

# Formation of an RNA polymerase II preinitiation complex on an RNA promoter derived from the hepatitis *delta* virus RNA genome

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Received March 28, 2008; Revised June 19, 2008; Accepted July 18, 2008

## ABSTRACT

Although RNA polymerases (RNAPs) are able to use RNA as template, it is unknown how they recognize RNA promoters. In this study, we used an RNA fragment derived from the hepatitis *delta* virus (HDV) genome as a model to investigate the recognition of RNA promoters by RNAP II. Inhibition of the transcription reaction using an antibody specific to the largest subunit of RNAP II and the direct binding of purified RNAP II to the RNA promoter confirmed the involvement of RNAP II in the reaction. RNA affinity chromatography established that an active RNAP II preinitiation complex forms on the RNA promoter and indicated that this complex contains the core RNAP II subunit and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, TFIIH and TFIIIS. Binding assays demonstrated the direct binding of the TATA-binding protein and suggested that this protein is required to nucleate the RNAP II complex on the RNA promoter. Our findings provide a better understanding of the events leading to RNA promoter recognition by RNAP II.

## INTRODUCTION

Cellular DNA-dependent RNA polymerases (RNAPs) are able to use RNA as template. T7 RNAP was shown to replicate a 64-nt RNA contaminant of unknown genetic origin in a commercial sample of the enzyme, and at high concentrations, is able to transcribe from a large variety of different RNA species (1,2). Another RNAP frequently associated with RNA-dependent RNA polymerization is the *Escherichia coli* RNA polymerase. This RNAP was shown to be capable of amplifying selected small, random, RNA polymers (3,4), to specifically initiate transcription from RNA templates derived from a stem-loop domain of the peach latent mosaic viroid RNA genome (5,6), and was recently reported to synthesize short RNA

products from endogenous bacterial 6S RNA (7). Similarly, the small noncoding RNA genomes of plant viroids are replicated either in the nucleus by DNA-dependent RNAP II (8) or in the chloroplast by chloroplastic RNAP (9). Among the known mammalian RNA pathogens, the human hepatitis *delta* virus (HDV) is the only one known to utilize this potential of RNAP II to replicate itself (10).

HDV is the smallest known animal virus. Its genome consists of a small (~1700 nt) single-stranded, circular RNA molecule, and is thought to fold into an unbranched, rod-like structure (11). HDV contains two self-cleaving motifs (i.e. *delta* ribozymes) and encodes a single open reading frame (ORF). There are two viral proteins (HDAg) encoded by this ORF (i.e. HDAg-S and HDAg-L; 12,13). The large HDAg (HDAg-L) contains an additional 19 amino acids at its C-terminus resulting from RNA editing of the termination codon of the small HDAg (HDAg-S) gene (14). Although they are mostly identical in sequences, each protein has distinct functions. HDAg-S (195 amino acids) is essential for HDV replication (15), while the HDAg-L (214 amino acids) is necessary for virion assembly (16).

Replication of HDV is considered to take place in the nucleus by a symmetrical, rolling circle mechanism. During this replication, infecting HDV genomic circular monomer is replicated into linear multimeric minus strands which are then cleaved and ligated, yielding antigenomic circular monomers (17). Using the latter RNA as template, the same three steps are then repeated to produce the genomic progeny. During this process, RNAP II is believed to be involved in the transcription of the HDAg mRNA because the mRNA is posttranscriptionally processed with a 5'-cap and a 3'-poly(A) tail (18,19). Furthermore, studies using cultured cells and nuclear extracts (NE) have reported that low levels of  $\alpha$ -amanitin, a known inhibitor of RNAP II, inhibits the accumulation of HDV mRNA and genomic HDV RNAs (20–23). Recently, we used a monoclonal antibody specific to the carboxy terminal domain (CTD) of the largest subunit of

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RNAP II, to establish the association of RNAP II with both polarities of HDV RNA in HeLa cells (24). This analysis revealed that RNAP II associates with the terminal stem-loop domains of both polarities of HDV RNA (24). In addition, RNAP I or an RNAP I-like polymerase might be involved in HDV replication (25–27). The accumulation of the antigenomic species is resistant to higher doses of  $\alpha$ -amanitin and synthesis of HDV RNA was affected by an anti-SL1 antibody (27).

RNAP II is a multisubunit enzyme that is known to catalyze the synthesis of mRNAs from DNA templates (28,29). The two large subunits of human RNAP II [i.e. RPB1 (~220 kDa) and RPB2 (~140 kDa)] form the catalytic domain through which the DNA–RNA assembly takes place. Initiation of DNA-templated transcription by RNAP II involves multiple events, including decondensation of the locus, nucleosome remodeling, histone modification, binding of the activator and coactivator to the promoter elements, and recruitment of the general transcription factors to the promoter (30). The specific binding of the polymerase to the promoter requires the coordinated assembly of RNAP II and the six general transcription factors [i.e. TFIIA, TFIIB, TFIID, TFIIIE, TFIIF and TFIIH; (31–33)]. In addition, sequence-specific transcriptional activators play an important role in the recruitment of the RNAP II holoenzyme complex to the promoter region. In particular, TFIID and TFIIB were shown to play critical roles in the recognition of DNA promoter motifs by directly interacting with the TATA box motif (34), which is located at a fixed distance upstream of the transcription start site. There is considerable evidence that TFIID also binds to the initiator element (Inr), encompassing the transcription start site, in a sequence-specific manner and to the downstream core promoter element (DPE) (35). In addition, TFIIB is able to bind directly to the TFIIB recognition element (BRE), located immediately upstream of some TATA boxes (36).

Although many studies have investigated DNA-dependent RNA polymerization and DNA promoter recognition by RNAP II, little is known regarding how this enzyme recognizes an RNA template, and what the composition of the RNAP II preinitiation complex (PIC) on such RNA promoter might be. Thus, HDV offers a perfect model to study this unconventional use of RNAP II and the molecular mechanism underlying RNA template recognition by RNAP II. The present study was undertaken to (i) investigate the direct interaction of the RNAP II holoenzyme with an HDV-derived RNA template and (ii) to define the RNAP II subunit(s) involved in the formation of the PIC on the RNA promoter. For this purpose, we used an RNA fragment derived from the HDV RNA genome that was previously reported to include the initiation site for HDV mRNA transcription (18) and confirmed that RNAP II binds directly to the RNA molecule and initiates transcription. RNA affinity chromatography was then used to identify the components of the RNAP II PIC forming on this unusual promoter, which led us to identify the TATA-binding protein (TBP) as an RNAP II subunit directly interacting with the RNA promoter. Together, our results support a model in which an RNA promoter is recognized by RNAP II in the same way

as a DNA promoter. Moreover, the mechanism of promoter recognition likely requires the involvement of the TBP-containing complex TFIID to nucleate the RNAP II complex on the HDV-derived RNA promoter.

