Comparative Expression of the Hepatitis B Surface Antigen Gene in Biochemically Transformed Human, Simian and Murine Cells

By FLORENCE COLBÈRE-GARAPIN, FLORIAN HORAUD, PHILIPPE KOURILSKY AND AXEL GARAPIN

1 Unité de Virologie Médicale and 2 Unité de Biologie Moléculaire du Gène, Institut Pasteur, 75724 Paris Cedex 15, France

(Accepted 15 April 1985)

SUMMARY

In this paper, we show that the pattern of expression of the human hepatitis B surface antigen (HBs Ag) gene, transfected along with a dominant selectable marker into mammalian cells, is complex. In human hepatoma (HepG2) cells, late transient expression occurs and permanent expression takes place at high frequencies in the selected clones. In HeLa and human xeroderma pigmentosum (GM4312A)-derived cells, the late transient expression is barely seen or absent and permanent expression is only seen in a few selected clones. In monkey kidney Vero cells, late transient expression has been described and we show in this report that only 5% of the selected clones are capable of expressing HBs Ag in a permanent manner. In most of the Vero clones, the absence of HBs Ag expression is mainly due to HBs Ag gene rearrangements. We have selected and amplified more than 500 transfected Vero clones and have characterized in detail one clone (GAR1412) which is a permanent high-level HBs Ag expressor.

INTRODUCTION

Hepatitis B viruses are non-cytopathic DNA viruses which replicate in a mode similar to that of retroviruses (Summers, 1984). The human hepatitis B virus (HBV) causes polymorphic liver diseases in man and certain non-human primates (Szmuness, 1975). The host cell range of HBV is more extensive than had been previously thought, since HBV DNA has been detected not only in hepatocytes but also in bile duct epithelium, endothelial cells, smooth muscle cells of blood vessel walls (Blum et al., 1983), and lymphoblastoid cells from bone marrow (Romet-Lemonne et al., 1983). There is no in vitro cellular system available in which the replication of HBV can be studied. The expression of the human hepatitis B surface antigen (HBs Ag) has, however, been studied in vitro in some human hepatoma cell lines which spontaneously produce the HBs Ag (Alexander et al., 1976; Koike et al., 1983) and by gene transfer. Transient and/or stable expression have been developed using virus vectors (Moriarty et al., 1981; Liu et al., 1982; Stratowa et al., 1982; Crowley et al., 1983; Smith et al., 1983; Stenlund et al., 1983; Wang et al., 1983) or recircularized HBV DNA (Hirschman et al., 1980; Wang et al., 1982). Stable expression of the HBs Ag has been obtained in rodent cells following transfection with a selective marker (Dubois et al., 1980; Gough & Murray, 1982; Christman et al., 1982). Stable expression of HBV core Ag takes place in human cells (Yoakum et al., 1983).

We have shown that plasmids bearing the aminoglycoside-3'-phosphotransferase [APH(3')] dominant selectable marker and the HBs Ag gene induce in Vero cells an HBs Ag expression during the first weeks following transfection (Colbère-Garapin et al., 1983) by either the calcium phosphate technique (Graham & van der Eb, 1973) or the protoplast fusion technique (Schaffner, 1980). This expression in Vero cells was termed 'late transient' (Colbère-Garapin et al., 1983) because its maximum occurs 3 to 14 days after transfection and then levels off. A similar kind of expression has been described in NIH 3T3 cells after transfection with a bovine papillomavirus vector carrying the HBs gene (Wang et al., 1983). Late transient expression is...
very weak or even undetectable in murine L cells in the first weeks after transfection (Colbère-Garapin et al., 1983) but occurs with recircularized HBV DNA after transfection into NIH 3T3 cells (Wang et al., 1982).

We report an investigation of the pattern of HBs Ag gene expression in a variety of cells which includes HBV-free human cells of liver (HepG2) and non-liver origin (HeLa and GM4312A). We also extend our previous observations on Vero cells to show that rare stable transformants expressing the HBs Ag gene can be obtained, and describe in detail one of them. Comparison of the results obtained in primate and murine cell lines shows important differences that suggest complex control mechanisms for the expression of the HBs Ag gene under its own regulatory sequences.

