9. For details on the model and validation studies, and more information on the climate effects for various smoke masses, see R. C. Malone *et al.*, *J. Geophys. Res.*, in press.
10. Smoke or passive tracer is injected over the United States, Europe, and the western Soviet Union at a rate that is maximum at day 0 and decreases linearly to 0 at day 7; half the mass is injected during the first 2 days. We take the solar absorption coefficient of smoke to be 2 m^2/g (*3*). Both precipitation scavenging and gravitational sedimentation are treated (*9*). We assume a particle radius of 1 μm in calculating the gravitational fall velocity, which is about 3×10^{-4} m/sec at an altitude of 10 km (*13*).
11. In the unperturbed atmosphere this stably stratified layer is the normal stratosphere, but in the perturbed atmosphere, it is the heated smoke layer itself.
12. The 1/*e* residence time is the time required for the mass of smoke to decrease further by a factor of 1/*e* ($e \simeq 2.72$).
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Deletion in Chromosome 11p Associated with a Hepatitis B Integration Site in Hepatocellular Carcinoma

Abstract. *Hepatitis B virus (HBV), a virus with known carcinogenic potential, integrates into cellular DNA during long-term persistent infection in man. Hepatocellular carcinomas isolated from viral carriers often contain clonally propagated viral DNA integrations. As small chromosomal deletions are associated with several types of carcinomas, the occurrence of chromosomal deletions in association with HBV integration in hepatocellular carcinoma was studied. HBV integration was accompanied by a deletion of at least 13.5 kilobases of cellular sequences in a human hepatocellular carcinoma. The viral DNA integration and deletion of cellular sequences occurred on the short arm of chromosome 11 at location 11p13-11p14. The cellular sequences that were deleted at the site of HBV integration were lost from the tumor cells, leaving only a single copy of the remaining cellular allele.*

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Hepatitis B virus (HBV) and Woodchuck hepatitis virus (WHV), two of four members of the hepadna virus family (*1*), exhibit characteristics associated with oncogenic viruses. Chronic infection with either of these viruses incurs a high risk of hepatocellular carcinoma (approximately 40 percent for HBV and 90 percent for WHV) (*2–4*). The finding of viral integrations in genomic DNA prepared from primary hepatomas of chronically infected humans and woodchucks (*3, 5–7*) has suggested a possible role of these integrations in hepatocarcinogenesis. Integration can occur at variable sites for both the cellular and viral DNA's (*5, 8–13*), similar to findings observed for integrated SV40 DNA in transformed eukaryotic cell lines (*14, 15*). However, in the case of HBV and WHV, an oncogene has not yet been identified (*8–10*).

In addition to searching for common integration sites, we have investigated the possibility that HBV integration may function in hepatocellular carcinoma by causing chromosomal deletions or translocations. The present study reports that a large genomic DNA deletion is associated with an HBV integration isolated from a primary liver tumor. We also demonstrate that the deleted sequences are lost from the tumor cells, leaving only a single copy of the normal cellular allele.

DNA was obtained from tumor HL70 (*6*), a postmortem specimen of primary hepatocellular carcinoma from a chronic HBV surface antigen (HBsAg) carrier (*6*). A Southern blot of Hind III-digested HL70 DNA, hybridized with cloned HBV DNA, showed three bands containing HBV DNA sequences. Two of these integrations were cloned into the Hind III site of bacteriophage Charon 30 (*5*). The restriction endonuclease map of one of the clones, HL70-3, is shown in Fig. 1A. This Hind III fragment was 10 kilobases (kb) in length and contained a 2.8-kb segment of integrated HBV DNA. The coding sequences for the entire X gene (function unknown) and the viral core antigen gene (HBcAg) of HBV were present as integrated sequences. The location of these genes in the clone (Fig. 1A) spans the 1.2-kb HBV DNA segment that contains two Ava I sites. As complete cloned HBV genomes contain only one Eco RI site (*16*), the presence of two Eco RI sites in the integrated HBV sequence could be due to various causes, including the generation of a new Eco RI site by base mutation or a rearrangement of viral sequences resulting in duplication of the Eco RI site. The latter is more likely as a duplication would be consistent with the structure of integrated viral DNA that we and others have observed (*5, 8, 11*). The restriction map also indicates that HBsAg gene sequences are present in this clone; however, on the basis of the published restriction endonuclease map of HBV (*16*), the entire surface antigen gene is not present.

