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Preferred Translation of Human Hepatitis B Virus Polymerase from Core Protein- but Not from Precore Protein-Specific Transcript

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In the human hepatitis B virus (HBV) genome, the 5′ end of the polymerase coding sequence overlaps with the 3′ end of the core protein coding sequence. Recent results obtained from genetic studies have suggested that translation of HBV polymerase initiates from the first ATG codon of the polymerase reading frame and is not a result of frameshift translation from the core protein reading frame, as in the case of retroviruses. By using in vitro-synthesized SP6 RNA transcripts, we now demonstrate that HBV core protein-specific mRNA can direct the synthesis of polymerase from the internal polymerase ATG codon in rabbit reticulocyte lysates and *Xenopus* oocytes. A related message with an additional 60 nucleotides at the 5′ end (pre-core protein mRNA) was not as efficient as the core protein mRNA for translation of polymerase. Furthermore, translation of polymerase from the core protein mRNA was not inhibited by the cap analog m^7GpppG. This result, together with the results described above, indicates that translation of HBV polymerase occurs in a novel, cap-independent manner.

Hepatitis B virus (HBV) as well as a group of closely related animal viruses belong to the hepadnavirus family (for reviews, see references 2 and 16). The genomic DNA of these viruses has a size of about 3.2 kilobases (kb) and replicates through a reverse transcription pathway. The HBV genome encodes four genes: the *S* gene encodes envelope proteins (also known as HBV surface antigen); the *C* gene encodes core protein (the major capsid protein) and precore protein (the precursor of a serum HBV antigen known as e antigen); the *X* gene encodes a transcriptional *trans*-activator protein; and the *pol* gene encodes the DNA polymerase (a reverse transcriptase) (Fig. 1).

It is generally believed that HBV polymerase is translated from the 3.5-kb polycistronic *C* gene transcript which contains the entire coding sequence of the *pol* gene. As illustrated in Fig. 1, the polymerase reading frame overlaps the carboxy terminus of the *C* gene sequence. Recent genetic studies with *cis*-rescue (18) and *trans*-rescue (1, 15, 19) strategies have indicated that translation of HBV polymerase initiates from the internal ATG codon of the polymerase reading frame rather than by ribosomal frame-shifting from the *C* gene reading frame, as in the case of retroviruses (6).

It has been shown that the *C* gene transcript has multiple transcription start sites (2, 23). The majority of *C* gene transcripts start one nucleotide downstream of the first ATG codon of the C gene and thus can only direct translation from the second ATG codon to produce the 21-kilodalton (kDa) core protein. A small fraction of *C* gene transcripts start upstream of the first ATG codon of the reading frame and can encode the 25-kDa precore protein containing the entire core protein sequence plus an amino-terminal extension of 29 amino acid residues (11, 17). Both precore and core protein transcripts contain the entire coding sequence of polymerase. As an effort to further understand the mechanism that regulates the expression of the polymerase gene, we have investigated whether these two transcripts could serve as templates for polymerase translation.

As illustrated in Fig. 1, three different HBV transcripts containing the entire polymerase coding sequence were synthesized in vitro by using SP6 RNA polymerase. The structures of the first two transcripts were similar to those of precore and core protein mRNAs: they began either 60 nucleotides upstream (PC mRNA) or one nucleotide downstream (C mRNA) from the precore protein ATG codon and terminated near the major HBV poly(A) addition site. The third transcript was similar to the first two transcripts except that most of the preceding *C* gene coding sequence has been deleted in this transcript. Thus, in the third transcript, the first ATG codon of the *pol* gene becomes the 5′-most proximal ATG codon. This transcript served as a positive control for polymerase translation in our experiments. Besides the HBV sequences, the 5′ ends of these three transcripts also contained seven additional nucleotides derived from the SP6 plasmid vector.