METHODS

Cells. Vero cells of clone VC10 (Colbère-Garapin et al., 1983) and HeLa cells were passaged in Dulbecco's modified Eagle's medium, supplemented with 10% calf serum and 10% tryptose phosphate broth. The HBV-free human hepatoma HepG2 cells (Aden et al., 1979) were passaged in minimum essential medium supplemented with 10% foetal calf serum. The GM4312A cells were obtained from the NIGMS Human Genetic Mutant Cell Repository and are derived from human xeroderma pigmentosum cells transformed by simian virus 40 (SV40). They were cultivated in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum. Mouse L cells were cultivated in Leibovitz L15 medium supplemented with 10% calf serum.

Plasmid constructions. The recombinant plasmid pAG141 carrying the HBs gene and the dominant selective marker APH(3') (Colbère-Garapin et al., 1981) was derived from plasmid pAG66 previously described (Colbère-Garapin et al., 1983) by deletion of a 145 base pair (bp) Stul fragment in the 3' non-coding region of the gene, as shown on the HBs gene map of Fig. 1. Plasmid pAG66 was first digested with Stul which cleaves the HBs DNA at positions 526, 721, 1645 and 1790 and the vector was ligated to the 924 bp Stul fragment carrying the HBs coding region. The recombinant plasmid pAG140 having the HBs gene in the same orientation as in pAG66 was then cleaved with XbaI (position 925 on the HBs gene map, Fig. 1) and PvuI (position 3735 on the pBR322 map) and the 2160 bp fragment forming the HBs coding region was ligated to the 6000 bp XbaI-PvuI fragment of pAG66. The resulting plasmid, pAG141, has the HBs gene promoter (Cattaneo et al., 1983) and the pre-S region. Upstream of the HBs BglII site, a 440 bp BglII-SphI DNA fragment was shown by sequence analysis to be a fragment of herpes simplex virus thymidine kinase DNA and not a fragment of APH(3') DNA as previously reported for pAG66 (Colbère-Garapin et al., 1983). Most of the 3' untranslated region of the HBs gene is present in pAG141, except the deleted 145 bp Stul fragment, and 9 bp upstream of the presumed polyadenylation signal (Cattaneo et al., 1983).

Two other plasmids, pAG400-2 and pAG400-4, containing the HBs gene promoter and 700 bp upstream of it, the coding region and the entire 3' untranslated region, were constructed (Fig. 1). A 2744 bp BglII DNA fragment was isolated from plasmid pCP10 (Charnay et al., 1980; Dubois et al., 1980) by a partial BglII digestion. This DNA fragment, which does not contain the core antigen gene, was then ligated to the vector pAG60 linearized with BamHI and treated with alkaline phosphatase as previously described (Colbère-Garapin et al., 1981). The two recombinant plasmids pAG400-2 and pAG400-4 differ in the orientation of the HBs gene. In pAG400-2, the HBs gene and the APH(3') gene have the same orientation.

Cell transfection. Subconfluent cell monolayers (3 × 10⁶ cells) were transfected with 10 µg of plasmid, by calcium phosphate precipitation (Graham & van der Eb, 1973) in the presence of aprotinin (Sigma), followed by butyrate treatment as described by Gorman & Howard (1983). Twenty-four h after transfection, cell culture medium containing G418 (Geneticin, Gibco) was added to the cells. The pure G418 concentration used was 150 µg/ml for VC10, HeLa and L cells, 100 µg/ml for GM4312A cells and 300 µg/ml for HepG2 cells.

Radioimmunoassay. HBs antigen detection in cell supernatants was performed by solid phase radioimmunoassay using an Austria II kit (Abbott Laboratories, North Chicago, Ill., U.S.A.). A purified HBs Ag preparation containing 7 µg of protein per ml was used as a reference. To determine the amount of intracellular HBs antigen, cells were washed three times in phosphate-buffered saline (PBS), scraped off with a rubber policeman, centrifuged, lysed by sonication and the cellular extract was clarified at 5000 g for 10 min at 4°C before HBs Ag assay. To test the immunogenicity of the excreted HBs Ag, a Merck, Sharpe & Dohme HBs immunogenicity reference sample containing 20 µg of HBs antigen per ml was used as a standard.

