The gene S promoter of hepatitis B virus confers constitutive gene expression

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ABSTRACT

The properties of the promoter of the hepatitis B surface antigen (HBsAg) were studied using recombinants containing either this promoter or the SV40 early promoter. Mouse L cells were transfected with these recombinants and the levels of gene expression obtained with the two promoters were compared. The level of expression of a cellular gene, the human fibroblast interferon gene, obtained with the HBsAg promoter was comparable to that obtained with the SV40 early promoter. Similarly when the HBsAg gene was controlled by the SV40 early promoter the level of HBsAg synthesis is in the same range as that observed with its own promoter. Together these results suggest that although the HBsAg gene codes for a structural viral protein, its expression is constitutive as for an early gene. The implications of these observations on the synthesis of HBV particles in vivo are discussed.

INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus which infects only man and a few other primates. Its genome is a circular molecule about 3,200 nucleotides long with a single-stranded region of variable length. The outer envelope of the virus which carries the surface antigen (HBsAg) contains one major polypeptide which exists in both glycosylated and unglycosylated forms. The inner nucleocapsid which carries the core antigen (HBcAg) contains also one major polypeptide (see Tiollais et al. (1) for a review).

Cloning and sequencing of the HBV genome have provided information on the genetic organization of the virus (2,3,4,5,6). The coding sequences of the major polypeptide of the envelope (gene S) and of the major polypeptide of the nucleocapsid (gene C) were mapped. Gene S is preceded in the same reading frame by an open region called pre-S. Although this region is conserved in the six sequenced HBV genomes no direct proof of its translation has been obtained. The lack of a cell culture system for propagating HBV has greatly hampered the study of its molecular genetics. Using transfection of animal cells with cloned HBV DNA only the transcription unit of the major polypeptide of the envelope was mapped (7).

In order to study the regulation of gene S expression, we have compared: (i)
the expression of a cellular gene, the human fibroblast interferon (HIFN-β) gene controlled either by the gene S promoter or by the SV40 early promoter, (ii) the expression of the gene S controlled either by its own promoter or by the SV40 early promoter. The results show that expression of a gene placed under the control of the gene S promoter is constitutive.

MATERIALS AND METHODS

Construction of the recombinant plasmids:

Plasmid pAC contains a 2800 bp HBV DNA fragment, positions 2423 to 1984, following the nomenclature of P. Tiollais et al. (1) including the gene S, the pre-S region and a putative promoter. This plasmid was derived from plasmid pAC2 (7) by deleting the TK gene. Plasmid pCP10 (8) has two HBV genomes integrated in tandem in the EcoRI site of pBR322. Plasmid pIFC15 (9) contains a 1800 bp human genomic fragment including the HIFN-gene. Plasmid pSV2-gpt (10) contains a 335 bp PvuII-HindIII SV40 DNA fragment including the early promoter, positions 253 to 5154 following the nomenclature of Fiers et al. (11). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs and were used as recommended by the supplier. EcoRI octanucleotide linkers (from BRL) were phosphorylated in 20 mM Tris-HCl (pH 7.6), 9 mM MgCl2, 5 mM DTT and 1mM ATP with 20 units of T4 polynucleotide kinase (Boehringer). Filling-in with Klenow polymerase (Boehringer) was performed in 50 mM Tris-HCl (pH 7.9) 6 mM MgCl2, 5 mM mercaptoethanol and 0.1 mM each dXTP for 1 hour at 25°C. All fragments were purified by electrophoresis on 1.2 % low-melting agarose (Biorad).

