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## Detection and Characterization of Subgenomic RNAs in Hepatitis A Virus Particles

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Defective viral particles containing deleted genomes were detected in harvests of cell cultures infected with various HAV isolates. The most prominent deletions were identified within the region of the genome coding for structural proteins. In this location three different deletions spanning nts 930-4380 (A), 1140-3820 (B), and 1370-3240 (C) were characterized. In addition to these internal deletions, various truncated RNAs were detected lacking either partially or completely the 3' terminal region which is supposed to code for viral replicase. RNA molecules with internal deletions as well as those with 3' terminal truncations could also be extracted directly from infected cells. During multiple consecutive passages of a given HAV strain, deletions A, B, and C accumulated and a quantitative increase of deleted RNAs occurred. Type and predominance of deletions varied with virus strains (CLF, GBM, MBB11/5, HM175, CR326, H141) and with the type of cells used for propagation (MRC-5, BGM, HELF, PLC/PRF/5). However, within the limits of the reliability of S1 analysis the endpoints of deletions A, B, and C were conserved. The mechanisms leading to formation of deletions remain unclear. Yet, some sequences flanking internal deletions showed homology with common splice signals and 3' terminal truncations proved to be confined to a distinct region within the genome. © 1988

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### INTRODUCTION

Hepatitis A virus is a small spherical virus with a genome of single-stranded linear RNA. It has recently been classified among the picornaviruses (Melnick, 1982; Gust *et al.*, 1983). In contrast to the situation with well-known members of the Picornavirus family (e.g., poliovirus), replication of HAV in cell culture is protracted and in most cell culture systems replication of wild-type (wt)-virus terminates in persistent rather than in acute lytic infection (Provost and Hilleman, 1979; Frösner *et al.*, 1979; Gauss-Müller and Deinhardt, 1984; Siegl *et al.*, 1984; Bradley *et al.*, 1984; Wang *et al.*, 1985; Friedmann-Alvermann *et al.*, 1985).

Observations with various viruses provided evidence that persistent infection can be brought about by the occurrence of defective interfering (DI) particles (Huang and Baltimore, 1970; Popescu and Lehmann-Grube, 1977; Spandidos and Graham, 1976; Holland *et al.*, 1980). These particles are known to accumulate in the course of repeated *in vitro* passages of the respective virus at high multiplicity of infection. DI particles usually contain a normal capsid, whereas their genome is incomplete. Deletions may concern all major parts of the genome, yet, are frequently located in the region coding for structural proteins, whereas signals required for packaging of deleted genomes are usually unaffected. Therefore, formation of DI particles depends on synthesis of viral structural proteins and/or essential nonstructural functions directed by com-

plete (standard) viral genomes present in the infected cell. This dependence on functions of standard virus is thought to form the basis for interference of DI particles with standard virus.

Except for a few recent examples, (Venuti *et al.*, 1985; Anderson *et al.*, 1987; Cromeans *et al.*, 1987), adaptation of wt-HAV to cell culture always leads to persistent infection. Consequently, if DI particles play a role in the development of the noncytolytic HAV/cell relationship, they should be demonstrable in all these virus/cell systems. To investigate into this hypothesis, six different HAV isolates produced in four different cell lines were analyzed for the presence of particles with defective genomes. In each instance defective particles evidently were generated both at low passage level and after multiple consecutive passages of the virus. Using fragments of HAV cDNA and of HAV-specific RNA transcribed for hybridization and S1-mapping, deletions were detected in the putative capsid region (P1) and in the region of the viral genome coding for viral replicase (P3).

### MATERIAL AND METHODS

#### Viruses and cells

HAV strain CLF (originating from an isolated case of hepatitis A in Switzerland) (Siegl *et al.*, 1984), GBM (Germany) (Frösner *et al.*, 1977), MBB11/5 (North Africa) (Frösner *et al.*, 1979), H141 (India) (Widell *et al.*, 1986), CR326 (Costa Rica) (Provost and Hilleman, 1979), and HM175 (Australia) (Daemer *et al.*, 1981)

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were propagated in BGM (buffalo green monkey), MRC-5 (human diploid lung fibroblast), HELF (human embryonic lung fibroblast), or PLC/PRF/5 (human hepatoma-derived) cells. Serial 7-day passages of strain CLF were performed in confluent MRC-5 or BGM cells at a multiplicity of infection (m.o.i.) between 0.1 and 1 TCID<sub>50</sub>/cell at 32°. Large-scale production of viruses was accomplished in Nunc cell factories infected at m.o.i. 10<sup>-3</sup> to 10<sup>-4</sup> (Siegl *et al.*, 1984).

### Isolation and purification of virus

Virus was extracted from infected cells by three cycles of freezing and thawing, concentrated by precipitation with PEG 6000, and purified by treatment with DNase (10 µg/ml) and RNase (20 µg/ml), trypsin (0.5 mg/ml), 1% SDS, as well as by banding in CsCl/sucrose gradients (Siegl *et al.*, 1984). Pure virus particles were obtained by velocity sedimentation through additional sucrose gradients (Siegl and Frösner, 1978).