## MATERIALS AND METHODS

### Synthesis of RNAs

RNA molecules were synthesized by *in vitro* run-off transcription using T7 RNA polymerase (New England Biolabs; Pickering, Ontario, Canada NEB), as previously described (24). Sense (5'-GGAATTCTAATACGACTCACTATAGGG<sup>1541</sup>ACTGCTCGATCTCTT<sup>1558</sup>-3'; underlined nucleotide sequence indicates T7 promoter) and antisense (5'-GGAATTC<sup>60</sup>ACATCCCTCTCGGGTAC<sup>43</sup>-3') oligonucleotides were used during PCR amplification to generate R199G cDNA templates from a derivative of pBluescriptKS + (Stratagene, La Jolla, CA, USA) containing a dimeric EcoRI-flanked HDV cDNA insert [pHDVd2; (37)]. To generate X-R199G cDNA templates, 5'-GGAATTCTAATACGACTCACTATAGGGGGGAACAAGGG<sup>1541</sup>ACTGCTCGATCTCTT<sup>1558</sup>-3' was used as the sense oligonucleotide. X-R199G $\Delta$ UUA cDNA was derived from X-R199G cDNA by PCR amplification using several oligonucleotides, in order to delete nucleotide 1629–1631 to remove the UUA sequence at the initiation site. R38G, R38GPU and R38GSW were produced from double-stranded DNA oligonucleotides possessing a T7 RNA promoter (24). P11.60 RNA was generated as previously described (6). Following the transcription reactions, the products were digested with DNase I (Promega, Madison, Wisconsin, USA) for 30 min at 37°C, fractionated by denaturing polyacrylamide gel electrophoresis (PAGE) in 1  $\times$  TBE buffer (100 mM Tris–borate, pH 8.3, 1 mM EDTA) and 7 M urea, visualized by UV shadowing, excised, and eluted overnight at 4°C in 500 mM ammonium acetate, 0.1% SDS. The RNAs were then precipitated in ethanol, resuspended in H<sub>2</sub>O, desalted by Sephadex G-50 columns (GE Healthcare, Baie d'Urfé, Québec, Canada), and precipitated in ethanol. The purified RNAs were then resuspended in H<sub>2</sub>O, quantified spectrophotometrically at 260 nm and stored at –20°C for further use.

### Transcription assay

RNA templates (50 pmol) were resuspended in 25  $\mu$ l of transcription buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 6.0 mM MgCl<sub>2</sub>, 0.5 mM DTT and 20% glycerol) containing 0.4 mM NTPs. Either HeLa NE (Cedarlane, Burlington, Ontario, Canada) or HeLa NE preincubated with 2.5  $\mu$ g of anti-RNAP II antibody (CTD4H8; Upstate Lake Placid, NY, USA) for 1 h on ice, was added and the reaction was incubated at 30°C for 60 min. The transcription reactions were stopped by adding 175  $\mu$ l of HeLa extract stop solution (0.3 M Tris–HCl pH 7.4, 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA and 3  $\mu$ g/ml tRNA), subjected to phenol–chloroform extraction, ethanol precipitation and resuspended in 40  $\mu$ l H<sub>2</sub>O. RNA products were subjected to reverse transcription, as described previously (24),

using primer X (5'-GGGGGGAACAAGGGAC-3'). PCR was performed using primer X and either primer B (5'-<sup>1631</sup>TAAGAGTACTGAGGACTGCC<sup>1612</sup>-3') or primer A (5'-<sup>1651</sup>CTCCTCTTTACAGAAAAGAG<sup>1632</sup>-3'), the DNA molecules resolved on a 2% agarose gel and the bands visualized using SYBR green. The 5'-end of the RNA product was determined using the '5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0' (Invitrogen, Burlington, Ontario, Canada) and primer X, and the PCR products were sequenced directly by Bio Basic Inc. (Markham, Ontario, Canada).

### Electrophoresis mobility shift assay

RNA molecules were 5'-end radiolabeled with [ $\gamma$ -<sup>32</sup>P]ATP (GE Health Sciences) by dephosphorylation using calf intestinal phosphatase and phosphorylation using T4 polynucleotide kinase according to the manufacturer's recommended protocol (NEB, Pickering, Ontario, Canada). The 5'-end radiolabeled RNA molecules were incubated with either purified RNAP II, TFIID or GST-TBP (ProteinOne) in the presence or absence of either 50-fold molecular excess of P11.60, unlabeled R199G or a double-stranded DNA fragment containing the CMV promoter (5'-CCAAAATCAACGGGACTTCCAAAATGTCGTAACAAGTGGGGCCCATGAGGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAG-3') in 20  $\mu$ l of binding buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol). Unless otherwise indicated, 0.1 pmol of 5'-end radiolabeled RNA, 50 ng of RNAP II, 50 ng of TFIID or 64 ng of GST-TBP were used. After incubation at 30°C for 30 min the complexes were resolved on 5% PAGE (*bis*-acrylamid:acrylamide, 1:49) under native conditions at room temperature in 1  $\times$  TBE. The gels were then exposed to a phosphor screen overnight and scanned using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