HBs antigen purification and immunoprecipitation. The HBs Ag in the supernatant of confluent cell cultures was clarified at 10000 g for 10 min at 4°C, pelleted at 22000 r.p.m. for 24 h at 4°C with a SW41 rotor, and purified in a CsCl step gradient (10, 20, 30 and 40% w/w in PBS) at 30000 r.p.m. for 22 h at 4°C in a SW41 rotor. The HBs Ag peak was localized by radioimmunoassay. Fractions containing the HBs Ag were pooled, diluted threefold in H₂O, and precipitated with monoclonal antibodies overnight at 20°C. The immune precipitate was then pelleted at 12000 g for 45 min at 4°C.
HBs antigen expression in animal cells

Fig. 1. Plasmids carrying the HBs gene and the dominant selective marker APH(3'). The HBs coding region is indicated with a thick line, and the non-coding region with a broken line. Coordinates of the HBs gene region are numbered from the RsaI site upstream of the pre-S region. This RsaI site corresponds to coordinate 680 on the DNA sequence of Galibert et al. (1979). On the HBs gene map, the TATA box upstream of the pre-S region, the HBs gene promoter (Pr), the start (ATG) and stop codon (TAA) of the coding region, and the polyadenylation signal (TATAAA) are shown. The HBV DNA fragments used to construct the recombinant plasmids are indicated. Plasmid pAG141 has a 145 bp deletion in the 3' non-coding region of the gene (see Methods). In the recombinant plasmids, APH(3') sequences are indicated with a double line, and thymidine kinase sequences with dots. The direction of gene transcription is indicated by arrows. EcoRI sites (O) used in Southern blot experiments (Fig. 5) are shown. The ampicillin resistance gene (Ap') is indicated.

Southern blot hybridization. Cellular DNA was extracted by treatment with proteinase K (Merck; 500 μg/ml) overnight at 50 °C in the presence of 0.2% SDS followed by phenol–chloroform–isoamyl alcohol extractions (Colbère-Garapin et al., 1981), and cleaved with restriction enzymes. DNA fragments were separated by gel electrophoresis in 0.6% agarose, and transferred to a nitrocellulose filter according to Southern (1975). The probe consisted of the 2.3 kb BglII DNA fragment carrying the HBs gene. It was isolated from pAG83 (Colbère-Garapin et al., 1983) and labelled by nick translation (sp. act. 5 × 10^6 c.p.m./μg) as described (Colbère-Garapin et al., 1981). Hybridization was carried out overnight at 68 °C. A Kodak X-Omat XAR-5 film was used for autoradiography.

Northern blot hybridization. Poly(A)+ mRNA was extracted (Auffray & Rougeon, 1980) from cell clones and isolated by oligo(dT)-cellulose column chromatography. RNA was denatured for 5 min at 65 °C in the presence of glyoxal and dimethylsulphoxide and electrophoresed on a horizontal 1.1% agarose gel at 4 °C with constant recirculation of the buffer. RNA transfer to nitrocellulose filters and hybridizations were performed by the method of Thomas (1980). The prehybridization and hybridization steps were performed at 42 °C for 16 and 20 h, respectively. The 2.3 kb BglII DNA fragment carrying the HBs gene was labelled by nick translation and used as a probe (Colbère-Garapin et al., 1983). The filter was autoradiographed with Kodak X-Omat XAR-5 film.

RESULTS

Construction of plasmids carrying the HBs gene

The HBs Ag gene transcription unit covers approximately 2700 bp of the HBV genome (Pasek et al., 1979). The major HBs Ag transcript depends on a promoter located in the pre-S region. This major promoter shows some DNA homology with the late promoter of SV40 (Cattaneo et al., 1983). The TATA box found upstream can act as a promoter (our unpublished results) but at a low level. The polyadenylation site and the processing site for the major HBs Ag transcript lie within the core region (Pourcel et al., 1982; Cattaneo et al., 1983).
Fig. 2. HBs Ag expression by HepG2 cells transfected with pAG400-4. Flasks of HepG2 cells containing $3 \times 10^6$ cells were transfected with 10 µg of pAG400-4, using the calcium phosphate technique (Graham & van der Eb, 1973). The amount of HBs Ag excreted in the cell supernatant was determined by radioimmunoassay 1, 3, 8, 12, 14, 16 and 17 weeks after transfection. Each value represents the average of two flasks.

