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Translation of the Hepatitis B Virus P Gene by Ribosomal Scanning as an Alternative to Internal Initiation

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The hepatitis B virus (HBV) P gene which encodes the reverse transcriptase and other proteins required for replication is expressed on the bicistronic mRNA pregenome which also encodes the capsid protein in its first cistron. Recent results have suggested that the hepadnaviral P gene is translated by internal entry of ribosomes upstream from the P gene, in the overlapping C gene. Using a reporter gene fused to the HBV C or P gene, we demonstrate that the C sequence does not allow internal initiation of translation. Alternatively, our results support a model in which the HBV P gene is translated by ribosomes which scan from the capped extremity of the bicistronic mRNA pregenome. The mechanism by which the ribosomes scan past four AUGs before they initiate translation at the P AUG was analyzed. Our results show that these AUGs are skipped via two mechanisms: leaky scanning on AUGs in a weak or suboptimal initiation context and translation of an out-of-C-frame minicistron followed by reinitiation at P AUG. The minicistron translation allows ribosomes to bypass an AUG in a favorable context that would otherwise be used as a start codon for translation of a truncated capsid protein. Our results suggest that this elaborated scanning mechanism permits the coordinate expression of the HBV C and P genes on the viral bicistronic mRNA pregenome.

The hepadnaviruses are a group of enveloped DNA viruses which replicate their genome by reverse transcription of the RNA pregenome (51). Genetic studies have revealed that the viral reverse transcriptase and several other enzymatic activities required for replication of hepadnavirus genomes are encoded by the P gene (4, 9, 42). Recently, the P gene product was demonstrated to be essential for the encapsidation of RNA pregenome molecules (5, 24).

The P open reading frame (ORF) extends over approximately 80% of the genome and overlaps the 3′ end of the C ORF which encodes the capsid protein and the e antigen (17). As no specific transcript of the P gene has ever been identified thus far in infected cells, it is believed that the P gene is translated from the RNA pregenome which also includes the C gene in its 5′ part. Previous studies have shown that the P gene product is not synthesized as a capsid-polymerase fusion protein but is translated from the first AUG of the P ORF (10, 28, 48). Different mechanisms could account for P gene translation, but two of them had been considered as unlikely possibilities: (i) ribosomal backwards scanning (38), because the distance between the C gene stop codon and the P gene initiation codon is too long (44); (ii) leaky scanning (31), because the P ORF is preceded by several AUGs on the pregenome mRNA, 4 for hepatitis B virus (HBV) subtype ayw and up to 13 for duck hepatitis B virus, some of which do not seem to serve as initiation codons, being in a favorable context for translation initiation (30). Thus, it has been suggested (8, 28, 48) that translation of the hepadnaviral P gene could be initiated by a mechanism similar to that reported for the translation of the picornavirus polyproteins (7, 12, 26, 29, 40), i.e., by direct entry of ribosomes in a specific sequence of the C region located upstream from the P gene AUG.

To substantiate this latter hypothesis for HBV, we undertook experiments based on the in vivo expression of bicistronic mRNAs. None of the strategies we used led to the conclusion that the HBV C region allows internal entry of ribosomes. Alternatively, our results suggest that the HBV P gene may be translated by a scanning mechanism through the C gene. A similar conclusion has been recently reported by Lin and Lo (32) using an in vitro translation system. Deletion analyses and site-directed mutagenesis were performed to understand how some scanning ribosomes could skip the four AUGs encountered in the C region. Our results show that, in addition to leaky scanning, translation of a short ORF upstream from the P AUG has a preponderant role in HBV P gene translation. This analysis allows us to present a model of translation regulation in which the HBV C and P genes are coordinately expressed.

MATERIALS AND METHODS

Plasmid constructions. DNA isolations, restriction enzyme digestions, enzymatic DNA modifications, ligations, *Escherichia coli* HB101 cells transformations, and large-scale preparation of plasmid DNA were done by the procedures described by Sambrook et al. (46). Double-stranded DNA sequencing was performed as described by Hattori and Sakaki (21). Nucleotide positions refer to the sequences of the GenBank data bank.

(i) Bicistronic *lacZ-cat* plasmids. The parent plasmid pMLPCAT is derived from the original adenovirus-based eukaryotic vector pMLP10 (3). It contains, downstream from the adenovirus 2 major late promoter (MLP) and tripartite leader sequences, a part of the multiple cloning site of pUC18 (from the *Hin*dIII site to the *Bam*HI site), the *cat* gene of *E. coli*, the intron of the small t antigen gene, and the polyadenylation signal of the simian virus 40 genome. Plasmid pZCAT was obtained by inserting the *Sal*I fragment of

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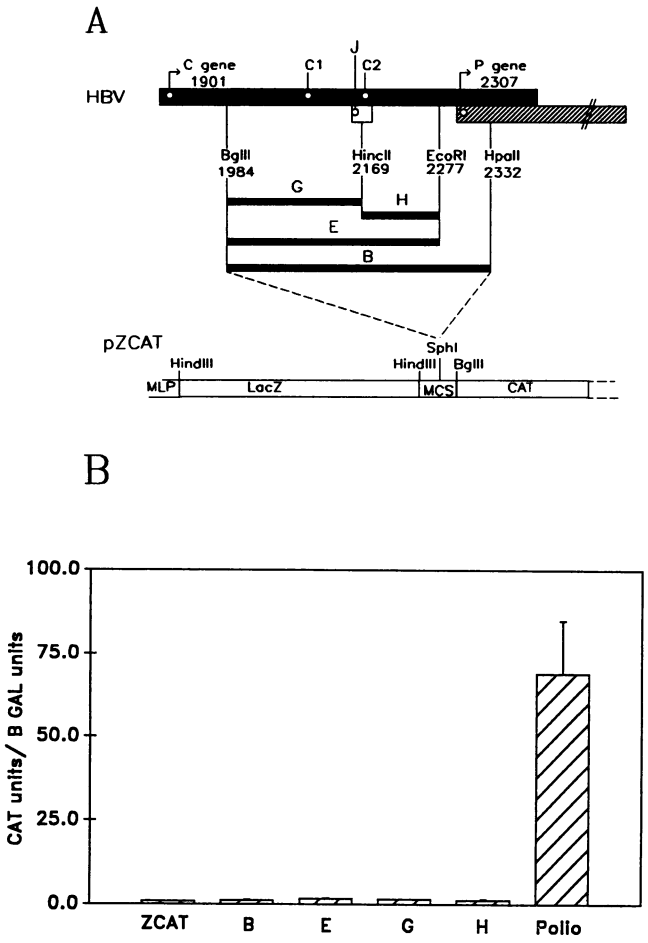