These three different transcripts were translated in vitro with the rabbit reticulocyte lysate to test their ability to express polymerase. As shown in Fig. 2 (lanes 1 and 2), two major protein species with relative molecular masses of about 25 and 21 kDa were translated from the PC and C mRNAs, respectively. These were the predicted sizes of full-length precore and core proteins. Similarly, *pol* mRNA produced a major protein band of about 90 kDa. This was also the predicted size of polymerase. This protein species as well as a minor protein species with a slightly faster mobility on the gel (Fig. 2, denoted by a dot) could be immunoprecipitated by an antibody made against polymerase (data not shown). The nature of this minor protein species is unclear. It had an estimated size of about 75 kDa and could be the result of translational initiation from the downstream ATG codon of the polymerase reading frame. As shown in Fig. 2, lane 2, a small amount of polymerase was also produced from the C mRNA. Interestingly, no detectable amount of the polymerase was produced from PC mRNA (Fig. 2A, lane 1). This indicates that the extra 60 nucleotides at the 5′ end

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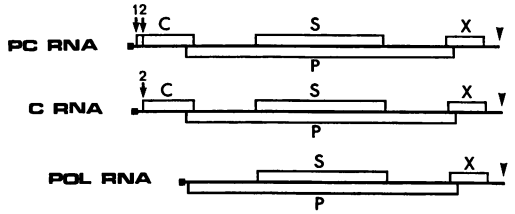

FIG. 1. Schematic illustration of the structures of PC, C, and *pol* RNAs synthesized in vitro. The procedures for the construction of plasmids pSP-PC and pSP-C have been described before (12). pSP-PC contains the entire genomic information of HBV starting from nucleotide 1757 (63 nucleotides upstream from the precore ATG) and terminates at nucleotide 1940 near the HBV poly(A) addition site. pSP-C is similar in structure to pSP-PC except that the 5′ end of the HBV sequence starts at nucleotide 1821, one base downstream of the precore ATG. pSP-Pol was constructed by deleting the sequence from 1821 to 2253 (*Hin*dIII-*Ssp*I fragment) of pSP-C. Thus, in pSP-Pol, the 5′ end of the HBV sequence is 57 nucleotides upstream of the first ATG codon of the *pol* coding sequence. Procedures for in vitro transcription were as described before (12). Briefly, plasmids were linearized with *Sal*I and transcribed in vitro by SP6 RNA polymerase in the presence of the methylated cap analog m^7GpppG. The capping efficiency under this condition is between 90 and 95% (22). The solid boxes indicate a sequence of seven nucleotides derived from the vector as a result of transcription. The arrows indicate the first and second ATG codons of the *C* gene, and the arrowhead indicates the position of the major HBV poly(A) addition site. Open boxes indicate the reading frames of the *C*, *S*, *X* and *pol* (P) genes.

of PC mRNA somehow inhibits translation of the downstream polymerase sequence (see below).

The results presented in Fig. 2 were confirmed in a *Xenopus laevis* oocyte system. As reported previously (12),

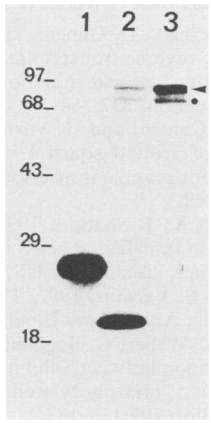

FIG. 2. Translation of PC, C, and *pol* mRNAs in rabbit reticulocyte lysates. Rabbit reticulocyte lysates were purchased from Promega Corp. Translation reactions were carried out as recommended by the manufacturer. Protein products were analyzed on a 12.5% acrylamide Laemmli gel. Lane 1, PC mRNA; lane 2, C mRNA; lane 3, *pol* mRNA. The arrowhead indicates the position of the full-length polymerase protein. The dot indicates the minor 75-kDa polymerase-related protein. Note that both C and *pol* mRNAs produced detectable amounts of polymerase. A small amount of the polymerase was also detected in lane 1 after a prolonged exposure. Taking into consideration the number of methionine residues in the precore and core proteins, densitometer scanning indicated that the translation efficiency of PC mRNA for precore protein synthesis was approximately sevenfold higher than for synthesis of core protein from C mRNA. Numbers on the left of the gel indicate the sizes of molecular mass markers (in kilodaltons).