Fig. 3. Level of stable HBs Ag expression by pAG400-2 or pAG400-4 transformed HepG2, Vero and mouse cells. The proportion of HepG2 cell clones (open bars), Vero cell clones (solid bars) and mouse L cell clones (dashed bars) expressing various amounts of HBs Ag are indicated by vertical bars.

A 2744 bp BglII fragment obtained from plasmid pCP10 (Dubois et al., 1980) was cloned in both orientations in plasmid pAG60 (Colbère-Garapin et al., 1981) to give pAG400-2 and pAG400-4 (Fig. 1). In addition, starting from pAG66 (Colbère-Garapin et al., 1983), plasmid pAG141 (Fig. 1) was obtained by deleting a 145 bp StuI-StuI DNA fragment in the 3' untranslated region of the HBs gene (for the details of the construction, see Methods). The level of HBs Ag expression induced by pAG141 is three- to fivefold lower than that of pAG66. The deletion of the 195 bp StuI-StuI DNA fragment at the 5' end of the HBs gene, which removes the HBs gene major promoter, reduced the HBs Ag expression over tenfold. The plasmids pAG66, pAG141, pAG400-2 and pAG400-4 carry the dominant selective marker APH(3') and transformed cells can be screened in the presence of the antibiotic G418 (Colbère-Garapin et al., 1981).

Transient expression of the HBs Ag gene in a variety of cells

Three human cell lines (HeLa, GM4312A and HepG2), simian (Vero) cells and murine (L) cells were transfected with the plasmids pAG400-2 and pAG400-4. GM4312A is a human xeroderma pigmentosum cell line transformed by SV40. The HepG2 line is, so far, the only human hepatoma cell line (Knowles et al., 1980) that retains the normal biochemical functions of human parenchymal cells and is devoid of the HBV genome (Simon et al., 1982).

One week after transfection, the amount of excreted HBs Ag was measured in the supernatant of cells selected by G418. High transient expression was obtained in two instances: in Vero cells as previously reported (Colbère-Garapin et al., 1983) and in HepG2 cells (Table 1, column 1 and Fig. 2). Transient expression was weak or undetectable in HeLa, GM4312A or L cells. The interpretation of this result is not straightforward because it depends on the level of expression...
Table 1. *Late transient and stable HBs Ag expression in mammalian cells transfected with the entire HBs Ag gene*

<table>
<thead>
<tr>
<th>Transfected cells*</th>
<th>Late transient HBs Ag expression† (ng/ml)</th>
<th>Transformation efficiency (colonies/µg of DNA)</th>
<th>HBs Ag+ clones/No. of clones tested (%)</th>
<th>HBs Ag expression among HBs Ag+ clones‡ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero VC10</td>
<td>17</td>
<td>8</td>
<td>16/316</td>
<td>5</td>
</tr>
<tr>
<td>HepG2</td>
<td>23</td>
<td>5</td>
<td>7/8</td>
<td>87</td>
</tr>
<tr>
<td>HeLa</td>
<td>2.5</td>
<td>5</td>
<td>1/12</td>
<td>8.3</td>
</tr>
<tr>
<td>GM4312A</td>
<td>1.6</td>
<td>2</td>
<td>1/8</td>
<td>12.5</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>150</td>
<td>28/30</td>
<td>93</td>
</tr>
</tbody>
</table>

* Cells were transfected with pAG400-2 or pAG400-4.
† One week after transfection.
‡ Average HBs Ag concentration for isolated HBs Ag+ clones, 1 week after trypsinization.
§ The HBs Ag expression of this HeLa clone disappeared after five cell passages.

as well as on the efficiency of DNA uptake, which could be different in the different cell lines. However, in these experiments, stable resistance to G418 was acquired by a similar number of clones of primate cells (Table 1). The efficiency was 1 to 2 orders of magnitude higher in murine L cells.