For rapid analysis of particles in viral harvests, extracts prepared by repeated freezing and thawing in 0.1% Triton X-100 were optionally treated with DNase/RNase and cleared by low-speed centrifugation, and virus was pelleted by ultracentrifugation (SW 50.1 rotor, 35,000 rpm, 4°, 14 hr).

### Extraction and characterization of RNA

Virus particles were digested with proteinase K (20 µg/ml) for 2 hr at 37° and RNA was extracted with phenol/chloroform under standard conditions. The molecules were separated by electrophoresis under denaturing conditions in 0.8% agarose gels in the presence of 0.5 M glyoxal/50% DMSO (McMaster and Carmichael, 1977). The nucleic acids were then transferred onto nitrocellulose filters and bound to the membranes by crosslinking under uv irradiation (2 min) and subsequent baking for 2 hr at 80° under vacuum (Alwin *et al.*, 1977). For isolation of whole-cell RNA, cells were lysed in the presence of 0.5% SDS, 50 mM Na-acetate at pH 5.2, and nucleic acids were extracted twice with phenol at 60° (Scherrer, 1969).

The hybridization probes are derivatives from cDNA clones of HAV strain HM175, which were generously provided by John Ticehurst (NIAID) (Ticehurst *et al.*, 1983). They were subcloned in Gemini vectors (Promega Biotech) and <sup>32</sup>P-labeled RNAs were synthesized by SP6 or T7 polymerase *in vitro* (Melton *et al.*, 1984). The 5' end-labeling of cDNA fragments with [ $\gamma$ -<sup>32</sup>P]ATP was achieved after treatment with phosphatase by use of T4-polynucleotidekinase (Maxam and Gilbert, 1980). For labeling of the 3' ends, [ $\alpha$ -<sup>32</sup>P]dCTP and the Klenow fragment of DNA polymerase I was used in fill-in synthesis on 5' overhangs in restricted cDNA. In addition, blunt-ended fragments

were labeled at the 3' terminus by replacement syntheses with T4 DNA polymerase according to Deen *et al.* (1983).

Hybridization was performed in 50% formamid, 100 mM Na-PO<sub>4</sub>, pH 6.5, 5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 5× SSC (1× SSC = 150 mM NaCl, 15 mM Na-citrate), 1 mM EDTA, 0.1% SDS at 50° overnight. Filters were washed for 1 hr in 0.3× SSC/0.1% SDS at 65° and for 1 hr in 0.1× SSC/0.03% SDS at 65°.

*S1-mapping.* Purified HAV RNA, 50 to 100 ng, was coprecipitated with 0.001 to 0.005 pmol of <sup>32</sup>P-end-labeled HAV cDNA, resuspended in 10 µl 80% formamide, 400 mM NaCl, 40 mM Mops (3-*N*-morpholinopropane-sulfonic acid), pH 6.7, 1 mM EDTA, denatured for 5 min at 100°, and hybridized overnight at 50°. After hybridization 200 µl 30 mM Na-acetate, pH 4.6, containing 250 mM NaCl, 1 mM ZnSO<sub>4</sub>, 10 µg/ml salmon sperm DNA, and 1000 units S1 nuclease were added and digestion was carried out at 37° for 90 min. After extraction with phenol:CHCl<sub>3</sub> (1:1; v/v) and precipitation with ethanol, probes were electrophoresed on 8% PAGE in TBE (90 mM Tris, pH 8.5, 10 mM boric acid, 3 mM EDTA) and 7 M urea (Berk and Sharp, 1977; Weaver and Weissmann, 1979).

*Exonuclease VII mapping.* ExoVII digestion of heteroduplexes was performed as described for S1-mapping, but instead of S1 digestion buffer, 200 µl of a solution containing 67 mM KPO<sub>4</sub>, pH 7.9, 8.3 mM EDTA, 10 mM DTT, 20 µg/ml salmon sperm DNA, and 0.5 units of exonuclease VII were used. Digestion was performed at 37° for 45 min (Berk and Sharp, 1977).