Two restriction fragments, corresponding to a portion of the left-end cellular sequence (pS8-1) and the right-end cellular sequence (HL70-3-4) of clone HL70-3 were identified and shown to be free of highly repeated DNA (they did not hybridize to a probe made from total human DNA). These sequences were subcloned into plasmid vectors. The subclones were used as probes to construct a restriction endonuclease map of cellular DNA sequences at the HBV integration site in the original tumor HL70, and cellular DNA sequences comprising the normal homologous cellular allele. The probes hybridized to two bands in the tumor DNA and one band in the normal human DNA (Fig. 1B). The band unique to tumor DNA represents the DNA that had been modified by HBV integration.

The sizes of those restriction fragments that corresponded to the normal and tumor DNA sequences hybridizing to each flanking probe and the known restriction map of clone HL70-3 were used to construct a restriction map of the normal and tumor loci (Fig. 1C). This method allowed us to map only from the probe site to the first restriction endonuclease site outside the probe in either direction for any particular enzyme. The boundaries of the deleted region represent the HBV integration site. The restriction map of the normal cellular sequences extending within the deleted region 5.5 kb to the right and 8.0 kb to the left of the HBV integration site did not show any homology to each other (Fig. 1C, bottom line). For example, the Hind

III site in the 8.0-kb region is adjacent to a Hpa I site, which is not the case in the 5.5-kb region. Since the left- and right-hand loci could not be linked by restriction mapping, they were judged to be at least 13.5 kb apart in the normal genome. This is consistent with either a major rearrangement or a deletion of cellular sequences associated with viral DNA integration. Deletions of large segments of genomic DNA up to 800 kb have been associated with viral integrations (*17*).

An alternative interpretation of these data is that a chromosomal translocation occurred during or after HBV integration. We addressed this question by hybridizing pS8-1 and HL70-3-4 to a panel of human-mouse somatic cell hybrid DNA's (Fig. 2A). The probes hybridized to Bgl II fragments of the predicted size in the human DNA sample and did not hybridize to control mouse DNA (Fig. 2A). The hybridizing bands for both probes cosegregated in all instances and were correlated only with the presence of chromosome 11 in the hybrid panel (Table 1 and Fig. 2A). No chromosome-specific isozyme marker cosegregated with either of the probes. As both flanking sequence probes were located on chromosome 11, we concluded that a deletion (with a minimum size of 13.5 kb), rather than a translocation, had occurred at the site of HBV integration in tumor HL70.

The deletion was mapped to the short arm of chromosome 11 by means of somatic cell hybrids containing different regions of chromosome 11 (Table 1). The cell hybrid XER 7 lacked a normal chromosome 11 and 70 percent of the cells retained the recombinant chromosome that included the 11p11–11qter region (*18*). Neither probe hybridized to XER 7 and must therefore represent sequences in the absent 11p11–11pter region. Hybrid EXR-5CSAz, which retains the 11q11–11qter region but not an intact chromosome 11 (*18*), also did not hybridize with the probes. These data suggested that the sequences recognized by the probes were located within the 11pter–11p11 region.

In situ cytological hybridization was conducted independently to localize the normal map position of pS8-1. The only region of the chromosome complement that consistently showed positive deviation of grain counts from a random (with grains equally distributed over the chromosome length) distribution were those in the centromeric to p-arm of chromosome 11. Grains over the p-arm of chromosome 11 were analyzed by determining the grain counts over the region just distal to the centromere on 11q, the centromere, and three areas within the p-arm. Grain density over the centromere and q-arm was low. Within the p-arm, the region of highest grain density was in the middle third of 11p or approximately bands 11p13–11p14. Figure 2B shows chromosomes after hybridization and Fig. 2C represents the G-banding patterns of chromosome 11 and the grain counts observed in each position on the chromosome. These studies localize the position of the HBV integration to the 11p13–11p14 region.