**Stable expression of the HBs Ag gene**

After several weeks of selection in G418, G418-resistant (G418R) clones were isolated, amplified and tested for HBs Ag expression. The results are shown in Table 1. Only 5% of the G418R Vero clones were able to express the HBs Ag permanently. One out of 12 G418R HeLa clones was HBs Ag-positive (HBs Ag+) but its expression disappeared after five cell passages. As for GM4312A cells, one out of eight G418R clones expressed the HBs Ag in a stable manner. In striking contrast, seven out of eight G418R HepG2 clones were HBs Ag+. This proportion (87%) of HepG2 clones is close to the 93% of the G418R HBs Ag+ clones obtained in murine L cells. Finally (Table 1, column 5), the average value of HBs Ag expression for isolated clones clearly shows that the highest expression level is reached in the HepG2 cells (of hepatic origin) followed by the murine L cells (of non-hepatic origin).

**Comparative expression of the HBs Ag gene in HepG2, Vero and L cells**

In an attempt to understand the differences between the three cell lines in which a significant HBs Ag expression was found, further comparisons were carried out. More detailed kinetic studies were undertaken. The kinetics of HBs Ag expression in HepG2 cells is shown in Fig. 2. The HepG2 cells display a transient expression 1 to 2 weeks after transfection, then the HBs Ag excretion levels off for about 4 weeks to reappear as a permanent expression along with the growth of the G418R clones. By comparison, transfected Vero cells displayed a peak of expression at 1 or 2 weeks after transfection, followed by a decrease to an undetectable level, reflecting the low percentage of expressors (Colbère-Garapin et al., 1983). The murine L cells, in contrast, at 1 or 2 weeks after transfection exhibited a slow increase in synthesis, stabilizing at a plateau level after 4 weeks (Colbère-Garapin et al., 1983).

Secondly, we compared the distribution of HBs Ag expressors and non-expressors among stable G418R clones. The largest survey was carried out with Vero cells because of the paucity of expressors among such clones. The histograms in Fig. 3 show the distribution of expressors and non-expressors as percentages of the G418R clones for HepG2, Vero and L cells. The HepG2 data were included because, although the number was low, the proportion of permanent G418R HBs Ag+ clones is significant.

L and HepG2 cells exhibited similar distributions and the level of HBs Ag expression of the clones varied by a factor of 10. The Vero cells, on the other hand, displayed a different distribution with a vast majority of weak or non-expressors.
Fig. 4. (a) Kinetics of HBs Ag expression by clone GAR1412. GAR1412 cells were seeded in T25 flasks and fed with Dulbecco's modified Eagle's medium, supplemented with 10% calf serum and 10% tryptose phosphate broth. At days 1, 4, 7, 11, 14, 18, 21, 25 and 32, the supernatant medium of two flasks was removed and HBs Ag was quantified (○). The corresponding cell monolayers were trypsinized and cells were counted using a Coulter Counter (●). The amount of HBs Ag present in the cells was measured at days 5 and 19. (b) HBs antigen produced by GAR1412 cells purified in a CsCl gradient and immunoprecipitated by monoclonal anti-HBs antibodies. The bar marker represents 22 nm.

Table 2. Frequency of stable HBs Ag expression among Vero cell clones*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of clones tested</th>
<th>No. of HBs Ag+ clones</th>
<th>% HBs Ag+ clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAG66</td>
<td>173</td>
<td>10</td>
<td>5.7</td>
</tr>
<tr>
<td>pAG141</td>
<td>66</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>pAG400-2</td>
<td>217</td>
<td>13</td>
<td>5.9</td>
</tr>
<tr>
<td>pAG400-4</td>
<td>98</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>554</td>
<td>31</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* For comparison, 30 mouse L cell clones transformed by pAG400-2 or pAG400-4 were tested and 93% were found to be HBs Ag+