## RESULTS

### Characterization of subgenomic viral RNAs

HAV particles with defective genomes were detected for the first time in large-scale preparations of HAV strain CLF. Banding of such preparations in a CsCl/sucrose gradient (Siegl *et al.*, 1984) yielded an apparently homogeneous virus peak with a mean density around 1.34 g/ml. Upon sedimentation in sucrose, however, particles from this peak resolved into two populations with values ranging from 160 to 190 S (pool 1 in Fig. 1) and from 130 to 160 S (pool 1a in Fig. 1), respectively. As electrophoresis and Northern blotting of extracted RNAs revealed, both pools consisted of virions containing the full size ("standard") HAV genome and of particles with subgenomic ("defective") RNA molecules. The subgenomic RNAs in pool 1 appeared as a largely heterogeneous population of molecules about 2500 to 5000 nucleotides (nts) in length. In contrast, two distinct subgenomic RNAs of 3800

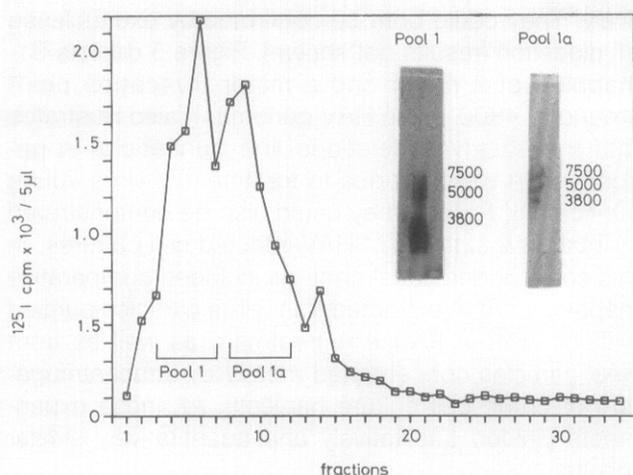


Fig. 1. Sedimentation profile in a sucrose gradient of HAV particles banding in a CsCl/sucrose step gradient between 1.33 and 1.35 g/ml. HAV was detected by RIA. In parallel experiments, radiolabeled poliovirus type 2 (160 S) sedimented in fraction 8. Autoradiographs show Northern blot analysis of RNA contained in pool 1 and pool 1a. RNA from pool 1 was detected by hybridization with a selection of negative-strand RNA probes individually spanning the 5' untranslated region (nt 140–680), VP2/VP3 (nt 1000–2310), and the P3 region (nt 6000–7500). RNA of pool 1a was hybridized to similar RNA probes spanning VP2/VP3 (nt 1000–2310) and 2C (nt 4300–4930) only.

and 5000 nts, respectively, were detected in defective particles from pool 1a.

To determine whether the subgenomic RNAs detected in the pools consisted of random breakdown products or of molecules with specific deletions, six identical Northern blots of such RNAs were hybridized to radiolabeled negative-stranded RNAs. The probes were derived from HAV cDNA clones representative

for distinct regions of the HAV genome (Fig. 2). Under standardized conditions (for details see legend to Fig. 2) hybridization of RNA from pool 1 with a probe complementary to the 5' terminus of HAV RNA (JK/EcoRI) detected the full spectrum of subgenomic molecules ranging in size from about 5000 to 3000 nts. Comparable results were obtained with probes 148H2/RI and 148K/EcoRI (representative for genome region 2C–3C). No hybridization signal, however, was detected with a probe specific for the capsid region (JBam/PstI) and only a relatively weak signal was observed following hybridization with a probe mostly representative for region 2AB (JG/PvuII). Finally, molecules < 3500 nts failed to show up when blots were examined for the presence of the 3' terminal part of the HAV genome. The 3.8-kb subgenomic RNA of pool 1a did not react with probes from the capsid region and the 5-kb species gave only a weak signal when tested under these conditions (data not shown). It was concluded from these data that the subgenomic vRNAs consisted of molecules with an internal deletion(s) between nucleotides 1000 and 4000 and, most likely, truncations in their 3' proximal part.

The exact size and location of deletions and truncations within the viral genome were determined by S1-mapping. The cDNA probes used for this purpose are indicated in the legend to Fig. 6, which summarizes the results of these and further mapping experiments. Results of S1 analysis were, whenever permitted by the quantity of viral RNA, confirmed by exonuclease VII mapping. This precaution was deemed necessary because CLF, the HAV strain under investigation, and strain HM175, of which the cDNA clones were derived, represent individual HAV isolates originating in