### RNA affinity column preparation

RNA molecules (200 pmol) were oxidized in 20 mM Tris-HCl pH 7.5 and 10 mM Na-m-periodate in a total volume of 100  $\mu$ l. The reaction mixtures were incubated for 1 h at 4°C in the dark, ethanol precipitated and resuspended in 60  $\mu$ l of 0.1 M sodium acetate (pH 5.0). Adipic acid dihydrazide agarose beads (400  $\mu$ l of 50% slurry; Sigma-Aldrich, Oakville, Ontario, Canada) were prepared by washing four times in 0.1 M sodium acetate. For direct coupling, the beads were resuspended in 600  $\mu$ l of 0.1 M sodium acetate and loaded onto filter tubes and incubated with the oxidized RNA overnight at 4°C. After washing four times with 2 M NaCl to remove unbound RNA, 15  $\mu$ g of the HeLa NE (Cedarlane) was added to the beads, and the mixture was incubated at room temperature for 30 min. The columns were washed three times with wash solution [50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl], eluted using SDS loading dye (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% BPB and 10% glycerol), and the eluted proteins were separated by 10% SDS-PAGE (38). After transfer onto a nitrocellulose

membrane, the proteins were visualized using X-ray films using the One-Step Western™ Complete Kit (Genscript, Piscataway, NJ, USA) according to the manufacturer's protocol. The following antibodies targeted against RNAP II CTD (CTD4H8; Upstate), TBP (Upstate), p55 (ProteinOne, Bethesda, MD, USA), p56 (ProteinOne), RAP74 (ProteinOne), SC35 (ProteinOne), TFIIB (IIB8; ABR—Affinity BioReagents, Golden, CO, USA), TCEA3 (Abcam, Cambridge, MA, USA), and Cdk7 (Abcam) were used. To perform the transcription initiation reactions, transcription buffer and 0.4 mM NTPs were added to the RNA-protein mixture following the last wash. The mixtures were incubated for 2 min at room temperature, the columns were washed and the samples were eluted and analyzed as above. When required for subsequent RT-PCR, the samples were eluted by the addition of transcription buffer, subjected to phenol-chloroform extraction, and ethanol precipitated. The RNA was then resuspended in 40  $\mu$ l H<sub>2</sub>O and analyzed by RT-PCR as above. For the experiments involving a control DNA promoter, two oligonucleotides were used to make the CMV DNA promoter, one of which was biotinylated at its 5'-end (Invitrogen). To guarantee that both DNA molecules forming the DNA promoter annealed, both strands were mixed, heated at 95°C for 10 min and incubated at room temperature for 20 min. The DNA promoter was then added to streptavidin beads (Sigma-Aldrich) that were prewashed three times with the wash solution. The mixture was incubated for 1 h in the presence of the binding buffer and washed four times with the same solution. Uncoupled streptavidin beads were used as control. HeLa NE proteins (15  $\mu$ g) were added and the streptavidin columns were processed in the same manner as the RNA affinity columns.

## RESULTS

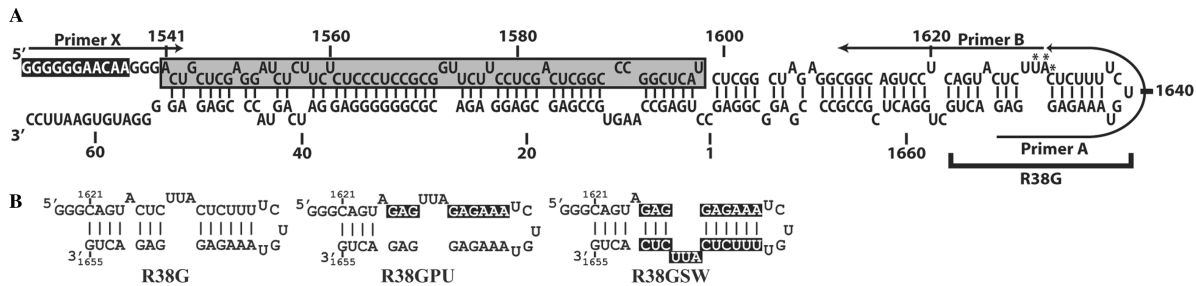
### The right terminal stem-loop domain of the genomic polarity of HDV contains an RNA promoter for RNAP II

To gain further insight into the mechanism by which RNAP II recognizes RNA promoters, we required the identification of a suitable RNA promoter for RNAP II. Previous works have shown that a region we refer to as the right terminal stem-loop domain of the genomic polarity of HDV (i) includes the reported initiation site for HDAG mRNA transcription (i.e. position 1630; 18), (ii) was speculated to be used by RNAP II because the HDAG mRNA is posttranscriptionally processed with a 5'-cap and a 3'-poly(A) tail (18,19), (iii) mutation analysis of this region revealed that the overall rod-like conformation was important for the accumulation of HDV RNA *in vivo* and for both RNAP II binding and transcription initiation *in vitro* (24,39).

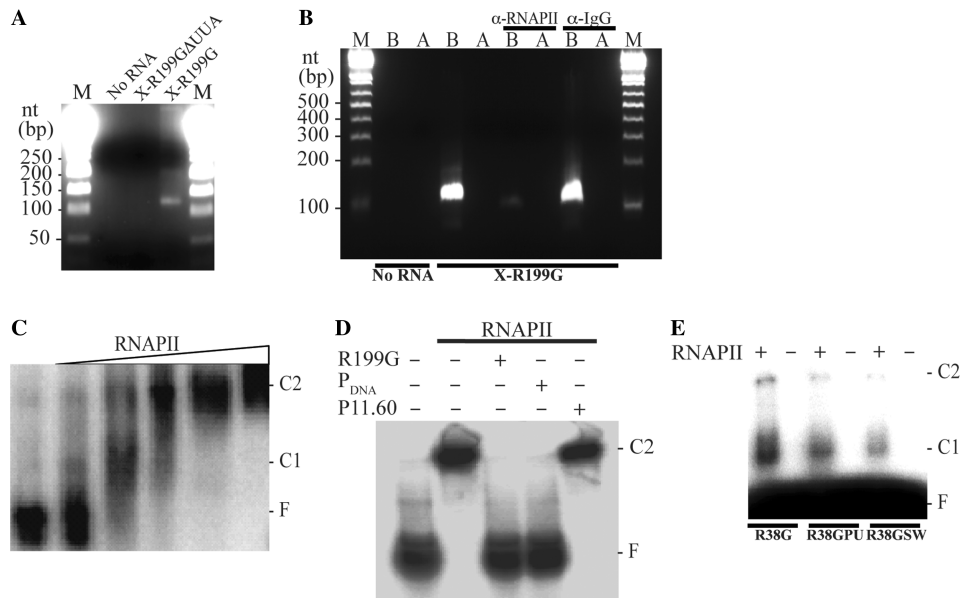
To corroborate that this segment of the HDV RNA genome acts as a promoter, we used an RNA fragment containing 199 nt corresponding to nucleotide 1541 to 60 of the genomic polarity of HDV RNA (Figure 1A; R199G), and performed transcription assays using HeLa NE proteins. To detect the product, RT-PCR was performed following the transcription reaction.

