosome entry site element for 3c translation must lie within a functional coding sequence. It is possible that direct competition between elongating and initiating ribosomes could be avoided if at some point in the virus replication cycle 3a-3b translation were inhibited in some way in order to open up the 3c ORF. However, if, as our data suggest, 3b is indeed translated relatively infrequently by leaky scanning past the 3a ORF, the density of the elongating ribosomes present on

the 3b region might be low enough to allow internal entry of ribosomes without interference.

ACKNOWLEDGMENTS

We are grateful to Richard Jackson, Ian Brierley, and Mike Boursnell for useful discussion.

This work was supported by AFRC Link scheme award LRG71, awarded to S.C.I. D.X.L. was supported by a Cambridge Overseas Trust Studentship.

REFERENCES

1. **Belsham, G. J., and J. K. Brangwym.** 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. **64**:5389–5395.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Blok, V. C.** 1989. Studies of the influenza virus RNA polymerase. Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.
4. **Borovjagin, A. V., A. G. Evstafieva, T. Y. Ugarova, and I. N. Shatsky.** 1990. A factor that specifically binds to the 5′-untranslated region of encephalomyocarditis virus RNA. FEBS Lett. **261**:237–240.
5. **Boursnell, M. E. G., M. Binns, and T. D. K. Brown.** 1985. Sequencing of coronavirus IBV genomic RNA: three open reading frames in the 5′ "unique" region of mRNA D. J. Gen. Virol. **66**:2253–2258.
6. **Boursnell, M. E. G., T. D. K. Brown, I. J. Foulds, P. F. Green, F. M. Tomley, and M. M. Binns.** 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J. Gen. Virol. **68**:57–77.
7. **Brierley, I., P. Digard, and S. C. Inglis.** 1989. Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot. Cell **57**:537–547.
8. **Cavanagh, D., D. A. Brian, L. Enjuanes, K. V. Holmes, M. M. C. Lai, H. Laude, S. G. Siddell, W. Spaan, F. Taguchi, and P. J. Talbot.** 1990. Recommendations of the coronavirus study group for the nomenclature of the structural proteins, mRNAs, and genes of coronaviruses. Virology **176**:306–307.
9. **Contreras, R., H. Cheroutre, W. Degrave, and W. Fiers.** 1982. Simple efficient in vitro synthesis of capped RNA useful for direct expression of cloned DNA. Nucleic Acids Res. **10**:6353–6362.
10. **Del Angel, R. M., A. G. Papavassilliou, C. Fernandez-Thomas, S. J. Silverstein, and V. R. Racaniello.** 1989. Cell proteins bind to multiple sites within the 5′ untranslated region of poliovirus RNA. Proc. Natl. Acad. Sci. USA **86**:8299–8303.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
12. **Dotto, G. P., V. Enea, and N. D. Zinder.** 1981. Functional analysis of bacteriophage f1 intergenic region. Virology **114**: 463–473.
13. **Hassin, D., R. Korn, and M. S. Horwitz.** 1986. A major internal initiation site for the in vitro translation of the adenovirus DNA polymerase. Virology **155**:214–224.
14. **Herman, R. C.** 1986. Internal initiation of translation on the vesicular stomatitis virus phosphoprotein mRNA yields a second protein. J. Virol. **58**:797–804.
15. **Herman, R. C.** 1987. Characterization of the internal initiation of translation on the vesicular stomatitis virus phosphoprotein mRNA. Biochemistry **26**:8346–8350.
16. **Inglis, S. C., D. J. McGeoch, and B. W. J. Mahy.** 1977. Polypeptides specified by the influenza virus genome. 2. Assignment of protein coding functions to individual genome segments by in vitro translation. Virology **78**:522–536.
17. **Jackson, R. J., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15**:477–483.
18. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63**:1651–1660.
19. **Jang, S. K., H.-G. Krausslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
20. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. **4**:1560–1572.
21. **Kaminski, A., M. T. Howell, and R. Jackson.** 1990. Initiation of encephalomyocarditis virus RNA translation: the authentic initiation site is not selected by a scanning mechanism. EMBO J. **9**:3753–3759.
22. **Kozak, M.** 1981. Mechanism of mRNA recognition by eukaryotic ribosomes during initiation of protein synthesis. Curr. Top. Microbiol. Immunol. **93**:81–123.
23. **Kozak, M.** 1986. Bifunctional messenger RNA in eukaryotes. Cell **47**:481–483.
24. **Kozak, M.** 1986. Regulation of protein synthesis in virus-infected animal cells. Adv. Virus Res. **31**:229–292.
25. **Kozak, M.** 1987. Analysis of 5′ noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**:8125–8149.
26. **Kozak, M.** 1989. The scanning model for translation: an update. J. Mol. Biol. **108**:229–241.
27. **Krieg, P. A., and D. A. Melton.** 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNA. Nucleic Acids Res. **12**:7057–7071.
28. **Kuhn, R., N. Luz, and E. Beck.** 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **64**:4625–4631.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lai, M. M. C.** 1990. Coronavirus: organization, replication and expression of genome. Annu. Rev. Microbiol. **44**:303–333.
31. **Lai, M. M. C., C. D. Patton, and S. A. Stohlman.** 1982. Further characterization of mRNA's of mouse hepatitis virus: presence of common 5′-end nucleotides. J. Virol. **41**:557–565.
32. **Liu, D. X., D. Cavanagh, I. J. Green, and S. C. Inglis.** 1991. A polycistronic mRNA specified by the coronavirus infectious bronchitis virus. Virology **184**:531–544.
33. **Liu, D. X., and S. C. Inglis.** 1991. Association of the infectious bronchitis virus 3c protein with the virion envelope. Virology **185**:911–917.
34. **Luz, N., and E. Beck.** 1991. Interaction of a cellular 57-kilodalton protein with the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **65**:6486–6494.
35. **Macejak, D. G., and P. Sarnow.** 1991. Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. Nature (London) **353**:90–94.
36. **Meerovitch, K., R. Nicholson, and N. Sonenberg.** 1991. In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5′ untranslated region. J. Virol. **65**:5895–5901.
37. **Meerovitch, K., J. Pelletier, and N. Sonenberg.** 1989. A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3**:1026–1034.
38. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
39. **Pelletier, J., and N. Sonenberg.** 1988. Internal binding of ribosomes to the 5′ noncoding region of a eukaryotic mRNA: translation of poliovirus. Nature (London) **334**:30–325.
40. **Pestova, T. V., C. U. T. Hellen, and E. Wimmer.** 1991. Translation of poliovirus RNA: role of an essential *cis*-acting oligopyrimidine element within the 5′ nontranslated region and involve-