FIG. 1. Expression of artificial *lacZ-cat* bicistronic plasmids in 293 cells. (A) Diagram of the *lacZ-cat* plasmid constructs. The upper scheme represents the HBV C gene with the restriction sites used for cloning indicated below (numbers correspond to the positions in the HBV sequence subtype ayw of the GenBank data bank). The black rectangles G, H, E, and B depict the restriction fragments inserted in the *Sph*I site of the intergenic region of plasmid pZCAT. Plasmid pZCAT contains, under the control of the adenovirus MLP, the *lacZ* gene separated from the *cat* gene by a multiple cloning site (MCS). (B) CAT/β-Gal ratio of cells transfected with the bicistronic plasmids containing in the intergenic region the fragments of the HBV C gene, or a part of the 5′ NTR of poliovirus as indicated. For each construction, the mean and standard deviation of six experiments are represented. For each experiment, the CAT/β-Gal ratio of the control plasmid pZCAT was set to 1.

the plasmid pGH101 containing the *lacZ* gene of *E. coli* (23) into the Klenow-filled *Hin*dIII site of pMLPCAT. HBV genome sequences were derived from a plasmid containing the HBV ayw subtype genome (16). The H, G, B, and E sequences of the HBV C gene described in Fig. 1A were blunt ended and inserted in the Klenow-filled and dephosphorylated *Sph*I site located between the *lacZ* and *cat* genes of pZCAT. Plasmid pZPOLIOCAT was obtained by inserting the Klenow-filled *Asp* 718-*Apa*I fragment of plasmid δP1 (subclone of the poliovirus type 1 [Mahoney strain] cDNA obtained from Sylvie Van der Werf, Institut Pasteur, Paris [unpublished construction]) into the Klenow-filled and dephosphorylated *Sph*I site of pZCAT. This fragment contains a large part of the 5′ nontranslated region (5′ NTR) of poliovirus from nucleotide 66 to nucleotide 743. Plasmids containing the inserts in the correct orientation were selected by restriction mapping.

(ii) Plasmids pCORT0, pCORT1, pCORT2, pRCORT0, and pRCORTHH. Plasmids pCORT0, pCORT1, and pCORT2 (28) contain, under the control of the MLP and tripartite leader, the HBV C gene and a part of the Moloney murine leukemia virus (MoMLV) *pol* gene fused to the C gene sequence at a *Bgl*II site, 25 nucleotides upstream from the end of the C gene. The MoMLV *pol* gene was inserted in the C gene frame in plasmid pCORT0 (see Fig. 3A), in the P gene frame in plasmid pCORT1 (see Fig. 3C), and in the third frame in plasmid pCORT2 (see Fig. 6A). The 5′ terminus of the HBV C gene sequence was inserted at a *Hin*dIII site at the end of the third leader. Plasmid pRSVCAT (18) was used to express the HBV C gene and MoMLV *pol* sequence under the control of the Rous sarcoma virus (RSV) promoter. The *Hin*dIII-*Nco*I fragment of pCORT0 containing the HBV C gene and MoMLV *pol* sequence was introduced into the *Hin*dIII-*Nco*I large fragment of pRSVCAT to obtain plasmid pRCORT0. Plasmid pRCORTHH was constructed by inserting a head-to-tail repeat of the *Hin*dIII-*Eco*RI 30-bp fragment of the multiple cloning site sequence of pUC8 (New England BioLabs) in the *Hin*dIII site of pRCORT0.

(iii) Plasmids pP2A and pRP2A. Plasmids pP2A and pRP2A contain the poliovirus protease 2A coding sequence derived from plasmid δP1 (see above) from nucleotide 3381 to the poly(A) tail of the poliovirus type 1 (Mahoney strain) cDNA. In this clone, a *Sma*I restriction site was fused upstream from the *Nde*I site (nucleotide 3381) located at the beginning of the protease 2A-coding sequence, and an *Eco*RI site follows the poly(dA) sequence. This *Sma*I-*Eco*RI fragment was introduced between the *Sma*I and *Eco*RI sites of the multiple cloning site of plasmid pSP18 (GIBCO BRL). The Klenow-filled *Sal*I-*Eco*RI fragment of the resulting plasmid was then introduced into the Klenow-filled and dephosphorylated *Nco*I site of plasmid pMLP14, a plasmid derived from pMLP10 and containing downstream from the MLP and tripartite leader an *Nco*I site (giving an ATG codon when filled). This led to plasmid pP2A, in which the protease 2A-coding sequence is fused in phase with an ATG and seven other codons just downstream from the MLP. The self-processing site at the N terminus of protease 2A is conserved. Plasmid pRP2A contains the poliovirus fragment in the reverse orientation. The MLP-poliovirus junctions of these plasmids have been sequenced.

(iv) X, X1, and M series of deleted plasmids. Two series of deletions were performed on plasmid pCORT0 DNA by *Bal* 31 exonuclease digestion. The first set of deleted plasmids (X series) was obtained by *Bal* 31 digestion of pCORT0 DNA linearized at a unique *Xho*I restriction site located at the end of the adenovirus third leader sequence. Five micrograms of *Xho*I-linearized pCORT0 DNA was incubated at 30°C with 1 U of *Bal* 31 in 100 μl of incubation buffer (20 mM Tris-HCl, 600 mM NaCl, 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$, 1 mM EDTA, pH 7.2). Twenty microliters was withdrawn every 3 min, and the reaction was stopped by the addition of 2.2 μl of 200 mM EGTA. After phenol extraction of proteins, DNA was precipitated in ethanol and collected. DNA fragments were Klenow filled, digested with *Pst*I, and ligated with the *Xho*I (Klenow-filled)-*Pst*I small fragment of pCORT0. The resulting deleted plasmids were selected on the basis of the size of the deletion by restriction mapping. The MLP-C gene junctions were then sequenced. Plasmids of the X1 series were obtained by ligation of the *Pst*I-*Mro*I small fragment of the plasmids of the X series with the *Mro*I-*Pst*I large

fragment of plasmid pCORT1. The deletion borders of the selected plasmids are shown in Fig. 3A and C.

The second set of deletions (M series) was obtained by *Bal* 31 digestion of pCORT0 DNA linearized at the unique *Mro*I restriction site located 22 nucleotides downstream from the HBV P gene ATG. The *Bal* 31 digestion was performed as described above. The DNA was then digested with *Pst*I and ligated with the *Mro*I (Klenow-filled)-*Pst*I large fragment of pCORT0. Deleted plasmids were selected on the basis of their size, and the regions surrounding the deletions were sequenced (see Fig. 4A). Plasmid pM9 is a plasmid derived from plasmid pM8, in which the reading frames were shifted by digestion of the *Mro*I site of pM8 (reconstructed after *Bal* 31 digestion and ligation), Klenow filling of the extremities, and religation.

