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Amplification and rearrangement in hepatoma cell DNA associated with integrated hepatitis B virus DNA

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DNA of hepatitis B virus is found to be integrated into the genome of infected human liver cells and may be related to the development of primary liver carcinoma. We have previously reported the cloning of cellular DNA with integrated HBV sequences from the PLC/PRF/5 cell line which derives from a human primary liver carcinoma. Two clones, designated as A-10.7 and A-10.5, and a third uncloned fragment are compared by restriction enzyme mapping, hybridization and nucleotide sequencing. The results indicate that amplification of integrated viral DNA and host flanking regions has occurred, followed by transposition and/or major deletions. The implications of these findings for the development of primary liver carcinoma are discussed.

Key words: amplification/hepatoma cell line/integrated hepatitis B virus DNA/rearrangement/transposition

Introduction

Hepatitis B virus (HBV) is considered to be aetiologically related to the development of primary liver carcinoma (Szmuness *et al.*, 1978; Beasley *et al.*, 1981). HBV DNA is found integrated in the DNA of infected human liver cells, in acute and chronic hepatitis (Brechot *et al.*, 1981), cirrhosis (Koshy *et al.*, 1981), and primary liver carcinoma (PLC) (Brechot *et al.*, 1980,1981; Chakraborty *et al.*, 1980; Edman *et al.*, 1980; Koshy *et al.*, 1981). To study structure and function of integrated HBV DNA we have cloned DNA of the human PLC-derived cell line PLC/PRF/5 (Alexander *et al.*, 1976) which has several integrated copies of HBV DNA (Brechot *et al.*, 1980; Chakraborty *et al.*, 1980; Edman *et al.*, 1980; Koshy *et al.*, 1981,1983). So far three HBV DNA-containing human sequences termed A-10.7, A-10.5 and A-6.0 have been isolated. Their restriction maps have been previously published (Koshy *et al.*, 1983) Here we describe the structural properties of two of these sequences, i.e., A-10.7 and A-10.5, and a further uncloned sequence which also contains integrated HBV sequences (Figure 1b,c,d). The results provide evidence for amplification and rearrangement of sequences composed of human and viral DNA.

Results

The conclusions presented in this paper are based mainly on nucleotide sequence analyses of the two cloned inserts A-10.7 and A-10.5 (Figure 1c,d). A third HBV DNA-containing fragment of 18 kb (Figure 1a), eluted from a preparative gel of *Hind*III-digested PLC/PRF/5 DNA, is also described (Figure 1b). A comparison with the restriction maps of viral genomic DNA (Galibert *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1980) shows that in A-10.7 and A-10.5 sequences of the hepatitis B surface (HBs) gene are present. As discussed below, this is also very likely to be the case in the 18-kb fragment. The restriction maps of the HBV parts in A-10.7 and A-10.5 are identical (Koshy *et al.*, 1983). Sequencing the region at the left *Hind*III site shows that the HBV DNA extends up to this site and that in both cases the site is at exactly the same position (Figure 1c,d). The presence of a *Hind*III site within HBV sequences was unexpected as none had previously been known to be present, hence the use of this enzyme in the cloning procedure (Koshy *et al.*, 1983). Viral sequences which lie leftward of this *Hind*III site and the other human flank have not yet been recovered by cloning. By sequencing rightwards from the viral *Acc*I site next to the viral-human junction (viral DNA is drawn as filled boxes, the human flanks as open boxes) it is proven that in both cases the integration sites are precisely the same, and further that human sequences immediately flanking these junctions are identical – except for three point mutations – for at least 89 bases in the two clones (Figure 1c,d: dashed boxes; Figure 2). This was unexpected because the human parts of A-10.7 and A-10.5 differ in their restriction sites (Figure 1c,d). Furthermore, subfragments of the two clones devoid of HBV sequences do not cross-hybridize (data not shown).

