JOURNAL OF VIROLOGY, Mar. 1990, p. 1063–1069
0022-538X/90/031063-07$02.00/0

Vol. 64, No. 3

cis Rescue of a Mutated Reverse Transcriptase Gene of Human Hepatitis B Virus by Creation of an Internal ATG

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Received 28 August 1989/Accepted 27 November 1989

Using mutational analysis, we have investigated the translation strategy of the reverse transcriptase gene (*pol*) of human hepatitis B virus. It has been proposed that this *pol* gene product is synthesized as a core-*pol* fusion protein from a polycistronic mRNA template via ribosomal frameshifting, a mechanism often seen in retroelements. Our data indicate that creation of a novel ATG initiation codon near the original ATG can compensate for a lethal missense mutation at the first ATG position of the *pol* open reading frame. Genetic analysis has rigorously ruled out the possibilities of frameshifting, non-ATG initiation, or RNA editing. These results are discussed in the context of a 5′-end entry model versus a novel model of direct internal entry of ribosomes.

Reverse transcriptase (*pol*) was independently discovered in retroviruses almost 2 decades ago by Baltimore (1) and Temin and Mizutani (39). It was demonstrated by Summers and Mason that DNA animal viruses such as duck hepatitis B virus (duck HBV) could also replicate via reverse transcription (38). The ubiquity of reverse transcriptase has also been supported by the recent discovery of this enzyme in procaryotes (11, 21). In many cases, the *pol* open reading frame (ORF) is overlapped by and out of frame to a 5′ upstream nucleocapsid ORF within the same polycistronic mRNA (e.g., for HBV, see Fig. 1). The biosynthesis of this enzyme in retroviruses involves the formation of a *gag-pol* fusion protein by either ribosomal frameshifting (fs) (12) or readthrough of an amber stop codon between the nucleocapsid (*gag*) and *pol* genes (44). For human HBV, it has been proposed that the synthesis of the *pol* gene product from a 3.5-kilobase (kb) polycistronic mRNA (Fig. 1) also involves the formation of a nucleocapsid-*pol* (designated c-*pol*) fusion protein via fs (43).

Identification and characterization of the *pol* gene product with anti-*pol* antibodies in hepatocytes by active HBV replication (2, 22) have proven difficult because of either poor immunogenicity or small amounts of the *pol* protein. One hypothesis concerning HBV *pol* gene expression via ribosomal fs has been examined by genetic analysis in the duck HBV system (4, 33). Using a *trans*-rescue genetic approach, a *pol*-defective mutant has been successfully complemented by cotransfection with a core-defective nonsense mutant, indicating that core and *pol* gene products can be synthesized separately (4, 33). Here, we report a complementary *cis*-rescue approach to investigate *pol* gene expression in the human HBV system. Our results provide further convincing evidence against the requirement of the formation of a core-*pol* fusion protein in HBV via ribosomal fs.

MATERIALS AND METHODS

Site-directed mutagenesis. The procedure for site-directed mutagenesis was adapted from that of Kunkel (18). Plasmid RG6 contains a full-length HBV monomer DNA in the minus orientation in the *Eco*RI site of vector M13mp18. A uracil-containing single-stranded DNA template of RG6 was prepared by infecting RG6 bacteriophage in a growing culture of CJ236 in the presence of uridine (0.25 μg/ml) and chloramphenicol (30 μg/ml). The oligonucleotides used for construction of the HBV mutants are shown below.

Nucleotide no. of mutant position	Oligonucleotide sequence
	5′ *
2306	GAC CAC TAA ATG CCC CTATC
	*
2310	GAC CAC CAA ACG CCC CTATC
	*
2471	TGG ACT CAT TAG GTG GGGAA
	*
305	CGT GTG TCT TAG CCA AAATT
	*
1314	AGC AGG TCT TGA GCA AACAT
	*
2299	CTC CAG CTT ATG GAC CACCA
	*
2342	CGG AGA CTA ATG TTG TTAGA

Approximately 100 to 200 ng of oligonucleotide was phosphorylated with T4 polynucleotide kinase and then annealed to 1 μg of uracil-containing, single-stranded RG6 template DNA. The resulting hybrid DNA was extended with T4 DNA polymerase and ligated with T4 DNA ligase. A sample of the ligation mixture (about 100 ng of DNA) was transformed into MV1304 *Escherichia coli* cells and then plated in the presence of MV1304. Mutant HBV clones were screened for the mutant sequence by the Sanger dideoxy technique (32) (Sequenase obtained from U.S. Biochemical Co.).