However, the RT reaction was not able to discriminate between the RNA product and the large molecular excess of its RNA template because they have very similar sequences (data not shown). Therefore, a non-HDV sequence was added to the 5'-end of R199G to generate an extended RNA template (i.e. X-R199G; inverted sequence in Figure 1A). Transcription reaction was performed on X-R199G, and the resulting RNA was subjected to reverse transcription using a primer complementary to the 3' extended extremity of the product (i.e. primer X; Figure 1A). The cDNA product was subjected to rapid amplification of cDNA ends (RACE) and resolved on an agarose gel. A specific RT-PCR product migrating between the 100- and 150-nt markers was detected when the RACE reaction was performed in the

presence of X-R199G (Figure 2A), indicating the production of an RNA species complementary to the upper strand of the RNA template (i.e. of antigenomic polarity). To locate the initiation site of the transcription, the RACE product was sent for sequencing. Analysis of the sequence indicated that transcription initiated near or at the proposed initiation site for HDVg mRNA (Figure 1A, the asterisks next to position 1630; 18). This result is in accordance with a previous report using a similar HDV-derived RNA molecule as template for *in vitro* transcription using HeLa NE proteins and showing that the initiation of synthesis occurred near this location (39). To further confirm the location of the initiation site, an RNA template with a deletion of nucleotide 1629–1631 was constructed to remove the UUA sequence at the initiation site



**Figure 1.** Representation of the predicted secondary structures of the RNA molecules used in this study. (A) Unbranched rod-like secondary structure of R199G and X-R199G. The initiation sites of transcription as determined in this study by 5' RACE are marked with asterisks. The gray box indicates the 5' extremity of the HDVg gene, the inverted characters represent the nucleotides that were added to R199G to form X-R199G, and the square bracket represents the location of R38G. Location of the primers used in this study is indicated. (B) Predicted secondary structures of R38G, R38GPU and R38GSW. Inverted characters represent mutations from R38G.



**Figure 2.** RNAP II interacts with and transcribes from the right terminal stem-loop extremity of the genomic polarity of HDV RNA. (A) 5' RACE reactions performed on the transcription product using either no RNA, X-R199G or X-R199GΔUUA. Lanes M contain MiniSizer 50-bp DNA ladder (Norgen, Thorold, Ontario, Canada). (B) Inhibition of the transcription reaction by a monoclonal antibody against the CTD of RNAP II. RT-PCR products were generated following transcription reactions performed on X-R199G using primer X with either primer B or primer A. Lanes M contain 1 Kb Plus DNA ladder (Invitrogen). (C) Titration of a constant amount of radiolabeled R199G with increasing amounts of purified RNAP II holoenzyme (0, 6.25, 12.5, 25, 50 and 100 ng). (D) EMSA of R199G bound to purified RNAP II holoenzyme in the presence of 50-fold molar excess of either unlabeled homologous competitor RNA (i.e. R199G), a control DNA promoter fragment (i.e. CMV promoter) or P11.60. (E) Binding of R38G and two RNA mutants to purified RNAP II holoenzyme in the presence of a 50-fold molar excess of P11.60. The free RNA (F) and both complexes observed (C1 and C2) are indicated.

(i.e. X-R199G $\Delta$ UUA). Following *in vitro* transcription and RACE, no HDV-dependent product was found using this construct (Figure 2A).

To verify the involvement of RNAP II, the transcription reaction was repeated in the presence of a monoclonal antibody specific for the CTD of RNAP II. However, the procedure described above was modified by performing PCR amplification on the cDNA using primer X and primers corresponding to sequences located on either side of the initiation site of transcription (Figure 1A, primers A and B). Using this approach, a specific RT-PCR product was detected when the transcription reaction was performed in the presence of X-R199G (Figure 2B). This product was not detected in transcription reactions where X-R199G was omitted, indicating the RNA template dependence of the reaction. More importantly, the product was only detected when primer B was used during the PCR amplification, in accordance with the initiation of synthesis occurring near or at position 1630. When the NE was preincubated with an antibody specific for the CTD of RNAP II, the production of the transcription product was strongly inhibited (Figure 2B). This inhibitory effect of the anti-RNAP II antibody is in accordance with previous studies reporting inhibition of DNA-directed RNAP II transcription by an antibody specific for the CTD of RNAP II (40). As a control for nonspecific effects of the antibody, the RNAP II antibody was replaced by an anti-IgG antibody. The addition of the anti-IgG antibody had no inhibitory effects on the transcription (Figure 2B). Together, these data indicate that the right terminal stem-loop domain of the genomic polarity of HDV contains an RNA promoter, and provide strong evidence that this RNA promoter is recognized by RNAP II. In addition, since the RNAP II antibody was targeted against the heptad repeats of the large subunit of RNAP II, our results indicate that the CTD of RNAP II is needed for the transcription from this RNA template.

### RNAP II binds directly to R199G

For initiation to occur, the RNAP II holoenzyme must bind to promoter sequences to form a PIC. To assess the direct binding of RNAP II to R199G, we performed electrophoretic mobility shift assays (EMSAs) using purified RNAP II holoenzyme. Radiolabeled R199G was allowed to bind to increasing amounts of RNAP II and the mixture was subjected to nondenaturing PAGE. RNAP II bound directly to R199G, as illustrated by the retardation of the migration of the complex when compared to free RNA (Figure 2C). Notably, two retarded species were detected, and their formation was dependent on RNAP II concentration; a fast migrating species first formed at low concentrations of RNAP II holoenzyme (Figure 2C, C1), followed by a slower migrating species formed at higher protein concentrations (Figure 2C, C2). R199G was completely complexed in the slower migrating species when the molar ratio for RNAP II and the RNA was approximately 1:1 (i.e. 50 ng of RNAP II; Figure 2C and D).

