
The genetic organization of integrated hepatitis B virus DNA in the human hepatoma cell line PLC/PRF/5

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ABSTRACT

Hepatitis B virus (HBV) DNA is often found integrated in the genome of infected human liver cells and is supposed to be related to the development of primary liver carcinoma (PLC). Four clones of HBV DNA-containing sequences derived from DNA of the human PLC-derived cell line PLC/PRF/5 are discussed. The viral sequences show no intricate rearrangements excepting for a duplication and an inversion in one case, and a deletion in another. In all cases integration of the viral DNA was seen to be in a region which is single-stranded in the unintegrated HBV DNA. Sequence homologies between human and viral DNA flanking the integration sites have been detected. That may have a functional role in integration. Nucleotide sequence analyses of regions encompassing the viral-human junctions reveal open reading frames which consist of viral and/or human information. The possible expression of chimeric or cellular proteins may play a role in tumour development, and offers directions for further investigations.

INTRODUCTION

HBV is widely believed to be a causative agent in the development of primary liver carcinoma (1, 2). HBV DNA has been found integrated in the DNA of infected human liver cells, in acute and chronic hepatitis (3), cirrhosis (4), and PLC (3-7). We have previously cloned (8) integrated HBV DNA of the human PLC-derived cell line PLC/PRF/5 (9) in bacteriophage lambda. Preliminary characterization of three of these clones has been previously reported (8). In this paper we present detailed structural analyses of the four inserts as deduced from DNA-sequencing and a consideration of their functions. All numbering of nucleotide positions given in the text refer to the circular HBV map seen in figure 1.

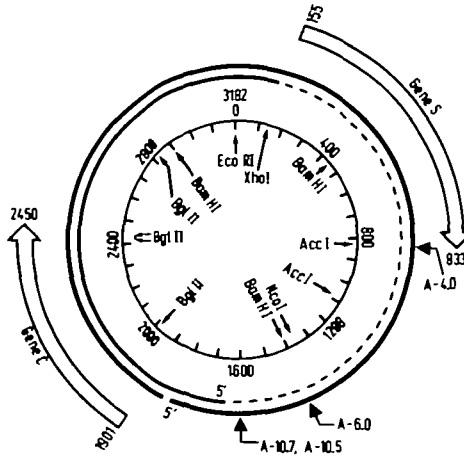


Fig. 1: Map of the circular HBV DNA of the subtype adw_2 .

The cleavage sites are composed of sites of the HBV subtype adw_2 (16) and ayw (in brackets) (18). The coding regions of HBsAg, HBeAg, P and X region are shown. The 6 and the 33 additional base pairs in the DNA of the subtype adw_2 , as well as the integration sites found in the cloned fragments of PLC/PRF/5 DNA are indicated. The dotted line represents the "gap".

METHODS

Extraction of cellular DNA, Southern blot analysis and cloning procedures were as previously described (8). The cloning and growth of recombinant phage and plasmids was carried out under L3B1 safety conditions as advised by the German Zentrale Kommission für biologische Sicherheit.

DNA Sequencing

Nucleotide sequencing was done following the method of Maxam and Gilbert (10). The recombinant plasmids were digested with Acc I, Bam HI, Bgl II, Eco RI, Hind III, Nco I, Rsa I and Tth I, respectively, and 5' end-labelled with ^{32}P -ATP using polynucleotide kinase. The subfragments were purified by preparative agarose gel electrophoresis. After base-specific chemical cleavage the samples were loaded on ultrathin (0.2 mm) 6%, 8% and 20% polyacrylamide gels (11). The sequenced regions are indicated by filled arrowheads in figures 5a-c.

Computer Analyses

Computer analyses of the DNA sequences were performed on a

VAX 11/782 computer of Digital Equipment Corporation using the FORTRAN-77 programs NAQ, RELATE, ALIGN and SEARCH (12).

