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NOTES

Internal Entry of Ribosomes and Ribosomal Scanning Involved in Hepatitis B Virus P Gene Expression

OLIVIER JEAN-JEAN,[1]* THOMAS WEIMER,[2] ANNE-MARIE DE RECONDO,[1] HANS WILL,[2] AND JEAN-MICHEL ROSSIGNOL[1]

Laboratoire de Biologie Moléculaire de la Réplication, Centre National de la Recherche Scientifique ER-272, BP no. 8, 94800 Villejuif, France,[1] and Max-Planck-Institut für Biochemie, 8033 Martinsried, Federal Republic of Germany[2]

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The recent demonstration that the synthesis of duck hepatitis B virus (HBV) reverse transcriptase does not require translational frameshifting and the finding that poliovirus mRNA translation occurs in a cap-independent manner by internal binding of ribosomes in the 5′ noncoding region led us to design experiments to test the hypothesis of internal entry of ribosomes on C gene mRNA for HBV P gene expression. We show that in human cells, translation can be initiated at the first AUG of the HBV P gene by entry of ribosomes in a region located upstream of the P gene. Moreover, the leaky scanning of ribosomes observed on the first AUG of the HBV P gene could be responsible for the synthesis of the two forms of reverse transcriptase described for HBV particles.

Hepadnaviruses are a family of small DNA viruses which replicate genomes via reverse transcription (28). Recently, the reverse transcriptase activity was found to be associated with two polypeptides with molecular masses of 90 and 70 kilodaltons (kDa) present in hepatitis B virus (HBV) particles and encoded by the P gene (2, 3). The initiation of reverse transcription is probably due to a nucleotide covalently linked to a protein attached to the 5′ end of the viral DNA minus strand (12, 20). This protein is encoded by the 5′ extremity of the P gene (1). One major species of genome-length mRNA identified in infected livers (4, 5, 10, 21, 30) serves for the synthesis of the core protein and as the template for reverse transcription. As neither spliced mRNA nor a specific transcript of the P gene has been identified, this genome-length mRNA could also be responsible for the synthesis of P gene translation products, i.e., reverse transcriptase, RNase H (18, 26), and genome-linked protein. However, the expression mechanism of the P gene still remains to be fully elucidated. Based on the finding of core-polymerase fusion proteins in hepatocellular carcinoma tissue (31) and on a C-P overlap reminiscent of *gag-pol* of retroviruses, the hypothesis was put forward that ribosomal frameshifting (7) could be involved in the synthesis of HBV polymerase. Recently, two groups have independently shown that synthesis of duck HBV reverse transcriptase is independent of the production of core-polymerase fusion proteins and have suggested that the P gene is expressed by internal initiation of translation at one of the AUGs of the open reading frame (6, 25).

We have designed experiments to obtain direct evidence on the mechanism of synthesis of HBV P gene-encoded proteins. Our strategy was to look at proteins synthesized in human cells after transient expression of plasmids containing the HBV C gene, the 5′ terminus of the P gene, and part of the Moloney murine leukemia virus (MoMLV) *pol* gene inserted in the C-P overlapping region (Fig. 1). The MoMLV *pol* sequence was fused to the HBV C gene at a *Bgl*II site located 25 nucleotides upstream from the stop codon. A few nucleotides of the *Bgl*II site were deleted to obtain the fusion of the MoMLV *pol* gene in the three frames of HBV DNA (Fig. 1B). For this construction, the *Bam*HI (3535)-*Hin*dIII (4894) blunt-ended restriction fragment of the MoMLV genome was inserted between the *Bgl*II-*Bam*HI sites of pMLP-C plasmid after mild digestion of the extremities with *Escherichia coli* DNA polymerase I in the presence of 0.2 mM deoxynucleoside triphosphate. After screening of recombinant plasmids, plasmids pCORT0, pCORT1, and pCORT2 were selected by sequencing of the HBV-MoMLV junction. The resulting open reading frames are shown in Fig. 1C. Thus, the MoMLV *pol* gene is in frame with the HBV C gene (C frame) in plasmid pCORT0, with the HBV P gene (P frame) in pCORT1, and with the third frame (Y) in pCORT2. As the MoMLV *pol* gene inserted sequence contains an ATG codon 20 nucleotides downstream of the *Bam*HI site used for cloning, the P frame of the pCORT1 plasmid contains two ATGs; the first represents the first ATG of the HBV P gene, and the second is from the MoMLV *pol* sequence. For the constructions, a eucaryotic expression vector was used in which the HBV C region (beginning at the second ATG of the C gene) is placed downstream of the adenovirus major late promoter (Fig. 1A, plasmid pMLP-C).