Cell transfections and gene expression assays:

Transfections were performed according to the calcium phosphate coprecipitation procedure of Graham and Van der Eb (12). About 10^6 mouse TK-L cells were transfected with 10 μg of the vector and 0.2 μg of the pAGO plasmid (13) containing the TK gene. After selection for two weeks in HAT medium, about 10 to 20 TK+ colonies were obtained per 10^6 transfected cells and subsequently cloned. About 3.10^6 cells from independent clones were maintained in 5 ml in the absence of HAT in Dulbecco modified Eagle's medium with 10 % calf serum. The biological activity of interferon accumulated during 72 hours was tested by the inhibition of the cytopathic effect (14) of vesicular stomatitis virus on amnion human cells AV3. Fibroblastic HIFN-β of known titer (from NIH) was used as a reference. As a control, the activity was checked by 1 hour incubation at 37°C of 100μl cell supernatant with 10 μl of anti-HIFN rabbit antiserum. The amount of HBsAg secreted during 24 hours accumulation in the cell culture supernatant was determined by radioimmunoassay (AUSRIA-II kit, Abbott Laboratories).
Northern blot analysis

About \(10^8\) cells at confluence were rinsed with PBS, scraped and lysed with 3 M LiCl, 6 M urea according to Auffray and Rougeon (15). Poly A+ RNA was purified from total RNA by oligo (dT) cellulose affinity chromatography (16). Tenug of poly A+ RNA were denatured following the procedure of McMaster and Carmichael (17) and electrophoresed through a 1.2 % agarose gel. RNA was then transferred and hybridized as described by Thomas (18) with nick-translated 1.8 kb HIFN-\(\beta\) EcoR1 restriction fragment (specific activity \(10^8\) cpm/\(\mu\)g).

RESULTS

Comparison of HIFN-\(\beta\) gene expression directed either by the gene S promoter or the SV40 early promoter:

Since the function of the pre-S region is unknown, two recombinants using the putative gene S promoter were constructed: one containing a large part of the pre-S sequence (plasmid pHII) the other one only the promoter region (plasmid pHDI) (Fig. 1).

Plasmid pHII was obtained by fusion of the HincII DNA fragment (containing the HIFN-\(\beta\) coding sequence) to the pre-S region. Synthetic linkers were used to conserve the reading frame at the gene junction. In order to conserve the complete 3' non-coding sequence of HIFN-\(\beta\) and especially the polyadenylation site, plasmid pHII was derived from pHII by insertion of the 900 bp BglII-EcoR1 DNA fragment purified from plFC15. The second construction (plasmid pHDI) was derived from plasmid pHII by deleting the BglII-EcoRI HBV fragment between position 2842 to 3178 (Fig. 1a). This deletion removed completely the pre-S region which starts at position 2848. As a reference we constructed a plasmid with the HIFN-gene placed under the control of the SV40 early promoter. This plasmid (pSV1911) contained a PvuII-HindIII SV40 DNA fragment (positions 253 to 515) including the early promoter. This regulatory sequence was fused to the HincII HIFN-\(\beta\) fragment carrying the coding sequence and the 3' non-translated region up to the poly A site (Fig. 1a). TK-L cells were transfected by the recombinant plasmids using a 50 fold excess of the HIFN-plasmids over the TK pAGO plasmid. TK+ clones (10 to 20 per 10\(^6\) transfected cells) were obtained after two weeks. Interferon production and titration were carried out as indicated in Materials and Methods. The supernatants of transfected L cells were submitted to gel filtration on Sephadex G100. Activity was detected as a single peak at 21K daltons (data not shown) corresponding to the MW of HIFN-\(\beta\). Additionally, the HIFN-\(\beta\) activity was neutralized by different anti-HIFN-\(\beta\) rabbit antisera. The product obtained is therefore indistinguishable from HIFN-\(\beta\) by size and immunological criteria. Arithmetical means of HIFN-production have been derived.
Figure 1: Construction of the recombinant plasmids. HBV DNA is represented by shaded boxes. The gene S promoter (P), the pre-S and the S regions are indicated. The coordinates of the restriction sites are according to Tiollais et al. (1). SV40 DNA is represented by cross-hatched boxes. HIFN-B gene and flanking regions are represented by open boxes. A) HIFN-expression plasmids. the sequence of the gene junction in pH1 is indicated. The deletion present in pHDI is underlined (positions 2842 to 3178). B) HBsAg expression plasmids.
from the histograms shown in figure 2 and gave 630, 380 and 1400 HIFN units/ml for the HBs promoter including the pre-S region, the deleted HBs promoter and the SV40 early promoter respectively. The values obtained vary within the same order of magnitude and may reflect some slight differences in the efficiency of expression of these constructions. No constitutive HIFN-β expression was detected in mouse L cells transfected with the plasmid (pIFC15) carrying the genomic HIFN fragment. In order to determine the size of the HIFN-β gene transcripts poly A+ RNA purified from transfected cells were analyzed by Northern blotting (Fig. 3). RNA of similar sizes (1.1 kb) were characterized in L cells transfected with pH1 or pHDI plasmids. Southern blot hybridization using the 1.8 kb EcoRI HIFN-genomic fragment showed integration of the recombinant plasmids (data not shown). It is generally admitted that in the conditions used the average number of integrated copies is about 5-10
Figure 3: Northern blot analysis of poly A+ RNA extracted from mouse L cells (lane a) and mouse L cells and plasmid pHDI (lane c). Arrows indicate migration of denatured DNA molecular weight markers (in kilobase pairs).