RESULTS

Origin of the Clones

The DNA of PLC/PRF/5 cells contains several copies of integrated HBV sequences (4-7) as seen in hybridizations of Hind III digested cellular DNA with a radiolabelled probe of cloned HBV DNA (pAO1-HBV; 13) (Fig. 2e). Hind III was also used in the cloning strategy because this enzyme was known not to cleave within HBV DNA of the cloned and sequenced HBV genomes of the subtypes adr (14, 15), adw (15), adw₂ (16), adyw (17) and ayw (18). Cloning was done in bacteriophage lambda using either Hind III digested total cellular DNA or gel fractionated DNA enriched for HBV-containing Hind III fragments (Fig. 2e; 8). Four different clones are described in detail here. They were derived as follows: One each from the 4 kb (insert A-4.0; fig. 2d) and the 6 kb band (insert A-6.0; fig. 2c) and two (inserts A-10.5 and A-10.7; fig. 2a,b) from the 11 kb band. Each of them was further subcloned into plasmid pBR327 (19). Three of the four clones unexpectedly contained a Hind III site within HBV sequences. However, a mutation of two bases can generate a Hind III site at the relevant position. Therefore only part of the viral sequences and one human flank were present in these three clones.

Identification of the HBV Subtype

The genomes of the HBV subtypes adr, adw, adw₂, adyw and ayw are easily distinguishable by the presence or absence of additional bases inserted in two specific regions. They do not disturb the reading frames as they are present in a multiplicity of three. The subtype adw₂ contains six additional bases (position 2356-2361, see figure 1) which are situated within the hepatitis B core (HBc) gene and 33 additional bases (position 2858-2890) directly at the beginning of the pre-s region. In subtype adw there are also the six bp but out of the 33 bp only 12 bp are present. Subtype adr contains only the 33 bp insert, whereas ayw and adyw lack both sequences completely. The subtype of the HBV DNA in the cloned inserts is suggested to be that of

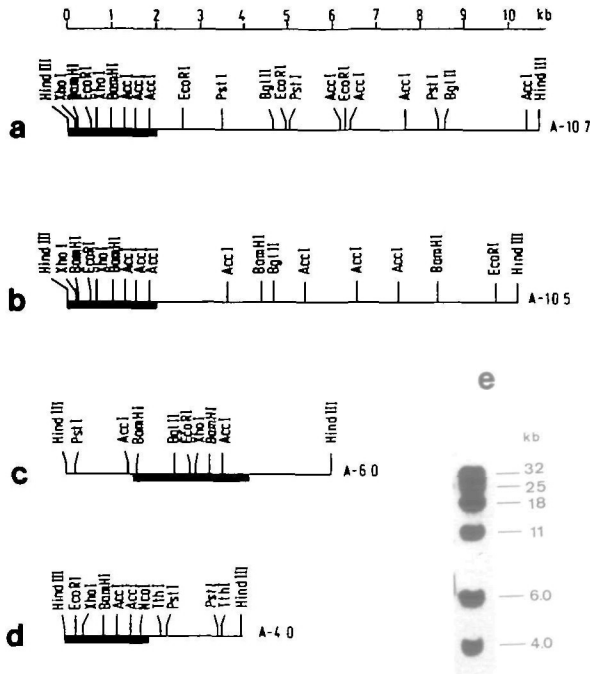


Fig. 2: Restriction maps and Southern blot analysis of integrated HBV sequences in the DNA of PLC/PRF/5 cells.

