

Production of immunologically active surface antigens of hepatitis B virus by *Escherichia coli*

(serology/animal viruses/recombinant DNA)

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ABSTRACT Several plasmids have been constructed which direct the synthesis of hepatitis B virus surface antigens in *Escherichia coli* either as the native polypeptide or fused to other plasmid encoded polypeptides. When injected into rabbits, extracts from bacteria carrying some of these plasmids induced the synthesis of antibodies to the antigens even though the extracts did not give satisfactory positive results in radioimmunoassay for them. Either the NH₂-terminal segment or the COOH-terminal segment of the surface antigens alone was sufficient to elicit the immune response, but antibodies against the two segments showed different specificities. The results emphasize the value of an *in vivo* assay for the presence of antigens in crude cell extracts and illustrate the feasibility of this type of screening with laboratory animals.

Hepatitis B virus (HBV) is widespread, but detailed studies of its molecular structure and of its pathobiology have been frustrated by the inability to propagate the virus in cultured cells (1). DNA from HBV has now been cloned and propagated in *Escherichia coli*, both as a series of fragments and as entire linear molecules after joining to plasmid or lambdaoid phage vectors (2-4), to provide HBV DNA for nucleotide sequence determination from which the general organization of the viral genome was deduced (5-7).

Several recombinants derived from HBV DNA fragments expressed the HBV core antigen (HBcAg) gene efficiently (2, 7), giving a product that was readily detectable by a solid-phase radioimmunoassay (8) and that induced antibody synthesis in rabbits (7). Production of the HBV surface antigen (HBsAg) gene product was more difficult to demonstrate by the radioimmunoassay (2, 7, 8). The HBV DNA sequence (7) shows that the genes for HBcAg and HBsAg are in different translational phases (1 and 3, relative to each other; see Fig. 1) and that the coding sequence for HBcAg (nucleotides 1-549) not only precedes that for HBsAg (nucleotides 1437-2114) by a considerable distance but also that, between the two genes, there are several translational termination signals in the phase of the HBsAg coding sequence. If expression of the HBV antigen genes in *E. coli* is regulated by *E. coli* control sequences, it is improbable that both HBcAg and HBsAg could be produced from a recombinant plasmid with these two genes in their native configuration.

Knowledge of the HBV DNA sequence (7) permits the design of new DNA molecules from which HBsAg should be expressed efficiently as either the native polypeptide or as a hybrid polypeptide fused to part of an *E. coli* gene product such as β -lactamase or β -galactosidase. Several such molecules have been constructed and shown to direct the synthesis of polypeptides that exhibit HBsAg activity. These should be useful as a basis

of vaccines against HBV because preparations of HBsAg subunits can protect chimpanzees against HBV infection (9).

MATERIALS AND METHODS

Recombinant Plasmids and Phage. The primary recombinant plasmids of HBV DNA in pBR322 described by Burrell *et al.* (2) were used as donors for further cloning experiments with the plasmid vectors pBR322 (10), pKT234 and pKT279 (11), and pEXlac205 (12) in the *E. coli* strain HB101. The λ T4 lig phage NM989 (13) was used as a vector into which recombinant plasmids were inserted after digestion with *Eco*RI. The transfection host strain and strain used for lysogenization were as described (13). Details of restriction and ligation reactions, polynucleotide terminal transferase reactions, and transformation and transfection operations have been reported (2).

Recombinant plasmids were characterized by their drug-resistance markers and their structures were confirmed by the fragments released on digestion with restriction enzymes and by nucleotide sequence analysis (14, 15). Recombinant phage were identified by plaque hybridization (16) against a ³²P-labeled probe made by nick-translation (17) of the plasmid pHBV114 (7). Most enzymes were prepared in the various laboratories or were purchased from New England BioLabs, Bethesda Research Laboratories (Rockville, MD), or Boehringer Mannheim AG.

Radioimmunoassays. Reagents used for the solid-phase radioimmunoassay adapted for direct screening of bacterial colonies or phage plaques (8) were as described (2). In double-antibody radioimmunoprecipitation assays (18), rabbit serum (50 μ l, diluted appropriately) was incubated with ¹²⁵I-labeled HBsAg (¹²⁵I-HBsAg; 50 μ l, containing 3000 cpm) at 4°C for at least 18 hr and complexes precipitated by addition of donkey anti-rabbit serum (DARS; 100 μ l, at the appropriate dilution) and pelleted by centrifugation after at least 18 hr at 4°C; supernatants were removed and the radioactivity in the pellets was counted. In competitive inhibition assays the appropriately diluted rabbit serum (50 μ l) was incubated with varying quantities of unlabeled HBsAg at 37°C for at least 3 hr before addition of ¹²⁵I-HBsAg for incubation at 4°C for at least 18 hr. Complexes were then precipitated with DARS (100 μ l) as before and the proportion of ¹²⁵I-HBsAg pelleted was determined. Results are expressed as the percentage of radioactivity precipitated rela-

Abbreviations: HBV, hepatitis B virus; HBcAg, HBV core antigen; HBsAg, HBV surface antigen; anti-HBcAg, antibodies against HBcAg; anti-HBsAg, antibodies against HBsAg; DARS, donkey anti-rabbit serum.