(19,20). In order to avoid statistical fluctuations of the number of integrated copies, HIFN-gene expression was measured on a large number of clones. As observed here, it has been reported that in similar transfection conditions more than 80% of the TK+ clones can express the non selectable marker (13).

Comparison of gene S expression directed by the gene S promoter and the SV40 early promoter:

Another approach to study the gene S promoter consisted in comparing gene S expression obtained either with the SV40 early promoter or its own promoter. For that purpose, we constructed a recombinant called pSVH4 containing the SV40 DNA fragment (positions 253 to 5154) fused to the 2350 bp BgIII fragment (positions 2837 to 1984). This recombinant similar to pSVI911 was obtained using plasmids pCP10 and pSV2-gpt (Fig. 1b). TK-L cells were transfected with either pAC or pSVH4 plasmids.

Figure 4: Histograms of the production of HBsAg by mouse L cells transfected with plasmid pAC (A) and plasmid pSVH4 (B). The amount of HBsAg is evaluated by RIA and expressed in ng/ml/24 h (1 ml of the cell supernatant corresponds to about 10^6 cells).
as described for the HIFN recombinant plasmids. The amount of HBsAg excreted in the cell supernatant was quantified by radioimmunoassay as described in materials and methods. Since intracellular and extracellular HBsAg are present in proportional amounts during the production cycle (8) accumulation of extracellular HBsAg was only used to quantify gene S expression. The expression of HBsAg was quantified by radioimmunoassay. Electron microscopy using negative staining showed that HBsAg was excreted as 22 nm particles identical to those found in human plasma (results not shown). As deduced from the arithmetical means of HBs antigen secretion, 160 ng/ml in both cases, no obvious differences appeared in the expression of S gene under the control of SV40 early promoter or under the control of its own promoter (Fig. 4).

DISCUSSION

The data presented in this report demonstrate that initiation of transcription of the HIFN-β sequence takes place in the 400 bp BglII fragment. This assumption is further supported by the absence of HIFN-synthesis in cells transfected with plasmids either containing the HIFN-gene in the wrong orientation or lacking the 400 bp BglII fragment. The length of the mRNA is in agreement with the size expected for a transcript starting immediately downstream to the putative TATA box at position 2776. The similar sizes of the transcripts encoded by the plasmids pHII and pHDI suggest the existence of a splice in the mRNA, of about 300 bp and encompassing the BglII-EcoRI fragment in the pre-S region. This is in agreement with recent observations of Stenlund et al. (21). Using the S1 mapping technique these authors have observed that a splicing event takes place during the HBsAg mRNA maturation and have localized an acceptor site at position 3160. The approximative length of the expected intron would therefore localize the donor site in the vicinity of the BglII site.