ment of a cellular 57-kilodalton protein. J. Virol. **65**:6194–6204.
41. **Pilipenko, E. V., A. P. Gmyi, S. V. Maslova, Y. V. Svitkin, A. N. Sinyakov, and V. I. Agol.** 1992. Prokaryotic-like cis elements in the cap-independent internal initiation of translation on picornavirus RNA. Cell **68**:119–131.
42. **Russel, M., S. Kidd, and M. R. Kelley.** 1986. An improved filamentous helper phage for generating single-stranded plasmid DNA. Gene **45**:333–338.
43. **Sarnow, P.** 1989. Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. Proc. Natl. Acad. Sci. USA **86**:5795–5799.
44. **Smith, A. R., M. M. Binns, M. E. G. Boursnell, T. D. K. Brown, and S. C. Inglis.** 1987. Identification of a new gene product encoded by mRNA D of infections bronchitis virus. Adv. Exp. Med. Biol. **218**:47–54.
45. **Sonenberg, N.** 1987. Regulation of translation by poliovirus. Adv. Virus Res. **33**:175–204.
46. **Stern, D. F., and B. M. Sefton.** 1984. Coronavirus multiplication: location of genes for virion proteins on the avian infectious bronchitis virus genome. J. Virol. **50**:22–29.
47. **Verver, J., O. Le Gall, A. Van Kammen, and J. Wellink.** 1991. The sequence between nucleotides 161 and 512 of cowpea mosaic virus M RNA is able to support internal initiation of translation in vitro. J. Gen. Virol. **72**:2339–2345.