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Expression of hepatitis B surface antigen in unselected cell culture transfected with recircularized HBV DNA

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Hepatitis B virus (HBV) DNA was isolated from the recombinant plasmid pA01-HBV and recircularized. Immediately after introduction of this DNA into mouse fibroblasts (NIH 3T3) we observed increasing release of hepatitis B surface antigen (HBsAg) into the culture medium. Later production of HBsAg declined to a lower but constant level. No dominant selective marker and foreign promoter were necessary in this system, which therefore can be used for the study of regulation of HBsAg expression.

Key words: gene transfer/hepatitis B surface antigen/mouse fibroblasts/rapid expression

Introduction

For the understanding of the molecular biology of the hepatitis B virus (HBV) it will be important to establish an *in vitro* system to study expression of the HBV genome. Until now, all attempts to achieve productive infection have failed. Provided that the non-permissiveness is due to the lack of HBV-specific receptors on cultivated cells, recombinant DNA technology could be used to circumvent this limitation. Cloned HBV genomic DNA has been introduced into mammalian cells and the expression of viral antigens is under investigation (Dubois *et al.*, 1980; Hirschman *et al.*, 1980; Moriarty *et al.*, 1981; Christman *et al.*, 1982; Pourcel *et al.*, 1982). However, so far the expression of hepatitis B surface antigen (HBsAg) has only been observed: (i) after time consuming selection for producing cells; (ii) after recombination with an SV40 viral vector. Here we report the rapid expression of HBsAg following transfection of NIH 3T3 mouse fibroblasts with cloned and recircularized HBV DNA. No selection system was applied. A high peak of HBsAg expression was observed within 6 days followed by a persistent production at a lower level. Additional experiments using defined subfragments show that the promoter for HBsAg can be localized on the viral genome by this approach.

Results

We made use of the plasmid pA01-HBV, which contains the HBV genome, subtype adw, cloned into the unique *Eco*RI site of plasmid vector pA01 (Cummings *et al.*, 1980). This results in a disruption of the pre-S region of HBsAg gene (Valenzuela *et al.*, 1980; Tiollais *et al.*, 1981). As expected, when this recombinant plasmid was transferred into NIH 3T3 mouse fibroblasts, no expression of HBsAg was observed. To restore the original genome organization the insert HBV DNA was isolated and recircularized as described in Materials and methods. As shown in Figure 1, lane b, the electrophoretic mobility of the molecules suggests that relaxed circles (upper band above linear form in lane e) and supercoils (lower bands) are present in the DNA preparation after ligation. This observation was confirmed by electron microscopy (Figure 2).