In the cloned inserts A-10.7 (a), A-10.5 (b), A-6.0 (c) and A-4.0 (d), the human flanks are drawn as thin lines and the HBV content, which was detected mainly by hybridization of restriction fragments (36), as filled boxes. Sizes are given in kilo bases (kb). Cellular PLC/PRF/5 DNA was cleaved with Hind III, electrophoresed in a 0.8% agarose₂ gel, blotted onto nitrocellulose filter and hybridized with ³²P labelled HBV DNA. The filter was exposed to x-ray film (e).

the adw₂ subtype, because the six and the 33 bp insert are detected. However, they are not present together in the same clone, since no one contains the complete viral information. The six bp are found in A-6.0 by sequencing from the Bgl II site at position 2408 (Fig. 3). The 33 bp are lost in this clone by a deletion of the relevant region. In A-10.7 and A-10.5 the viral part which contains the additional six bp has been missed due to the Hind III site within HBV DNA. However, 30 out of the 33 additional bases are seen at the expected sites in A-10.7 and A-10.5 (Fig. 4). The regions which contain the two additional sequences are not present in A-4.0 because of the viral Hind III

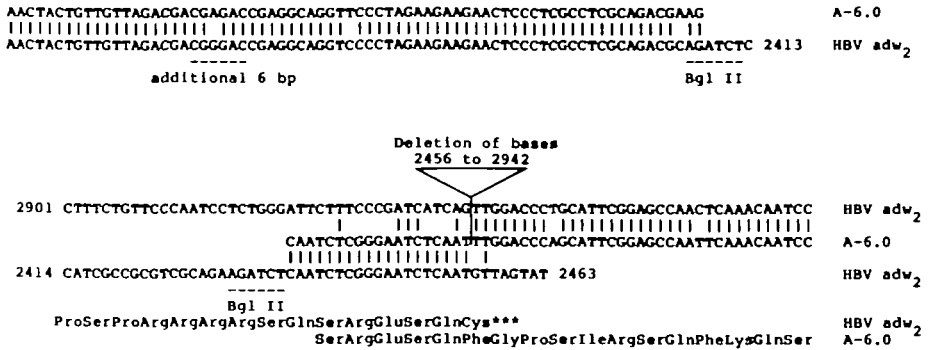


Fig. 3: Mapping the deletion in A-6.0.

The HBV DNA sequence of A-6.0 is aligned with the corresponding parts of the sequence of HBV subtype adw₂ DNA (16), to show the location of the deletion in A-6.0 which involves the bases from 2456 to 2942. The deletion removed the stop codon of the HBc gene and joins it to the pre-s region so that the reading frame of HBc is in phase with that of the P gene. The resulting amino acid sequence is shown below. The stop codon is marked by three asterisks. The two Bgl II sites, at which the DNA was labelled for nucleotide sequencing, and the additional six base pairs, only found in the subtype adw₂, are indicated. Bases in HBV DNA are numbered according to figure 1.

site and of a rearrangement. However, the comparison with the restriction maps of A-10.7 and A-10.5 suggests that this clone also contains HBV DNA of the adw₂ subtype. These data correlate with the observation that among the population of Southern Africa, from which the PLC/PRF/5 cell line was originally established, 97.7% of hepatitis B surface antigen (HBsAg)-positive sera contain HBV of the adw₂ subtype (20).

Colinearity of Integrated and Genomic Viral DNA

The HBV parts of A-10.7 and A-10.5 are identical as seen from the restriction maps (Fig. 2a,b), and are colinear with the cloned and sequenced HBV genomes. The insert A-4.0 is similar to A-10.7 and A-10.5 with respect to most of the restriction sites in the HBV part. However, there is a shorter distance between the unique viral Hind III and Eco RI sites (Fig. 2d) suggesting a rearrangement. Sequence analyses of this region clearly shows that the structure of the viral genome in A-4.0 is not as conserved as it is in A-10.7 and A-10.5, since there is an inversion (Fig. 5c, region Y) which affects a segment containing