Construction of MC-3 vector. A pSV2ANeo-HBV monomer vector was constructed as described previously (14, 35). This vector contains two *Eco*RI sites flanking the full-length 3.2-kb HBV DNA fragments (ayw subtype of HBV). The transcription of HBV pre-core promoter in this vector is in the same direction as that for the neomycin resistance gene. In order to dimerize the HBV genome efficiently, the downstream *Eco*RI site of the pSV2ANeo-HBV monomer was eliminated, resulting in a pSV2ANeo-HBV monomer with a

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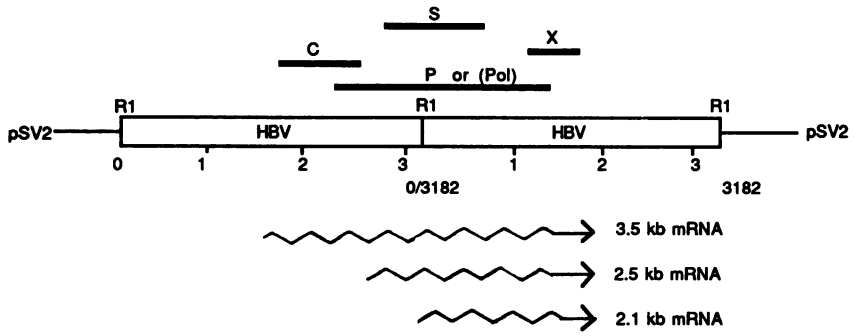

FIG. 1. Transcription map of HBV drawn with a DNA template in a head-to-tail tandem dimer configuration, which is like a circular permutation of a covalently closed circular form. The four different ORFs are shown by solid bars. The 2.5-kb mRNA encodes the pre-surface antigen (pre-S1), and the 2.1-kb mRNA encodes another pre-surface antigen (pre-S2) and surface antigen (S). The polymerase gene (*pol*) is in the +1 frame with respect to the overlapping nucleocapsid gene (C), and the X ORF encodes a putative transactivator (for a review, see references 7, 23, and 41).

single upstream *Eco*RI site. This vector was designated MC-3. Briefly, the pSV2ANeo-HBV monomer (35) was linearized by *Eco*RI partial digestion. The 9.5-kb DNA fragment of this linearized pSV2ANeo-HBV monomer was electroeluted from a 1.2% agarose gel. The *Eco*RI end of this eluted fragment was eliminated by Klenow repair. After blunt-end religation, the ligated DNA mixture was transformed into *E. coli*. Loss of the downstream or upstream *Eco*RI site was differentiated by cleavage with an *Acc*I or *Acc*I-plus-*Eco*RI double digest.

Dimerization of HBV DNA. The construction of a wild-type HBV dimer expression vector has been described elsewhere (35). For the dimerization of mutant HBV DNAs, the aforementioned MC-3 plasmid was used. The 3.1-kb mutant HBV DNA fragment was isolated from the phage DNA of the M13mp18-HBV mutant by complete digestion with *Eco*RI and was electroeluted from agarose gel. This purified mutant HBV DNA fragment was ligated to the MC-3 vector which had been linearized by *Eco*RI and dephosphorylated by alkaline phosphatase. A sample of the ligation mixture was transformed into *E. coli* MV1304. Head-to-tail tandem dimers were screened by *Xho*I or *Acc*I digestion of miniprep DNA. For the construction of double mutants, the uracil template of mutant 2310 was used. Double mutants were also confirmed by the Sanger dideoxy sequencing method (32).