Human 293 cells (14) were transfected with plasmid DNA (15 μg), using the calcium phosphate method as previously described (13). After transient expression of the HBV-MoMLV recombinant plasmids, cells were labeled with [^{35}S]methionine (New England Nuclear Corp., Dreieich, Federal Republic of Germany), and intracellular proteins (5×10^7 cpm) were immunoprecipitated with 5 μl of either a commercial anti-hepatitis B core antigen (HBcAg) antiserum (Dako Corp., Santa Barbara, Calif.) or an anti-MoMLV reverse transcriptase antiserum (17) as described previously

* Corresponding author.

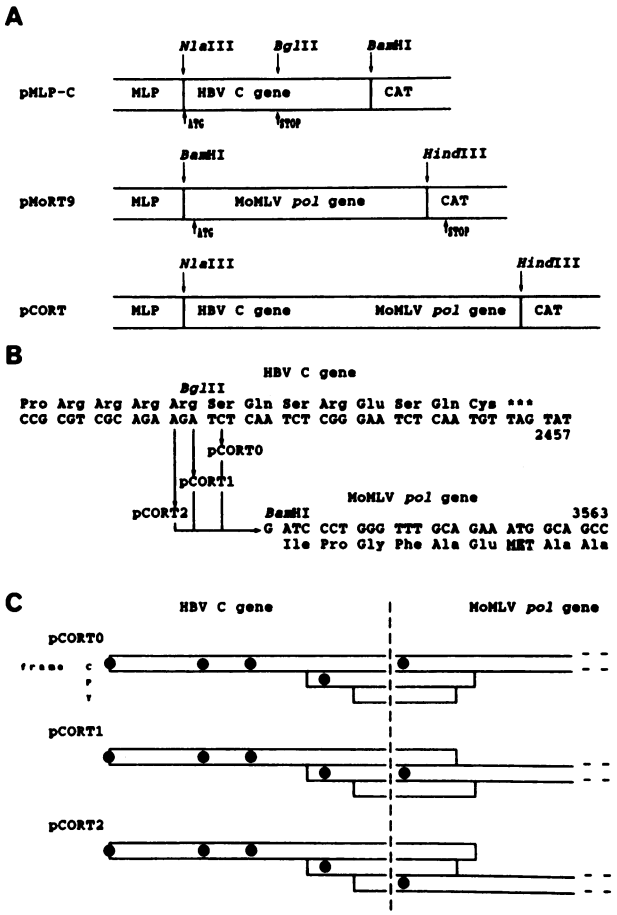

FIG. 1. Construction of HBV C gene-MoMLV *pol* gene recombinant plasmids. Nucleotide positions are derived from the DNA sequences as reported in the GenBank genetic sequence data bank. Restriction sites used in DNA manipulation are shown. (A) Schematic representation of plasmid pMLP-C, pMoRT9, and pCORT series. The adenovirus-based eucaryotic expression vector (pMLP-CAT) and plasmid pMLP-C have been previously described (16). Briefly, plasmid pMLP-C contains the *Nla*III (1902)-*Bam*HI (2906) fragment of the HBV C gene downstream from the adenovirus major late promoter (MLP). In this plasmid, the C region begins at the second ATG of the HBV C gene. Plasmid pMoRT9 contains the *Bam*HI (3535)-*Hin*dIII (4894) fragment of the MoMLV *pol* gene inserted in the same expression vector. Plasmids of the pCORT series are derived from the former constructions as shown in panel B. (B) Sequences of the HBV C gene (11) and of the MoMLV *pol* gene (27) in the region of the gene fusion. The fusions are indicated by arrows. (C) Diagram of the changes in reading frames of pCORT plasmids (named C, P, and Y, as explained in the text). ATG codons are indicated (●).