Recent work with retinoblastoma (*19*) and Wilms' tumor (*20*) has shown that the establishment of a homozygous state for a particular chromosomal region is associated with oncogenesis. The deletion data indicated that HBV integration could cause a similar homozygous condition for a particular DNA sequence. However, the deleted region could have been autonomously replicated in the form of a double minute, as observed in other solid tumors (*21*), or translocated to another location in the genome. In order to distinguish among these possibilities, we cloned cellular sequences homologous to the deleted region and used sequences from the clone HL-70-3 del. as a probe to determine the copy number of the deleted region DNA in the original tumor. A recombinant phage with an 8.5-kb Hind III fragment comprising part of the deleted region (HL70-3 del.) was isolated. The restriction map (Fig. 3A) was consistent with the original map for the deleted region (Fig. 1C).

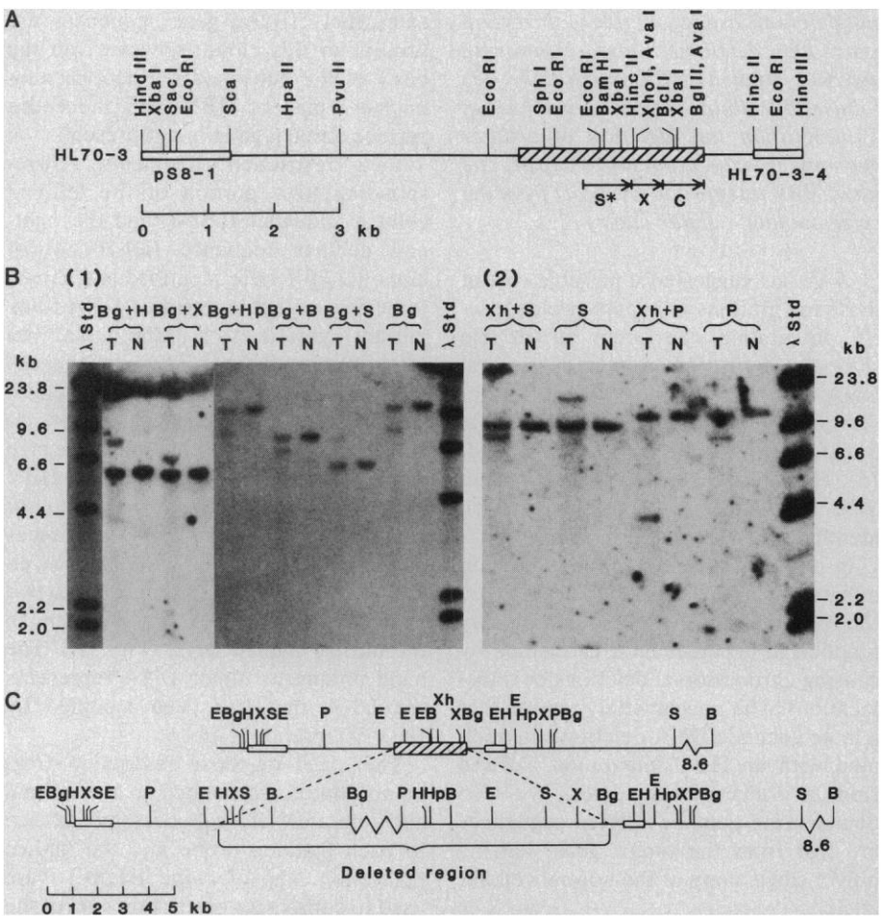

Fig. 1. Analysis of integrated sequences. (A) Restriction endonuclease map of clone HL70-3. Hatched box, integrated HBV sequences; solid line, cellular sequences; open box, single-copy genomic sequences subcloned into pBR322 and used to map normal and tumor loci; S*, incomplete HBsAg gene; X, X-gene open reading frame; C, HBcAg gene. Probe pS8-1 consisted of a 5-kb Sca I fragment from HL70-3, which contained 1.5-kb of human cellular sequences and 3.5-kb of the left arm of λ Charon 30, cloned in the Sca I site of pBR322. Probe HL70-3-4 consisted of a 0.8-kb Hind III–Hinc II human cellular fragment ligated into the Hind III site of plasmid pBH20 (*16*). (B) Southern blot analysis of tumor HL70 (T) and normal human (N) DNA's digested with restriction endonucleases and hybridized with flanking sequence probes pS8-1 and HL70-3-4 (*5*). Bg, Bgl I; H, Hind III; X, Xba I; B, Bam HI; S, Sac I; Xh, Xho I; P, Pvu II; Hp, Hpa I; E, Eco RI. (C) Restriction endonuclease maps of the normal (bottom line) and tumor (top line) loci mapped by Southern blot analysis with pS8-1 and HL70-3-4. Solid line, cellular sequence; hatched box, HBV sequences; open box, regions corresponding to the flanking probes used for mapping the cellular loci; jagged line, regions not drawn to scale; 8.6, size in kilobases. Restriction endonuclease abbreviations are as in (B).