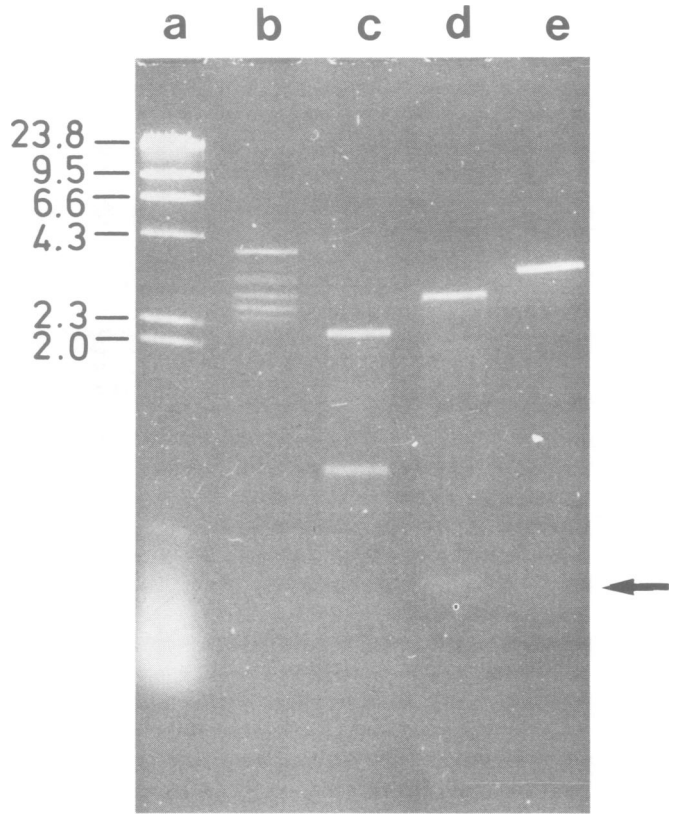

Fig. 1. Electrophoretic mobility of recircularized HBV DNA. **Lane a**, λ DNA digested with *Hind*III as mol. wt. marker (presented in kbp). **Lane b**, recircularized HBV DNA. **Lane c, d,** and **e**, digestion of recircularized HBV DNA with restriction enzymes *Aval* (two sites), *Bgl*II (two sites), and *Eco*RI (one site), respectively. Arrow shows the smaller *Bgl*II fragment.

The recircularized HBV DNA was characterized with several restriction enzymes. The electrophoretic mobilities of *Aval*, *Bgl*II, and *Eco*RI fragments are shown in Figure 1, lane c, d, and e. The size of these fragments compare well with those published elsewhere (Cummings *et al.*, 1980). A more detailed restriction map is shown in Figure 3, which is in agreement with restriction sites found by sequencing another cloned HBV DNA (Valenzuela *et al.*, 1980). In particular, the presence of the unique *Eco*RI site in the recircularized HBV DNA suggests that the original complete sequence of the HBV genome was preserved after the procedures described above.

After transfection of NIH 3T3 fibroblasts with this DNA, cells were kept as a monolayer for >1 month. During this time no cytopathic change was observed. The supernatants of

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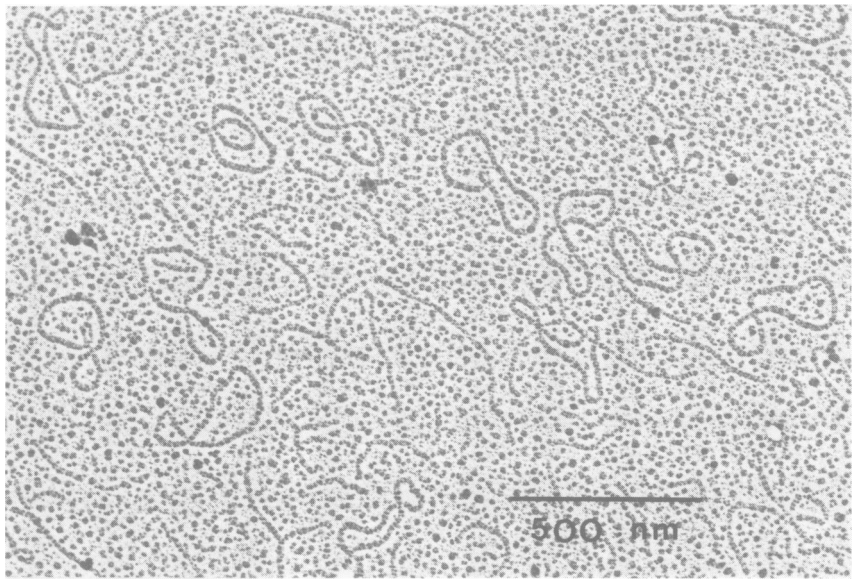

Fig. 2. Electron micrograph of recircularized HBV DNA (x 50 000). DNA was spread by the technique developed by Kleinschmidt (1968).