(16). Immunoprecipitates were analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide gels that were treated with En^{3}Hance (New England Nuclear), dried, and autoradiographed. The modified core proteins immunoprecipitated with the anti-HBcAg antiserum (Fig. 2A) had apparent molecular masses corresponding to the coding capacities of pCORT1 and pCORT2 C frames (22.5 and 24 kDa, respectively). A small amount of these proteins was also unspecifically coprecipitated with the anti-MoMLV reverse transcriptase antiserum (Fig. 2B). This unspecific coprecipitation was not seen when the cell extracts were preincubated with anti-HBcAg antibodies preadsorbed to protein A-Sepharose (Fig. 2C). As expected, the core-MoMLV reverse transcriptase fusion protein synthesized in cells transfected with plasmid pCORT0 was immunoprecipitated with anti-HBcAg antiserum as well as with anti-MoMLV reverse transcriptase antiserum (Fig. 2A and B). Although the entire core-reverse transcriptase fusion protein of 73 kDa was present in pCORT0-transfected cells, the major immunoprecipitated polypeptide had a size of 50 kDa, which was likely the proteolytically processed form of the 73-kDa protein. With the control plasmid pMoRT9 carrying only the *Bam*HI (3535)-HindIII (4894) restriction fragment of the MoMLV *pol* gene, the major immunoprecipitated protein corresponded to a 32-kDa polypeptide instead of the expected 54-kDa protein (Fig. 2B). This result was probably due to proteolysis of the C terminus of the MoMLV reverse transcriptase, as previously observed when this protein was synthesized as a chimeric protein (17, 29). In pCORT1- and pCORT2-transfected cells, C-P fusion proteins of 73 and 50 kDa, possibly created by ribosomal frameshifting, were not observed (Fig. 2A and B). Because of the absence of the last 25 nucleotides of the HBV C gene in our constructs, we cannot completely rule out that frameshifting does occur at a low level in the expression mechanism of the HBV P gene.

Interestingly, the 32-kDa polypeptide synthesized in pMoRT9-transfected cells was also observed in cells transfected with plasmids pCORT0, pCORT1, and pCORT2 (Fig. 2B), suggesting that translation was initiated at the AUG present at the 5′ end of the MoMLV *pol* sequence. In addition, in pCORT1-transfected cells, a protein of about 37 kDa was synthesized more abundantly than the 32-kDa species (Fig. 2B). A mutation changing the first ATG of the HBV P gene of plasmid pCORT1 into an ATA prevented the synthesis of the 37-kDa polypeptide (Fig. 2B, plasmid pCORT1HM), indicating that the synthesis of this protein was initiated at the first AUG of the P gene (mutagenesis was carried out by a standard protocol [32], and the fragment harboring the mutation was transferred into pCORT1, leading to plasmid pCORT1HM). Thus, in pCORT1-transfected cells, the presence of proteins initiated at both AUGs can be interpreted as a leaky scanning of the ribosome on the HBV P gene AUG.

To exclude the possibility that P gene products were synthesized under the control of promoter sequences present in the C gene, the adenovirus promoter region of plasmid pCORT1 was deleted in plasmid pΔCORT1. Cells transfected with this plasmid did not synthesize HBcAg- or MoMLV reverse transcriptase-related protein (Fig. 2A and B). By Northern (RNA) blot analysis of total cellular RNA probed with the *Bam*HI-*Hin*dIII MoMLV *pol* fragment, an mRNA 2,600 nucleotides in length, as predicted from the construct, was observed (data not shown), and none which could correspond to a P gene-specific unspliced or spliced mRNA was observed. This result argues against the existence of a P-specific mRNA made from a cryptic P gene promoter. Taken together, these results indicate that synthesis of the MoMLV *pol* gene-related proteins of 37 and 32 kDa occurs by internal initiation of translation on C gene mRNA and demonstrate that the HBV P gene is translated by initiation at the first AUG of the open reading frame. Since several preceding AUGs within the C gene were not recognized, as evidenced by the absence of the corresponding HBcAg-related proteins (Fig. 2A, lanes CORT1, CORT1HM, and CORT2; in lane CORT0, the polypeptide of 50 kDa cannot account for initiation at the internal C gene AUG because this would lead to a C-P fusion protein of 63 kDa), this result strongly suggests that initiation of transla-