A 2.0-kb Hind III–Xba I restriction fragment (Fig. 3A) lacked repeated sequences and was used as a probe to search for sequences homologous to the deleted region in tumor HL70. Restriction enzymes were chosen (Hind III, Pvu II, and Hpa I) that cut cellular sequences to the right of the HBV integration site and outside the probe (Figs. 1C and 3A). Bam HI, which cuts in the middle of the probe sequences, and Xba I, which cuts at the right end of the probe, were also used. These digests allowed us to distinguish the normal DNA sequences from any modified DNA sequences present in the tumor. If the deleted cellular sequences still remained in the cell, we expected to see a normal band plus a second band in the Southern blot of HL70 tumor DNA. The only bands observed in five separate restriction digests were those corresponding in size to the normal DNA sequences (Fig. 3, B and C). The sizes of the fragments were also the same as those predicted from our previous mapping data with probe HL70-3-4 (Fig. 1C). Two normal bands were observed in the Bam HI digestion, be-

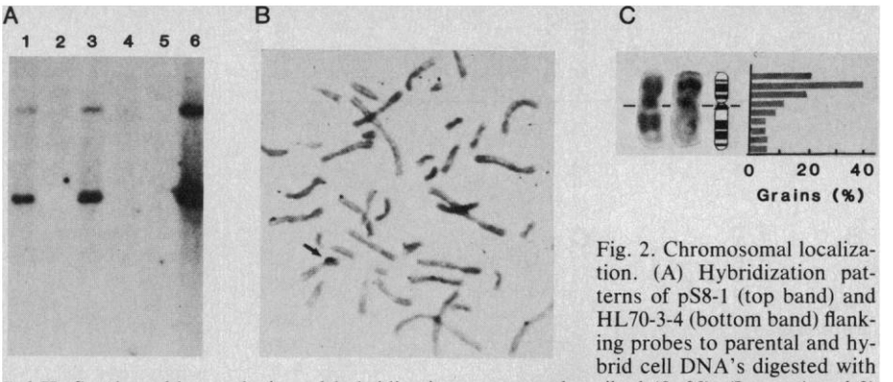

Fig. 2. Chromosomal localization. (A) Hybridization patterns of pS8-1 (top band) and HL70-3-4 (bottom band) flanking probes to parental and hybrid cell DNA's digested with Bgl II. Southern blot analysis and hybridizations were as described (*5, 29*). (Lanes 1 and 3) Hybrid cell line containing chromosome 11, (lanes 2 and 4) hybrid cell line lacking chromosome 11, (lane 5) mouse DNA, (lane 6) human DNA. (B) A portion of a metaphase plate after cytological hybridization, showing silver grains over 11p13 (arrow). (C) Grain distribution over chromosome 11 regions as delineated (left to right) by a G-banded chromosome 11, chromosome 11 after hybridization, and a diagrammatic representation of a G-banded chromosome 11. Analysis of grain distribution was as previously described (*30, 31*). Grain counts were made by region over identified chromosomes of 62 metaphase plates. The total number of grains over chromosome 11 in 54 metaphase plates in which chromosome 11 was analyzed in detail was 89. The χ^2 value (1 d.f.) over the general region of 11p relative to a random grain distribution in that region was greater than 50. Methods used for cytological hybridization have been described previously (*30*). Metaphase preparations from peripheral blood lymphocytes were G-banded prior to hybridization. Chromosomal DNA was denatured in 95 percent formamide in standard saline citrate (SSC) for 1.5 hours at 72°C. Hybridization was in 50 percent formamide in 3× SSC for 20 hours at 42°C. Autoradiographic exposure was for 21 days. The DNA probe was nick-translated with ^{125}I-labeled deoxycytidine triphosphate to a calculated specific activity of 8×10^8 dpm/μg and prepared for hybridization at a concentration of 100 ng/ml as described (*31*).