Analysis of the stably expressing Vero clones

We have previously shown that the synthesis of HBs Ag in simian Vero cells is markedly unstable (Colbère-Garapin et al., 1983). Following calcium phosphate transfection into Vero cells of pAG66, pAG141, pAG400-2 or pAG400-4, a total of 554 G418R clones was isolated and each clone was grown to confluence (Table 2). Only a low percentage of Vero G418R clones (about 5%) were able to express the HBs Ag in a stable manner. The results also show that none of the four plasmids, whether bearing a limited HBV fragment (pAG66), the same fragment with a deletion (pAG141), or a more extended sequence (pAG400-2 or pAG400-4), was capable of inducing, in Vero cells, a permanent HBs Ag expression at high frequency such as occurs in HepG2 cells (Tables 1 and 2).
HBs antigen expression in animal cells

Fig. 5. Blot hybridization of HBs DNA sequences in HBs Ag+ Vero cell clones. DNA fragments were separated in 0.6% agarose gel electrophoresis, transferred to a nitrocellulose filter (Southern, 1975) and hybridized with the HBs gene carried by a 2.3 kb BglII DNA fragment labelled by nick translation. The arrows indicate the positions of markers of 3.8, 2.5, 2.4, and 1.1 kb. Left panel: plasmid pAG400-2 cleaved with EcoRI (lanes a and d) and plasmid pAG400-4 cleaved with EcoRI (lanes e and h) were used as internal markers; EcoRI-cleaved DNA of Vero cell clones transformed by pAG400-2 (lanes b and c) and Vero cell clones transformed by pAG400-4 (lanes f and g). Right panel: GAR1412 DNA cleaved with HpaI (lane c), EcoRI (lane d) and BamHI (lane e); DNA fragments hybridizing specifically with the HBs gene were used as markers, i.e. 2.4 kb and 2.5 kb (faint band) (lane a), and 2.1 and 2.8 kb (lane b).

Only 0.5% of the G418R Vero clones expressed high levels (100 ng/ml to 1 μg/ml per day) of HBs Ag in a stable manner. One such clone, GAR1412, is a high-expressor Vero clone obtained by transfection with plasmid pAG141. We believe that the occurrence of GAR1412 is not related to the genetic structure of the HBs gene in pAG141 but is purely fortuitous, for many other pAG141-transformed Vero clones were weak or non-expressors. However, this clone was studied in greater detail because of its high level of expression (Fig. 4) and its potential interest for vaccine development. This level of expression is similar to the level obtained with the HBs Ag-producing hepatoma cell line described by Alexander et al. (1976) (not shown). Trypsinization of GAR1412 cells reduced HBs Ag synthesis to a low level which rises again steadily and reaches a peak level after cell confluence (Fig. 4a). A continuous level of expression of 500 ng to 1 μg/ml per day was obtained with clone GAR1412 over more than 6 months of culture during which the cells were fed twice weekly without passaging. The stability of HBs Ag expression has been studied on clone GAR1412 over a period of 7 months (100 cell generations).
Fig. 6. Northern blot analysis of HBs mRNA in HBs+ clones. Poly(A)+ mRNA was extracted from cell clones, denatured and electrophoresed on a horizontal 1.1% agarose gel at 4°C. RNA transfer to the nitrocellulose filter and hybridization were performed by the method of Thomas (1980). The HBV DNA probe was a 2300 bp BgII fragment isolated from plasmid pAG83 and labelled by nick translation. The arrows show DNA fragments obtained by digestion of pAG66 (Colbère-Garapin et al., 1983) with EcoRI, denatured and used as molecular weight markers of 4, 2.4 and 1.9 kb (a). Poly(A)+ mRNA was isolated from HBs+ monkey cell clones: (b) GAR66-17, 3 μg; (c) GAR141-13, 2 μg; (d) GAR1412, 0.4 μg; (e) GAR1412, 0.5 μg, and a mouse L cell clone transformed by pAG66, 1 μg of mRNA (f). In (d), mRNA was extracted from cells 4 days after trypsinization, while in (e) mRNA was extracted from 17-day-old cells. No mRNA homologous to the HBs gene was detected in non-transformed monkey cells (g).

No difference in the rate of synthesis was noted when the cells were grown in the presence or the absence of the G418 antibiotic. Over 85% of the antigen was found in the supernatant. The excreted antigen had the same buoyant density in CsCl as the antigen prepared from human plasmas and was visualized in electron microscopy as characteristic 22 nm spherical particles (Fig. 4b).