To show that the two clones are faithful copies of two different human restriction fragments containing integrated HBV sequences, rather than a result of cloning artifacts, the following experiment was done. The uncloned *Hind*III-derived '11-kb DNA' fragment (Figure 1a), from which the clones A-10.7 and A-10.5 were derived, was digested with convenient enzymes, blotted onto nitrocellulose filters and hybridized with labelled cloned HBV DNA (pAO1-HBV; Cummings *et al.*, 1980). After digestion with *Eco*RI, HBV-containing fragments of 2.04 kb and 0.47 kb (as seen in A-10.7; Figure 1c), and of 9.5 kb and 0.47 kb (as seen in A-10.5; Figure 1d) were expected to hybridize to the HBV probe. The presence of a 2.04-kb band (Figure 3, lane 8) indicates the faithfulness of A-10.7. Since the 9.5-kb band is not clearly distinguishable from undigested fragments of 10.7 kb or 10.5 kb, the '11-kb DNA' was also cleaved with *Bam*HI. Restriction with this enzyme should yield HBV DNA-containing fragments of 9.7, 0.76 and 0.17 kb corresponding to A-10.7 (Figure 1c) and of 3.4, 0.76 and 0.17 kb corresponding to A-10.5 (Figure 1d). The detection of the 3.4-kb and 0.76-kb bands (Figure 3, lane 9) indicates that the restriction pattern in the uncloned PLC/PRF/5 DNA is the same as in the cloned segment. The 3.4-kb band proves the faithfulness of the A-10.5 clone. The 0.17-kb band does not hybridize. This may be due to differences between the HBV DNA used as probe and HBV sequences in the clones, as discussed elsewhere (Koshy *et al.*, 1983).