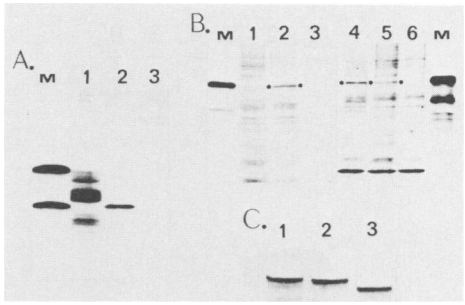

FIG. 3. Translation of PC, C, and *pol* mRNAs in *Xenopus* oocytes. (A) Immunoprecipitation of *C* gene products with rabbit anticore antiserum. Lane M, Full-length precore and core protein markers synthesized in vitro as described in the legend to Fig. 2; lane 1, oocytes microinjected with PC mRNA; lane 2, oocytes microinjected with C mRNA; lane 3, oocytes microinjected with *pol* mRNA. (B) Immunoprecipitation of *pol* gene product with rabbit anti-*pol* antiserum. Lanes 1 to 3 and 4 to 6 show the results of two separate experiments. Lane M, Polymerase synthesized in vitro as described in the Fig. 2 legend; lanes 1 and 6, PC mRNA-injected oocytes; lanes 2 and 5, C mRNA-injected oocytes; lane 3, mock-injected oocytes; lane 4, *pol* mRNA-injected oocytes. (C) Comparison of the stability of PC, C, and *pol* mRNAs in *Xenopus* oocytes. Lane 1, Oocytes injected with PC mRNA; lane 2, oocytes injected with C mRNA; lane 3, oocytes injected with *pol* mRNA. Procedures for oocyte microinjection were as described before (20). Approximately 20 to 40 nl of the capped mRNA (0.5 mg/ml in H_2O) was injected into each oocyte. After 16 h of incubation, oocytes were labeled with [^{35}S]methionine (ICN; Trans-^{35}S) at a concentration of 0.5 mCi/0.5 ml of MBS-H medium per 20 oocytes for 6 h. The oocytes, homogenized in 50 mM Tris, pH 8, containing 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride, were diluted in RIPA buffer for immunoprecipitation by our previously described procedures (13). Rabbit anti-*pol* antibody was made against a β-galactosidase-polymerase fusion protein. Briefly, an *Ava*I-*Taq*I DNA fragment containing the entire polymerase coding sequence less the amino-terminal 44 amino acid residues was fused to the β-galactosidase sequence at the *Hin*dIII site in the pUR292 vector (7). The recombinant DNA was then transfected into *Escherichia coli* DG101. The growth of the transformed *E. coli* cells and the purification of the fusion protein were done as described before (14). The rabbit anti-*pol* serum was prepared by Ben Yen at the University of California, San Francisco. The preparation of the anticore antiserum has been described before (13). For extraction of RNA from oocytes, 24 h after injection, five intact oocytes were selected and homogenized in 50 mM Tris (pH 8.0)–0.3 M NaCl–2% sodium dodecyl sulfate–1 mM EDTA. One-fifth of the homogenate was phenol extracted and analyzed by the Northern (RNA) blot procedure.

PC mRNA directed translation of the full-length precore protein and its proteolytically cleaved derivatives (Fig. 3A, lane 1). Similarly, C mRNA also directed translation of core protein (Fig. 3A, lane 2). *pol* mRNA, as expected, did not produce any *C* gene product since it lacked most of the *C* gene coding sequence (Fig. 3A, lane 3). When the *pol* gene products were analyzed by the immunoprecipitation procedure with a polyclonal antibody directed against polymerase, no detectable amount of polymerase was produced in oocytes microinjected with PC mRNA (Fig. 3B, lanes 1 and 6). On the other hand, a small amount of polymerase was detected in oocytes microinjected with either *C* or *pol* mRNA (Fig. 3B, lanes 2, 4, and 5). Failure of PC mRNA to produce the polymerase in oocytes was not due to its instability, because all three transcripts had similar stabilities in oocytes (Fig. 3C). Thus, our results confirmed those obtained from in vitro translation experiments, namely, C