(v) Plasmids pJM and pJM1. Plasmid pJM was constructed by site-directed mutagenesis by the polymerase chain reaction. The polymerase chain reaction was performed as described by Saiki et al. (45) on plasmid pCORT0 with a 17-mer primer (positions 9665 to 9681 of the MLP) and a 19-mer primer (positions 2153 to 2171 of the HBV C gene) in which the T at position 2168 was substituted by C; this replaces the J ATG codon with the threonine ACG codon. The *Xho*I-*Hin*dII fragment of the amplified DNA was introduced at the place of the original *Xho*I-*Hin*dII fragment in the parent plasmid pCORT0. The amplified fragment of pJM was sequenced to verify the presence of the J ATG mutation and the absence of unexpected ones. Plasmid pJM1 resulted from the ligation of the small *Pst*I-*Mro*I fragment of pJM with the large *Mro*I-*Pst*I fragment of pCORT1.

(vi) Plasmids pPCRT2 and pAERT2. Plasmid pPCRT2 is a plasmid derived from pCORT2 in which the pre-C sequence of the HBV C region was added. This was obtained by ligation of the small *Pst*I-*Mro*I fragment of plasmid pMLP-PC (27) with the large *Pst*I-*Mro*I fragment of pCORT2. Plasmid pAERT2 is a plasmid derived from pPCRT2 in which the CAA codon following the pre-C ATG was transformed in the TAA codon. Site-directed mutagenesis was performed by polymerase chain reaction with plasmid pPCRT2 and two primers: the one at positions 1800 to 1825 of the pre-C sequence has a substitution of C to T at position 1814, and the other (positions 2328 to 2354 of the C gene) contains an *Mro*I site. The amplified fragment was digested by *Mro*I and replaced the *Fsp*I-*Mro*I fragment of pPCRT2. The presence of the mutation was confirmed by sequencing.

Transfection of 293 cells, cell labelling, and immunoprecipitation. Adenovirus-transformed human embryo cells (line 293-31 [20]), were grown in 100-mm dishes in Dulbecco modified Eagle medium containing 10% fetal calf serum. Cells at 50% confluency were transfected by the calcium phosphate method (19) with 15 μg of plasmid DNA or 10 μg of each plasmid DNA in cotransfection experiments. Twenty-four hours later, the medium was removed and 10 ml of fresh medium was added. Twenty-four hours later (or 5 h later for the protease 2A experiment), cells were starved of methionine for 1 h and labelled with 500 μCi of [^{35}S]methionine for 3 h in 4 ml of methionine-free medium. Cells were washed with phosphate-buffered saline (PBS; 10 mM phosphate buffer [pH 7.4], 140 mM NaCl) and lysed with 2% Nonidet P-40 in PBS. Cell debris and nuclei were spun out, and supernatants (cytoplasmic lysates) were collected. Immunoprecipitations were done with 5×10^7 cpm of cytoplasmic lysate and 5 μl of anti-MoMLV reverse transcriptase antibodies or 5 μl of anti-HBV capsid protein antibodies as described previously (27). The immunoprecipitated proteins were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels were fixed in 30% methanol–10% acetic acid, treated with Amplify (Amersham), dried, and autoradiographed.

CAT and β-Gal assays. Forty-eight hours after transfection of the bicistronic *lacZ-cat* plasmids, the culture medium was removed and the cells were collected in PBS. The cells were lysed in 500 μl of buffer H (100 mM sodium phosphate [pH 7.5], 250 mM sucrose) by four cycles of freezing and thawing (3 min in dry ice-ethanol, 3 min at 37°C). After centrifugation at 12,000 × *g* for 10 min, the supernatant (cytoplasmic extract) was used for a β-galactosidase (β-Gal) assay as described previously (2). Five, 10, or 20 μl of cytoplasmic extract was incubated in a 1-ml final volume of buffer Z (100 mM sodium phosphate buffer [pH 7.5], 10 mM KCl, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol) with 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 100 mM sodium phosphate buffer, pH 7.5) at 37°C. Commercial β-gal (Sigma) was used as a standard, and the blank contained 20 μl of buffer H. The reaction was stopped by the addition of 500 μl of 1 M Na_2CO_3 when the yellow color appeared in the standard (approximately 5 min), and the optical density at 420 nm was measured.

Chloramphenicol acetyltransferase (CAT) activity was measured as previously described (49). Briefly, cellular acetylases were inactivated by heating the cytoplasmic extract described above at 65°C for 10 min. After centrifugation at 12,000 × *g* for 10 min, 5, 10, or 15 μl of the supernatant (blank was made of buffer H) was added to 10 μl of reaction mixture containing 0.225 mM acetyl coenzyme A, 4 mM chloramphenicol, and 1.0 μCi of [^{14}C]acetyl coenzyme A (60 mCi/mmol); the volume was then adjusted to 25 μl with 250 mM Tris-HCl (pH 8). The mixture was incubated at 37°C for 1 h, and 20 μl was withdrawn and placed in a scintillation vial containing 200 μl of water and 250 μl of ethyl acetate. The mixture was mixed vigorously, and 4 ml of Econofluor (Dupont, NEN Research Products) was added. After at least 2 h at room temperature, the radioactivity was determined by scintillation counting. The average value of blank and mock-transfected cell assays was 600 cpm. After subtraction of the blank value, the plot of counts per minute as a function of the cytoplasmic extract volume gave a linear result.

RESULTS

Insertion of HBV C gene sequences in a bicistronic transcript did not influence translation of the second cistron. To determine whether C gene sequences located upstream from the P gene could allow direct entry of ribosomes, we constructed a eukaryotic expression vector which produced a bicistronic mRNA after transfection into human cells (plasmid pZCAT). The first cistron placed under the control of the adenovirus MLP encoded the β-Gal of *E. coli*, and the second cistron encoded *E. coli* CAT. The intercistronic region contained a 30-nucleotide polylinker which was used to introduce various sequences of the HBV C gene as shown in Fig. 1A or a part of the poliovirus 5′ NTR that allows internal entry of ribosomes (40) as a control for efficient translation of the *cat* gene. Plasmid pZCAT and its derived plasmids were transfected in parallel cultures of 293 cells, and β-Gal and CAT activities were measured in cell extracts. For each plasmid transfection, CAT activity was normalized by β-Gal activity (CAT/β-Gal ratio), β-Gal activity being an internal control for transfection efficiency. For each series of transfection experiments, the CAT/β-Gal ratio of cells transfected by the bicistronic plasmids was calculated in relation

to the CAT/β-Gal ratio of cells transfected by pZCAT, which was arbitrarily set to 1.

In cells transfected by pZCAT, very weak CAT activity was detected, most likely due to a few ribosomes which after completing β-Gal synthesis resumed scanning and reinitiated translation at the *cat* gene AUG. CAT activity was not significantly increased (less than 1.7-fold) in cells transfected by the bicistronic plasmids containing the B, E, G, or H sequences of the HBV C gene (Fig. 1B). However, insertion of the 5′ NTR of poliovirus mRNA between the *lacZ* and *cat* genes (plasmid pZPOLIOCAT) leaded to a 60-fold increase of *cat* gene translation (Fig. 1B).

