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Recombinant retroviral DNA yielding high expression of hepatitis B surface antigen

Christian Stratowa*, Johannes Doehmer, Yuan Wang[1], and Peter Hans Hofschneider

Max-Planck-Institut für Biochemie, Am Klopferspitz, Martinsried, FRG

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A genomic fragment of hepatitis B virus encoding the surface antigen (HBsAg) was inserted into the proviral genome of Moloney mouse sarcoma virus (MSV), obtained from the mouse cell line G8-124. The recombinant DNA was introduced into NIH 3T3 mouse fibroblasts. Cells, morphologically transformed by the oncogene of MSV (*v-mos*^{M}) were selected, established as cell lines and tested for expression of HBsAg. An expression level of up to 4.5 μg/10^7 cells/day was detected.

Key words: eukaryotic gene transfer/gene expression/hepatitis B surface antigen/murine sarcoma virus DNA/recombinant DNA

Introduction

A variety of approaches has been employed to introduce foreign DNA into eukaryotic cells: transfer solely of the unselected gene, co-transfer with a selectable gene, use of animal viruses as eukaryotic vectors (for review, see Rigby, 1982). The DNA virus SV40 was the first virus to be used as a eukaryotic vector (Berg, 1981). Recently, the use of retroviruses for introducing the herpes simplex thymidine kinase gene (Wei *et al.*, 1981; Shimothono and Temin, 1981; Tabin *et al.*, 1982) and the rat growth hormone gene (Doehmer *et al.*, 1982) into cells has been reported.

A gene which can easily be used to study the properties of different vector systems is a part of the hepatitis B virus (HBV) genome encoding the surface antigen (HBsAg). This gene does not have a cellular counterpart, thus avoiding background problems. Expression can easily be checked because the product is released into the culture medium

Furthermore, the gene product is of medical interest. It is found as 22-nm spherical and filamentous particles in the serum of human HBV carriers and the potential of these particles as a vaccine has been shown (Cabral *et al.*, 1978). Therefore, since it is still difficult to express HBsAg in bacteria, several attempts have been made to establish eukaryotic cell lines that produce HBsAg. Head-to-tail tandems of the HBV genome have been introduced into mouse fibroblasts by co-transfection with herpes simplex thymidine kinase gene (Dubois *et al.*, 1980) or the methotrexate-resistant dihydrofolate reductase gene (Christman *et al.*, 1982) as selectable markers. Monkey kidney cells were infected with an SV40 recombinant carrying the HBV fragment encoding the mature HBsAg linked to the SV40 late promoter (Moriarty *et al.*, 1981). A similar HBV fragment was joined to the yeast alcohol dehydrogenase gene promoter to study the expression of HBsAg in yeast (Valenzuela *et al.*, 1982).

[1]Present address: Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China.

*To whom reprint requests should be sent.

We have recently used the transforming fragment of extrachromosomally replicating bovine papillomavirus DNA to introduce the HBV fragment containing the HBsAg gene and its regulation signals into mouse fibroblasts (Wang *et al.*, in preparation).

Here we report the use of the integrated proviral form of Moloney mouse sarcoma virus (MSV) as a eukaryotic vector for introducing the HBsAg gene into NIH 3T3 mouse fibroblasts. We describe the establishment of a cell line continuously expressing high amounts of HBsAg.

Results

Cloning of MSV proviral DNA

Integrated proviral DNA of MSV obtained from the mouse cell line G8-124 (Ball *et al.*, 1973) was cloned in bacteriophage lambda Charon 4A and subcloned in plasmid pBR322 as described in Materials and methods.