The same type of experiment reveals further a relationship

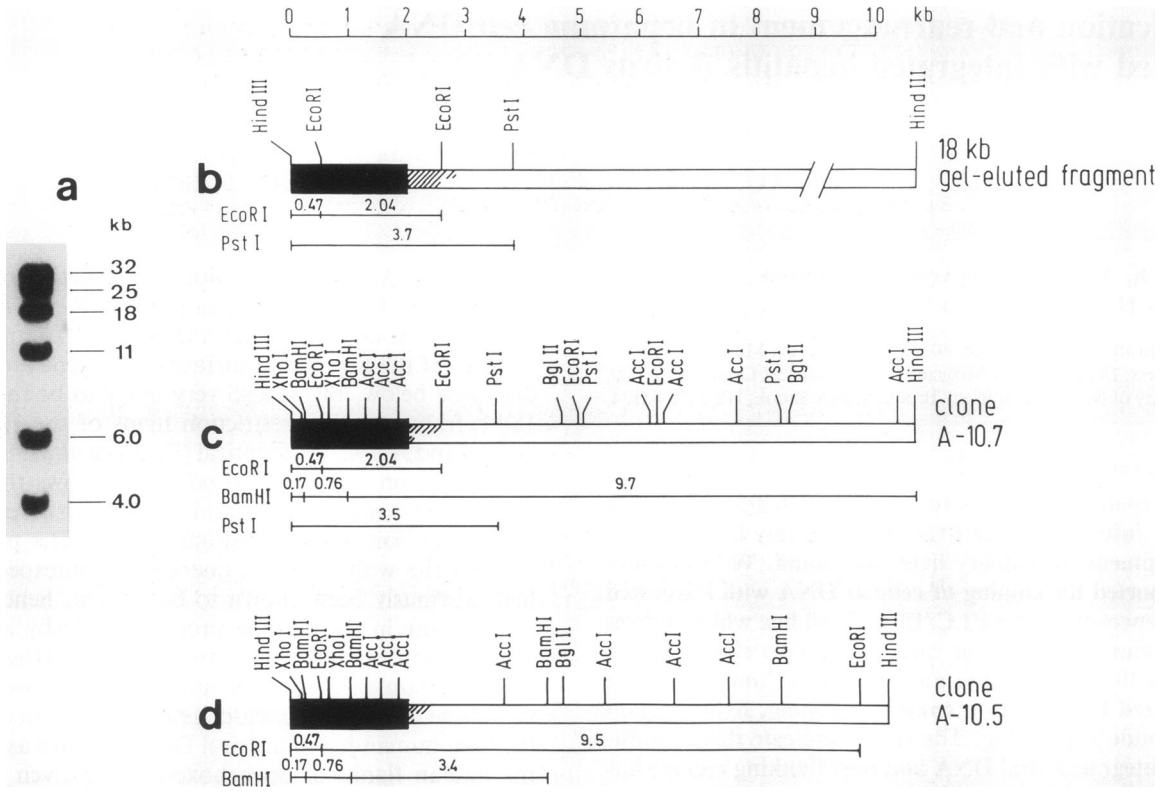

Fig. 1. Southern blot and restriction maps of integrated HBV sequences in DNA of PLC/PRF/5 cells. Cellular PLC/PRF/5 DNA was cleaved with *Hind*III, electrophoresed in a 0.8% agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labelled nick-translated HBV DNA **(a)**. In the restriction maps of the uncloned gel-eluted 18-kb fragment **(b)**, and the cloned inserts A-10.7 **(c)** and A-10.5 **(d)**, both of which were derived from the 11-kb band, the human flanks are drawn as open boxes and the HBV content as filled boxes. The dashed regions represent human sequences which are identical in the fragments. The subfragments which derive from cleavage with the indicated enzyme and which hybridize to HBV DNA are shown below the maps. Sizes are given in kb.

between A-10.7 and another human fragment with integrated HBV DNA. An HBV DNA-containing *Hind*III fragment of 18 kb (Figure 1a) eluted from a gel, cleaved with *Eco*RI, and hybridized with HBV DNA exhibits signals of 2.04 kb and 0.47 kb (Figure 1b; Figure 3, lane 7) exactly as seen in A-10.7 (Figure 1c; Figure 3, lane 8). This result indicates the presence of identical *Eco*RI sites in the 18-kb DNA as in A-10.7, and therefore strongly suggests a homology between the two that extends at least up to the first *Eco*RI site in the human flank but not up to the *Pst*I site in A-10.7, because the *Hind*III-*Pst*I subfragment of 3.5 kb seen in A-10.7 (Figure 1c; Figure 3, lane 11) is not found in the 18-kb fragment (Figure 1b; Figure 3, lane 12). Since the *Eco*RI-*Eco*RI subfragment of 2.04 kb in A-10.7 (Figure 1c) is not found in A-10.5 (Figure 1d), the homology between A-10.7 and A-10.5 does not extend as far as it does between A-10.7 and the 18-kb fragment (Figure 1b).

Discussion

Summarizing, direct nucleotide sequencing of the viral-cellular junction areas of the clones A-10.7 and A-10.5 proves that (i) integration in both cases is at the same viral nucleotide, and (ii) at least the first 86 out of 89 bases of human flanking DNA are identical. Furthermore, restriction mapping reveals identity in the extent and nature of the viral sequences. All these features clearly point to a close relationship between the two clones which have been proven to be faithful copies of the corresponding cellular sequences. A third fragment is also probably identical with respect to the viral content and human DNA at the region of the junction. Two possibilities can be considered to explain these results. First, the improbable case of three separate integration events involving the same position on the viral DNA and identical cellular sequences. Second, a situation where one of the integrated HBV sequences might have given rise to the others. We favour the interpretation that HBV DNA, after the initial integration event, has been involved in repeated amplification along with host DNA. These amplifications might have been followed either by transpositions or by extensive deletions of host DNA thus bringing sequences normally more distal closer to the junction. The deletions in such an event would have been rather large, i.e., several kb, as not only are the restriction sites very different in both flanking portions, but also there is no homology between them as tested by hybridization. Karyotype analyses of the cells, soon after they were established as a line, revealed chromosomal duplications and translocations as well as abnormalities in the chromosomal number (between 48 and 61; Alexander *et al.*, 1976). Thus, it is also possible that in one of the allelic multiple chromosomes which bear this particular integrated HBV DNA, human DNA next to HBV DNA was deleted or a segment containing viral and human DNA was excised and re-integrated at another site in the cellular DNA.