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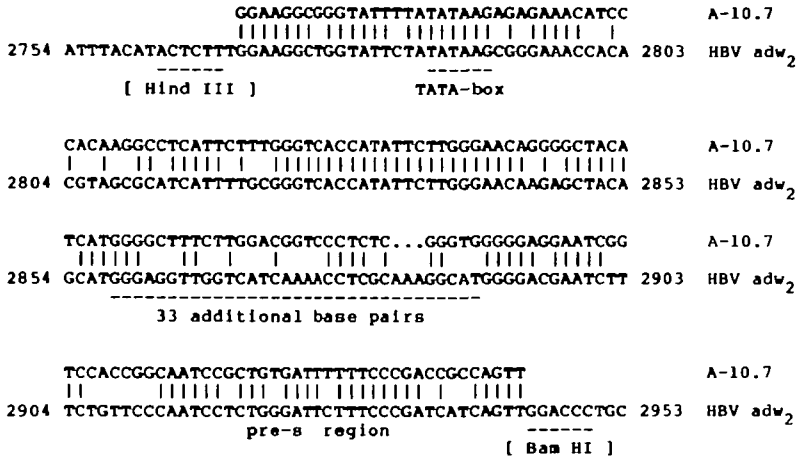


Fig. 4: The pre-s region in A-10.7 and A-10.5.

The sequence of A-10.7 aligned with the corresponding region of viral DNA of the subtype adw₂ (16) is shown. The sequence of A-10.5 is identical to that of A-10.7. In the sequence of the cloned inserts (upper string) there are 30 bases of the 33 additional base pairs only present in the HBV subtype adw₂ (lower string). The bases in HBV DNA are numbered according to figure 1. The restriction sites in brackets are found in the cloned inserts. They are not present in the published HBV sequences, but can be generated by base pair mutations.

the viral Hind III site.

The viral-human junction was sequenced rightward from an Nco I site. The DNA from this site up to the junction is seen to be composed of sequences normally situated at position 540-818 in the hepatitis B surface (HBs) gene, but present in this clone also as a duplication at the viral-cellular junction (Fig. 5c, region Z). The sequences to the left of and including the Nco I site (position 1374) are as expected from the known sequences of HBV subtypes adr, adw, adw₂, adyw and ayw. It is possible that the duplicated region was brought just next to this Nco I site. However, it is more likely that there are two Nco I sites very close to each other, since at the beginning of the duplicated part in the normal position there is a potential Nco I site which could be generated by mutation of two bases. The duplicated portion of the HBs gene mentioned above would start in the latter case between these two Nco I sites. This data

clearly shows that conclusions about integration sites cannot be based on restriction mapping and hybridization results alone.

Correlation of Structural Features and Expression of Integrated Viral Genes

The mapping data suggest that all four clones (Fig. 5a,b,c) encompass the coding region of the HBs gene. Two sequences have been reported to promote transcription of the HBs gene. One consists of the TATA-box (TATAAG, position 2786-2791) 592 bases in front of the HBs gene (21-23), the other of an SV40-like late promoter (CCTACTCC, position 3168-3175) situated within the pre-s region 210 bases upstream of the HBs gene (24). A-10.7 and A-10.5 contain both of these promoters. In A-4.0 the inversion (Fig. 5c, region Y) affected the TATA-box which is therefore not present in the cloned fragment. However, the SV40-like late promoter should be untouched. As we know from mapping data A-6.0 contains a deletion of about 0.5 kb which is in the region of the HBs promoters. By sequencing from the Bgl II site (position 2432), the deletion endpoints were determined (Fig. 3, 5b). The deletion removes the TATA-box but leaves the SV40-like late promoter unaffected.

After transfection into animal cells the inserts A-10.7, A-10.5 and A-4.0 lead to expression of HBsAg, whereas A-6.0 does not (Tested with Abbott-Ausria; Freytag von Loringhoven et al., submitted). In A-4.0 transcription of the HBs gene can only start at the SV40-like late promoter. In addition, we were able to prove this to be the case for A-10.7 by means of S1 nuclease analysis of the mRNA (Freytag von Loringhoven et al., submitted). Since the HBV part of A-10.5 is identical to that of A-10.7, this implies that transcription starts also in this clone at the SV40-like late promoter. All our data support the finding of Cattaneo et al. (24). The lack of HBsAg expression in A-6.0 may be due to a mutation in the SV40-like late promoter or in the gene itself, or to the absence of an appropriate polyadenylation signal. Since in all four cloned inserts the viral polyadenylation site is disconnected, the signal must be provided by the human sequences.