The comparison of gene expression obtained with either the gene S promoter or the SV40 early promoter indicates that the gene S promoter can elicit gene expression at a level comparable to that obtained with the SV40 early promoter. Since gene S codes for a structural polypeptide the properties of the gene S promoter are unexpected. Indeed it is generally admitted for DNA viruses that in non permissive cells containing integrated viral sequences early genes which code for regulatory proteins are expressed in large amounts whereas expression of late genes, which code for structural proteins, is very low (22). Concerning HBV, constitutive expression of gene S in mouse cells leads to the conclusion that whereas gene S codes for a structural protein and could be a priori regarded as a late gene, its expression mechanism is closer to that of an early gene. Recently, Summers et and Mason (23) proposed that HBV-like viruses despite their DNA genome replicate via an RNA
intermediate which is reverse transcribed into viral DNA. This important observation is another argument indicating that this virus does not behave like other animal DNA viruses.

In animals cells transfected with cloned monomeric HBV DNA and producing HBsAg, gene C is either not expressed (8, 24, 25) or expressed at a low level (26, 27). Moreover, Gough and Murray (26) have reported that HBeAg and HBcAg synthesis needs to transflect cells with a recombinant carrier four tandem head to tail copies of the HBV genome. The mechanism of gene S expression seems therefore to be very different of that of gene C. The lack of a cell culture system for HBV propagation does not permit analysis of the regulation of the viral genes expression during the virus cycle. Nevertheless the above results provided by cells transfected with cloned HBV DNA fragments could explain the synthesis of viral particles during the chronic infection in man. In chronic carriers, HBV DNA is present in two states. In the first situation observed in hepatocarcinoma or some cases of chronic hepatitis, HBV DNA is integrated into the host cellular DNA and the liver cells secreted only empty envelopes (28). Cloning and determination of the structure of integrated HBV sequences have only confirmed that the transcription unit of HBsAg is complete and functional (29). The second situation observed in some cases of chronic hepatitis is characterized by the presence of free HBV DNA corresponding to intermediate replicative forms (28). In this case, the liver cells secreted both empty envelopes and virions, with a large excess of empty envelopes. Although the results presented in this paper concern in vitro experiments and do not prove that the mechanism proposed is the same in vivo in the normal environment of viral DNA, the properties of the gene S promoter described here suggest the following hypothesis: in cells containing integrated HBV DNA sequences, gene S expression is constitutive while the others viral genes are not expressed. During the viral multiplication while gene S expression remains constitutive and not limited by regulatory factors, gene C expression depends on factors such as viral gene expression and/or DNA replication. This discrepancy between the expression of gene S and of the other viral genes would account for the large excess of empty envelopes in the blood of chronic carriers.

Eukaryotic promoters contain two well characterized regulatory sequences (30). The highly conserved "TATA" box sequence located at about 30 nucleotides upstream to the cap site and the "CAT" box usually found about 80 bp upstream to the cap site. A "TATA" box located at position 2776 is observed in the gene S promoter (7). In addition strong promoters as the SV40 early promoter contain activator sequences located around 100 to 200 base pairs upstream to the "TATA" box (31, 32). These activators contain the consensus sequence TGG\text{AAA} \text{TTT} (33). Such a sequence is present several times around 100 nucleotides upstream the "TATA" box of
the gene S promoter. The possible role of these sequences would be therefore interesting to investigate.

In previous reports concerning HIFN-β synthesis in transfected animal cells, HIFN-β gene expression was inducible (34-38)). The results presented here show that constitutive synthesis is also possible at a level comparable to that obtained after poly (rI:rC) induction. Until now most of the recombinant vectors constructed for eukaryotic gene expression in animal cells are dealing with papovaviruses or retroviruses promoters (39). Constitutive expression of the HIFN-β gene controlled by the gene S promoter shows that the 400 bp BgII HBV DNA fragment can be a valuable tool for expression of a foreign gene in mammalian cells.

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REFERENCES