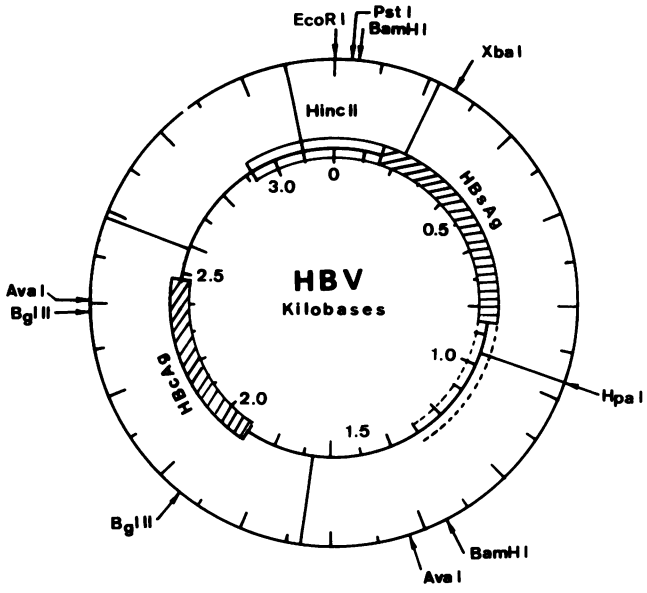

Fig. 3. Restriction map of recircularized HBV DNA. The restriction sites are indicated by arrows. DNA length is represented in kbp. The restriction map was aligned with the genomic organization using the data provided by Valenzuela *et al.* (1980).

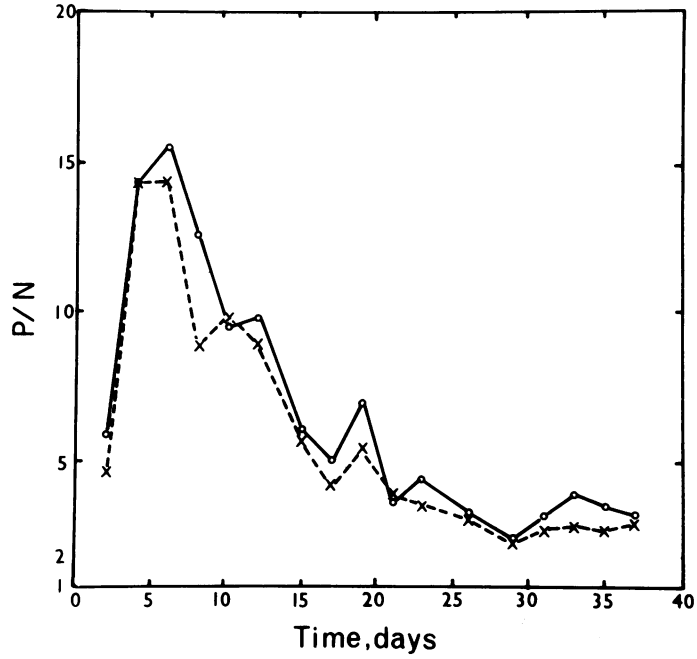

Fig. 4. Time kinetics for HBsAg expression. Transfection was carried out as described in Materials and methods with 2 μg of HBV DNA (○——○) and with 5 μg of HBV DNA (x - - - x). Medium was changed every 2 days. HBsAg was assayed by AUSRIA II (Abbott). P/N ≥2.1 is considered to be positive (P, c.p.m. measured in the samples: N, c.p.m. measured in negative control. The average in the negative controls was 90 c.p.m.).

the monolayers were checked for HBsAg every 2 days after transfection. Release of HBsAg into culture medium could be detected as early as 48 h after transfection with 2 or 5 μg of DNA (Figure 4). Increasing production of HBsAg was observed until the 6th day after transfection, reaching a maximum of P/N = 15 (P/N indicates the c.p.m. of the probe divided by c.p.m. of the negative control = relative value). The production of HBsAg declined gradually to a P/N level of 3−4 within the next 14 days. The expression of HBsAg was followed until the 37th day after transfection and found to persist at this lower level. Re-transfection of the transformed cells is under investigation. The expression of core and e antigen was studied by immunofluorescence and radioimmunoassay, respectively, but was found to be negative.