Cell culture and transfection. The human hepatoma cell line HepG2 was maintained in 10% fetal bovine serum in Dulbecco modified Eagle medium at 37°C in the presence of 5.5% CO_2. The calcium phosphate transfection procedure was followed as detailed elsewhere (35). Briefly, 7×10^5 cells per 10-cm dish were transfected with 5 μg of either wild-type dimer or mutant dimer DNA plus 30 μg of human genomic DNA as carrier. A control dish transfected with HBV monomer DNA was always included. Donor DNA was removed 4 h after transfection, and cells were fed with fresh Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Analysis of HBV expression. Five days after transfection, medium was removed and saved for further analysis (see below). Cells from one 10-cm dish (6×10^6 cells) were lysed to prepare intracellular, extrachromosomal HBV DNA by the Hirt method (10). Total cellular RNA was prepared from cells from another 10-cm dish by the method of Chirgwin et al. (5) (see Fig. 3b), and cells from a third 10-cm dish were used to prepare intracellular core particles for an endogenous-polymerase assay (15, 38; see below).

Core particle-associated endogenous-polymerase activity. The procedure for preparation of intracellular core particles was adapted from the method of Summers and Mason (38). Briefly, cells from a 10-cm dish (6×10^6 cells) were lysed in 0.5 ml of chilled extraction buffer (20 mM Tris hydrochloride [pH 7.4], 7 mM $MgSO_4$, 50 mM NaCl, 0.1% β-mercaptoethanol, 100 μg of bovine serum albumin per ml, 0.25 M sucrose) with a Dounce homogenizer. The homogenates were then spun in a Microfuge (Beckman Instruments, Inc.) at 4°C for 30 min to remove cellular debris and nuclei. The clear homogenates were loaded on a sucrose gradient (5 ml of 15 to 30% sucrose [wt/vol] in extraction buffer). The gradients were run at 32,000 rpm for 6 h at 4°C in a Beckman SW50.1 rotor. Fractions (200 μl) were collected from the bottom of the gradient, and core particle fractions were pooled. The particles from pooled fractions were pelleted by spinning them at 35,000 rpm for 14 h at 4°C in a Beckman SW50.1 rotor. The pellets were suspended in 100 μl of extraction buffer, and 40-μl samples were used for the polymerase assay as described elsewhere (35).

Preparation of extracellular HBV DNA. Extracellular DNA was prepared from 72-h-conditioned medium from three 10-cm dishes (total, 30 ml) collected 5 days after transfection. The medium was precleared by spinning it at 35,000 × *g* for 30 min at 4°C. Particles from the precleared medium were pelleted by spinning them at 25,000 rpm in a Beckman SW28 rotor for 16 h at 4°C. The pellets were suspended in 0.5 ml of 10 mM Tris hydrochloride–10 mM EDTA–0.6% sodium dodecyl sulfate buffer (pH 7.5) and digested with 400 μg of proteinase K per ml for 3 h at 37°C. After phenol and chloroform extractions, the DNAs were precipitated and redissolved in 30 μl of TE (10 mM Tris hydrochloride, 1 mM EDTA buffer [pH 7.5]) and analyzed on a 1% agarose gel by the Southern procedure (37).

RESULTS

Mutant 2310. A point mutation was introduced into the first ATG codon of the HBV *pol* ORF at nucleotide (nt) 2310, converting methionine to threonine while keeping the core protein unaltered (Fig. 2A). Upon tandem dimerization and transfection of this mutant into the human hepatoma HepG2 cell line, the functional activities of mutant 2310 were assayed in parallel with those of the wild-type HBV dimer control as described in the legend of Fig. 2. Neither HBV-specific DNA replicative intermediates, as measured by Southern blot analysis, nor polymerase activity could be detected.