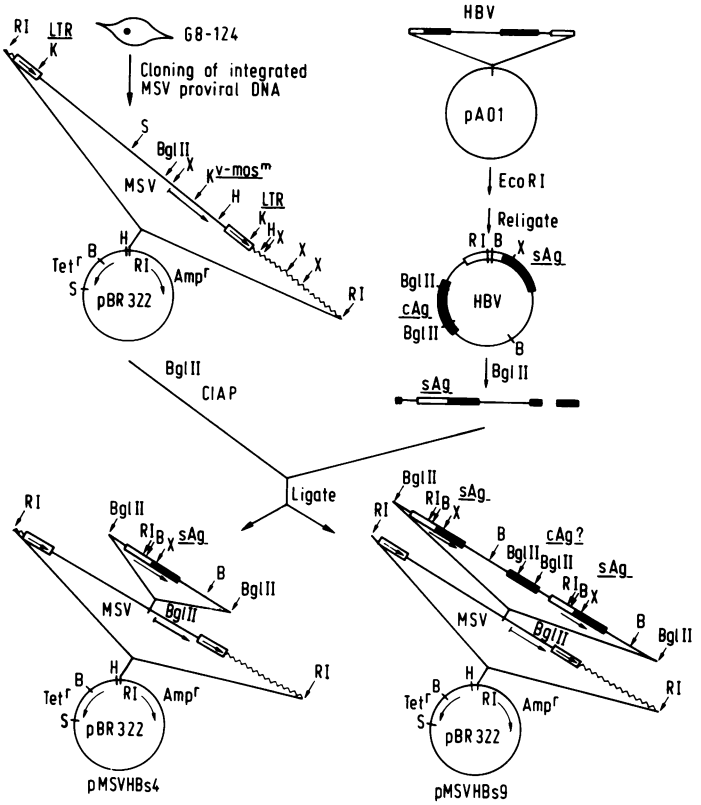

Fig. 1. Construction of recombinant plasmids used in gene transfer experiments. Integrated proviral MSV DNA from cell line G8-124 was cloned in lambda Charon 4A and subcloned in plasmid pBR322 as described in Materials and methods. Cloned HBV DNA was isolated from plasmid pA01-HBV and recircularized. The restored HBV genome was cleaved with *Bgl*II and inserted into the single *Bgl*II site of vector pMSV. Two recombinant plasmids were obtained. Plasmid pMSVHBs4 contains a single copy of the HBsAg gene, whereas plasmid pMSVHBs9 contains two copies of the HBsAg gene in a tandem arrangement. Moreover, *Eco*RI cleavage shows an additional band in the clone pMSVHBs9 which has the size of the complete linear HBV genome. The following restriction endonucleases were used for characterization: RI: *Eco*RI, B: *Bam*HI, H: *Hind*III, X: *Xba*I, S: *Sal*I, K: *Kpn*I.

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The clone obtained was characterized by restriction endonuclease mapping. As shown in Figure 1, the cloned DNA retains its proviral form and the restriction pattern is analogous to the sequence data (Van Beveren *et al.*, 1981). The cloned fragment contains a further 100-bp cellular sequence at the 5′ end of the provirus and a 2.1-kbp cellular flanking sequence at its 3′ end.

To check for transforming activity, cloned MSV DNA was transfected in NIH 3T3 mouse fibroblasts by the calcium phosphate method (Graham and Van der Eb, 1973). Foci of morphologically transformed cells were scored microscopically 3–4 weeks later. The efficiency of transformation was ~2000 focus forming units (f.f.u.)/μg of MSV insert DNA. Harvey sarcoma virus DNA (kindly provided by E.Scolnick) gave the same result. From transfected cells MSV could be rescued upon superinfection with Moloney murine leukemia virus (MLV) as helper virus.

Construction of the recombinants

The construction of MSV-HBV recombinants carrying the HBsAg gene from HBV is illustrated in Figure 1. We used cloned HBV genome, subtype adw, inserted into the *Eco*RI site of plasmid pAO1 (Cummings *et al.*, 1980). To overcome the permutation of the HBsAg gene caused by cloning, the insert DNA was isolated and recircularized as described by Wang *et al.* (1982). The restored HBV genome was cleaved with *Bgl*II. The *Bgl*II fragment encoding the HBsAg was ligated into the unique *Bgl*II site of the retroviral MSV vector. This fragment is known to contain all promoter and termination signals necessary for expression (Wang *et al.*, 1982). *Escherichia coli* HB101 was transformed with the ligation products and checked for recombinant plasmids. We found not only a recombinant, pMSVHBs4, having the single *Bgl*II fragment inserted into the provirus, but also a second clone, pMSVHBs9, containing the large *Bgl*II fragments in tandem, with the same orientation of the HBsAg gene, separated by the small *Bgl*II fragment of which the orientation is not yet known. Thus it is possible that clone pMSVHBs9 contains one copy of the complete HBV genome.