For further characterization, the HBsAg from the cell culture supernatant at the 6th day was applied to a CsCl buoyant density gradient. HBsAg was found in the fraction with a buoyant density of 1.20 g/ml, which is identical to the antigen found in the serum of patients (Figure 5). The peak fraction of the gradient was submitted to electron microscopy. Micrographs show HBsAg-forming spherical particles of an average diameter of 22 nm (Figure 6). The size and form is identical with those particles found in human serum (Gerin *et al.*, 1971).

Further experiments were carried out to verify that the ge-

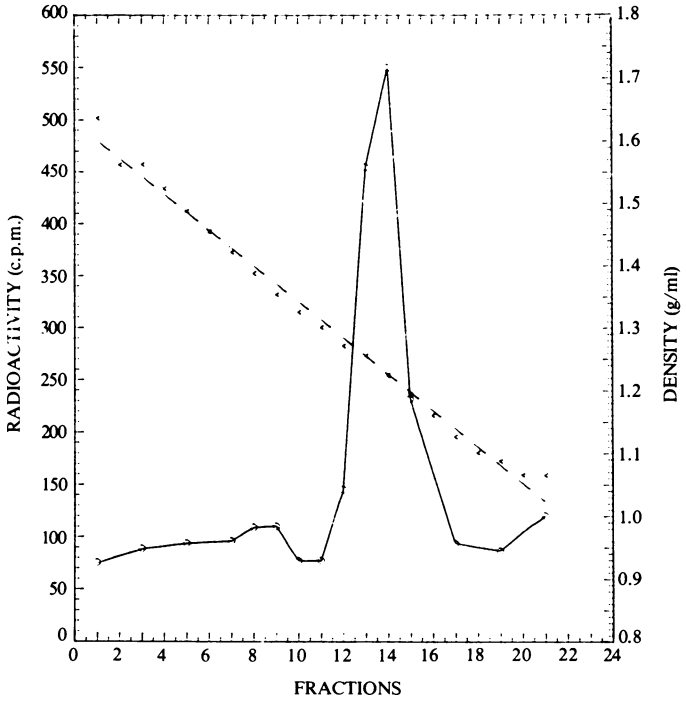

Fig. 5. Isopycnic centrifugation of HBsAg in a CsCl gradient. After a low speed centrifugation 20 ml of supernatant was ultracentrifuged at 4°C and 35 000 r.p.m. for 20 h (rotor Ti 60). The pellet was resuspended in 0.5 ml of TE buffer (10 mM Tris/1 mM EDTA, pH 7.5) and layered on a six-step gradient of CsCl (1.1 – 1.6 g/cm³) and ultracentrifuged for 18 h at 4°C and 34 000 r.p.m. (rotor SW 41). Fractions (0.5 ml) were collected and assayed for HBsAg by AUSRIA II (negative control was 85 c.p.m.).