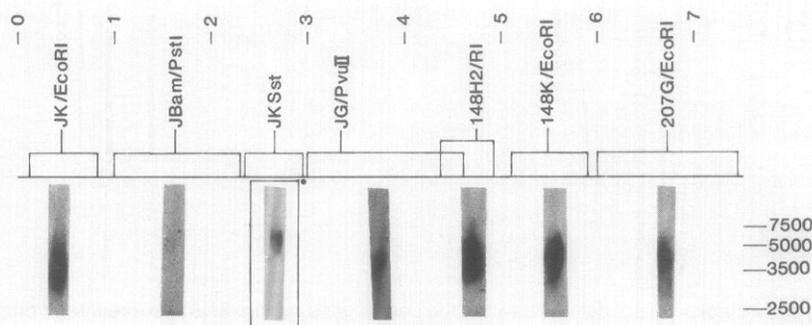


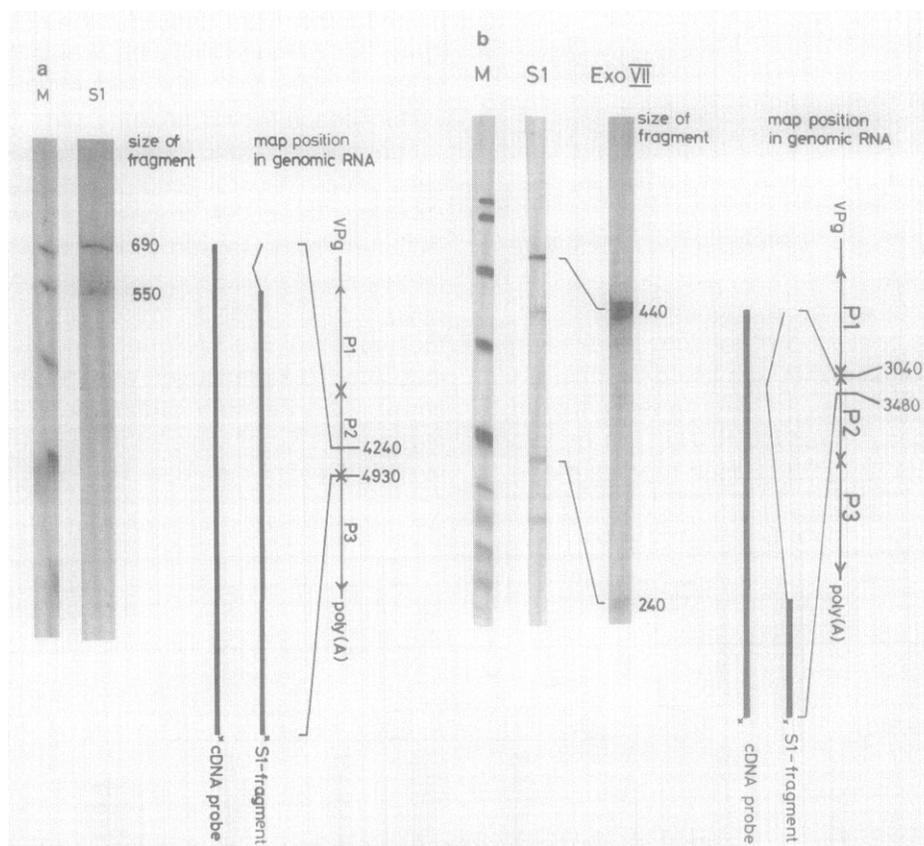
Fig. 2. Detection of deletions in subgenomic HAV RNA molecules by hybridization. Hybridization of viral RNA was performed with anti-sense RNAs transcribed from cDNA in Gemini vectors and representing various regions of the HAV genome. RNA from pool 1 of Fig. 1 was electrophoretically separated and transferred to nitrocellulose membranes before hybridization. Hybridization was performed in 1.5 ml hybridization mix, containing  $1 \times 10^6$  cpm each of the respective  $^{32}\text{P}$ -labeled probes. Map positions of probes are indicated as restriction fragments on the genome. The 5' end of the genome is orientated to the left. Autoradiographic exposure was standardized to allow for optimal detection and quantitative comparison of subgenomic RNAs. With extended exposure, all probes detected also the standard HAV genome. The insert (\*) shows such an overexposed autoradiograph of hybridization in this region of the genome and underlines the detectability of full-length HAV RNA. Efficiency of hybridization (e.g., with probe JBam/PstI) was assessed by dot blot hybridization.

Switzerland and Australia, respectively. Differences in nucleotide sequence of the individual genomes can be expected to occur and have in fact been demonstrated (Weitz and Siegl, 1985). Incomplete base pairing during hybridization resulting from such differences would lead to the appearance of multiple bands during S1 mapping, yet, would barely affect the result of exonuclease VII digestion.

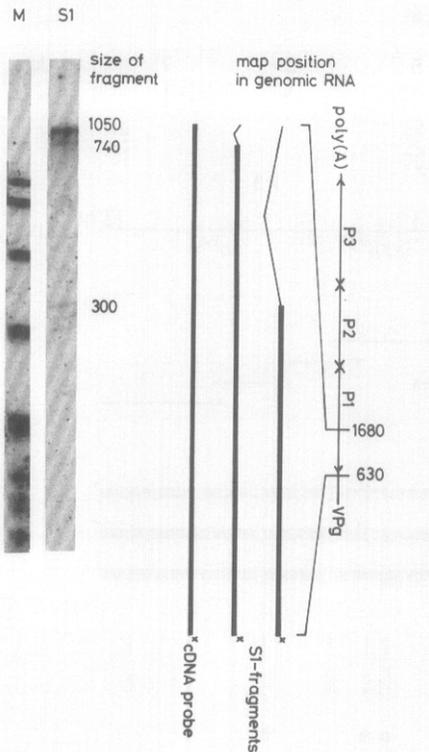
Examples of S1 analysis and of the outcome of exonuclease VII controls are given in Figs. 3–5. For instance, S1 mapping alone yielded a clear result in the identification of the deletion point at nt 4380 (Fig. 3a). Digestion by exonuclease VII, however, proved to be irrevocable to determine the true deletion endpoint at nt 3240 out of three possibilities put forward by S1 mapping. (Fig. 3b). Figure 4 illustrates attempts to locate two deletion points at nts 930 and 1370, respec-