These results with this mutant suggest that ribosomal fs is

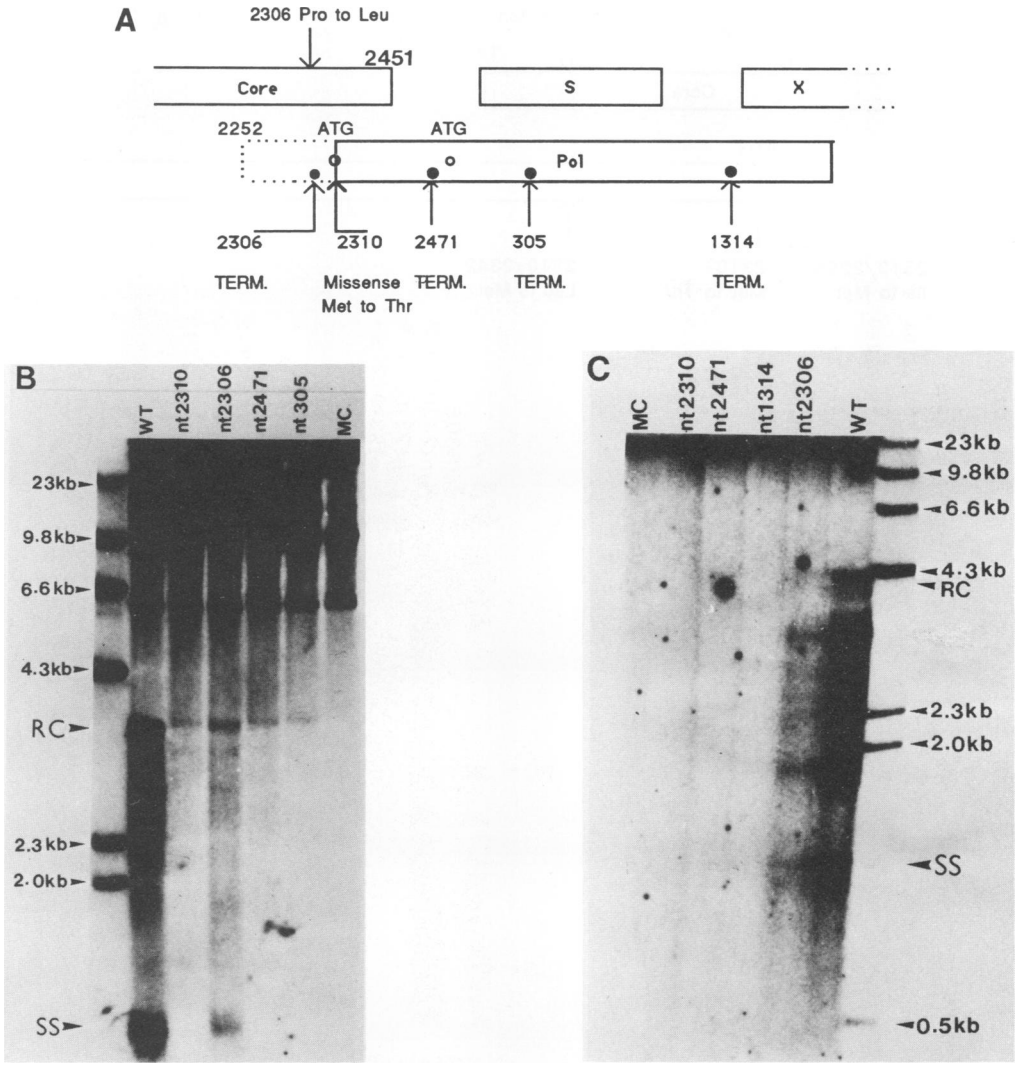

FIG. 2. (A) Schematic representation of different mutation positions in the *pol* ORF. The nucleotide number (HBV subtype ayw) indicates the position of the base change. None of the mutations except that at nt 2306 change the coding capacity of any overlapping ORF. For the sake of clarity, this figure is not drawn to scale. Symbols: ○, ATG; ●, stop codon. S, X, and *pol* are explained in the legend to Fig. 1. (B) Southern blot analysis of wild-type and mutant HBV DNA replicative intermediates. Both relaxed-circle and minus-strand, single-stranded forms are characteristic replicative-intermediate molecular forms of HBV that occur during reverse transcription (7, 23, 41). All the bands above the 6.0-kb position represent the residual donor DNA of the HBV dimer in the pSV2A vector. These "contaminating" bands serve as convenient internal references to assess transfection efficiency and the relative amount of donor DNA applied to each recipient culture. (C) Core particle-associated endogenous polymerase activity of wild-type and mutant HBV. Base substitution mutants 1314 and 2471, which contain a truncated *pol* gene, are included as negative controls. With a longer chase, more full-length open circles can be seen at positions near 4.3 kb. RC, Relaxed circle; SS, single stranded; WT, wild-type HBV. Lanes MC, MC-3 vector (see Materials and Methods).

not likely to occur downstream from the ATG codon. If fs does occur downstream from this position, the change from methionine to threonine at position 2310 would not change the amino acid sequence of the c-*pol* fusion protein; therefore, mutant 2310 should not be lethal. The fact that mutant 2310 exhibits no detectable *pol* activity (Fig. 2B and C) suggests that fs cannot occur downstream from position 2310.