Out of the four clones only A-6.0 encompasses the HBc coding region. In the other clones this part of the viral genome was

lost due to the internal Hind III site. Sequencing and mapping data suggest that the HBc gene in A-6.0 is intact with the exception of a deletion of the stop codon. This deletion joins the HBc gene with the pre-s region. In this part of the HBV genome the two reading frames of the pre-s and the P region overlap each other (Fig. 1). The deletion is in a way that the reading frame of HBc is in phase with that of the P region (Fig. 3, 5b) which codes for a putative polymerase (16). However, although the putative HBc promoter (TACATAAG, position 1653-1660, 249 bp in front of the HBc gene; 16, 23) should be present in the cloned DNA as deduced from restriction mapping, no expression of HBcAg is detected after transfection of A-6.0 DNA into animal cells (Tested with Abbott-HBe; unpublished observations). There are many explanations for this phenomenon. One of them is that HBc expression in A-6.0 is present but not detected because the putative long fusion protein does not react well with anti-HBe used in the radio immune assay. However, the most trivial one is a mutation within the gene. Other studies suggest that HBc expression is somehow regulated by methylation (25) and that expression can be induced by cultivating PLC/PRF/5 cells in nude mice (26). Gough (22) gives evidence that in the unintegrated HBV DNA molecule a transcript which starts at one of the HBs promoters and includes the HBc gene gives rise to HBc expression. In A-6.0 HBV DNA is integrated in such a way that

Fig. 5: Structure of HBV DNA and the human sequences close to the junction in the cloned inserts A-10.7, A-10.5 (a), A-6.0 (b) and A-4.0 (c).

The HBV genome of the subtype adw₂ (16) is aligned to the viral sequences in the cloned inserts. Filled boxes indicate HBV DNA and thin lines human sequences. Spotted boxes represent homologies between the human parts of A-10.7 and A-10.5. Inverted and duplicated viral parts are drawn as hatched arrows. The filled arrowheads show the direction of sequencing. Reading frames are drawn as open arrows. They are dotted, if they presumably extend further into the unsequenced human regions. Reading frames marked P', X' and HBs' are in phase with those of the P, X or HBs coding regions. Polyadenylation sites are drawn as crosses, promoters (the putative HBc promoter and the HBs TATA-box and the SV40-like promoters) as open arrowheads. The restriction sites in brackets are found in the HBV subtype ayw (18), e.g. (Bam HI), or can easily be generated by base pair mutations, e.g. [Hind III]. Sizes of the enlarged parts are given in base pairs.

transcription starting from an HBs promotor cannot lead to a mRNA which contains the HBc gene.

In all four clones analyzed integration is such that the polyadenylation site necessary for the processing of HBs transcripts is disconnected. Therefore the mRNA must stop at a human site and consequently contain viral as well as human information. Computer analyses of the relevant human sequences extending from the junction to the first Eco RI site in A-10.7 reveal the presence of polyadenylation sites (AATAAA) which are 222 bp and 433 bp from the viral-human junction (Fig. 5a). In A-4.0 there is a polyadenylation site 273 bp from the junction (Fig. 5c). In A-10.5 (Fig. 5a) and A-6.0 (Fig. 5b) no such signals are present in the sequenced regions. In the case of A-10.7, the only insert studied for transcription products, HBV-related mRNA is indeed composed of viral and human sequences (Freytag von Loringhoven et al., submitted).

In all cases viral reading frames extend into human sequences. They could yield fusion-proteins composed mainly of viral information. In A-10.7, A-10.5 (Fig. 5a) and A-6.0 (Fig. 5b) the reading frames are those of the putative P and X proteins (16). P is thought to code for a DNA-polymerase, whereas the X product is possibly the protein covalently linked to the 5' end of the long strand in the virion DNA (27). In the clones the amount of C-terminal amino acids encoded by human DNA varies from 5 to 34 amino acids.