Transfection of mouse fibroblasts with the MSV-HBV recombinants

The recombinant plasmids pMSVHBs4 and pMSVHBs9 were transfected into NIH 3T3 mouse fibroblasts by the calcium phosphate method (Graham and Van der Eb, 1973; Stow and Wilkie, 1976). Focus formation appeared 3 weeks after transfection. Foci were grown for another week to a certain size. Individual foci were picked using cloning cylinders, grown in mass culture and subsequently subcloned by limiting dilution.

To study the organization of pMSVHBs4 and pMSVHBs9 DNA in the transformed cells, cellular clones were grown and their DNAs were analyzed by blot hybridization. As indicated in Figure 1, *Bgl*II cleavage could be used to determine the number of *v-mos*^{M} copies integrated into the DNA of transformed cells, when a *v-mos*^{M}-specific probe is used. Cleavage with *Eco*RI could show if the HBsAg gene and *v-mos*^{M} are integrated in co-linear form. From the autoradiograms shown in Figure 2, it can be seen that the transformants contain reactive sequences when hybridized to HBsAg- or *v-mos*^{M}-specific probes. DNA from untransfected NIH 3T3 cells do not show any hybridization to the HBsAg probe (lanes h and q) but a 23-kbp *Eco*RI fragment and a 4-kbp *Bgl*II fragment could be identified by the *v-mos*^{M} probe representing the cellular homologue *c-mos*^{M} (lanes n and c). Hybridization of the *Bgl*II- or *Eco*RI-cleaved DNAs from the transformed cell lines J1 and J2 to the *v-mos*^{M}-specific probe repeatedly show mainly a band corresponding to the size of the cellular homologue of the *v-mos*^{M} sequences (lanes a, b, l, and m). This has to be studied further. From the intensity of the bands it can be assumed that 5–10 copies of the foreign DNA are integrated into the host genome. *Bgl*II-cleaved DNA from J1 and J2 should show a 2.8-kbp fragment when hybridized to the HBsAg probe. This band can be seen in lanes f and g of Figure 2. The additional bands seen in these lanes reflect the fact that some of the recombinant MSV DNAs, which were introduced into the mouse fibroblasts in circular form, have opened in the HBV region, between the *Bgl*II sites, before integration into the host genome. This may also be the explanation for the pattern obtained when *Eco*RI-cleaved DNAs from J1 and J2 were hybridized to the HBsAg probe (lanes o and p).

Expression of the HBsAg gene

To test whether the integrated HBsAg gene is expressed, we tested for HBsAg secreted into the medium using a radioimmunoassay (AUSRIA II, Abbott Laboratories). Eighteen clones were picked. Five of them produced high amounts of HBsAg. Cell line J1, derived from cells transfected with plasmid pMSVHBs4, and cell line J2, obtained after transfection with plasmid pMSVHBs9, were further analyzed. HBsAg production from cell lines J1 and J2 was measured and compared to an established human hepatoma cell line (PLC/PRF/5, Alexander *et al.*, 1976) under conditions where cells were kept in maintenance medium, in accordance with the conditions of Skelly *et al.* (1979) for the production rate of PLC/PRF/5. During exponential cell growth HBsAg accumulated in the medium at a rate proportional to the increase in cell number. From day 3 the culture medium is progressively depleted of nutrients and cells cease to produce HBsAg and die (Figure 3a), whereas PLC/PRF/5 cells stay alive for 6 days without feeding. J1 and J2 cells could be kept alive and producing HBsAg for at least 7 days when the medium was changed every 24 h. For J1 and J2 cells, the rate of HBsAg expression increased till the cells reached their saturation density, then the level of surface antigen released daily into the medium remained constant at the maximum level (Figure 3b).