tively. They could both be confirmed by exonuclease VII digestion (results not shown). Figure 5 depicts S1-mapping of a major and a minor truncation point around nt 4800 of the HAV genome. It also illustrates that the observed deletions and truncations in genomic RNA were not due to treatment of virus during purification. Rather, they could also be demonstrated in total RNA extracts of HAV-infected cell cultures. In this context, additional controls included comparative mapping of RNA extracted from virus particles purified with or without RNase A treatment as well as from HAV particles concentrated merely by ultracentrifugation of crude cell culture harvests. All these experiments yielded qualitatively and quantitatively similar results.

In summary of the above attempts, two deletions spanning from nt 930 to nt 4380 (deletion A in Fig. 6)



**Fig. 3.** Mapping of the 3' terminal breakpoints of deletions within the capsid gene region. RNA was prepared from isolated HAV particles and hybridized to cDNA probes labeled at the 5' end of the minus strand. Hybrids were digested either with S1 nuclease or with exonuclease VII as indicated under Materials and Methods and protected cDNA fragments were resolved on a sequencing gel. The mapping strategy is presented schematically. (a) The hybridization probe was 5' end-labeled at the *Eco*RI site at nucleotide position 4930. Full-size vRNA protected this probe over its entire length of 690 nucleotides whereas hybridization to defective RNA allowed digestion down to a single protected fragment of 550 nts which is indicative for a breakpoint at nt 4380. (b) The hybridization probe was labeled at the *Av*II site (nt 3480). After hybridization to HAV RNA, S1 digestion gave rise to a 440-nt fragment protected by standard vRNA and to three smaller fragments. Of these, only a fragment of 240 nts remained detectable during *Exo*VII mapping and, thus, identified a single breakpoint at nt 3240. Videodensitometric analysis of HAV RNA protected and unprotected cDNA probes in S1 mapping experiments showed an at least five times greater signal for protected bands. M, DNA size marker (pBR322/*Hpa*II). X, position of the label on the S1 hybridization probe.

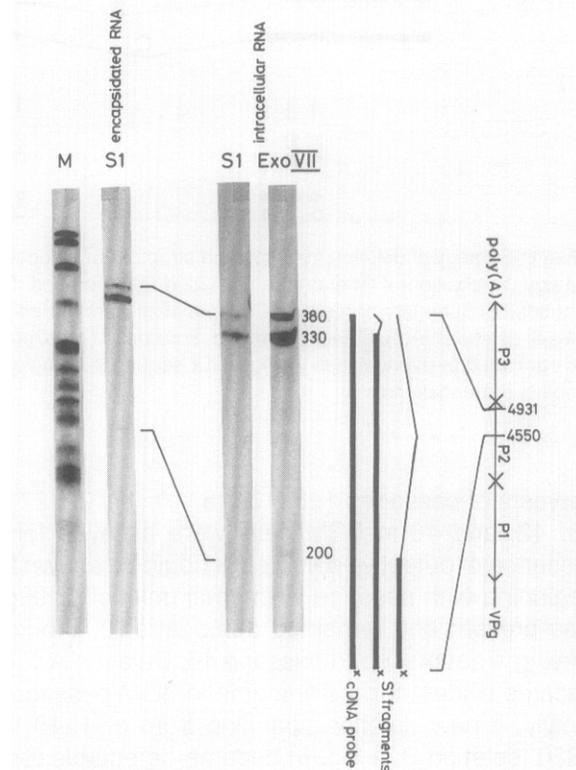


**Fig. 4.** Mapping of the 5' terminal breakpoints of internal deletion at the beginning of the capsid gene region of the HAV genome. vRNA was isolated from purified HAV particles and hybridized to a cDNA probe labeled at the 3' end of the minus strand at the *Bam*HI site at nt position 630. During digestion with S1 nuclease, protection by standard vRNA yields a fragment of 1050 nts in length whereas partial protection by defective genomes results in two smaller fragments consisting of 300 and 740 nts, respectively. The corresponding deletion breakpoints are located at nt 930 and nt 1370, respectively. Both breakpoints could be confirmed by *Exo*VII mapping. Videodensitometric analysis of HAV RNA protected and unprotected cDNA probes showed an at least five times greater signal for protected bands. M, DNA size marker (pBR322/*Hpa*II). X, position of the label on the S1 hybridization probe in the schematic presentation of the analysis.