Mutant 2306. A ribosomal fs occurring upstream from the first ATG of *pol* in wild-type HBV will generate a core-*pol* fusion protein with an internal methionine residue at nt 2310. A missense mutation at this position might abolish the enzymatic activity of *pol* completely. To rule this out, a stop codon was introduced at position 2306, which is in frame with and immediately adjacent to the first ATG codon of *pol* (Fig. 2A). If fs occurs upstream from position 2306, the frameshifted ribosome should be arrested when it encounters the created stop codon at nt 2306. Functional analysis of this nonsense mutant 2306 indicated that both DNA replicative intermediates and polymerase activity are clearly detectable (Fig. 2). Although the level of activity appeared to be significantly lower than that of the wild type, it is clearly significantly above the background level. This difference between wild-type and mutant 2306 cannot be attributed to a concurrent structural alteration of the core protein by mutation 2306, which converts a proline residue into a leucine, since cotransfection of mutant 2306 with other *pol* mutants producing wild-type core protein failed to restore the polymerase activity to the wild-type level (see Discussion). Examination of the steady-state mRNA levels by Northern

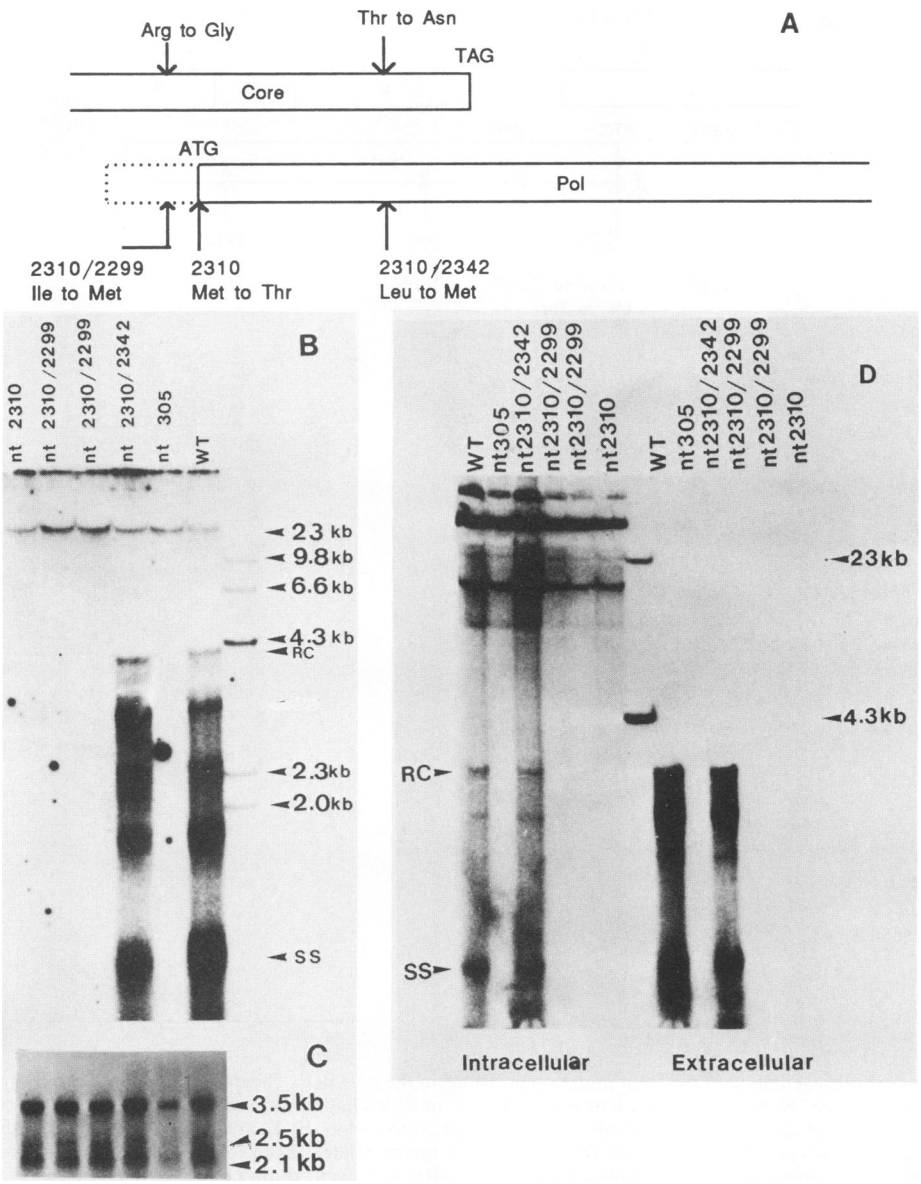

FIG. 3. (A) Schematic representation of the positions of newly created methionine codons in the *pol* ORF. The nucleotide number indicates the position at which the base has been changed. The concurrent change of amino acid residue in the core protein is indicated. (B) Core particle-associated endogenous polymerase activity of wild type and 2310/2342 double mutant. The duplicated lanes of 2310/2299 in panels B, C, and D are two independent isolates of mutant 2310/2299. (C) Northern blot analysis of the steady-state level of HBV-specific transcripts in HepG2 cells transiently transfected with double mutants. (D) Southern blot analysis of intracellular and extracellular HBV-specific DNAs from HepG2 cells transiently transfected with 2310/2342 double mutant. WT, Wild-type HBV; RC, relaxed circle; SS, single stranded.