The quantity of HBsAg in the medium (ng/ml) was determined from a curve obtained with the reference standard (HBsAg/adw from human serum, 17 μg/ml, from Max von Pettenkofer-Institut, Munich). Independently, aliquots were assayed by the Paul-Ehrlich-Institut, Frankfurt (National Reference Center) and the results obtained here were confirmed. Quantitation for cell lines J1 and J2 yields a production rate of 400 ng/ml/day (i.e., 4.5 μg/10^7 cells/day) and 60 ng/ml/day (0.8 μg/10^7 cells/day), respectively, under maintenance conditions (see first 3 days in Figure 3a). When medium was exchanged daily the production rate at cell saturation density was 280 ng/ml/day for J1 and 60 ng/ml/day for J2 (beginning with day 4 in Figure 3b). In maintenance medium the human hepatoma cell line PLC/PRF/5 produces 10 ng/ml/day (i.e., 0.2 μg/10^7 cells/day, see Figure 3a). When medium was changed each day (Figure 3b) the quantity of HBsAg secreted into the medium per day was 40 ng/ml on the first day and then it declined to 6 ng/ml on the seventh day.

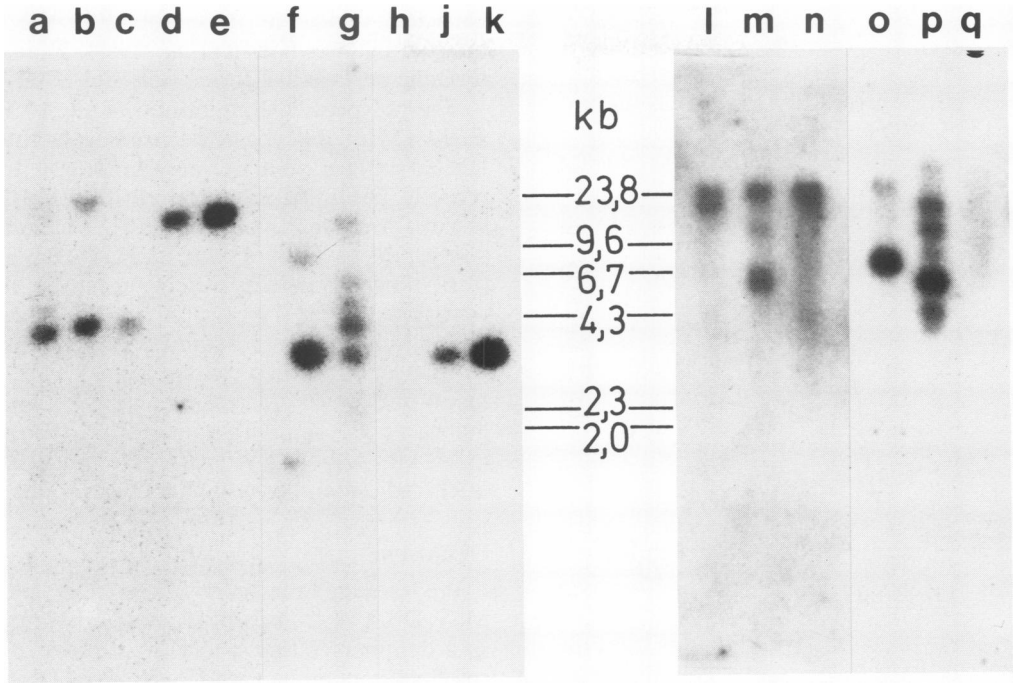

Fig. 2. Southern blot analysis of restriction endonuclease-digested high mol. wt. cellular DNA. Genomic DNA was cleaved with *Bgl*II and *Eco*RI and hybridized either to *v-mos*^{M} probe or to HBV probe as described in Materials and methods. DNA from **(a)** cell line J1, **(b)** cell line J2, **(c)** NIH 3T3 cells, **(d)** plasmid pMSVHBs4, **(e)** plasmid pMSVHBs9, **(f)** J1, **(g)** J2, **(h)** NIH 3T3, **(j)** pMSVHBs4, **(k)** pMSVHBs9, cleaved with *Bgl*II; DNA from **(l)** J2, **(m)** J1, **(n)** NIH 3T3, **(o)** J2, **(p)** J1, **(q)** NIH 3T3, cleaved with *Eco*RI. DNA seen in lanes **a**–**e** and **l,m,n** was hybridized to *v-mos*^{M}; DNA seen in lanes **f**–**k** and **o,p,q** was hybridized to the *Bgl*II fragment of HBV containing the HBsAg gene.