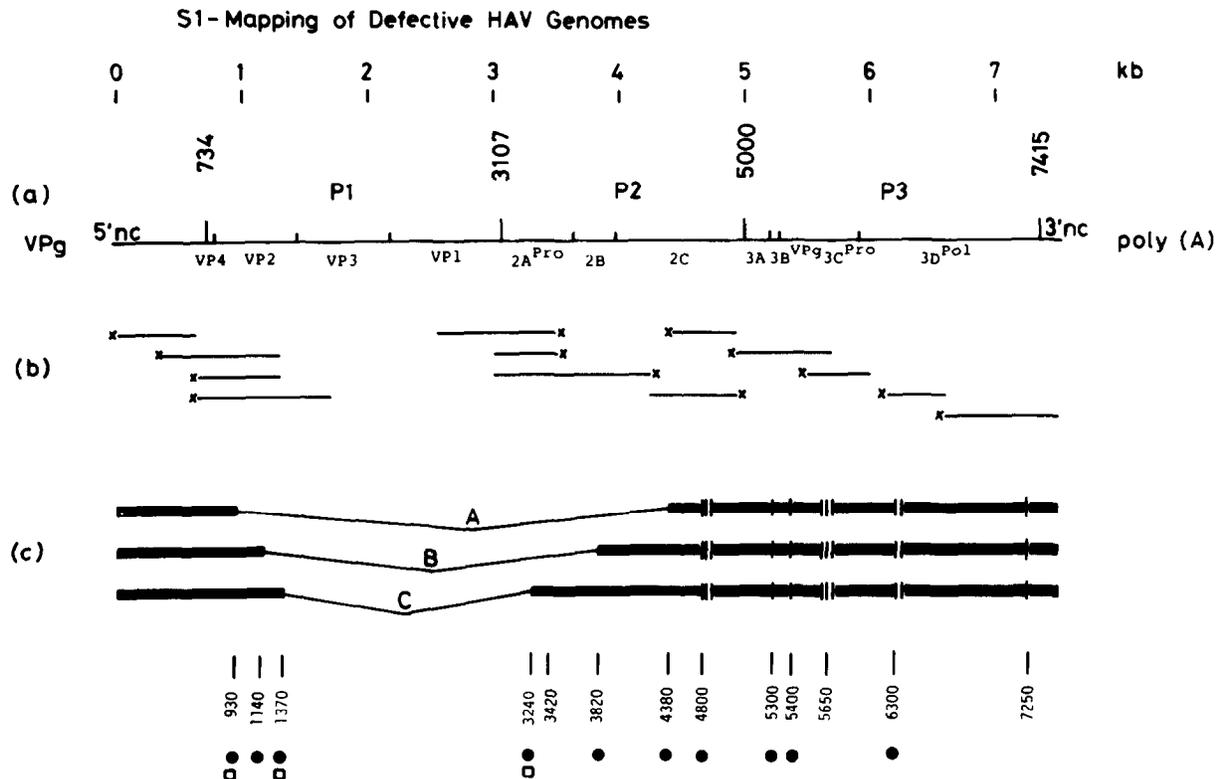
and from nt 1370 to nt 3240 (deletion C in Fig. 6), respectively, were mapped in the subgenomic RNAs of particles contained in pools 1 and 1a. Given the size of 7450 nucleotides of standard HAV RNA (Baroudy *et al.*, 1985), these deletions result in molecules of 4000 and 5580 nts. Such values are compatible with the size of the two distinct species of subgenomic vRNAs detected by Northern blotting in pool 1a particles (see Fig. 1). S1-mapping experiments in the 3' terminal region of RNA from pool 1 particles revealed truncation points clustering around nts 4800, 5350, 5650, 6300, and 7250 of the HAV genome. In combination with the presence of the internal deletions, this multitude of truncations explains the apparently continuous size spectrum of subgenomic RNA molecules present in the latter pool.

### Occurrence of defective HAV genomes during consecutive passages in different cell systems

The results presented so far were obtained with the CLF strain originally isolated from a fecal sample in MRC-5 cells, passaged 10 times in this cell line before adaptation to BGM cells, and carried through 18 consecutive passages in the latter culture system at a m.o.i. of 0.1 to 1. Published observation with well-known picornaviruses suggest that appearance and accumulation of virus particles containing defective genomes may vary with the cell system and fluctuate with consecutive passages (McClure *et al.*, 1979, Holland *et al.*, 1980). To probe into the influence of passage number on generation of deleted HAV genomes



**Fig. 5.** S1 analysis of truncations located within the P3 region of the HAV genome. Viral RNA was extracted from purified particles of HAV strain MBB11/5 ("encapsidated RNA") or directly from cells infected with CLF ("intracellular RNA"). Both types of RNA were hybridized to the *Hinf*I fragment of cDNA labeled at the 3' end of the minus strand at position 4550. Standard size vRNA in both samples protected the complete fragment of 380 nts from digestion with S1 nuclease. Truncated vRNAs, however, gave rise to two shortened fragments of 330 and 200 nts in length. The latter fragments were also resolved by *Exo*VII mapping and correspond to truncation points at nt 4750 and 4880, respectively. Videodensitometric analysis of HAV RNA protected and unprotected cDNA probes showed an at least five times greater signal for protected bands. M, DNA size marker (pBR322/*Hpa*II). X, position of the label on the S1 hybridization probe in the schematic presentation of the mapping strategy.