Evidence for cap-dependent translation of a gene located downstream of the HBV C gene in a bicistronic mRNA. As documented by studies on picornaviruses (25), internal initiation in eukaryotic cells is a cap-independent mechanism. In poliovirus, ribosomes enter the viral uncapped RNA in its 5′ NTR and scan to the initiation codon (40), while translation of cellular mRNAs is inhibited by inactivation of the cap-binding complex eIF-4F, a process mediated by poliovirus protease 2A (22). Sun and Baltimore (52) have previously shown that transient expression of protease 2A is sufficient to inhibit cap-dependent translation. The same approach was used to determine whether cap-independent entry of ribosomes can occur in the HBV C gene. For this purpose, human cells were cotransfected with an HBV C gene-derived construction and a plasmid expressing the poliovirus protease 2A. As we had no efficient way to characterize the HBV P polypeptide(s), we used a reporter gene inserted downstream from the HBV C gene. If internal entry of ribosomes occurs in a C gene sequence, translation of the first cistron (C gene) should be inhibited by protease 2A, whereas translation of the second cistron (reporter gene) should not. On the other hand, if the two genes are translated by ribosomes scanning from the 5′ end of the mRNA, protease 2A should inhibit the synthesis of both proteins. However, due to the inefficiency of the calcium phosphate method we used for cell cotransfection, a portion of the cells received only the HBV C gene-derived plasmid. Thus, we could not expect to observe a total inhibition of cap-dependent translation by protease 2A. Consequently, interpretation of the results was based on a similar or dissimilar decrease of syntheses of the C and reporter proteins. We used a construction in which the reporter gene was fused in frame with the HBV C gene. In this way, the antiserum raised against the reporter protein immunoprecipitated the fusion C-reporter protein translated from the C AUG as well as the reporter protein translated from its own AUG. The reporter gene we used was a part of the MoMLV *pol* sequence, the first AUG of which is in a favorable context for initiation of translation. Expression of the MoMLV *pol* gene from such a construction is made possible because the P gene AUGs of hepadnaviruses are in weak initiation contexts and allow leaky scanning to a downstream AUG as previously reported (8, 28).

The poliovirus protease 2A-coding region was inserted downstream from the adenovirus MLP and its tripartite leader (see Materials and Methods). The adenovirus tripartite leader confers to its mRNA the ability to be translated independently of the formation of the cap-binding complex (13) and allows its translation in poliovirus-infected cells (14). The plasmid pP2A contained the insert in the correct orientation with respect to the promoter and allowed the expression of protease 2A. The plasmid pRP2A containing the insert in the reverse orientation served as a control. The C gene-MoMLV *pol* gene fusion was inserted downstream from the RSV long terminal repeat (plasmid pRCORT0, Fig. 2A). Translation of mRNAs synthesized under the control of this promoter is cap dependent and is inhibited by poliovirus protease 2A expression (52). The same construction was also inserted downstream from the MLP as a reference of cap-independent translation.

The results of cotransfection experiments are shown in Fig. 2A. In the control experiment (lane RP2A), essentially two polypeptides of 50 and 32 kDa were immunoprecipitated with the anti-MoMLV reverse transcriptase antiserum, corresponding to the proteolytically processed forms of the 73-kDa fusion protein translated from the C AUG and to the protein synthesized from the reporter gene AUG (RT AUG), respectively (28). When protease 2A was coexpressed, translations of both fusion and reporter proteins were similarly decreased (lane P2A, Fig. 2A). As a control, when expressed in a cap-independent manner under the control of the MLP, syntheses of the C and RT proteins were unaffected by protease 2A (data not shown). These results are indicative of a cap-dependent translation of the reporter gene.

Stable hairpin structures inserted in the 5′ noncoding region of mRNAs have been shown to prevent scanning ribosomes from initiating translation at downstream AUG codons (31, 39). In a bicistronic mRNA, such a hairpin inhibits translation of both cistrons except if the second cistron is translated by ribosomes binding directly to an internal site downstream from the hairpin (33, 40). Accordingly, an inverted tandem repeat of the 30-bp *Hin*dIII-*Eco*RI fragment from pUC8 (33) was inserted into the unique *Hin*dIII site of plasmid pRCORT0 (plasmid pRCORTHH, Fig. 2B). mRNA analysis confirmed that the presence of this secondary structure did not modify the transcription efficiency at the RSV promoter (data not shown). As shown in Fig. 2B, the hairpin structure inhibited the synthesis of the proteins initiated at the reporter gene AUG as well as at the C AUG (compare lane HH to lane T0). This result suggests that the reporter protein, like the C protein, was translated by ribosomes scanning from the 5′ end of the bicistronic messenger and eliminates the possibility of a jump of ribosomes from the cap to the reporter gene AUG as reported for Sendai virus X protein translation (11).

Taken together, the results of the three experimental approaches indicate that the translation of a gene located downstream from the HBV C gene cannot be attributed to a mechanism of internal entry of ribosomes followed by a scanning to the initiation codon, as reported for poliovirus (40), but suggest most likely that the translation of the second cistron can be initiated by ribosomes scanning from the 5′ end of the C mRNA.

Influence of C-region AUGs on downstream translation. To determine how some scanning ribosomes can reach the reporter gene, we analyzed translation initiation at the internal AUGs C1, J, and C2 (Fig. 3A, plasmid pCORT0). The X series of plasmids was obtained from plasmid pCORT0 by nuclease *Bal* 31 digestion from a unique *Xho*I site. Deleted plasmids were selected on the basis of their size in order to have one to four AUGs deleted (Fig. 3A). The X plasmids were transfected in 293 cells, and ^{35}S-labelled proteins were immunoprecipitated with the anti-MoMLV reverse transcriptase antibodies. The results of these experiments are shown in Fig. 3B. When the C AUG was deleted (plasmid pX4), a faint amount of the shortened fusion protein translated from the C1 AUG was observed (Fig. 3B, lane X4). In cells transfected with plasmid pX10, in which both the C and C1 AUGs were deleted, the fusion protein