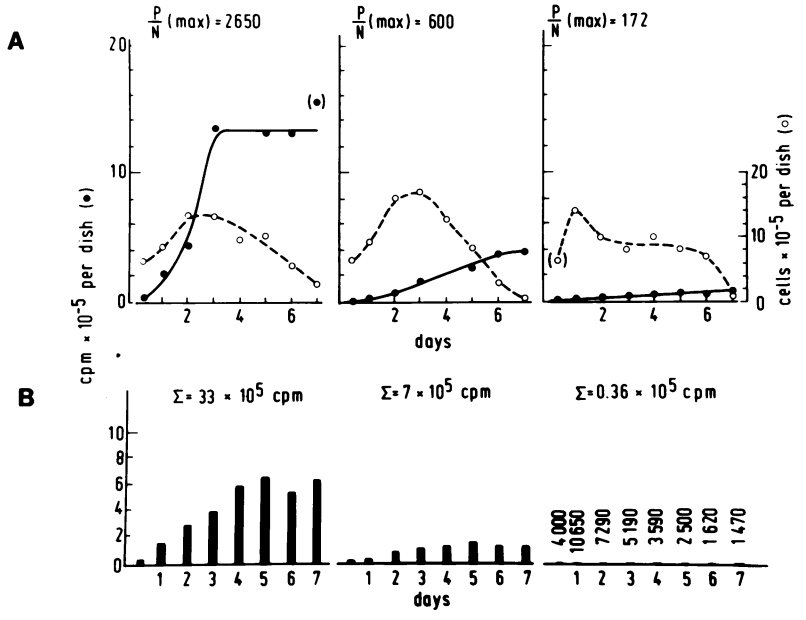

Fig. 3. Production kinetics for cell lines J1, J2, and PLC/PRF/5 (from left to right). **A**: Cells were seeded at 5 x 10^5 per 35 mm Petri dish in DME medium/10% newborn calf serum. Previously trypsinized cells were fed with 2 ml medium after overnight cultivation (time = 0). At the indicated times, medium was removed, volume was measured, and medium assayed for HBsAg by radioimmunoassay (AUSRIA II); maximum yield is expressed as the ratio of ^{125}I c.p.m. in the sample (P) relative to the negative control (N; 91 c.p.m.). The cells were removed from the same dish by trypsinization and counted with a hematocytometer. Values shown are the average of two determinations. **B**: Cells were seeded at 5 x 10^5 per 35 mm Petri dish in 2 ml DME medium/10% newborn calf serum. At the indicated times, medium was changed each day and assayed for HBsAg.

Characterization of the gene product

The nature of the HBsAg released into the medium was examined further. The supernatants of cultures of J1 and J2 cell lines were centrifuged and the pellets were analyzed in CsCl buoyant density gradients. As shown in Figure 4, all of the HBsAg banded at a buoyant density value of 1.2 g/cm^3, identical to that of the 22-nm particles found in human serum (Gerin *et al.*, 1969). Examination of the surface antigen by electron microscopy of the pooled and concentrated peak fractions from the CsCl gradient revealed 22-nm spherical