Instability of integrated viral genomes has been reported for SV40 and adenovirus (Mougneau *et al.*, 1980; Tooze, 1980; Hiscott *et al.*, 1981; Clayton and Rigby, 1981) which readily induce malignant transformation in appropriate cultured cells. It was possible in those cases to study integration events early after infection and transformation. However, with HBV no experimental systems are available and studies

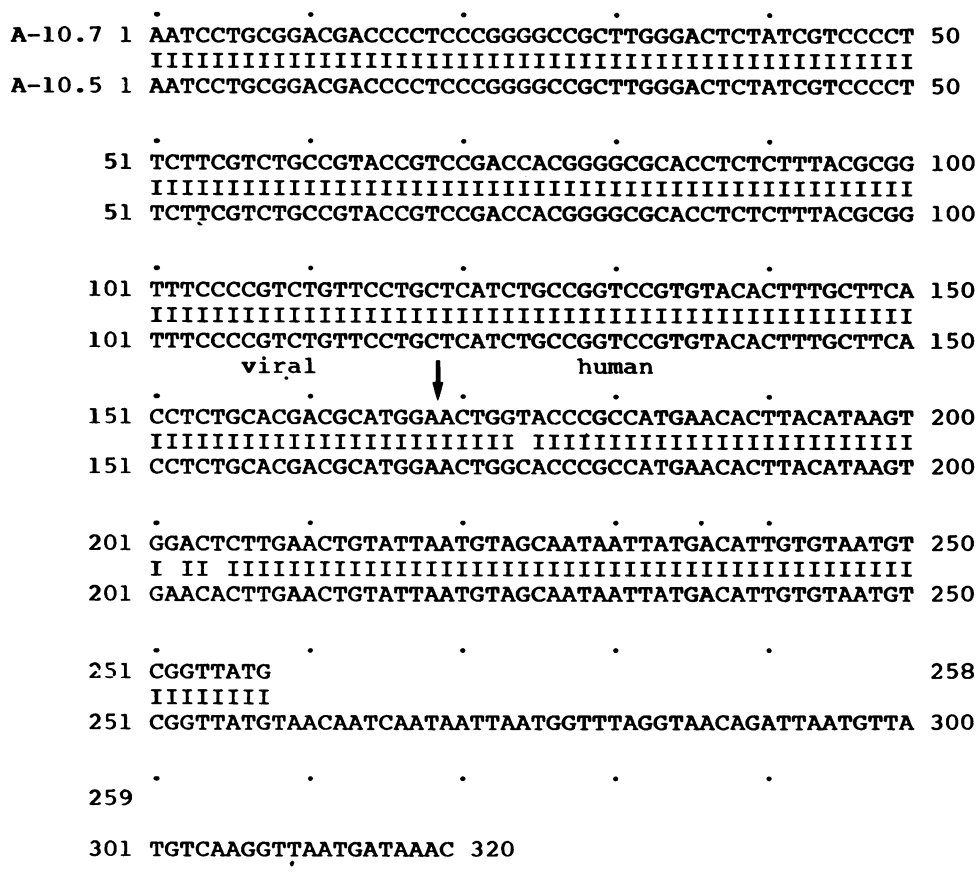

Fig. 2. Aligned nucleotide sequences of the viral-human junctions of A-10.7 and A-10.5. The junctions, which were determined by direct comparison of the sequences of A-10.7 and A-10.5 with the published sequences of HBV genomic DNA (Galibert *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1980), are identical in the two clones, and indicated by the arrow. Integration took place at nucleotide 1614/1615 of the viral genome, taking the single *Eco*RI site as position 0. The junction sites and 86 out of the 89 bases sequenced of the human parts are identical in the clones.