In A-10.7 there is a reading frame of exclusively human information (Fig. 5a). By S1-mapping of the mRNA, which was isolated from mouse Ltk cells transfected with A-10.7 (Freytag von Loringhoven et al., submitted), it was shown that this reading frame is transcribed, since many transcripts reach the second polyadenylation signal (Fig. 5a, nucleotide 621). In A-10.5 (Fig. 5a) and A-6.0 (Fig. 5b) the beginning of putative human reading frames are detected which presumably end somewhere in the unsequenced part of the human DNA region.

In A-4.0 (Fig. 5c) two reading frames begin with start codons within the P and HBs gene. The resultant proteins would have 12 or 10 C-terminal amino acids coded by human DNA. A third reading frame lies completely within HBV DNA and is normally

found in the cloned and sequenced HBV genomes. The P reading frame left of the Nco I site at position 1374 is in phase with one of these three, unless there is a stop codon between the two Nco I sites mentioned above.

Integration Sites and Human Flanking Sequences

The restriction maps of the HBV sequences in A-10.7 and A-10.5 are identical suggesting that integration of the viral DNA might have taken place in the same region of the HBV genome. To analyze this in more detail the two viral-human junctions were sequenced starting from the third Acc I site (Fig. 5a). It is apparent that in both cases the integration sites are precisely the same and further that human sequences of 187 bp flanking these junctions are identical with the exception of three bases. This is unexpected because the human parts of A-10.7 and A-10.5 are totally different in their restriction maps. This phenomenon is discussed elsewhere (28) with respect to amplification and rearrangement of cellular DNA containing integrated HBV sequences.

A comparison between the sequences of the different junction sites does not reveal integration at specific human sequences (Fig. 6). All clones, however, have in common the fact that at least one of the sites of integration are located in the "gap" region of the viral genome which consists of a large single-stranded part in more than 99% (29) of the unintegrated circular HBV DNA molecules (Fig. 1). Setting the single Eco RI site as 0 the gap extends variably between position 140 and 1690 (29). The integration sites are at position 1614/1615 in A-10.7 and A-10.5, at position 818/819 in A-4.0 and at 1408/1409 and 1417/1418 in A-6.0 (with a concomitant deletion of bases 1409 to 1417). This numbering incorporates a seven base correction from our previously reported integration site for A-6.0 (8).

In order to find some features which can contribute to recombination and integration of the viral DNA into human sequences, the areas of the junctions were searched for any similarities. In A-6.0 the presence of inverted repeats of "AAA" and "TTT" on each side of the integrated HBV DNA is interesting (Fig. 6). Since in the other clones one human flank each is lost due to the Hind III site within HBV DNA, we cannot tell whether



Fig. 6: Nucleotide sequences of the junctions.

The viral-human junctions are indicated by arrows. In A-6.0 the inverted repeats "AAA" and "TTT" on both sides of the integrated HBV DNA are indicated and the nine base pairs missing at the junction site are underlined. The bases in HBV DNA are numbered according to figure 1.

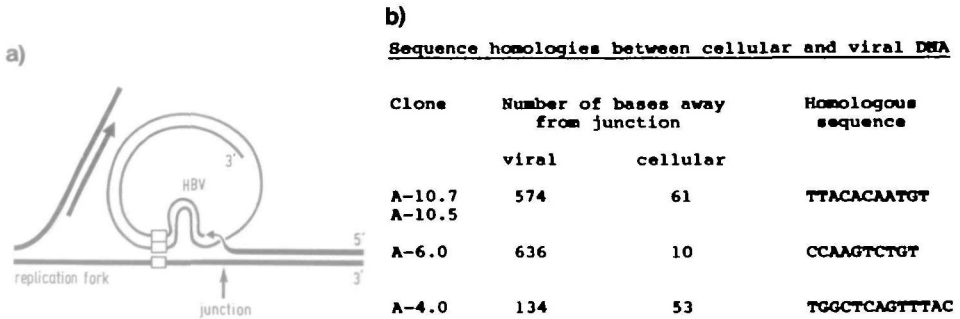


Fig. 7: Model for the mechanism of integration of HBV DNA.