**Fig. 6.** Strategy of deletion mapping and structure of defective HAV genomes. (a) The most likely structure of the HAV genome. (b) Mapping strategy. X, position of label on the S1 hybridization probes. Only the minus strand of cDNAs was labeled as indicated under Materials and Methods. (c) Summary of results of S1 mapping. Bars represent RNA present in defective particles, carets represent deleted regions. Three types of deletions (A, B, C) were detected. Positions of breakpoints are given in nucleotide numbers. ●, breakpoints determined by S1 mapping and confirmed by exonuclease VII digest. □, sequence homologies of breakpoints to 5'/3' splice sites, as determined by sequence comparison. |, points of truncations.

harvests of passages 1, 5, 10, 15, 16, 17, 22, 27, 32, 35, 42, and 46 in BGM cells were assayed for the presence of defective particles. It could be shown that, beginning with passage 5, internal deletion C (Fig. 6) was present and remained demonstrable, though in varying amounts, up to passage 46. Deletion A (Fig. 6) became evident for the first time in BGM passage 16. Finally, a new deletion spanning from nt 1140 to nt 3820 (deletion B in Fig. 6) became detectable for the first time in passage 41. Particles containing the same defective genomes (deletion type A, B and C) were also found in harvests of passages of HAV strain CLF in MRC-5 cells. In this virus/cell system, however, both deletions B and C (Fig. 6) could be detected as early as passage 5. Subgenomic RNA with deletion A had accumulated to detectable levels in passage 20. From then on and up to passage 94 deletions A, B, and C were simultaneously present (see also Table 1).

The quantity of defective HAV particles evidently fluctuated throughout consecutive passages. Large amounts were recorded in passages 31, 50, and 70 in

**TABLE 1**

DETECTION OF DELETED GENOMES IN VIRUS PARTICLES HARVESTED FROM VARIOUS HAV/CELL SYSTEMS

HAV strain	Cell system	Number or range of passages examined	Deletion detected	In passage number
CLF	MRC-5	1-94	B, C	5 <sup>b</sup>
CLF	MRC-5	1-94	A, B, C	20 <sup>b</sup>
CLF	BGM	1-46	C	5 <sup>b</sup>
CLF	BGM	1-46	A, C	16 <sup>b</sup>
CLF	BGM	1-46	A, B, C	41 <sup>b</sup>
GBM	MRC-5	11, 21	A	21
H141	MRC-5	8, 23	A, B	23
HM175	MRC-5	17	A, B, C	17
MBB11/5	HELFL <sup>a</sup>	21, 36	C	21 <sup>b</sup>
CR326	PLC/PRF/5	5	C	5

*Note.* Deletion A, nt 930-4380; deletion B, nt 1140-3820; deletion C, nt 1370-3240.

<sup>a</sup> Human embryonic lung fibroblasts.

<sup>b</sup> First passage in which deletion was detected.

MRC-5 as well as in passage 10, 17, 26, and 46 in BGM cells. In general, however, production of deleted HAV genomes was less efficient in MRC-5 cells than in BGM cells. Analysis of total encapsidated RNA by Northern blotting suggested that the quantity of HAV particles containing subgenomic RNA was at least equal and frequently higher than the quantity of virions with standard genomes (data not shown).

#### Defective particles associated with various HAV isolates

In addition to HAV strain CLF from Switzerland, strains originating in Australia (strain HM175), India (H141), Costa Rica (CR326), North Africa (MBB11/5), and Germany (GBM) were assayed for the presence of defective, encapsidated genomes in cell culture harvests. RNA molecules with internal deletions and those with 3' proximal truncations were readily demonstrated in such samples. Most interestingly, the internal deletions and 3' proximal truncations mapped at identical locations with all the viruses (Table 1; Fig. 5), but type and number of deletions varied among different strains.

### DISCUSSION

Particles containing defective genomes are commonly found among both DNA and RNA animal viruses (for references see Holland, 1986). Therefore, the detection of similar structures in harvests from HAV-infected cell cultures is not unexpected. The observation that major deletions within the defective HAV genomes include sequences coding for viral structural proteins also correlates to findings in other picornaviruses e.g., polio- (Cole *et al.*, 1971), coxsackie-, echo-, and mengoviruses (Holland and McLaren, 1974). The genomes of most of the defective viruses investigated in greater detail lack the information for their own capsid proteins. This is believed to form part of the basis for interference with replication of a coinfecting standard particle (Choppin, 1969; Cole and Baltimore, 1973; Kingsbury and Portner, 1980; Perrault and Holland, 1972). Our studies of HAV show that, with exception of the sequences required for initiation of replication and for packaging, the vast majority of the virus genome can be deleted (Levis *et al.*, 1986). However, whether defective HAV particles interfere with virus replication remains to be shown.