We next investigated the specificity of the interaction between R199G and RNAP II by challenging the formation of the complex with various competitors. As an

unrelated RNA competitor, we added a small RNA hairpin derived from the left terminal stem-loop domain of the peach latent mosaic viroid genome (i.e. P11.60; 5). This small RNA folds into a hairpin structure, thus providing an excellent competitive RNA to test for nonspecific binding of RNAP II to double-stranded RNA molecules or stem-loop secondary structures. As shown in Figure 2D, 50-fold molar excess of P11.60 did not displace RNAP II from R199G. However, inclusion of 50-fold molar excess of either unlabeled homologous competitor RNA (i.e. R199G) or a control DNA promoter fragment (i.e. CMV promoter) greatly inhibited formation of the radiolabeled R199G–RNAP II complexes. Finally, to provide evidence that RNAP II was binding to an internal site on R199G close to the initiation site, we performed EMSA with R38G, which corresponds to a 38-nt RNA located at the tip of the hairpin (Figure 1; 24). As observed for R199G, two retarded species were detected when radiolabeled R38G was allowed to bind to RNAP II (Figure 2E). These results are in accordance with our previous report indicating that R199G can be reduced to a smaller hairpin without significantly affecting its interaction with RNAP II in HeLa NE (24).

EMSAs were performed with two R38G derivatives (Figure 1B; 24). R38GPU contains mutations changing the pyrimidine-rich sequence located upstream of the terminal loop to purines, thus destabilizing the rod-like secondary structure. R38GSW is a ‘flip mutant’ of the region containing the initiation site. These two mutants are deficient in their interaction with the largest subunit of RNAP II in NE (24), and no transcription products were detected from these RNA species (data not shown). For both mutants, the amount of two retarded species was reduced, as compared to when R38G was used (Figure 2E). The relative intensities of the fast migrating species were reduced to 65 and 31% for R38GPU and R38GSW, respectively. The slower migrating species were reduced to 25 and 4% for R38GPU and R38GSW, respectively. These results correlate with our previous report suggesting that the maintenance of the rod-like conformation and the polarization in the purine/pyrimidine content near the tip of the rod of both polarities of HDV RNA might be required for RNAP II interaction and HDV replication (24). Taken together, these results provide direct evidence that the RNAP II holoenzyme interacts specifically with an internal site within R199G and warrant further investigation into PIC formation on R199G.

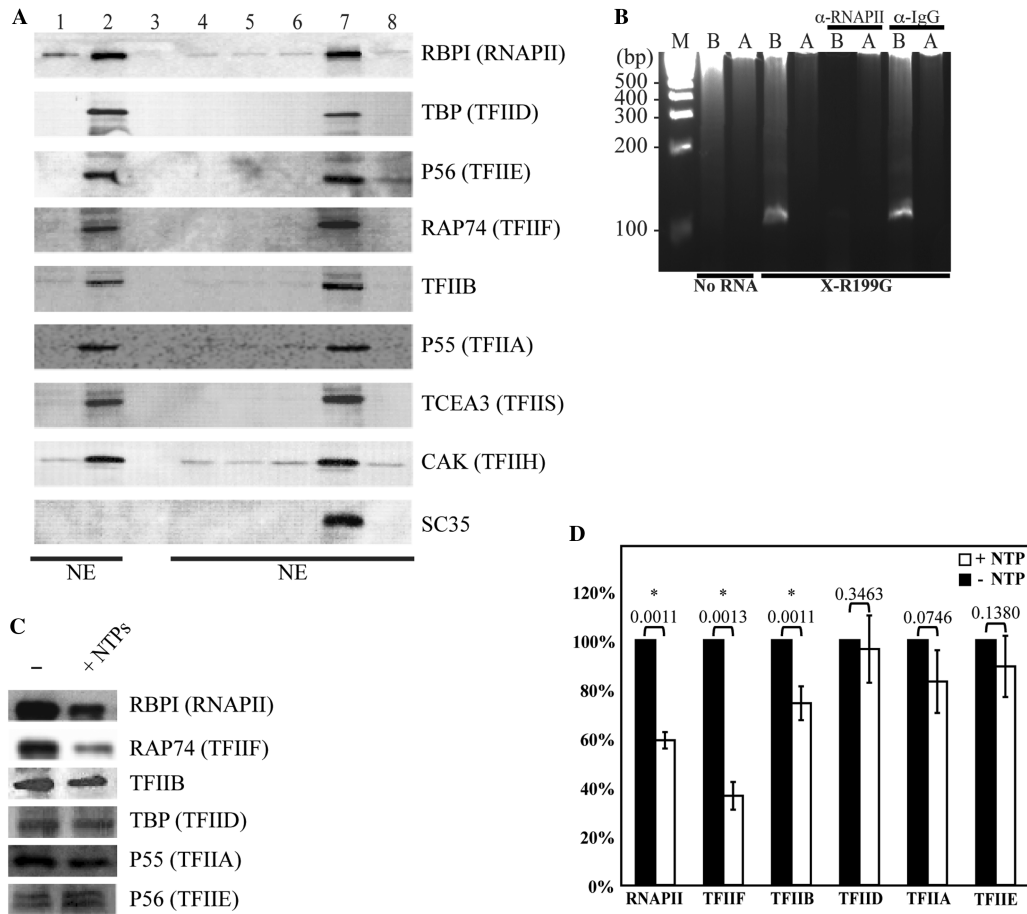
### An active PIC forms on R199G

If similar mechanisms exist between DNA- and RNA-directed transcriptions by RNAP II, the specific binding of the polymerase to the HDV RNA promoter should require the coordinated assembly of RNAP II and general transcription factors. To identify the transcription machinery recruited to form a PIC on R199G, we used an affinity purification procedure that involves the cross-linking of R199G to adipic acid dehydrazide agarose beads (41). Such an approach allowed us to monitor the entire transcription machinery assembly on R199G using

active HeLa NE proteins. To compare PIC formation between R199G and a control DNA promoter, the CMV DNA promoter was biotinylated and immobilized on streptavidin agarose beads. As a negative RNA control, P11.60 was also immobilized on adipic acid dehydrazide agarose beads and subjected to the same treatment as R199G. In addition, the complexes on R199G were challenged with a large molecular excess of either the DNA promoter fragment or R199G to assess of the specificity of PIC formation. Finally, unbound adipic acid dehydrazide agarose and streptavidin agarose beads were tested for protein interaction to rule out nonspecific binding of NE proteins to the beads.