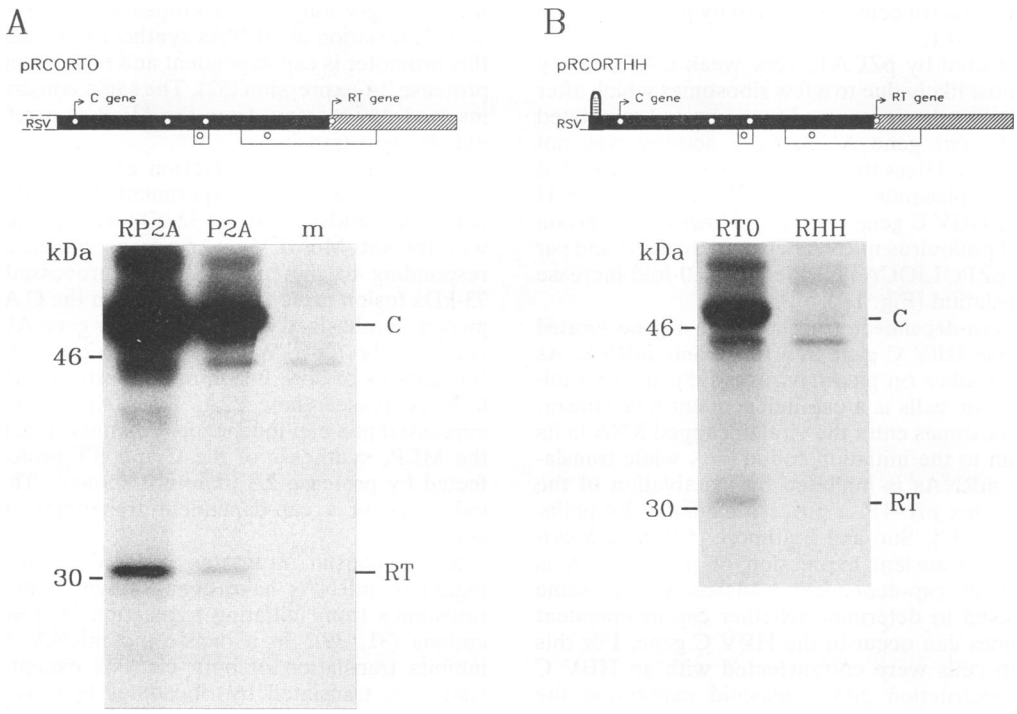

FIG. 2. (A) Effect of poliovirus protease 2A expression on the translation of a bicistronic C-MoMLV *pol* mRNA. Plasmid pRCORT0 contains downstream from the long terminal repeat sequence of RSV the C gene of HBV (black box) fused in frame with a portion of the MoMLV *pol* gene (hatched box) starting with an AUG codon. Plasmid pP2A expresses poliovirus protease 2A under the control of the adenovirus MLP; pRP2A has the p2A sequence in the reverse orientation as a control. Proteins expressed in cells cotransfected with the plasmid pRCORT0 and either the plasmid pRP2A or the plasmid pP2A, as indicated above each lane, were immunoprecipitated with anti-MoMLV reverse transcriptase antibodies and analyzed on by SDS-polyacrylamide gel electrophoresis (PAGE). Lane m, mock-transfected cells. Positions of standard molecular size markers are indicated on the left. The fusion C-RT protein (C) and the reporter protein (RT) are indicated on the right. (B) Inhibition of C-MoMLV *pol* mRNA translation by introduction of a hairpin at its 5′ extremity. Plasmid pRCORTHH is identical to pRCORT0 except that it contains a head-to-tail repeat of 30 bp inserted in the *Hin*dIII site located downstream from the RSV promoter. Immunoprecipitated proteins from pRCORT0 (lane T0)- and pRCORTHH (lane HH)-transfected cells were analyzed by SDS-PAGE. Molecular size markers are indicated on the left. The 50-kDa fusion protein (C) and the 32-kDa reporter protein (RT) are indicated on the right.

translated from the C2 AUG was barely detectable (Fig. 3B, lane X10). The extension of the deletion to the J AUG (plasmid pX11), which precedes the C2 AUG by only a few nucleotides, led to the efficient synthesis of the fusion protein initiated at the C2 AUG and to a concomitant decrease of the synthesis of the reporter protein (compare lane X11 with lane X10). Another deletion eliminating the four AUGs preceding P AUG (plasmid pX12) resulted in a large increase of RT protein synthesis. To have a direct examination of events occurring at the P AUG, we engineered new constructs (X1 series). The X1 series of plasmids are similar to the X series except that the reporter gene is in frame with the P ORF instead of the C ORF (Fig. 3C). As shown in Fig. 3D, with the control plasmid (lane T1), two proteins of 37 and 32 kDa were specifically immunoprecipitated with the anti-MoMLV reverse transcriptase antibodies, corresponding, respectively, to the fusion protein translated from the P AUG and to the protein translated from the RT AUG. Deletions of the C AUG (plasmid X14) or of the C and C1 AUGs (plasmid X110) resulted in an increase of the amounts of both proteins. As anticipated, from the results of the X series, neither fusion protein nor reporter protein was detected in cells transfected with plasmid pX111 in which the J AUG was deleted. Maximal synthesis of fusion and reporter proteins was obtained when all the AUGs preceding the P AUG were deleted (plasmid X112). These results were in agreement with the respective Kozak's context of the different initiation codons (weak for C1, gaa cua AUG a; optimal for C2, acu aau AUG g) and suggest (i) that translation of the J ORF prevents translation from the C2 AUG from which a truncated capsid would be synthesized and (ii) that as the J ORF stops upstream of P gene, its translation allows reinitiation at the downstream P AUG (plasmids X11 and X111).

To confirm these hypotheses, we constructed another set of deleted plasmids using *Bal* 31 deletions starting from the unique *Mro*I site. Plasmids in which the reporter gene was in frame with the C gene were selected on the basis of their size in order to have one to three AUGs deleted (M series, Fig. 4A). In addition, a plasmid (pM9) in which the reporter gene was fused to the J ORF was constructed. As shown in Fig. 4B, deletion of the P AUG and of the 90 upstream nucleotides (plasmid pM5) did not prevent translation initiation at the RT AUG (compare lane M5 with lane T0), as expected if this region would permit internal entry of ribosomes. In pM8 (C2 and P AUGs deleted)-transfected cells, the synthesis of the reporter protein was much lower than in pM5- or pCORT0-transfected cells (compare lane M8 with lanes T0 and M5). The most likely explanation for this observation is that the J ORF is fused to a downstream ORF which