The existence of defective genomes which, in addition to the internal deletions, have truncations of variable lengths in their 3' terminal part is a unique feature of HAV among the picornaviruses (Lundquist *et al.*, 1979; Nomoto *et al.*, 1979; McClure *et al.*, 1980).

Such genomes seem to be exclusively present in particles sedimenting with  $>160$  S (cf. Fig. 1). In contrast, particles sedimenting with an S value characteristic for the mature hepatitis A virion (156–160 S) (Siegl *et al.*, 1981) contain either the full size viral genome or vRNA with distinct internal deletions. We presume that the  $>160$  S particles are closely related to the so-called high-density ( $\geq 1.44$  g/ml) components demonstrated in harvests of several enteroviruses as well as of HAV (Rowlands *et al.*, 1975; Siegl and Frösner, 1978; Siegl *et al.*, 1981). High-density particles evidently differ from the mature picornavirion in buoyant density ( $\geq 1.44$  g/ml), sedimentation behavior (200–220 S), stability, and, most likely, capsid structure (cf. Wieggers *et al.*, 1977). If such particles lack a major part of the viral genome it is conceivable that they band close to mature virions in CsCl and, under the conditions of moderate resolution existing in the CsCl/sucrose gradients used as a primary step in HAV isolation, are harvested together with them. Whether the high-density picornavirus component represents a necessary intermediate or an aberrant end product of picornavirus maturation is not yet clear. There is good evidence, however, that the truncated genomes revealed in our studies were not generated during virus isolation. They were present in HAV particles purified according to widely differing protocols and, also, could be extracted directly from HAV infected cells. Furthermore, the 3' terminal truncations do not occur at random. Rather, they start in several apparently restricted regions between nucleotide 4800 and the 3' terminus of the molecule.

The three types of internal deletions as well as the multiple 3' proximal truncations were found in all strains tested and independent of the cell system used for propagation of the viruses. This conservation of deletion points suggests that some "signal" or "effector" sequence(s) exist, which favor shortening of HAV genomes during replication or subsequent processing *in vitro*. For instance, visual inspection of the HAV RNA sequence as reported by Najarian *et al.* (1985) revealed short complementary stretches flanking deletions that might induce "ring closure" events. Parts of the HAV genome would then be omitted by "intersegment transfer" of the viral RNA polymerase at intramolecular crossover points (cf. Berg *et al.*, 1983; Perrault 1981; Lazzarini *et al.*, 1981). Recent work with corona- (Baric *et al.*, 1985) and polioviruses (Kirkegaard *et al.*, 1986; Agut *et al.*, 1987) points to recombination as another possible mechanism for production of defective genomes. However, characteristics of replication and especially copy numbers of intracellular viral RNA of polio- and hepatitis A viruses

are quite different. We also detected splicing consensus sequences of eucaryotic pre-mRNAs in some of the locations flanking internal deletions in HAV RNA (see Fig. 6). Therefore, a splicing mechanism comparable to the "lariat model" (Grabowski *et al.*, 1984) should be taken into consideration. Splicing signals were not found in defective influenza and vesicular stomatitis virus RNAs (Chanda *et al.*, 1983; De and Perrault, 1982; Yang and Lazzarini, 1983).

The 3' truncations of encapsidated viral RNAs occurred in confined regions of the genome. In these locations primary and/or secondary structures of the molecule could be responsible for termination of RNA synthesis. Previous studies in one of the HAV/cell systems under investigation have shown that active synthesis of HAV RNA declines significantly when infected cells enter into the persistent state of infection (de Chastonay and Siegl, 1987). This could be interpreted as inefficiency of viral RNA polymerase, yet, might also result from a depletion of complete templates during RNA replication.

The present search for defective (possibly interfering) particles in cell cultures inoculated with HAV was undertaken to explain persistent infection in the majority of HAV/cell systems. The presence of defective particles in all HAV/cell systems investigated would make their involvement in persistence indeed likely. Yet, neither quantitative differences nor type of deleted genomes appeared to influence establishment of persistent HAV infection during early or late *in vitro* passages. Therefore, the role of defective particles with internal deletions of capsid genes in this virus/cell relationship is at least questionable. On the other hand, and as discussed before, 3' truncations of the HAV genome may be related to a specific block in HAV replication (de Chastonay and Siegl, 1987) and to establishment of persistent infection. Future experiments probing into appearance and accumulation of truncated viral RNAs during one-step replication of HAV in cell cultures may shed some light onto this problem.

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