(RNA) blot analysis did not reveal any differences among wild-type and any mutant HBV (data not shown). Taken together, these data strongly suggest that fs does not occur either downstream or upstream from the first ATG position of *pol* within the c-*pol* overlap. Nevertheless, it is still possible that fs occurs in the vicinity of positions 2306 to 2310. Another similar possibility is that the fs signal necessary for the fs event (12) is located near this position. A missense mutation at the first ATG may therefore have abolished the fs signal.

One prominent band near the 4.3-kb position of the relaxed circle of HBV can also be detected in other mutants, including mutant 2310 (Fig. 2B). This band does not appear to represent a bona fide replicative intermediate, since neither a positive *pol* assay result (Fig. 2C) nor the presence of a characteristic single-stranded replicative intermediate (Fig. 2B) has ever been observed in mutant 2310, 2471, or 305. In fact, when less donor DNA was used, this pseudosignal disappeared (e.g., see Fig. 3).

Creation of internal novel ATG codons. To better understand translational initiation of the *pol* gene, we created novel ATG codons either 3 amino acids upstream (position 2299) or 11 amino acids downstream (position 2342) from the mutated ATG codon in mutant 2310 (Fig. 3A). These double mutants were analyzed in a manner similar to that described previously (Fig. 3B and D). Both *pol* enzymatic assay and

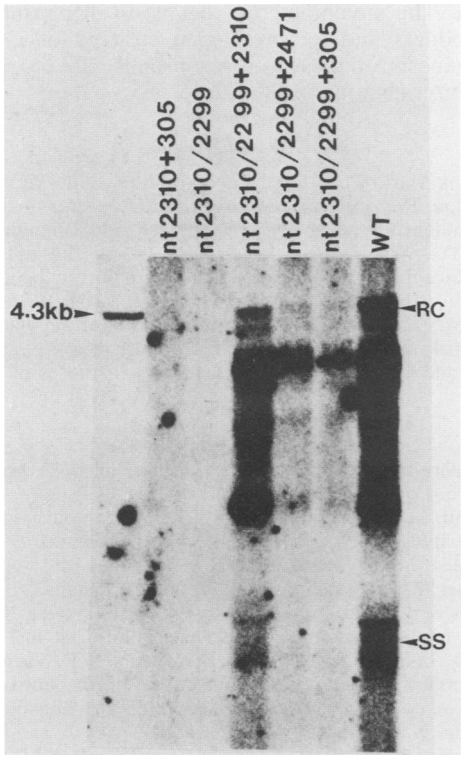

FIG. 4. Restoration of polymerase activity of mutant 2310 by creation of an upstream ATG codon at nt 2299 after cotransfection with *pol*-defective mutant such as 305, 2471, or 2310. Controls to test for recombination were always included by cotransfecting two different *pol*-defective mutants (e.g., 305 and 2310). RC, Relaxed circle; SS, single stranded; WT, wild-type HBV.