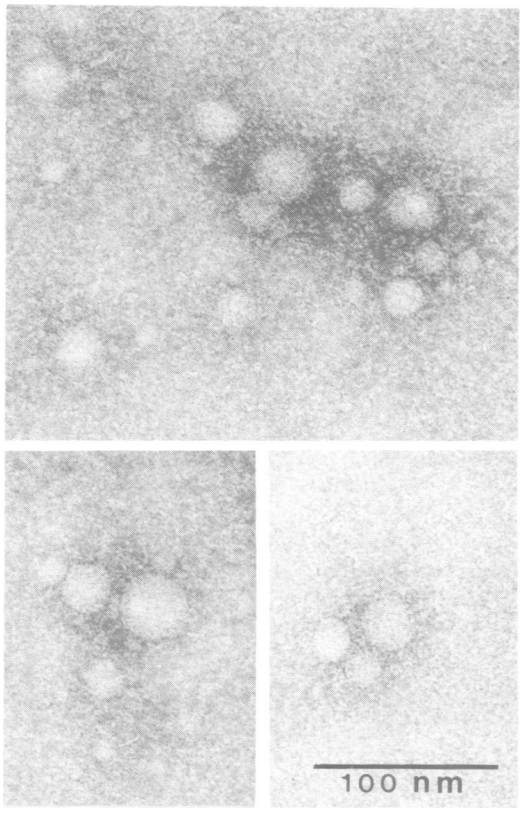

Fig. 6. Electron micrograph of spherical HBsAg particles (x 240 000). The peak fraction from the CsCl gradient was collected and dialysed thoroughly against TE buffer. HBsAg particles were stained with 1% phosphotungstic acid.

nuine viral promoter was utilized. The *Bgl*II fragment of recircularized HBV DNA, which contains the pre-S region and the structural gene for HBsAg, was subcloned into the *Bgl*II site of plasmid vector pKK92c-2 as described in Materials and methods. Transfection with the recombinant plasmid, pKKHBs34, showed time kinetics identical to those obtained with the complete recircularized HBV genome within 2 weeks (data not shown). The *Bam*HI fragment of recircularized HBV DNA, which essentially contains only the structural gene for HBsAg, was subcloned into the *Bam*HI site of plasmid pBR322. No production of HbsAg was found after transfection with the hybrid plasmid pBRHBV9.

Discussion

Recircularized HBV DNA has already been introduced into HeLa cells by Hirschman *et al.* (1980). A weak expression of HBsAg was observed after cytopathic changes only several weeks after transfection. Our results clearly show that a rapid expression of HBsAg with recircularized HBV DNA can be detected in unselected NIH 3T3 cells. We also checked for expression of HBsAg in HeLa cells immediately after transfection. With our method we found an early expression within 6 days but at a 10-fold lower level than found in NIH 3T3 cells. This may be due either to a better uptake of DNA by NIH 3T3 cells or because HeLa cells in general express HBsAg at a lower level.

In several reports it was claimed that an efficient selection system is necessary for detection of HBsAg expression. Expression was only found in cells selected for the presence of herpes simplex thymidine kinase gene (Dubois *et al.*, 1980) or in methotrexate-resistant cell colonies (Christman *et al.*, 1982). In that case a dimeric head-to-tail tandem construction of HBV DNA was necessary to overcome the permutation of HBV genome caused by cloning in the *Eco*RI site interrupting the coding sequence for HbsAg. In unselected African green monkey kidney cells expression of HBsAg was found but under control of the late promoter of SV40. The advantage of the method described here is the very early detection of HBsAg, made under the promoter of the HBsAg gene, which can be used for model studies; expression of the antigen is an easy indicator of successful transfection.

The transient high expression between 2 and 6 days shown in Figure 4 could be explained by episomal expression. The persistant expression at a lower level later on could reflect the stable integration of HBV DNA into the host chromosomal DNA or, less likely, episomal replication in a few cells. These possibilities as well as the expression of other viral antigens should be studied in further experiments.

The transfection experiments with *Bgl*II and *Bam*HI fragments of HBV DNA show that the promoter region is present within the *Bgl*II/*Bam*HI fragment containing the pre-S region and is sufficient for the rapid expression of HBsAg. This was also found for subtype ayw (Pourcel *et al.*, 1981). We therefore conclude that, in our experiments, HBsAg expression is under the control of the genuine viral promoter. Sequencing data of HBV DNA (Galibert *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1980) suggested the presence of signals necessary for HBsAg expression. We provide here a biological assay system which can be exploited to study the function of these signals in the regulation of transcription and translation of the HBsAg gene by using