During replication of cellular DNA, a DNA polymerase switches to the single-stranded region of the HBV DNA molecule. Followed by recombination, this leads to integration of viral DNA. Sequence homologies between cellular and viral DNA in the neighbourhood of the integration sites which are indicated by open boxes might determine the positioning of the viral DNA upon cellular DNA. Recombination and integration into the cellular DNA then takes place (a). Sequences homologous between human and HBV DNA are indicated for each clone. Their respective distances from the point of integration is also given (b).

there also exist comparable sequences. Large homologies close to the viral-human junction which have been reported in recombination of SV40 DNA with cellular sequences (30, 31) are not found at the integration sites of the clones. To investigate whether there are larger homologies in distal regions of the different human flanks of each clone or of these flanks and HBV DNA, the corresponding sequences were compared by computer. This analysis revealed homologies between the human flanks and HBV DNA close to the relevant junction sites to the "left" of the junction as seen in figure 7a. Their size range varies from 10 to 13 bp and their distances from the junctions are from 10 to 61 bp in the human DNA and from 131 to 636 bp in the viral DNA (Fig. 7b). To obtain an estimation of the frequency of such homologies, they were compared with the nucleic acid sequence database of the National Biomedical Research Foundation (Georgetown University, Washington, D.C.). In this database, which contains 1.3 million bases, the homologous strings were found without any mismatch only in HBV sequences.

DISCUSSION

Four cloned fragments of PLC/PRF/5 DNA containing HBV sequences have been analyzed. In contrast to integrated woodchuck hepatitis virus DNA (32), rearrangements in integrated HBV DNA are less complex. Two of the integrated copies (in A-10.7 and A-10.5) show no obvious rearrangements. There is a deletion in A-6.0 and an inversion and a duplication in A-4.0. The viral information is relatively stably conserved as is also shown by the ability of three of the four clones to direct expression of HBsAg.

As we have previously discussed (8) the gap may play a functional role in integration. We suggested that the circular viral DNA molecule interacts with cellular replication processes. DNA polymerase using the cellular DNA as template switches to the single-stranded part of the HBV genome and thus joins human and viral DNA. Followed by recombination this leads to the integration of viral DNA (Fig. 7a). The hypothesis would predict that integration of HBV is favoured in actively replicating liver cells. It is known that about 95% of very

young children infected with HBV become carriers. In adults the rate of carriers and the risk of developing PLC is significantly lower (2). This fact strengthens the hypothesis presented for integration coupled with replication.

Computer analyses revealed homologies between viral and human sequences "left" of the junction. These homologies are not directly involved in recombination and integration, but they may serve by means of base pairing to position the viral DNA on the cellular DNA and therefore facilitate recombination. The homologous strings are suggested to be present only in a limited number in the human genome since they were not found by comparison with a nucleic sequence data base of 1.3 million bases. Therefore integration would not be totally random, if such homologies would direct the integration event close to the corresponding regions in the human genome. Since similar homologies have also been found in other integrated viruses, e.g. adenoviruses (33, 34), the positioning of viral DNA distant from the point of integration could be a general feature of the integration of animal DNA viruses.

In all four clones integration is such that transcription has to stop at a human termination signal. Reading frames in human sequences as well as those which should yield viral-human fusion-proteins are found by nucleotide sequence analyses. In the case of A-10.7 a chimeric mRNA transcript has been detected (A. Freytag von Loringhoven et al., submitted). It will be interesting to determine whether these hypothetical proteins are expressed. The strong and constitutive HBs promotor (35) might alter or stimulate expression of adjacent cellular sequences, contributing thereby to the transformed state of the cell.

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