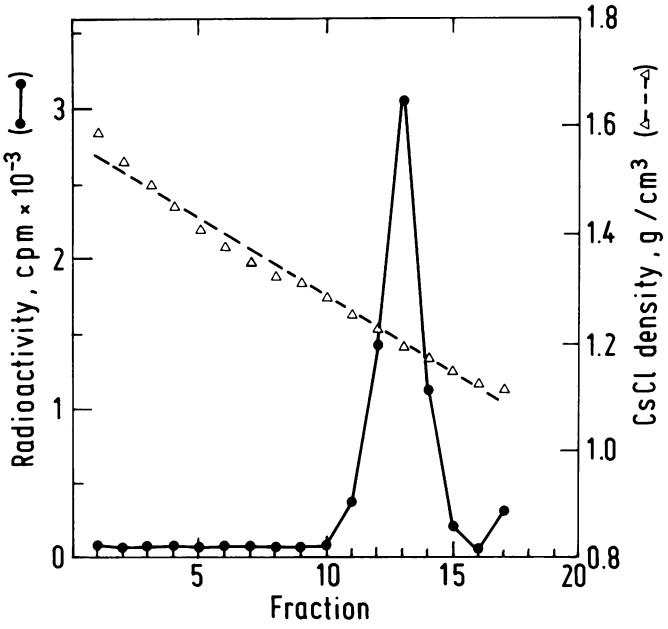

Fig. 4. Biophysical properties of HBsAg particles present in the medium of J1 cells. After low speed centrifugation to remove cell debris, 25 ml of J1 culture medium was centrifuged at 73 000 *g* at 4°C for 23 h. The pellet was resuspended in 0.5 ml 10 mM Tris, pH 7.5. 200 μl were layered onto a six step gradient (1.1 – 1.6 g/cm³) of CsCl in 10 mM Tris, pH 7.5 in a nitrocellulose tube of the Spinco SW41 rotor. The sample was centrifuged with 34 000 r.p.m. for 20 h at 14°C. Fractions (0.5 ml) were collected by bottom puncture. Aliquots were assayed for HBsAg by radioimmunoassay (AUSRIA II) after 1:50 dilution in medium. CsCl density was determined by refractometry.

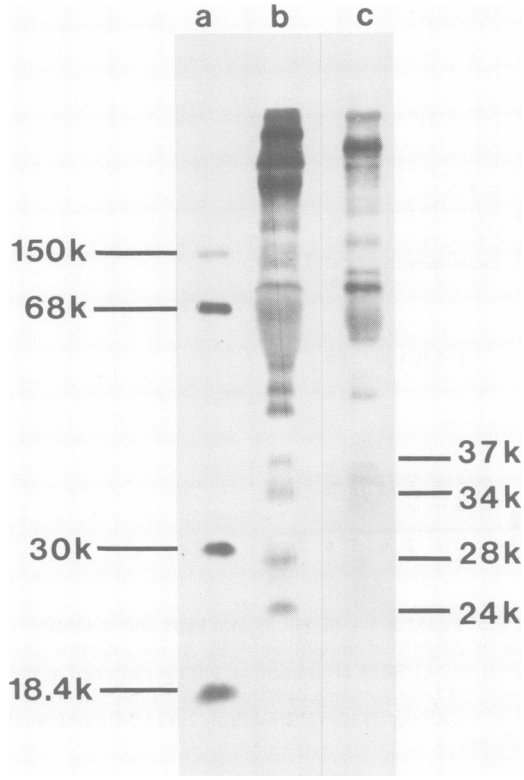

Fig. 5. Electrophoretic analysis of HBsAg polypeptides. Proteins were run on 12.5% polyacrylamide gel as described in Materials and methods. **Lane a**, ^{14}C-labeled protein standards – globulins (150 K), bovine serum albumin (68 K), carbonic anhydrase (30 K), and lactoglobulin A (18.4 K); **lane b**, proteins from cell line J1; **lane c**, proteins from untransfected NIH 3T3 cells.

particles, similar to those found in the serum of HBV-infected patients (Gerin *et al.*, 1969). Filamentous structures or Dane particle-like forms were not observed.

The polypeptide composition of the secreted HBsAg was analyzed by analytical electrophoresis of biosynthetically labeled antigen on a polyacrylamide gel. In agreement with recently published data for purified HBsAg from human serum (Stibbe and Gerlich, 1982), we could recognize four polypeptides with mol. wts. of 24, 28, 34, and 37 K. In contrast, no such proteins were found in the medium from untransfected NIH 3T3 cells (Figure 5).

For immunogenicity studies, guinea pigs were inoculated with 20 μg of purified HBsAg from cell line J1 twice at an interval of 2 weeks. Increasing antibody titers in the serum of guinea pigs could be detected by radioimmunoassay (AUSAB, Abbott). After 6 weeks, anti-HBs titers of >512 URI (radioimmunoassay units) were found.

Discussion

We have used the ability of the integrated proviral form of MSV DNA to transform mouse fibroblasts morphologically as a dominant selective marker in order to construct mouse cell lines containing the HBsAg gene. DNA blot analysis of the transformed cells indicated that HBsAg-related sequences are localized in high mol. wt. DNA and that independent transformants have integrated the HBsAg gene and *v-mos*^{M} into the host genome.