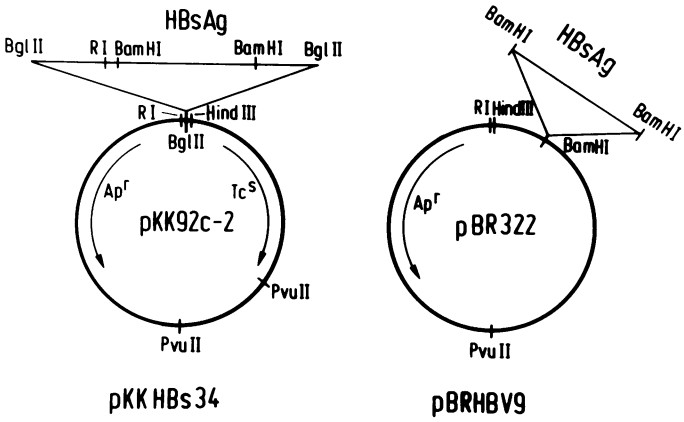

Fig. 7. Recombined plasmids containing subfragments of recircularized HBV DNA. For details see Materials and methods.

defined subfragments and other modifications.

Furthermore, HBsAg can be easily detected by radioimmunoassay. Therefore, HBsAg expression may be used as a marker to study eukaryotic vectors, promoters, and new transfection methods.

Materials and methods

Preparation of HBV DNA

The plasmid pA01-HBV contains the genome of HBV, subtype adw, inserted into the *Eco*RI site of the plasmid vector pA01 (Cummings *et al.*, 1980). The HBV DNA was cleaved from plasmid pA01-HBV using restriction enzyme *Eco*RI. The insert DNA was separated from the plasmid vector by 1% agarose gel chromatography and recovered by electrophoresis. After phenolization and ethanol precipitation, insert DNA was recircularized by T4 DNA ligase (Boehringer) in highly diluted solution (10 μg of DNA/ml). Recircularized DNA was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in 10 mM Tris/1 mM EDTA, pH 7.5.

Restriction mapping

Recircularized HBV DNA was digested with different restriction enzymes under standard conditions. Several double digestions were performed to determine the restriction sites. The electrophoretic mobility of the fragments was determined by 1% agarose gel electrophoresis. λ DNA digested with *Hin*dIII and M13 DNA digested with *Hae*III were used as mol. wt. markers.

Cloning of subfragments

The *Bgl*II fragment and *Bam*HI fragment of recircularized HBV DNA were subcloned as follows. The recircularized HBV DNA was digested with *Bgl*II and ligated into the plasmid pKK92c-2, a modified pBR322 (a kind gift of Jürgen Brosius), which was cleaved at its unique *Bgl*II site and treated with alkaline phosphatase. One unit of phosphatase (Boehringer) was incubated with 2 μg of DNA at 65°C for 30 min in 10 mM Tris, pH 9.0. The ligation mixture was used to transform *Escherichia coli* HB 101. Ampicillin-resistant transformants containing the large *Bgl*II fragment of HBV DNA were identified by using the screening method of Birnboim and Doly (1979). One such transformant, pKKHBs34 (Figure 7), was selected for the transfection experiments.

The recircularized HBV DNA was also digested with *Bam*HI and ligated to the *Bam*HI site of plasmid pBR322 (as described above). The recombinant plasmid, pBRHBV9 (Figure 7), containing the smaller *Bam*HI fragment of HBV DNA was used for transfection.

Cell culture

NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's minimal medium supplemented with heat-inactivated 10% calf serum (Gibco) and kanamycin at 50 μg/ml.

Transfection

Transfections were carried out essentially as described (Graham and Van der Eb, 1973; Stow and Wilkie, 1976). Briefly, 1 – 5 μg of recircularized HBV DNA or recombinant plasmids containing subfragments of HBV DNA, and 10 μg of calf thymus DNA as carrier were added to 5 x 10^5 cells per 6 cm Petri dish. After 6 h of incubation at 37°C the cells were washed once with medium and then incubated with fresh medium. Cells were not treated with dimethyl sulphoxide. The transfection was repeated several times yielding the same results.

Assay for HBsAg

HBsAg was assayed in culture medium by the radioimmunoassay AUSRIA II (Abbott).

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