Nucleic acid-coupled beads were incubated with HeLa NE proteins and were washed to remove unbound proteins. The ribonucleoprotein complexes were eluted with SDS-loading dye, separated by SDS-PAGE, and analyzed by western blot using antibodies raised against various

proteins involved in RNAP II transcription initiation. Using this approach, we detected the presence of RBPI (RNAP II), p55 (TFIIA), TFIIB, TBP (TFIID), p56 (TFIIE), RAP74 (TFIIF), CAK (TFIIH) and TCEA3 (TFIIS) in association with both R199G and the DNA promoter (Figure 3A). As expected, no significant binding of the RNAP II transcriptional machinery to either P11.60 or to the beads alone was observed, indicating that the interactions with R199G were specific. Furthermore, molecular excess of the DNA promoter and R199G was able to abolish the protein interactions with R199G. In addition, because HDV was previously suggested to be associated with SC35-containing nuclear speckles (42), we tested for the presence of SC35 to serve as a control differentiating between proteins binding to R199G but not to the control DNA promoter. As shown in Figure 3A, SC35 interacted with R199G but not with the DNA promoter.



**Figure 3.** Formation of an active PIC on R199G. (A) Composition of a PIC on R199G. The samples were incubated with HeLa NE proteins in the presence or absence of either molecular excess of free DNA promoter or R199G. After elution, the proteins were detected using antibodies specific for RNAP II and its general transcription factors. Lane 1 contains the streptavidin agarose beads; lane 2 contains the biotinylated control DNA promoter bound to streptavidin agarose beads; lanes 3 and 8 contain adipic acid agarose beads; lane 4 contains P11.60 bound to adipic acid agarose beads; lane 5 contains R199G bound to adipic acid agarose beads in the presence of molecular excess of free control DNA promoter; lane 6 contains R199G bound to adipic acid agarose beads in the presence of molecular excess of free R199G; lane 7 contains R199G bound to adipic acid agarose beads. With the exception of lane 3, all the samples were incubated with HeLa NE. (B) Initiation of transcription on the bound PIC following the addition of NTPs. Following transcription initiation on X-R199G-coupled beads, RT-PCR products were generated using primers complementary to sequences located either before (i.e. primer B) or after (i.e. primer A) the proposed initiation site for HDAG mRNA transcription (18). Lanes M contain 1 Kb Plus DNA ladder (Invitrogen). For some reactions, monoclonal antibodies against either the CTD of RNAP II or IgG were used. (C and D) Decrease in the levels of various PIC components following initiation of transcription. The experiment was repeated three times and *P*-values (Student's *t*-test) were obtained (indicated above the bars). Asterisks indicate significant differences ( $P < 0.05$ ).

Although R199G is an RNA promoter for RNAP II, it was possible that the observed interactions of R199G with RNAP II and its general transcription factors were not related to PIC formation and transcription initiation. To test this possibility, X-R199G-coupled beads were incubated with HeLa NE proteins, as above. After removing unbound proteins, the ribonucleoprotein complexes were equilibrated with transcription buffer and free NTPs were added to allow initiation of transcription to occur. After elution of the sample, RT-PCR was performed as above. The specific RT-PCR product was detected when the transcription reaction was performed in the presence of X-R199G (Figure 3B), demonstrating RNA synthesis by the immobilized RNAP II complex. As observed above, the transcription product from the bound RNA template was only detected when primer B was used during the PCR amplification, indicating that the initiation of synthesis occurred near the proposed initiation site for HDAG mRNA. In addition, no product was detected in absence of the X-R199G template, or when the NE was preincubated with anti-RNAP II, indicating the involvement of both RNAP II and the RNA template in this process. To further confirm the location of the initiation site of the transcription, the cDNA product was subjected to RACE, as above. Analysis of the sequence of the RACE product indicated that transcription initiated at the right location (data not shown). More importantly, these results indicate that an active RNAP II PIC forms on the bound RNA template and that this complex is able to perform the transcription reaction.

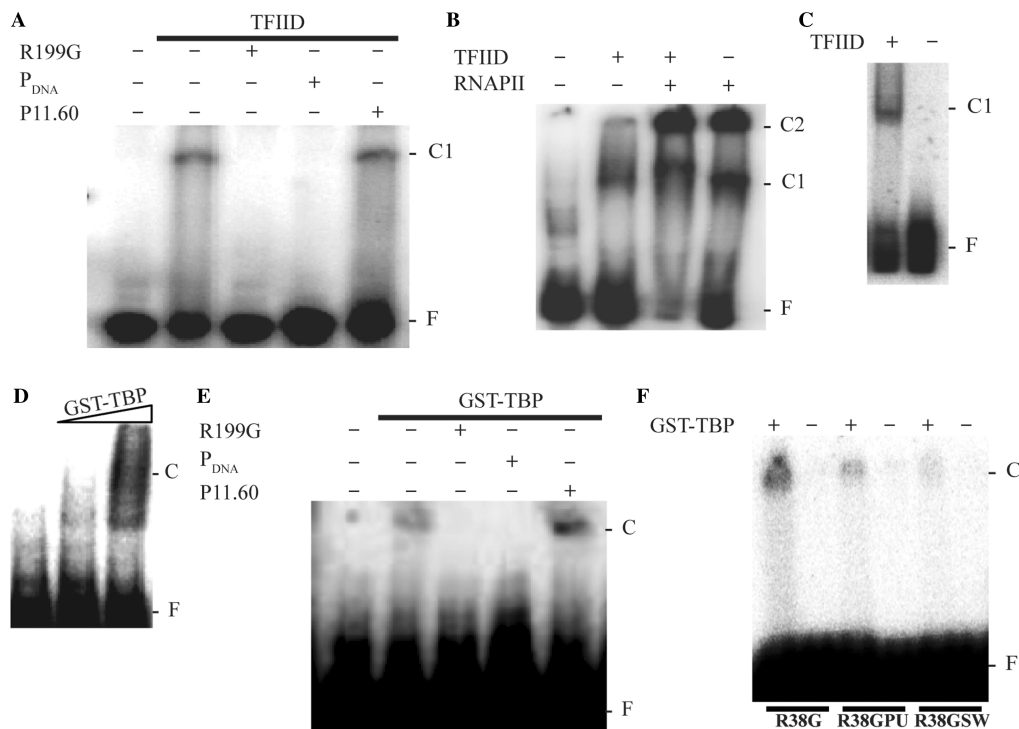
Using the same procedure, we examined the constituents of the RNAP II complex following transcription initiation. Addition of free NTPs significantly reduced the level of RPB1 (RNAP II), TFIIB and RAP74 (TFIIF) retained on R199G (Figure 3C and D). In contrast, no significant release of either p55 (TFIIA), TBP (TFIID) or P56 (TFIIE) was observed (Figure 3C and D). Such a phenomenon is in agreement with what was observed to occur on canonical DNA promoters (32). Specifically, the core subunits of RNAP II and various transcription factors leave the DNA promoters after initiation, whereas a subset of the transcriptional machinery (i.e. TFIIA, TFIID, TFIIE and TFIIF) remains to form a scaffold for assembly of a second transcription complex (32). Taken together, these results indicate that an active RNAP II PIC forms on R199G and that this complex contains the core RNAP II subunit and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIF and TFIIS. Analogous to what is observed for DNA promoters, our results also suggest that a re-initiation complex remains on the RNA promoter following initiation of transcription.