Southern blot analysis clearly indicated that the missense mutant 2310 can be rescued by providing a novel downstream ATG codon at nt 2342 (Fig. 3B and D). In order to be certain that the difference between these mutants indeed reflects a difference in translational control rather than in transcriptional control or mRNA stability, we examined the steady-state level of the 3.5-kb *pol*-specific mRNA in these mutants by Northern blot analysis (Fig. 3C). Our results indicate that these mutants do not differ from each other regarding levels of all species of HBV-specific mRNAs. One explanation for the failure to rescue the *pol* activity in double mutant 2310/2299 is a concurrent structural alteration of the core protein (from Arg to Gly at position 2299). Although the core gene product of mutant 2310/2299 is detectable by immunoprecipitation with anti-core antibody (data not shown), it is possible that this mutated core protein is no longer functional. In an attempt to complement the potential defect of the core protein in double mutant 2310/2299 with a functional core protein from mutant 2310, we cotransfected this double mutant 2310/2299 with single mutant 2310. Neither single mutant 2310 nor double mutant 2310/2299 alone was sufficient to produce detectable *pol* activity (Fig. 4). When cotransfected with both mutant 2310 and double mutant 2310/2299, cells showed wild-type levels of *pol* activity. Similar results (Fig. 4) were obtained when DNA replicative intermediates were assayed by Southern blot analysis (data not shown). The possibility of rescue by DNA recombination between a core-producing plasmid (mutant 2310) and a *pol*-producing plasmid (mutant 2310/2299) can be excluded since in a control experiment, *pol*-truncated mutant 305 did not rescue mutant 2310. This result proves not only that rescue of mutant 2310 with an upstream ATG codon is possible, but also that the core and *pol* proteins of HBV can be synthesized separately.

DISCUSSION

Potential mechanisms of HBV *pol* gene expression. The genetic data presented in this paper argue convincingly against ribosomal fs or non-ATG initiation as a mechanism of *pol* gene expression in HBV. Furthermore, they strongly support the conclusion that the first ATG in the *pol* ORF is essential for translational initiation. Several different mechanisms can explain this observation (Fig. 5). First, translation may occur by direct internal initiation, as recently described for the picornavirus system (13, 29). A major difference between picornavirus and 3.5-kb HBV-specific mRNAs is that the former are naturally uncapped, while the latter are believed to be capped (19, 20). The likely presence of a capped structure at the 5′ end of the 3.5-kb mRNA of HBV does not argue against the possibility of direct internal initiation, since it has been shown that methylated capped poliovirus mRNA can be translated in vitro at an efficiency

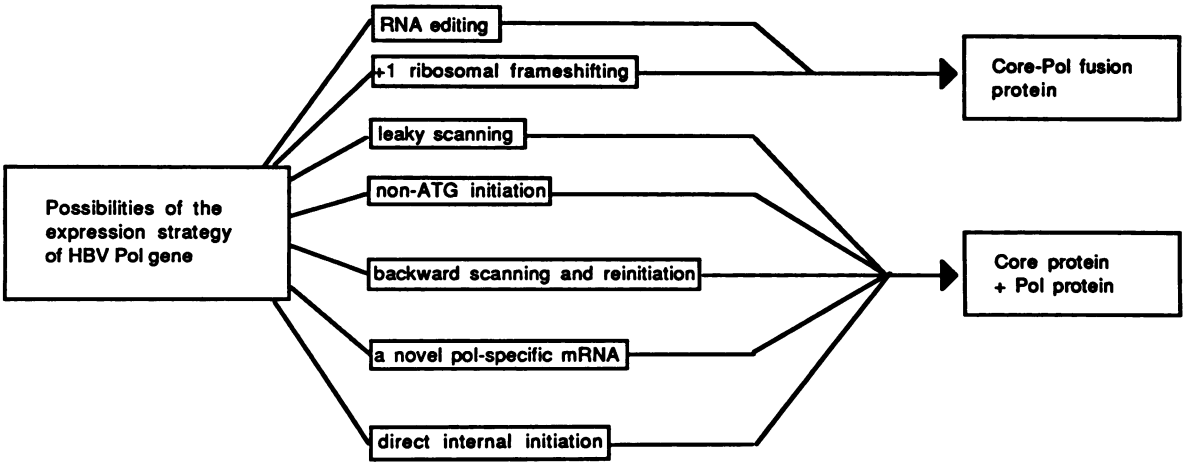

FIG. 5. Potential strategies of HBV *pol* gene expression. See text for references.