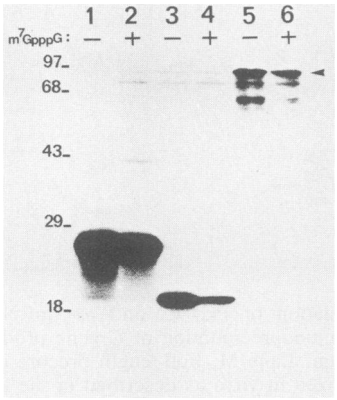

FIG. 4. Translation of polymerase in the absence or presence of the cap analog m^7GpppG. Lanes 1 and 2, PC mRNA; lanes 3 and 4, C mRNA; lanes 5 and 6, *pol* mRNA. Lanes 1, 3, and 5, No m^7GpppG added; lanes 2, 4, and 6, 1 mM m^7GpppG added. The same amount of RNA was used in each lane for protein translation. Densitometer scanning indicated that in the presence of m^7GpppG, the amounts of precore and core proteins synthesized were reduced by 43 and 80%, respectively. On the other hand, in lanes 3 to 6, the cap analog had no significant effect on the amount of polymerase synthesized. A printing artifact has caused the polymerase signal in lane 6 to appear weaker than that in lane 5. A shorter exposure of the film was used for quantitating the amounts of precore and core proteins synthesized.

mRNA, besides being the mRNA of core protein, can also serve as the template for translating polymerase; PC mRNA, on the other hand, directs translation of precore protein but not polymerase.

The 3.5-kb core protein mRNA of the related duck hepatitis B virus has been shown to be capped (9). Thus, we decided to examine whether translation of polymerase from c mRNA is cap dependent. PC, C, and *pol* mRNAs were translated in reticulocyte lysates in the presence or absence of the cap analog m^7GpppG. m^7GpppG can compete for the initiation factor eIF-4F and inhibit translation of cap-containing mRNAs (5). As shown in Fig. 4 (lanes 1, 2, 3, and 4), as expected, translation of precore and core proteins from PC and C mRNAs was suppressed by this cap analog by 43 and 80%, respectively, indicating that translation of precore and core proteins is cap dependent. On the other hand, translation of polymerase from C and *pol* mRNAs was not significantly affected by m^7GpppG, suggesting that translation of polymerase is cap independent. Interestingly, translation of polymerase from PC mRNA was stimulated by this cap analog. Thus, translation of polymerase appears to be effected by a cap-independent mechanism. We have attempted similar experiments in *Xenopus* oocytes with capless mRNA or mRNA capped with an inactive 2,2,7-trimethylguanosine cap (21). Unfortunately, both types of transcripts were unstable in oocytes, and thus the experimental results could not be analyzed (data not shown).

In conclusion, our results demonstrate that the 3.5-kb C mRNA can serve as the template for translation of core protein and polymerase. PC mRNA, on the other hand, although similar in structure to C mRNA, is not as efficient as C mRNA for polymerase synthesis. The reason for this is unclear. It may be due to higher translation efficiency of PC mRNA for precore protein synthesis compared with synthesis of core protein from C mRNA (approximately sevenfold difference) (Fig. 2). Efficient translation of precore protein from PC mRNA may interfere with translational initiation of the downstream polymerase sequence. This type of elongation interference has been suggested to be responsible for regulating translation of a bicistronic reovirus mRNA (3). This may explain why there was an increase in polymerase translation from PC mRNA when translation of precore protein was suppressed by m^7GpppG (Fig. 4).

Translation of most eucaryotic mRNAs is believed to be initiated by a "scanning" mechanism (8). For this mechanism, the 40S ribosomal subunit first binds to the 5′ cap of the mRNA and then scans along the template from the 5′ end until it encounters an ATG codon flanked by a favorable sequence (A/GXXATGG); at this moment, the 60S ribosomal subunit will attach to the 40S subunit and translation begins. Translation of picornavirus mRNA represents a unique case for which translation initiates from an internal ATG codon and is cap independent (10). Our results indicate that translation of HBV polymerase is also regulated by a novel, cap-independent mechanism. It is interesting that the first initiation codon of the HBV polymerase reading frame is flanked by an unfavorable sequence, CCAAATGC. Thus, it is likely that some other translation factors may be involved to ensure successful initiation of translation from this reading frame. Experiments are in progress to investigate this possibility.

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