of this sort are only possible in natural infections of man. HBV infection can lead to early integration as observed in acute hepatitis (Brechot *et al.*, 1981), but it is extremely difficult to obtain sufficient material from such sources for the kinds of experiments discussed here. Moreover, we suggest that early integration events are not necessarily relevant to liver tumour development, for two reasons. First, only a tiny fraction of HBV-infected individuals develop tumours and second, the latency between infection and tumour development is many years. These facts are not yet explained in molecular terms. The results reported here provide possible explanations. We propose that HBV DNA, after integration into the hepatocellular genome, is involved in repeated relocations which are tolerated by the cell. The frequency of these rearrangements is likely to be a determining factor in the progression to malignancy. Thus, in a stably transformed cell line derived from a tumour, the state of the HBV sequences may reflect a situation more relevant to the transformed state than at any time early after infection. The possibility that rearrangements might have taken place subsequent to transformation cannot be ruled out. However, in the transformed cells there should be a selection for the transforming event which should be genetically stable in the cells under study. Since the first Southern blot analyses of integrated HBV sequences in PLC/PRF/5 cells, reported in 1980 (Brechot *et al.*, 1980; Chakraborty *et al.*, 1980; Edman *et al.*, 1980), similar analyses repeated several times in different laboratories have not revealed variant patterns of integrated HBV DNA (Koshy *et al.*, 1981,1983; Monjardino *et al.*, 1983). It is therefore reasonable to assume that the integrations have now been stable for at least some years.

Major rearrangements of cellular sequences are known to be specifically related to the development of some cancers. Translocations or transpositions can bring cellular *onc* genes under the influence of strong promoters (Klein, 1981) or enhancers. A limited survey by dot hybridization of cellular flanking DNA of A-10.7 and A-10.5 did not show homology to any of *myc, erb, sis, src* or *fes* v-*onc* genes. Similar experiments using PLC/PRF/5 RNA spotted on nitrocellulose membranes and probed with *src, erb, fes, myb,* K-*ras,* H-*ras, abl* and *sis v-onc* genes indicated that there was no expression of the related cellular *onc* genes. A low signal was obtained with the *myc* probe (unpublished observations). Further experiments are needed using other known *onc* probes. However, the involvement of cellular sequences yet unknown, which may be affected by HBV-induced rearrangement as described here, may play a role in transformation. Similarly, deletions can affect DNA sequences which normally suppress potential *onc* genes, thus leading to tumour formation (Benedict *et al.*, 1982; Sandberg, 1983).

Materials and methods

The cloning of A-10.7 and A-10.5 has been previously reported (Koshy *et al.*, 1983). Methods concerning extraction of cellular DNA and Southern blot