equal to that of the uncapped counterpart in a cap-independent fashion (28). As described in the legend to Fig. 2, the diminished *pol* activity of mutant 2306 cannot be explained by the concurrent structural alteration of the core gene product. If *pol* expression indeed involves direct internal initiation, diminished activity of *pol* in mutant 2306 (from CC AAATG to CTAAATG) can be explained by a perturbation of unknown signals for direct internal initiation. Second, the *pol* protein may be encoded by an as yet unidentified mRNA species which exposes the ATG at nt 2310 as the 5'-most proximal initiation codon. The scanning 40S ribosome subunit would thus commence initiation at nt 2310 when it encountered this first ATG of *pol*. It should be noted that this hypothetical transcript has never been identified by primer extension-nuclease S1 mapping (3, 6, 24, 42). Third, it has previously been proposed that ribosomes, after reaching the stop codon, may be able to scan backwards and reinitiate at the once-bypassed, upstream ATG codon (26, 27, 40). We consider this a less likely possibility because the distance between the end of the core gene (nt 2451) and the first ATG of *pol* (nt 2310) is approximately 140 nt. This distance appears to be too long for this type of reinitiation to take place (26, 27). Another possibility, such as leaky scanning (16, 17), is unlikely because within the 3.5-kb mRNA, there are four or five ATG codons 5' upstream from the first ATG of *pol* at nt 2310 in HBV subtype ayw. Moreover, some of these upstream ATG codons are in optimal sequence context for translational initiation, while the authentic ATG codon of *pol* at nt 2310 and the created ATG codons at nt 2299 or 2342 are in poor sequence context, with a cytosine rather than a purine at the −3 position (17). Neither RNA editing (36) nor non-ATG initiation (9, 25, 31) appears to be consistent with the data presented in Fig. 2, 3, and 4.

Although our studies of HBV *pol* gene translation are not consistent with a previous report of detection of c-*pol* fusion proteins in certain human hepatoma tissue (43), it should be noted that human hepatoma tissues often contain integrated and rearranged HBV DNA (34). Therefore, one likely interpretation of this discrepancy is that the reported c-*pol* fusion protein is actually produced from the rearranged HBV DNA rather than synthesized as a natural product during the normal life cycle of wild-type HBV.

Our observation of genetic complementation between core-defective and *pol*-defective HBV plasmids argues against c-*pol* fusion (Fig. 4) and substantiates an earlier report on the duck HBV system (4, 33). As discussed in the legend to Fig. 2, by itself the missense mutation of the first ATG of *pol* at nt 2310 does not provide conclusive evidence against fs occurring downstream from this position, since the potential perturbation of a +1 fs signal cannot be excluded. Our report here of the successful rescue of the missense mutant 2310 by creation of an internal ATG constitutes strong support for either direct internal initiation or the existence of a novel species of mRNA responsible for *pol* translation which may have escaped detection.

Packaging strategy of *pol* gene product. Of potential biological significance are the formation of a *gag-pol* fusion protein in retroviruses and its ability to target and compartmentalize the *pol* gene product in the core particles during packaging. Another aspect of potential significance is the stoichiometric regulation of the *gag* and *gag-pol* products in a proper ratio (for a review, see reference 8). However, the biosyntheses of the nucleocapsid and the reverse transcriptase appear to be separable in HBV (Fig. 3 and 4). This is also the case in the closely related duck HBV (4, 33) and even in cauliflower mosaic virus (30), another retroelement in plants. The stoichiometric regulation of capsid and *pol* gene products and the packaging strategy of *pol* during morphogenesis of these viruses promise to be rewarding areas of investigation in the future.

ACKNOWLEDGMENTS

We thank Mario Chen for the construction of the MC vector for dimerization, Richard Greenberg for the RG6 vector for mutagenesis and mutant 305 as a negative control, and Duanqing Pei for mutant 1314. We also thank Jonathan Israel, Steve Liebhaber, Vincent Racaniello, William Mason, John Taylor, and colleagues in our laboratory for careful reading of the manuscript. We are grateful to Malia McCarthy for her help in preparation of the manuscript.

This work was funded by Public Health Service grants ROl CA43835 and CA 48198 from the National Institutes of Health to C.S.

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