#### The TATA box-binding protein binds directly to R199G

In DNA-directed RNAP II transcription, the binding of TFIID to the DNA promoter is the first step in the assembly of an active PIC (43). Because our results suggested that RNAP II is forming a PIC on R199G analogous to

that observed on DNA promoters, we investigated the binding of TFIID to R199G using EMSA experiments. Radiolabeled R199G was allowed to bind to purified TFIID, and the samples were subjected to nondenaturing PAGE. Purified TFIID bound directly to R199G, as illustrated by the retardation of the migration of the complex when compared to free RNA (Figure 4A). To test the specificity of this interaction, the binding of TFIID to R199G was challenged by competition with the various competitors used above. The 50-fold molar excess of P11.60 was not able to compete for TFIID binding to R199G. However, either 50-fold molar excess amounts of nonradiolabeled R199G or the control DNA promoter fragment greatly inhibited the formation of the R199G-TFIID complex (Figure 4A). Noteworthy, when the migration of the R199G-TFIID complex was compared to the complexes formed between R199G and the RNAP II holoenzyme, we found that the migration of R199G-TFIID corresponded to the fast-migrating species (Figure 4B, C1), suggesting formation of the R199G-TFIID prior to the assembly of the RNAP II complex (Figure 4B, C2). In addition, TFIID was able to bind to R38G, suggesting that TFIID was binding to R199G close to the initiation site, as observed above with RNAP II (Figure 4C).

It is well established that TFIID interacts with DNA promoters through its TBP subunit (43); it is therefore possible that TBP serves a similar role with respect to R199G. To test this hypothesis, the formation of a complex between purified GST-tagged TBP and either R199G or R38G were analyzed by EMSA. A typical gel showing a titration of a constant amount of radiolabeled R199G with increasing amounts of purified GST-TBP is shown in Figure 4D. Purified GST-TBP bound directly to R199G only when high amount was used (i.e. 64 ng), suggesting a very low affinity when it is not part of the TFIID complex. Such low affinity was also reported between purified TBP and DNA promoters (44). Despite this low affinity, the binding of GST-TBP to R199G was shown to be specific when the interaction was challenged with various competitors. A 50-fold molar excess of P11.60 was not able to compete for GST-TBP binding to R199G. Excess of nonradiolabeled R199G or the control DNA promoter fragment inhibited the binding of GST-TBP to radiolabeled R199G (Figure 4E). Moreover, purified GST protein alone was not able to bind to R199G (data not shown) and GST-TBP was able to bind to R38G (Figure 4F). In correlation with the binding efficiency of both mutants to the RNAP II holoenzyme, the binding of GST-TBP to R38GPU and R38GSW were found to be reduced to 36 and 20%, respectively (Figure 4F). Taken together, these results provide direct evidence that TBP interacts specifically and directly with R199G close to the initiation site of the transcription. Although we cannot exclude the possibility that other TFIID component(s) might also bind directly to the RNA promoter, our results suggest that this part of TFIID is involved in the nucleation of the RNAP II PIC on R199G, similar to what occurs on DNA promoters.



**Figure 4.** Specific binding of purified TFIID and GST-tagged TBP to HDV-derived RNAs. (A) EMSA of R199G in the presence of purified TFIID with 50-fold molar excess of either unlabeled homologous competitor RNA (i.e. R199G), a control DNA promoter fragment (i.e. CMV promoter) or P11.60. (B) EMSA of R199G in the presence purified TFIID or RNAP II. (C) EMSA of R38G with purified TFIID in the presence of 50-fold molar excess of P11.60. (D) Titration of a constant amount of radiolabeled R199G with increasing amounts of purified GST-tagged TBP (0, 32 and 64 ng). (E) EMSA of R199G bound to purified GST-TBP in the presence of 50-fold molar excess of either unlabeled homologous competitor RNA (i.e. R199G), a control DNA promoter fragment (i.e. CMV promoter) or P11.60. (F) Binding of R38G and two mutants with purified GST-TBP in the presence of 50-fold molar excess of P11.60.

## DISCUSSION

RNAP II has been proposed to be involved in RNA-directed RNA polymerization of both HDV and many viroids (i.e. pospiviroids), principally based on the sensitivity of their replication/transcription to  $\alpha$ -amanitin (21–23,45). In addition, both tomato and human RNAP II were reported to specifically coimmunoprecipitate with citrus exocortis viroid (CEVd) and HDV RNA genomes, respectively (24,46). However, these findings did not reveal either the direct interaction of RNAP II or the involvement of RNAP II transcription factors in RNA promoter recognition. To obtain insight into the mechanism of RNA promoter recognition by RNAP II, we used a transcription assay with HeLa NE proteins to demonstrate that an RNA fragment derived from the right terminal stem-loop domain of the genomic polarity of HDV acts as an RNA promoter, and that the initiation of synthesis occurred near the proposed initiation site for HDV mRNA (18). The confirmation of RNAP II involvement was achieved by the demonstration of direct binding of the purified human RNAP II holoenzyme and the inhibition of transcription by a monoclonal antibody specific for the CTD of RNAP II.