Table 1. Distribution of cellular flanking sequences (pS8-1 and HL70-3-4) in human-mouse cell hybrids. The human-mouse cell hybrid panel was derived from independent sets of hybrids constructed with four mouse cell lines and 12 human fibroblast lines (*32*). Chromosomes of hybrid cells were karyotyped and banded by Giemsa-trypsin staining (*33*). Enzyme markers assigned to each chromosome except Y have been tested on each cell hybrid (*34*) to confirm the chromosome analysis. Chromosomes, enzymes, and the probes were tested on the same cell passage for each hybrid. Most hybrids were derived from karyotypically normal human parental cells. The others were derived from parental cells with well-defined translocation chromosomes for regional chromosome mapping. Both right- and left-hand cellular sequences were tested simultaneously for each cell hybrid. The hybridizing bands cosegregated in each cell hybrid.

Cell hybrids	Probe*	Human chromosomes																						Translocations
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
WIL-7	+	−	+	+	−	+	+	−	+	−	+	+	−	+	+	−	−	+	+	−	−	+	−	+	
WIL-8X	+	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	
WIL-13	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	−	
WIL-15	+	−	+	+	+	−	−	+	−	−	+	+	+	+	+	−	−	+	+	−	+	+	−	+	
REW-7	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	
REW-8D	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	+	+	+	+	
REW-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	
REW-11	+	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	+	−	−	−	+	+	−	+	
REX11B-SAgB	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−	
REX11BSHF	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	22/X
TSL-1	+	−	−	+	+	−	−	−	−	−	+	+	−	+	+	−	+	+	+	−	+	−	−	−	
SIR-8	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	
DUM-13	+	+	+	+	−	−	+	+	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	−	X/15, 15/X
DUA-3BSAgA	−	−	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	
DUA-5BSAgA	+	−	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−	+	+	−	−	+	−	−	
ATR-13	−	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	5/X
1CL-15CSBF	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−	
JSR-29	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
JWR-22H	+	−	−	−	+	−	+	−	−	−	+	+	−	−	+	+	−	+	+	−	+	+	−	−	2/1
HSL-9	−	−	−	−	−	+	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	+	+	−	17/9
XTR-22	+	−	+	−	+	+	+	−	+	−	+	+	−	−	−	+	−	−	+	+	+	+	+	−	X/3
XER-7	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+	−	−	−	+	11/X
EXR-5CSAz	−	+	−	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+	−	X/11
% Discordancy		48	30	30	30	43	39	43	52	56	35	0	43	43	43	48	43	35	30	43	30	26	56	35	

*Both right (pS8-1) and left (HL70-3-4) cellular sequences were used as probes simultaneously for each cell hybrid. The hybridizing bands cosegregated in each cell hybrid.