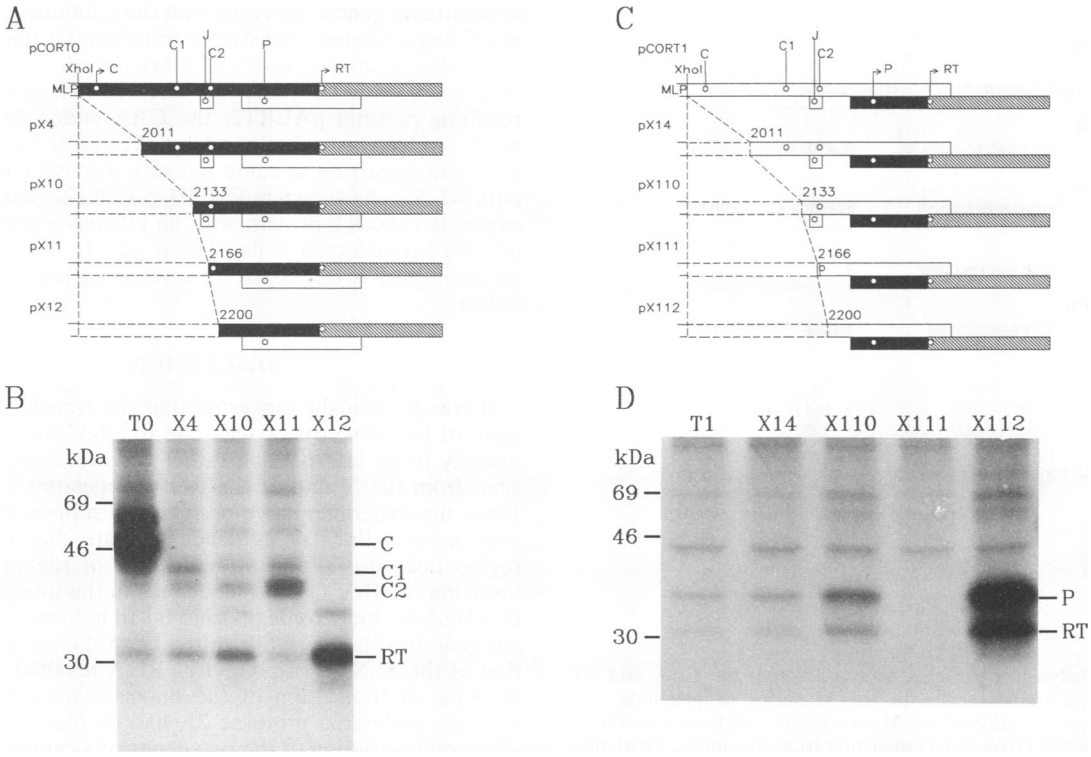

FIG. 3. Analysis of proteins translated from C, C1, C2, and RT AUGs in cells transfected with the X and the X1 series of plasmids. (A) Schematic representation of plasmids pX4, pX10, pX11, and pX12. These plasmids were obtained from pCORT0 by *Bal* 31 digestion from the unique *Xho*I site. The borders of the deletions refer to the positions in the HBV sequence. Wild-type or 5′-truncated C genes (black boxes) were directly under the control of the promoter (MLP) and were fused with the MoMLV *pol* gene sequence (hatched boxes). Empty boxes represent the J and P ORFs. (B) SDS-PAGE analysis of immunoprecipitated proteins from cells transfected with pCORT0 (lane T0) and cells transfected with the X series of plasmids as indicated above each lane. Positions of standard molecular size markers are indicated on the left. The fusion proteins translated from the C, C1, or C2 AUG and the 32-kDa reporter protein (RT) are indicated on the right. (C) Schematic representation of plasmids pX14, pX110, pX111, and pX112. These plasmids were identical to the X plasmids except that the reporter gene (hatched box) was in frame with P ORF (black box). Empty boxes represent the C and J ORFs. (D) SDS-PAGE analysis of immunoprecipitated proteins from cells transfected with pCORT1 (lane T1) and cells transfected with the X1 series of plasmids as indicated above each lane. Positions of standard molecular size markers are indicated on the left. The 37-kDa fusion protein translated from the P AUG and the 32-kDa reporter protein (RT) are indicated on the right.

overlaps the reporter gene (Fig. 4A), thus eliminating the possibility of reinitiation at the reporter gene AUG after completion of J ORF translation. This result strongly suggests that the reporter gene would be translated on the parent CORT0 mRNA by ribosomes reinitiating after J ORF translation. This hypothesis is supported by the results with pM9-transfected cells (pM9 contains the same deletion as pM8 but the frames were shifted to create a fusion between J and the reporter ORFs). In this case, a protein longer than RT protein, probably corresponding to the J-RT fusion product, was synthesized in large amounts, indicating that the J ORF was actually and efficiently translated despite the nonoptimal context of the J AUG (uca guu AUG u). Synthesis of RT protein in pM9-transfected cells was greatly increased (compare lanes T0 and M9), most likely because ribosomes reinitiate translation at the reporter gene AUG after completion of C ORF translation (Fig. 4A). This increase of RT protein was also observed when cells were transfected with other plasmids of the M series in which the C ORF ended before the RT AUG (data not shown). Further deletion eliminating the J AUG (Fig. 4A, pM10) resulted in a large increase of RT protein synthesis (Fig. 4B, compare lanes M10 and T0). These data emphasized those obtained with the X series of plasmids which suggested that the J ORF modulates the rate of translation initiation at the reporter gene AUG.

Influence of the J ORF on downstream translation. To demonstrate that the translation of the J ORF influences downstream translation, we mutated the J initiation codon into the threonine ACG codon in plasmid pCORT0 (plasmid pJM) or in plasmid pCORT1 (plasmid JM1). If ribosomes actually translate the J ORF, this mutation would lead to an increase of protein synthesis initiated at the C2 AUG and to a concomitant decrease of the translation of downstream genes. As expected, in cells transfected with plasmid pJM in which the reporter gene was in frame with the C gene, synthesis of the fusion protein translated from C2 AUG was significantly increased with a concomitant decrease of RT protein synthesis compared with pCORT0-transfected cells (Fig. 5A). Similarly, when the reporter gene was in frame with the P AUG (plasmids pCORT1 and pJM1), the mutation of the J AUG in plasmid pJM1 led to a decrease of the synthesis of the polypeptides translated from the P and RT AUGs (Fig. 5B). In a control experiment, the capsid protein expressed from either plasmid pCORT1 or pJM1 was immunoprecipitated with anti-C antibodies. No difference in the rate of synthesis of the capsid protein was observed (data not shown). These experiments demonstrate that translation of