Transfection of NIH 3T3 mouse fibroblasts with a MSV-HBV recombinant resulted in the establishment of a cell line, J1, that produces high amounts of HBsAg, i.e., ~4.5 μg/10^{7} cells/day. This result was compared to the production rate of the well established human hepatoma cell line PLC/PRF/5 (Alexander *et al.*, 1976). J1 was shown to produce 20 times more HBsAg than did PLC/PRF/5 under identical conditions. The amount of HBsAg produced by J1 is similar to that obtained with bovine papilloma virus DNA as vector in this laboratory (Wang *et al.*, in preparation), or in the case of co-transfection of the HBsAg gene with the dihydrofolate reductase gene (Christman *et al.*, 1982). In the latter two cases, the high production rate of HBsAg is mainly due to amplification of the HBsAg gene, whereas in the case of the MSV-HBV recombinants the high production rate might be explained by the presence of the retroviral large terminal repeats (LTR) that have been shown to contain a so-called enhancer sequence (Levinson *et al.*, 1982).

The HBsAg synthesized by mouse fibroblasts is identical with respect to mol. wt. with the HBsAg polypeptides derived from human serum. As reported by Peterson (1981) for HBsAg from human serum, the 28-K molecule is the glycosylated form of the single 24-K HBsAg molecule. It was shown to be antigenetically indistinguishable from (Cabral *et al.*, 1978), and to contain the same amino acid composition as, the 24-K form (Peterson, 1981; Shih and Gerin, 1977). Stibbe and Gerlich (1982) showed that the 34-K and 37-K polypeptides are also glycosylated. These data indicate that the HBsAg obtained from mouse fibroblasts is also glycosylated.

Unlike yeast cells, which can also express cloned HBsAg (Valenzuela *et al.*, 1982), mouse fibroblasts secrete the HBsAg into the culture medium. The HBsAg produced by mouse fibroblasts is indistinguishable in its physical properties from HBsAg found in human serum: (i) 22-nm spherical particles are found; (ii) it is glycosylated; (iii) it is immuno-

genic in guinea pigs.

Considering these results, constructions such as the one described here might be useful as a source for HBsAg for vaccination purposes. With respect to basic research, the combination of MSV and the HBsAg gene offers possibilities for studying gene expression.

Materials and methods

Cloning of the provirus

The cloning procedure used was as described by Verma *et al.* (1982). High mol. wt. DNA was extracted from the G8-124 cell line which contains three integrated copies of MSV. The cellular DNA was cleaved by *Eco*RI and size fractionated by gel electrophoresis. DNA from one lane was transferred to a nitrocellulose filter and hybridized to a *v-mos*^{M}-specific probe (Verma *et al.*, 1980). Autoradiography showed four bands with sizes of ~14, 12, 10, and 8 kb. The 14-kb fragment contains the cellular oncogene, *c-mos*^{M} (Jones *et al.*, 1980). The other three bands originated from cellular DNA containing the integrated provirus. The shortest fragment was eluted from agarose gel using the glass-powder procedure (Vogelstein and Gillespie, 1979) and ligated to the vector arms of *Eco*RI-cleaved lambda Charon 4A. The ligation mixture was then packaged *in vitro* into infectious lambda particles and plated on *E. coli* DP50supF according to the method of Blattner *et al.* (1977). Phage plaques were assayed *in situ* for lambda-MSV recombinants. Insert DNA was further subcloned into the *Eco*RI site of pBR322. The nature of the cloned 8-kbp DNA fragment was examined by restriction endonuclease mapping.

Construction of MSV-HBV recombinants

Plasmid pA01-HBV was cleaved with *Eco*RI and the HBV DNA was isolated by electroelution after preparative electrophoresis in a 1% agarose gel. HBV DNA was recircularized by T4 DNA ligase (Boehringer Mannheim) in a highly diluted solution (10 μg DNA/ml). Recircularized DNA was purified by phenol/chloroform extraction and ethanol precipitation; resuspended in 10 mM Tris/1 mM EDTA, pH 7.5. Plasmid vector pMSV was digested with *Bgl*II and treated with calf intestine alkaline phosphatase (CIAP Grade 1, Boehringer Mannheim). Religated HBV DNA was cleaved with *Bgl*II, generating two fragments of 2.8 and 0.5 kb. Without prior separation the fragments were ligated to the *Bgl*II-cleaved and CIAP-digested pMSV vector. Ligation mixture was used to transform *E. coli* HB101. Ampicillin-resistant transformants were analyzed for recombinant plasmids using the screening method of Birnboim and Doly (1979). Clones containing the HBsAg gene were identified by restriction mapping with *Bgl*II and *Bam*HI.