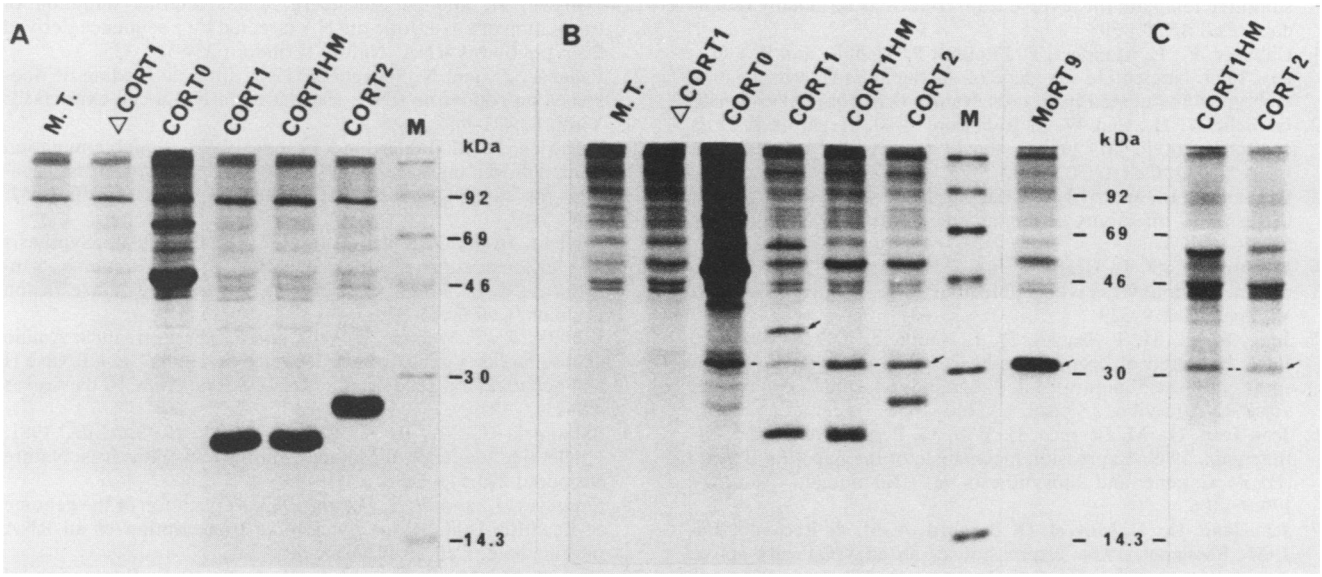

FIG. 2. Analysis of intracellular proteins immunoprecipitated by an anti-HBcAg antiserum (Dako) (A) or by an anti-MoMLV reverse transcriptase rabbit antiserum (17) (B and C); in panel C, the cell extracts were incubated for 2 h with the anti-HBcAg antiserum preadsorbed to protein A-Sepharose, and after brief centrifugation, the supernatant was immunoprecipitated by the anti-MoMLV reverse transcriptase antiserum. Plasmids used for transfection are indicated above each lane. Lanes M.T., Mock-transfected cells; lanes M, ^{14}C-labeled molecular mass markers. In panels B and C, arrows indicate the 37- and 32-kDa polypeptides described in the text.

tion at the first P gene AUG does not involve ribosome scanning along the C gene sequence of the C mRNA but is mediated by specific recognition by the ribosome of a region immediately adjacent to the first P gene AUG. The initiation of translation by internal entry of ribosomes has been demonstrated for the translation of poliovirus mRNA (22, 23), for Sendai virus (8), and for encephalomyocarditis virus (15). Furthermore, initiation at an internal AUG of mRNA was suggested for the synthesis of reverse transcriptase of cauliflower mosaic virus (24) and duck HBV (6, 25). MoMLV *pol* proteins synthesized by internal initiation of translation in the human cells correspond to not more than 1% of core protein. If one assumes a similar translation efficiency in vivo for the HBV P gene, one would expect approximately three reverse transcriptase molecules per HBV core particle. This is a smaller amount than observed in most of the retroviruses (9) and could partially explain the failure to isolate active reverse transcriptase from HBV virions.

Another conclusion of our results is that according to the rules of Kozak (19), the unfavorable context surrounding the P gene AUG (5′ CAAAUGC 3′) allows some ribosomes to migrate through to initiate translation at the next downstream AUG, 330 nucleotides downstream. Thus, two polypeptides with different amino termini could be synthesized. Consistent with this conclusion, two forms of reverse transcriptase, of 90 and 70 kDa, have been identified in HBV virions (2, 3). As most of the reverse transcriptase activity is associated with the 70-kDa polypeptide, it is possible that this protein is initiated at the second P gene AUG and represents the major reverse transcriptase, whereas the amino-terminal domain of the polypeptide initiated at the first AUG could be the genome-linked protein (1).

In conclusion, hepadnaviruses may use both ribosomal scanning and scanning-independent entry of ribosomes at internal AUGs for the coordinated synthesis of the proteins involved in viral replication. In contrast, synthesis of the two C gene products (core protein and hepatitis B e antigen) is regulated by the production of two separate mRNAs (16).

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