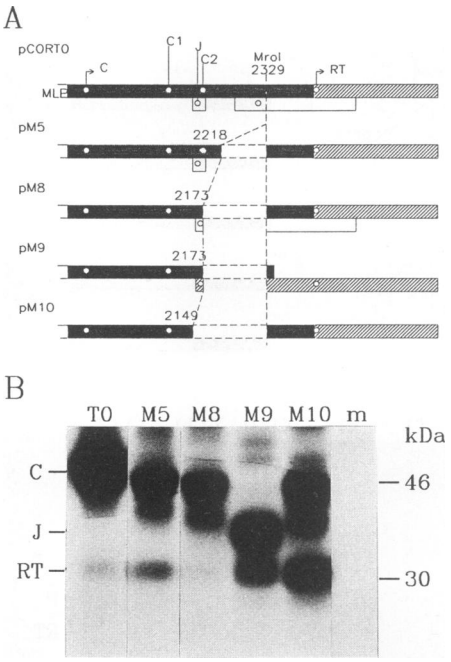

FIG. 4. Analysis of proteins translated from the C, J, and RT AUGs in cells transfected with the M series of plasmids. (A) Schematic representation of the M series of plasmids obtained from the plasmid pCORT0 by *Bal* 31 digestion from the unique *Mro*I site. Upstream limits of the deletions are indicated and numbered according to their positions in HBV sequence. Wild-type or deleted C genes (black boxes) are fused in frame with the reporter gene (hatched boxes) in plasmids pCORT0, pM5, pM8, and pM10 and in the +1 frame in plasmid pM9. (B) SDS-PAGE analysis of immunoprecipitated proteins from cells transfected with pCORT0 (lane T0), cells transfected with the M series of plasmids as indicated above each lane, and mock-transfected cells (lane m). The fusion proteins translated from the C or J AUG and the 32-kDa reporter protein (RT) are indicated on the left. Positions of standard molecular size markers are indicated on the right.

the seven-codon minicistron beginning at the J AUG allows ribosomes to bypass the C2 AUG to reinitiate translation at a downstream AUG while preventing translation of a truncated capsid protein.

The pre-C AUG of the HBV C region did not allow leaky scanning of ribosomes. It has been previously demonstrated that the pre-C mRNA which contains the entire pre-C/C region and the P gene is responsible for the synthesis of the HBV e antigen but not of the capsid protein (27, 50). This observation could be explained by the context surrounding the pre-C AUG, which has a good match with the consensus sequence (agc acc AUG c) and does not allow leaky scanning. Therefore, it is expected that an mRNA containing the pre-C sequence cannot serve for translation of a gene located downstream from the C gene, except if this gene is translated by direct entry of ribosomes. To demonstrate this hypothesis, we constructed a plasmid containing the pre-C/C region of HBV followed by the MoMLV *pol* gene (plasmid pPCRT2, Fig. 6A). This plasmid and the control plasmid pCORT2 have the reporter gene in −1 frame with respect to the C gene to avoid immunoprecipitation of the processing intermediates of the secreted HBV e antigen with the anti-MoMLV reverse transcriptase antibodies. As shown in Fig. 6B, plasmid pPCRT2 did not express the RT protein, confirming that the pre-C sequence does not allow translation of downstream genes. To verify that the inhibitory effect of the pre-C sequence on downstream translation is due to efficient translation from the pre-C AUG, we introduced a premature stop codon in pPCRT2 by site-directed mutagenesis. In the resulting plasmid pAERT2, the CAA codon following the pre-C AUG was substituted by the TAA stop codon, a mutation occurring in some recently discovered HBV variants (47, 53). As shown in Fig. 6B, pAERT2-transfected cells expressed the RT protein with an efficiency comparable to pCORT2-transfected cells. These results confirm that the reporter gene is translated by a scanning reinitiation mechanism.

DISCUSSION

It was previously suggested that the translation of the P gene of hepadnaviruses was due to ribosomes which bind directly to an internal site rather than to ribosomes which scan from the 5′ end of the RNA pregenome (10, 28, 48). From the experiments we designed to support this hypothesis, several lines of evidence indicate that the HBV C region does not allow internal entry of ribosomes. First, insertion of HBV C gene sequences in the intergenic region of a *lacZ-cat* bicistronic plasmid failed to increase efficiently *cat* gene translation, while in the control experiment, insertion of the 5′ NTR of poliovirus RNA resulted in a 60-fold increase of translation of the downstream coding region. Second, poliovirus protease 2A had an identical inhibitory effect on translation of the two genes of a capped bicistronic C gene-MoMLV *pol* mRNA, demonstrating the cap-dependent translation of the reporter gene. Third, an RNA hairpin inserted close to the cap inhibited translation of the reporter gene. Except if this secondary structure has unanticipated effects on adjacent structural elements, this result suggests that the reporter gene is translated by scanning ribosomes from the 5′ end of the mRNA. Fourth, deletion of sequences upstream from the P AUG (X, X1, and M series) never abolished translation of the reporter gene. Thus, these results are consistent with a model in which the translation of the P gene is likely to be mediated by a scanning mechanism through the C gene, a conclusion recently reached by Lin and Lo (32), who used an in vitro transcription and translation coupled system.

Since the X1 series of deletion demonstrates that MoMLV *pol* translation reflects P gene translation, our results are consistent with the following model for HBV P gene translation. First, most of the ribosomes scanning from the 5′ end of the RNA pregenome initiate translation at the C AUG. However, a few ignore this initiator codon and continue scanning toward the C1 AUG. Second, most likely because of the unfavorable context surrounding this AUG, almost all ribosomes scan past the C1 AUG toward the J AUG, which is out of the C reading frame, 11 nucleotides upstream from the C2 AUG. Our results clearly demonstrate that the efficient translation of the seven-codon J ORF allows ribosomes to bypass the C2 AUG, which is in a strong context and otherwise would allow efficient initiation of translation. Third, after completing the translation of the J minicistron, ribosomes resume scanning and reinitiate translation at the P AUG.

The analysis of HBV C gene sequences available in the GenBank data base (Fig. 7) showed that the J ORF is ubiquitous with respect to position and length in all strains of HBV and in the chimpanzee virus genomes. While many point mutations are found in the internal codons of this ORF, none of them affect either the J AUG or the position of the J