Our results are in accordance with a previous report showing initiation of transcription from a similar HDV-derived RNA (39). In both cases, HDVAg-S was not required for the reaction, and the size of the RNA

products suggested that the initiation of synthesis occurred near the proposed initiation site for HDV mRNA (i.e. position 1630; 18). Interestingly, a different transcription mechanism was reported using an RNA template derived from the left terminal stem-loop domain of the antigenomic polarity of HDV (21). In that case, the transcription reaction yielded a chimeric template/transcript product, and the reaction stopped after an elongation of only 41 nt. Addition of HDVAg-S allowed the elongation to resume by binding to RNAP II directly and by displacing the elongation repressors NELF and DSIF (15,21,47). Although not tested because it was not required in our system, it is possible that the addition of HDVAg-S might stimulate RNA synthesis from R199G.

Using RNA affinity chromatography, we demonstrated that RNAP II, along with general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIS, binds to the RNA promoter to form a PIC similar to those observed on canonical DNA promoters (32). Moreover, this PIC was active, since addition of free NTPs resulted in production of the transcript and the release of the large subunit of RNAP II, along with both the TFIIF and TFIIB subunits. Similar to what was reported for a DNA promoter (32), our results suggest that TFIIA, TFIID and TFIIE might be part of a scaffold for a re-initiation complex. However, although the level of RNAP II, TFIIF and TFIIB were reduced,



they were not reduced to the extent reported for a DNA promoter (32). This is consistent with our observation that *in vitro* transcription initiation is reduced on such an RNA template as compared to a DNA template (i.e. it is a very weak promoter). In addition, although not required for transcription in our system using HeLa NE, additional host proteins, or the involvement of HDAG, might be required for efficient transcription initiation and/or elongation *in vivo*.

In mammalian RNAP II transcription, the formation of the TBP/DNA promoter complex is the first step in the assembly of a set of transcription factors necessary for the initiation of transcription (48). Following this binding, RNAP II and other general transcription factors are recruited to form a PIC (49). By analyzing the formation of a complex between R199G and RNAP II holoenzyme, we have observed that a fast-migrating species first formed at low concentrations of RNAP II, followed by a slower migrating species formed at higher concentrations of the enzyme. When the migration of the R199G-TFIID complex was compared to those formed between R199G and the RNAP II holoenzyme, the migration of R199G-TFIID corresponded to the fast-migrating species. Accordingly, our results suggest that formation of the R199G-TFIID complex is required to nucleate the RNAP II PIC. Because we found that purified GST-TBP binds directly and specifically to R199G, it is tempting to suggest that RNA promoter recognition by RNAP II occurs primarily through TBP.

Typically, DNA promoters recognized by RNAP II have several sequence motifs, each having specific functions that relate to the transcription process (28). Despite clear association of TBP (or TFIID) with R199G, no consensus sequence similar to the TATA box was found. However, TBP binds to canonical DNA target sites through the minor groove (50). Proteins that bind the DNA double helix through the minor groove must operate mainly by indirect readout since the symmetric positioning of donor and acceptor groups in this groove makes it difficult to differentiate an A-T base pair from a T-A pair (and likewise for G-C and C-G base pairs; 51). It is proposed that the structure of the DNA promoter region might produce intrinsic structural characteristics favoring TBP binding (52). Since TBP induces significant bending at the TATA box, sequences that are already bent, or are more flexible in a particular orientation could be energetically favored. Such a phenomenon was reported to occur in DNA templates constructed with arbitrary nonpromoter sequences and small single-stranded bubbles (53). In this case, core RNAP proteins were capable of directing RNA synthesis. Rather than being a major contributor to core promoter recognition, the Inr-like motifs found near the transcription initiation site on R199G might therefore represent preferred initiation sites for RNAP II after binding at its initial interaction site.

We recently reported that RNAP II interacts with HDV-derived RNAs at sites located within the terminal stem-loop domains of both polarities of HDV RNA (24). Analysis by base pair covariation and site-directed mutagenesis of this region revealed a strong selection to

maintain a hairpin conformation (24,39,54). Wheat germ RNAP II was shown to bind to the large terminal loops of potato spindle tuber viroid genome (55), and it was recently reported that potato NE was able to initiate transcription of this viroid within a terminal stem-loop region of the RNA genome (8). Thus, it seems that a defined hairpin domain is a common feature of RNA promoters for RNAP II. Based on the conserved features of HDV RNA domains interacting with RNAP II, which are located near large terminal loops and present bulged initiation sites within a thermodynamically unstable stem (i.e. few G-C base pairs; 24), those RNA regions might mimic a prebent conformation giving those RNA molecules a general affinity for the TBP-containing TFIID. Since much of the affinity of TBP for DNA templates is mediated by electrostatic interactions with the phosphate backbone (56), an RNA template could preserve these interactions.

Further investigation is required to determine the significance of the association of TBP (and probably TFIID) with RNA promoters and to identify the RNA features involved in PIC formation. More importantly, our findings propose potential evolutionary relationships between DNA- and RNA-dependent RNAPs. Despite the unusual nature of the HDV-derived RNA promoter, our results demonstrate that an RNA template uses the same RNAP II general transcription factors in promoter recognition. Crystal structures of RNA-dependent RNAPs of positive-strand RNA and dsRNA viruses show structural similarity not only to each other, but also to DNA-dependent RNA and DNA polymerases, and reverse transcriptases (57-63). All of these polymerases share a structure similar to that of a right hand with palm, thumb and finger domains. The palm domain structure is particularly conserved and contains four sequence motifs preserved in all RNA and DNA polymerases (64,65). Because these features have been conserved throughout evolution, it is not excluded to find reminiscent RNA promoters in cellular RNAs, which therefore, would have the potential for initiation of transcription from RNA rather than DNA templates.

## ACKNOWLEDGEMENTS

This work was funded by a grant from The Canadian Institutes of Health Research (CIHR) awarded to M.P. A.A. is supported by a doctoral award from the Libyan government. Funding to pay the Open Access publication charges for this article was provided by CIHR.

*Conflict of interest statement.* None declared.

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