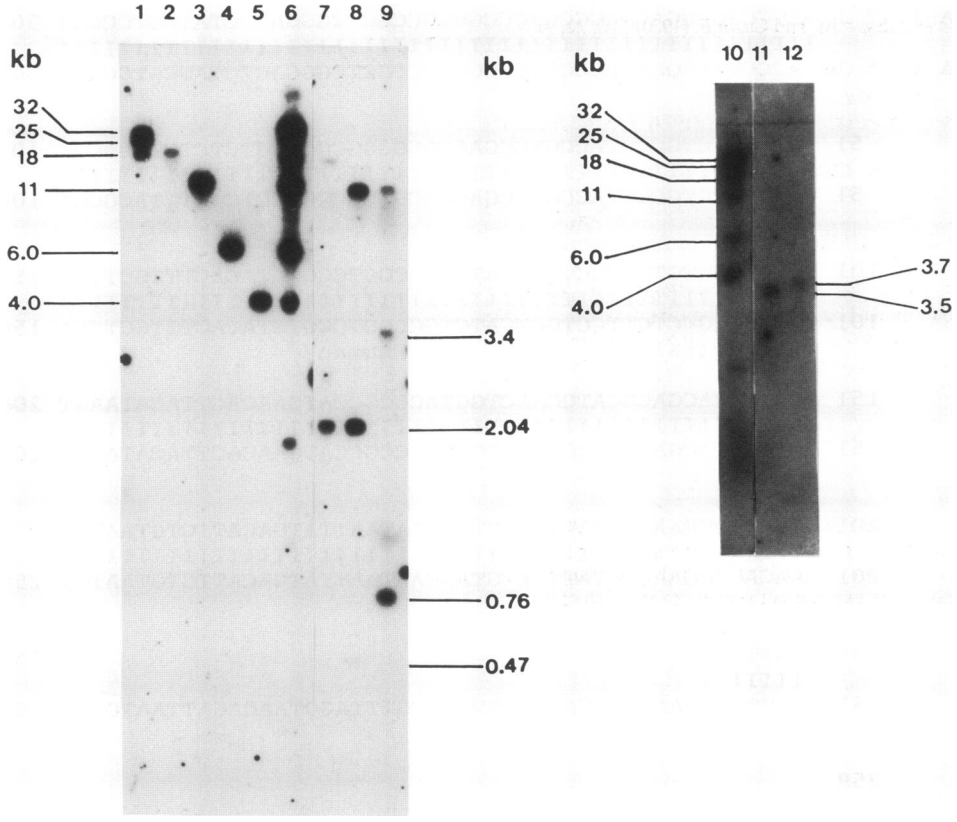

Fig. 3. Southern blot of HBV DNA-containing fragments derived from *Hind*III-digested PLC/PRF/5 DNA. HBV DNA-containing fragments, eluted from a preparative agarose gel with *Hind*III-digested PLC/PRF/5 DNA, were either loaded directly or after being digested with a second restriction enzyme on an analytical 0.8% agarose gel. As reference, *Hind*III-digested PLC/PRF/5 DNA was used. Band-sizes were determined by comparison with *Hind*III-digested bacteriophage lambda DNA and *Hae*III-digested pBR327 DNA. The gel was blotted and hybridized to ^{32}P-labelled HBV DNA probe. **Lanes 6** and **10** show PLC/PRF/5 DNA cleaved with *Hind*III. The other lanes show HBV-containing fragments of the same DNA which were eluted from a preparative agarose gel: 32 and 25 kb (accidentally contaminated with 18 kb) **(lane 1)**, 18 kb **(lane 2**, *Eco*RI-digested: **lane 7**, *Pst*I-digested: **lane 12)**, 11 kb **(lane 3**, *Eco*RI-digested: **lane 8**, *Bam*HI-digested: **lane 9**, *Pst*I-digested: **lane 11)**, 6 kb **(lane 4)** and 4 kb **(lane 5)**.

analysis are contained in the above reference.

Isolation of HBV DNA-containing fragments

*Hind*III-digested PLC/PRF/5 DNA was electrophoresed in a 0.8% preparative agarose gel from which a part was blotted onto nitrocellulose (Southern, 1975) and hybridized with ^{32}P-labelled nick-translated (Rigby *et al.*, 1977) HBV DNA (sp. act. 2 x 10^8 c.p.m./μg). The regions which correspond to the positive bands (as seen in Figure 1a) were cut out and the DNA electroeluted. This DNA was used for the restriction analyses seen in Figure 3 and also for cloning of some of these fragments.

DNA sequencing

Nucleotide sequencing was done following the method of Maxam and Gilbert (1980). The DNA was digested with *Acc*I, labelled using polynucleotide kinase and eluted from agarose gels. After base-specific chemical cleavage the samples were loaded on ultrathin (0.2 mm) 6%, 8% and 20% polyacrylamide gels (Garoff and Ansorge, 1981).

Computer analyses

Computer analyses of the DNA sequences were performed on a VAX 11/782 computer of Digital Equipment Corporation. The FORTRAN-77 programs NAQ, RELATE and ALIGN (Dayhoff *et al.*, 1981) were used for all computations.

Viral onc genes

Hybridization experiments referred to in the Discussion were done utilizing the following viral *onc* probes purified from the plasmids. The source of each is indicated in brackets: *src* (pSRA-2; Dr J.M.Bishop), *erb* (pAE-11; Dr J.M. Bishop), *fes* (pFeSV; Dr C.J.Sherr), *myb* (pHax-4; Dr M.Baluda), K-*ras* (Ki-ras; Dr R.A.Weinberg), H-*ras* (Ha-ras; Dr D.DeFeo), *abl* (pABL-Sub9; Dr J.Y.J.Wang), *sis* (pC14; Dr E.P.Gelmann), *myc* (pMCO; Dr T.Papas).

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Note added in proof

The extent of homology between the human parts of A-10.7 and A-10.5 has now been fully determined by further DNA sequencing. 187 bp, starting from the viral-human junction are identical with the exception of three bases. Beyond this point the two human sequences are different.