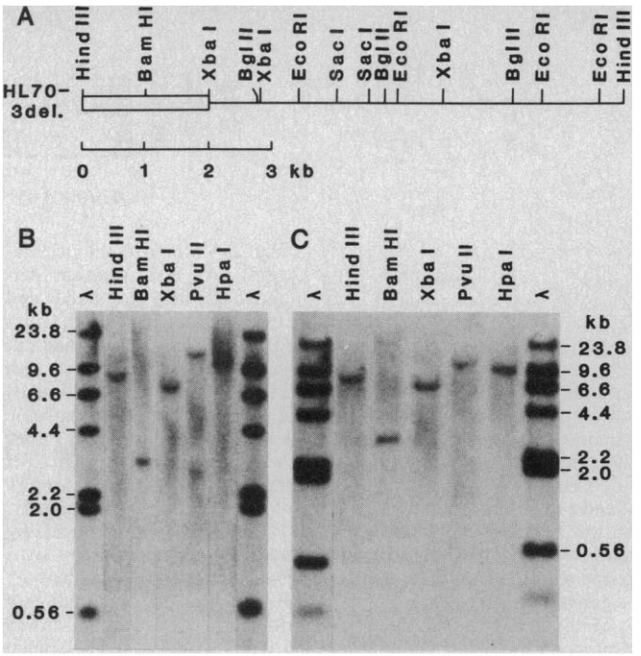

Fig. 3. (A). Restriction endonuclease map of clone HL70-3 del. containing cellular sequences from the deleted region mapped in Fig. 1C. Restriction endonuclease abbreviations are as in Fig. 1B. Southern blots of tumor HL70 DNA (B) and normal human white blood cell DNA (C) digested with various enzymes and hybridized with the ^{32}P-labeled 2.0-kb Hind III–Xba I DNA fragment from the deleted region clone, HL70-3 del. Hybridizations were as in Fig. 1.

cause the probe was cut by Bam HI. The larger band was >23 kb and appeared very faintly on the Southern blot. This band corresponded to the same fragment that had hybridized with probe HL70-3-4. Thus, the deleted region was lost from the tumor cells, and only a single copy of the normal cellular sequences remained.

The most common defects observed in leukemias and lymphomas are specific chromosomal translocations (*22*). This is in contrast to studies with carcinomas, in which deletions of specific bands or segments of chromosomes are the most common chromosomal abnormality (*22*). The significance of the deletion we have observed at 11p13–11p14 in hepatocellular carcinoma is unknown. However, the loss of heterozygosity of DNA sequences in chromosome 11p has recently been specifically correlated with the occurrence of hepatoblastoma, rhabdomyosarcoma, and adrenal carcinoma in patients with Beckwith-Weidemann syndrome (*23*). Also, in the case of Wilms' tumor, individuals with a constitutional deletion at 11p13–11p14 in one chromosome have a strong predisposition to the aniridia-Wilms' tumor syndrome (*20*). The deletion itself is not sufficient for tumor development, because only a minority of individuals (40 percent) with aniridia and the chromosomal deletion actually develop tumors. This strongly implies that a second event, either at the same or at a distant locus, is required for neoplasia to occur. Our data for 11p13–11p14 in human hepatocellular carcinoma HL70 show that one normal allele still remains in the tumor cells. Therefore, a second event would presumably be necessary if this locus were to participate in oncogenesis, analogously to the Wilms' tumor model. In any event, this individual having incurred an HBV-induced deletion was predisposed to effects of mutations at this locus.

Integrated HBV sequences and flanking cellular sequences are amplified and transposed after the initial HBV integration event in the PLC/PRF/5 cell line (*24, 9*). There is evidence for rearrangement of cellular sequences at another HBV integration site in the PLC/PRF/5 cell line (*11*), and an inverted duplication of viral and cellular sequences is present in a cloned integration from the huSP cell line (*10*). We have also identified and characterized a chromosomal translocation at an HBV integration site in a primary hepatocellular carcinoma (*25*). Therefore, the deletion reported here appears to be one example of a general phenomenon involving many types of chromosomal rearrangements associated with HBV integration.

Oncogenic viruses in general cause a variety of chromosomal aberrations (*26*), and deletions at viral integration sites are not unique to HBV. Both SV40 and Epstein-Barr virus DNA integrations cause deletions of up to 15 kb of cellular DNA in transformed cells (*27, 28*). Even though the function of these deletions is currently unknown, the high correlation between chromosomal abnormalities and carcinogenesis supports the idea that deletions may be part of a multistep mechanism leading to cell transformation. Therefore, the ability of HBV integrations to mediate deletions and other chromosomal aberrations may be related to the oncogenic potential of this virus.

Note added in proof: Analysis of (i) additional human-hamster somatic cell hybrid DNA's containing defined deletions in human chromosome 11p and (ii) hybrid DNA's containing chromosome 11 from Wilms' tumor patients with constitutional deletions spanning 11p13 are consistent with our localization of the HL70-3 deletion to 11p13.

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