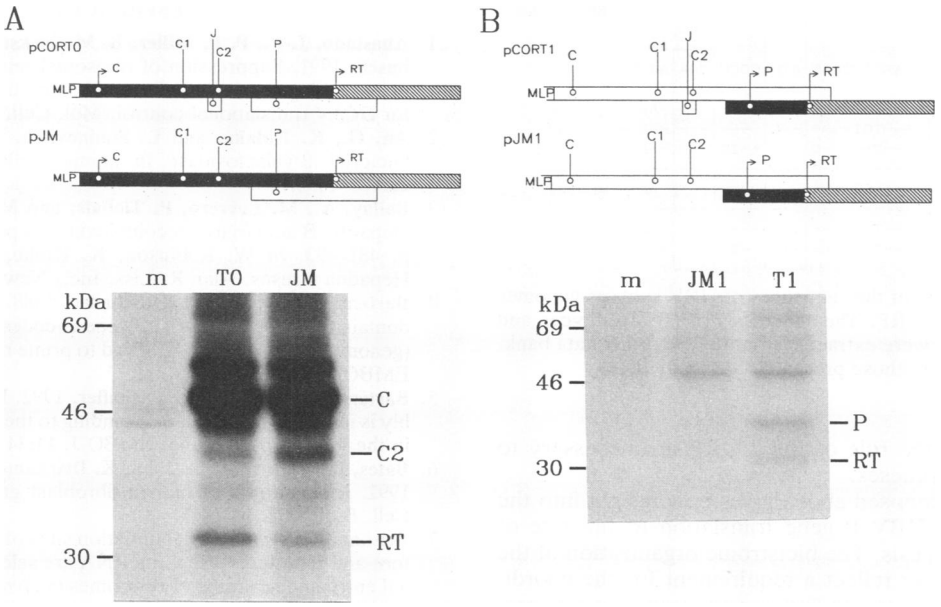

FIG. 5. Influence of the J ORF on the P and reporter gene translations. (A) Plasmid pJM is derived from plasmid pCORT0 by site-directed mutagenesis: the J AUG codon was mutated into the threonine ACG codon. Immunoprecipitated proteins of cells transfected with the plasmids pCORT0 (lane T0) and pJM (lane JM) or of mock-transfected cells (lane m) were analyzed by SDS-PAGE. Proteins initiated at the C, C2, and RT AUGs are indicated on the right. Standard molecular size markers are indicated on the left. (B) Plasmid pJM1 is identical to plasmid pCORT1 except that the J AUG codon was replaced by a threonine ACG codon. Immunoprecipitated proteins of cells transfected with the plasmids pCORT1 (lane T1) and pJM1 (lane JM1) or of mock-transfected cells (lane m) were analyzed by SDS-PAGE. Proteins initiated at the P and RT AUGs are indicated on the right. Standard molecular size markers are indicated on the left.

stop codon. Because of the degenerate structure of the genetic code, these conservations are not necessary to maintain the C amino acid sequence. Moreover, the same initiation context surrounds the J AUGs, and no additional minicistrons are found in the different subtypes of HBV. These observations raise the question of the function of this small ORF. The first possible interpretation is that translation of the J ORF prevents the efficient synthesis of a truncated capsid protein initiated at C2 AUG. However, the lack of the C2 AUG in two HBV subtypes (Fig. 7) raises the possibility of another function of the J minicistron. The second possible interpretation is that, as observed from deleted plasmids pX12, pX112 (Fig. 3), and pM10 (Fig. 4), the J ORF has an inhibitory effect on downstream translation either by its coding sequence or by its peptide product. This latter possibility seems to us very unlikely since the J amino acid sequences of the different HBV subtypes (data not shown) are quite different in terms of their chemical properties. A more likely explanation is that the translation of the J ORF may be a means of derailing ribosomes and decreasing translation of the downstream P cistron. Precedents to this hypothesis have been reported in different systems in which the down-regulation of translation is due to the efficiency of translation of a short ORF in the 5′ leader of the RNA and not to the encoded peptide (1, 6, 15, 41). However,

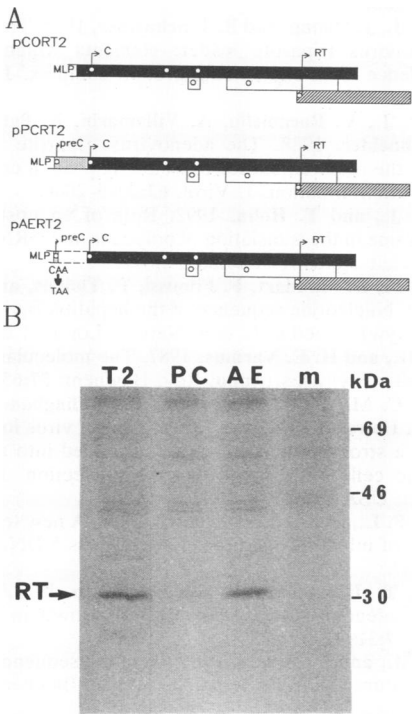

FIG. 6. Analysis of reporter gene expression from pre-C mRNA. (A) Schematic representation of the plasmids used in the experiment. Plasmid pCORT2 is identical to pCORT0 except that the reporter gene (hatched box) is in the −1 frame with respect to the C gene (black box). Plasmids pPCRT2 and pAERT2 are derived from pCORT2 and contain the HBV pre-C sequence upstream from the C gene. In plasmid pAERT2, the CAA codon following the pre-C ATG was changed into the TAA stop codon by mutagenesis. (B) SDS-PAGE analysis of the 32-kDa reporter protein (RT) (indicated by an arrow on the left) from cells transfected with pCORT2 (lane T2), pPCRT2 (lane PC), or pAERT2 (lane AE) and from mock-transfected cells (lane m). Positions of standard molecular size markers are indicated on the right.

HBV subtype		J		C2		stop		References
ayw	AGTT	ATG	TCAACACTAAT	ATG	GGCC	TAA	AGTTC	16
adr	--C-	---	----TGT----	---	----	---	-AA--	36
adr1	--C-	---	----TGT----	--C	----	---	-AA--	43
adw	-A--	---	-T--T-----C	---	--TT	---	--A--	36
adw1	-A--	---	-T--------C	---	----	---	--A--	35
adw2	--C-	---	-----GT----	---	----	---	-AA--	35
adyw	----	---	----------	G--	----	---	-----	37
ayr	--C-	---	----TGT----	---	----	---	-AA-T	34
Chimpanzee	-A--	---	----T--C---	---	--TT	---	--A--	54

FIG. 7. Comparison of the sequences of HBV subtype genomes in the region of the J ORF. The sequences of HBV subtypes and chimpanzee genomes were extracted from the GenBank data bank. Nucleotides identical to those presented are omitted.

further studies on the role of the J ORF are necessary to evaluate these hypotheses.

The model we proposed above gives new insight into the fine adjustment of HBV P gene translation to the rate of capsid protein synthesis. The bicistronic organization of the RNA pregenome may reflect a requirement for the coordinate regulation of the capsid and reverse transcriptase synthesis. However, we cannot extend our model for HBV P gene translation to all hepadnaviruses because (i) the C genes of the other members of the family contain numerous AUG codons (8 to 15) between the C and P AUGs and (ii) Chang et al. (8) have shown that the synthesis of the duck hepatitis B virus P gene product was not affected by modifications of the C AUG context. It would be of interest to determine why two members of the same family use two different mechanisms for the translation of an essential gene.

It is tempting to speculate that in HBV-infected cells, an increase of P gene product synthesis would lead to an increase of replication and virion production and thus could influence the severity of the illness. Such deregulation could be the consequence of mutations affecting the different elements involved in the down-regulation of P gene expression. For example, it has been reported for a plant virus mRNA that the increase in RNA replicase translation provokes the systemic infection of the plant (41). The possibility that the C and P genes can be translated on two mRNA species when a non-sense mutation is introduced downstream from the pre-C AUG could lead to the same effect and could explain the selective advantage of these e minus mutants on wild-type viruses in chronically infected patients (47).

In conclusion, we presented in this report some evidence that the C and P genes are coordinately expressed from the HBV pregenome. This regulation involves a mechanism of translational control in which leaky scanning is associated with translation of a minicistron.

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