The immunogenicity of the HBs Ag was tested in BALB/c mice. Seroconversion of 50% of the animals was induced by 60 ng of purified HBs Ag produced by GAR1412 or by the same amount of the reference antigen used as a positive control (not shown). Subcutaneous inoculation of 4 x 10^6 GAR1412 cells or of the same amount of Vero cells did not induce tumours in nude mice.

In view of the paucity of HBs Ag+ Vero clones, it was of interest to study the pattern of integration of the plasmid, to look for possible DNA rearrangements. Southern blots were performed with the cellular DNA of G4188 HBs Ag+ clones cleaved with EcoRI, BamHI (Fig. 5) or HindII (not shown) and hybridized with a 2.3 kb BgII-BgII probe. No DNA rearrangements were detected (Fig. 5). Most of the hybridization bands corresponded to the homologous plasmid bands. The HBV DNA bands which did not correspond to plasmid bands are most probably junction fragments between plasmid and cellular DNA. The enzyme HpaI which does not cleave plasmid pAG141 produced one major band of hybridization with
HBs antigen expression in animal cells

GAR1412 DNA cleaved by HpaI. This indicates that there is one major integration site of the HBs gene in GAR1412 DNA (Fig. 5). This pattern was stable for at least 1 year after cloning. The absence of rearrangements in G418\(^{R}\) HBs Ag\(^{+}\) clones is in sharp contrast with what occurs in most of the HBs Ag-negative clones, in which the HBs gene region has undergone some degree of rearrangement (Colbère-Garapin et al., 1983).

The major species of poly(A\(^{+}\)) mRNA specific to the HBs gene was 2350 nucleotides (nt) long in monkey and in mouse HBs Ag\(^{+}\) clones transformed by pAG66 (Fig. 6). The mRNA was shorter in pAG141-transformed cells (about 2200 nt) due to the 145 bp deletion in pAG141 (Fig. 1). When the mRNA of GAR1412 cells cultured for 17 days without renewal of the medium was compared to the mRNA of 4-day-old GAR1412 cells, there seemed to be an enrichment of specific HBs mRNA in the 17-day-old cells (Fig. 5, lanes d and e) as judged by the relative intensities of the bands.

DISCUSSION

In this report, we describe the expression of the HBs Ag gene under control of its own regulatory sequences and under non-lytic conditions in human, simian and murine cells. Our purpose was to study and compare the different phases of expression of the HBs Ag gene from the time of transfection up to the establishment of the clones, in cells of both hepatic and non-hepatic origin.

In the human hepatoma HepG2 cells, the HBs Ag gene is expressed after transfection during a late transient phase, levels off, then is expressed again in a permanent phase coincident with the growth of transformed colonies. This permanent expression is obtained at a high level in the majority of the selected clones. Thus, the HBs Ag gene is expressed both transiently and in stable transformants of human hepatoma HepG2 cells. This is not unexpected, since the hepatitis B virus is predominantly replicated and expressed in human liver during acute infection.

This contrasts sharply with the pattern of expression observed in the other cell lines studied: in the human HeLa and xeroderma pigmentosum GM4312A cells, using the same plasmids, we found very weak or no late transient expression, and a very low frequency of stable expressors. We have recently shown, by Southern blots, that in these two human cell lines of non-liver origin, the HBV DNA is lost or rearranged (F. Colbère-Garapin & A. Garapin, unpublished results).

For mouse L cells, late transient expression is barely detectable. This suggests that HBs Ag expression might be species- and/or tissue-specific. However, when integrated stably into the mouse chromosomes, the HBs Ag gene seems to be triggered for permanent synthesis since the mouse clones are good expressors, albeit not to the level obtained in the HepG2 cells. It is not yet clear whether the late transient expression is due to plasmid molecules which are autonomous after transfection or to an early integration of the gene.

The histograms (Fig. 3) show a similar distribution for the L and HepG2 clones with levels of HBs Ag expression varying over a tenfold range, as in the case of transfected APH(3\(^{'}\)) gene (Colbère-Garapin et al., 1981). This probably reflects a difference in the site of chromosomal integration from clone to clone.