All experiments were performed in a L3 facility, in compliance with the rules of the Zentrale Kommission für die Biologische Sicherheit.

Cell culture

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's minimal (DME) medium supplemented with heat-inactivated 10% newborn calf serum (Gibco) and kanamycin at 50 μg/ml. Cellular clones were free of mycoplasma contamination (Russel *et al.*, 1975).

Transfection of cells

Transfections were carried out essentially as described by Graham and Van der Eb (1973), and by Stow and Wilkie (1976). Briefly, 1 μg of purified plasmid DNA in 3.3 μl of 10 mM Tris/0.1 mM EDTA was diluted into 246.7 μl of Hepes-buffered saline (137 mM NaCl/6 mM dextrose/5 mM KCl/0.7 mM Na_2HPO_4/20 mM Hepes adjusted to pH 6.98 with 0.5 M NaOH) and 25 μg of NIH 3T3 DNA/ml as carrier was added. The DNA was precipitated by addition of 13.2 μl of 2.5 M $CaCl_2$ (final concentration 125 mM), and the mixture was incubated at room temperature for 25 min and then added to 3 x 10^5 NIH 3T3 cells in a 35 mm Petri dish. After 4 h of incubation, the cells were treated with 15% glycerol (v/v) in DME medium for 2 min and then washed twice with DME medium. After overnight incubation in DME medium/10% calf serum, the cells were trypsinized and seeded into three 50 mm Petri dishes. Foci visible 3 weeks after transfection were picked by cloning cylinders after 4 weeks, grown in mass cultures, and subsequently subcloned by limiting dilution.

Nucleic acid analysis

High mol. wt. DNA from cells was prepared and analyzed by the Southern blot transfer procedure (Southern, 1975). Briefly, 1 μg of genomic DNA, cleaved with *Bgl*II or *Eco*RI, was electrophoresed in 0.7% agarose gels (mini-gel 6 × 4 cm²) and transferred to nitrocellulose filters. Blotted DNA was hybridized to the ^{32}P-labelled *Hind*III/*Xba*I fragment of MSV, encoding *v-mos*^{M}, or to the ^{32}P-labelled *Bgl*II fragment of HBV, encoding HBsAg. Hybridizations were performed in 6 x SSC, 50% formamide, 1 x Denhardt's solution, and 100 μg/ml salmon sperm DNA at 45°C for 18 h. Filters were washed in 2 x SSC and 0.1% SDS for 15 min with three changes and in 0.1 x SSC for 15 min with two changes at 50°C. Autoradiography was carried out by exposure of Kodak X-Omat film to dried filters at −70°C, in conjunction with a Dupont Lightening Plus intensifying screen.

Protein analysis

The presence of HBsAg in the culture medium and in CsCl gradient fractions was detected with a radioimmunoassay (AUSRIA II, Abbott Laboratories) and quantitated by a parallel-line assay with a known standard. To determine the polypeptide composition, proteins were biosynthetically labeled. Cells were grown to ~80% confluency. Then culture medium was replaced with 5 ml of methionine-free medium containing 400 μCi of [^{35}S]methionine and 400 μCi of [^{35}S]cysteine. After overnight incubation, the medium was collected. Particles were pelleted down by centrifugation (100 000 *g*) and aliquots were made 5% in SDS and 0.5 M in dithiothreitol (DTT) in 30 μl, boiled for 4 min, and electrophoresed in 0.75 mm thick slabs of 12.5% polyacrylamide, 0.4% Bis, using Laemmli's buffer system (Laemmli, 1970; Stibbe and Gerlich, 1982). Gels were stained with Coomassie Blue, dried, and autoradiographed with X-ray film (Kodak XAR).

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