In the case of Vero cells, we have previously shown that HBV DNA rearrangements often take place (Colbère-Garapin et al., 1983). We have so far found no DNA sequence, either in the 5\(^{'}\) region upstream of the promoter or in the 3\(^{'}\) untranslated region, which, when deleted, allows both stable integration and HBs Ag expression in Vero cells. Point mutations and small deletions have been observed in shuttle vectors transfected into simian cells (Razzaque et al., 1984). About 5% of the G418\(^{R}\) Vero clones express the HBs Ag stably and the expression level is generally low in these cells (Tables 1 and 2). These results are in agreement with those of Carloni et al. (1984). Only 0-5% of the G418\(^{R}\) Vero clones express more than 100 ng of HBs Ag per ml per day. The high HBs gene expression level in some Vero clones might depend on the HBs gene copy number and on the integration site. One clone, GAR1412, which synthesizes as much HBs Ag as the human hepatoma cell line isolated by Alexander et al. (1976), has been studied in detail. Besides its high level of expression (500 ng to 1 \(\mu\)g of HBs Ag per ml per day) over at least
6 months during which the cells were fed without passaging, its pattern of HBs Ag synthesis is remarkable for a non-liver cell: trypsinization of GAR1412 cells reduces the expression of HBs Ag to a low level which rises again steadily and reaches its maximum after cell confluence (Fig. 4). Similar kinetics of synthesis have been observed in human hepatoma HBs Ag+ cell lines (Aden et al., 1979). Recently, a reciprocal modulation of the cellular growth functions and of the differentiated functions of mature rat hepatocytes has been demonstrated (Nakamura et al., 1983). The differentiated functions in primary rat hepatocytes are, therefore, maximum when cell-to-cell contact increases. A similar result has been reported for ß-foetoprotein (Aden et al., 1979) synthesized by human hepatocytes. In the last two cases, the differentiated functions govern the expression of secreted liver proteins.

This pattern of synthesis might also reflect the physiological properties of a secreted protein in a liver or non-liver cell. No difference from the plasma 22 nm particles has been noted in the quaternary structure, the antigenicity or the immunogenicity of the HBs Ag produced by GAR1412.

We find no apparent simple relationship between the late transient expression of the HBs Ag gene and its long-term stable expression in eukaryotic cells. The hepatoma HepG2 cell line exhibits the expected two-phase kinetics of expression from the time of transfection to the time of clone growth. The stable expression of the HBs Ag in HepG2 cells is reminiscent of the immunoglobulin and ß-globin genes whose expression is tissue-specific (Rice & Baltimore, 1982; Chao et al., 1983). It is conceivable that a particular identifier sequence is required for tissue-specific HBs Ag expression in primate cells, as shown by Sutcliffe et al. (1984) for genes transcribed in the brain. The stable or unstable expression of the HBs Ag in Vero cells and, perhaps, in other primate cells, might be the consequence of two different phenomena: the rearrangement of the transfected DNA before or after integration into cellular DNA and a modulation of transcription suggested by a low level of synthesis in most of the G418 R HBs Ag+ Vero clones.

We thank Dr B. Knowles for her permission to use HepG2 cells, Dr R. Bertolotti for giving us the HepG2 cells, M. C. Dauguet for electron microscopy, Dr R. Crainic and Mrs A. Candréa for immunization of mice, Dr J. Pilot for monoclonal anti-HBs antibodies, Dr M. Ferguson (National Institute for Biological Standards and Control, London, U.K.) and Dr P. Adamowicz (Institut Pasteur Production) for antigen standards, Dr P. Daniels (Schering Corporation) for providing G418, Dr C. Tram for PPLO testing and Dr P. Candido for critical reading of the manuscript. We are grateful to Mrs J. Lavalé and M.-L. Ryhiner, Miss P. Brisset and I. Stephany for skilful technical assistance and to Mrs N. Perrin and V. Caput for typing the manuscript. This work was supported by grants from C.N.R.S. (95.5097 and 033916), I.N.S.E.R.M. (124006 and 135042, as well as S.C. 20), M.I.R. (82V1379) and from C.C.A.R. Institut Pasteur, Paris (grant 7920).

REFERENCES


HBs antigen expression in animal cells

1751


*(Received 3 December 1984)*