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tive to that in a control reaction in the absence of blocking antigen (100%). The blocking reaction was used to determine type specificities of the rabbit antisera by using unlabeled HBsAg of *ad* or *ay* subtypes (10 ng per reaction) with ^{125}I -HBsAg of either type.

Inoculation of Rabbits with Bacterial Extracts. Pellets of bacterial cells were resuspended, in 1/10th of the original harvested volume, in 0.05 M Tris-HCl/0.062 M EDTA, pH 8.0, containing 1% Triton X-100 and 0.4% sodium deoxycholate and lysed at 0°C (19). The clarified lysate was free from viable bacteria and was used for inoculation of rabbits either freshly prepared or after storage at -20°C. Three injections of the extract (1 ml or 0.2 ml) were given intramuscularly at 6-week intervals or, in later experiments, at 4-week intervals; the sample for the first injection was mixed with Freund's complete adjuvant and subsequent ones were mixed with buffer or incomplete adjuvant. Serum samples were taken for analysis before the injections and 2 weeks after each injection.

Polypeptide Synthesis in Minicells. Recombinant plasmids carrying the gene for HBsAg—pHBsAg4 and pHBsAg57—were transformed into *E. coli* strain DS410 from which minicells were prepared and separated from nucleated cells by sedimentation through sucrose gradients (20, 21). Minicells (2×10^9) were suspended in M9 minimal salts medium (100 μl) containing glucose (0.2%) and cycloserine (20 $\mu\text{g}/\text{ml}$), incubated at 37°C for 30 min with Difco methionine assay medium (5 μl) containing L-[^{35}S]methionine (0.5 μl ; 1 Ci/ μmol ; 1 Ci = 3.7×10^{10} becquerels), harvested by centrifugation, and resuspended in 40 μl of gel electrophoresis buffer (22). Samples were heated at 100°C for 1 min and electrophoresed in a 12.5% polyacrylamide gel containing NaDodSO₄ which was then stained with Coomassie brilliant blue, destained, dried, and radioautographed.

RESULTS

Construction of HBV DNA Derivatives for HBsAg Expression. The organization of the HBV genome and the HBV DNA contained in some primary recombinant plasmids (2, 7) are il-

lustrated in Fig. 1; the corresponding DNA sequence was published by Pasek *et al.* (7). Digestion of the plasmids with restriction enzymes gave fragments containing the HBsAg gene, in whole or in part, which were inserted into various plasmid and phage vectors. The new DNA molecules were recovered by transformation and propagated in the usual way. Nucleotide sequences in the region of the new fusions were established by the technique of Maxam and Gilbert (14, 15).

The restriction enzymes *Ava* I, *Xho* I, and *Taq* I cut the HBV DNA sequence 26 or 27 nucleotides ahead of the initiation of the HBsAg coding sequence. *Hha* I cuts the DNA six nucleotides ahead of this point, but none of these enzymes cuts the DNA within the HBsAg encoding sequence. Digestion of pHBV114 with these enzymes generated fragments that contained the entire HBsAg gene and were inserted into suitable vectors (Fig. 2). The recombinant pHBV20 contained the HBV sequence between nucleotides 1447 and 2199, which includes all but the first 10 nucleotides of the HBsAg gene, inserted via 3' oligo(dG-dC) tails into the *Pst* site of pBR322. Digestion of pHBV20 with *Pst* thus gave a HBV DNA fragment lacking the coding information for the first three amino acids of HBsAg and substituting valine for isoleucine in the fourth. However, the HBV DNA sequence has an additional target for *Pst* within the HBsAg gene (nucleotide 1796, Fig. 1) so that partial digestion of pHBV20 with *Pst* was necessary to provide a DNA fragment carrying as much of the coding sequence as possible for this antigen. Such digestion products were inserted, by ligation, into derivatives of pBR322 that contained a target for *Pst* early in the gene for β -lactamase (11).

Analysis of Expression of the HBsAg Gene. Colonies of transformed cells were screened for HBsAg production by solid-phase radioimmunoassay (8) but, as in previous experiments (2, 7), responses were weak and variable and results generally were inconclusive. Similar assays, but using microtiter wells coated with anti-HBsAg (23), of clarified lysates (19) of small cultures of cells also gave erratic results. Alternative methods therefore were used; in one, radioactively labeled polypeptides

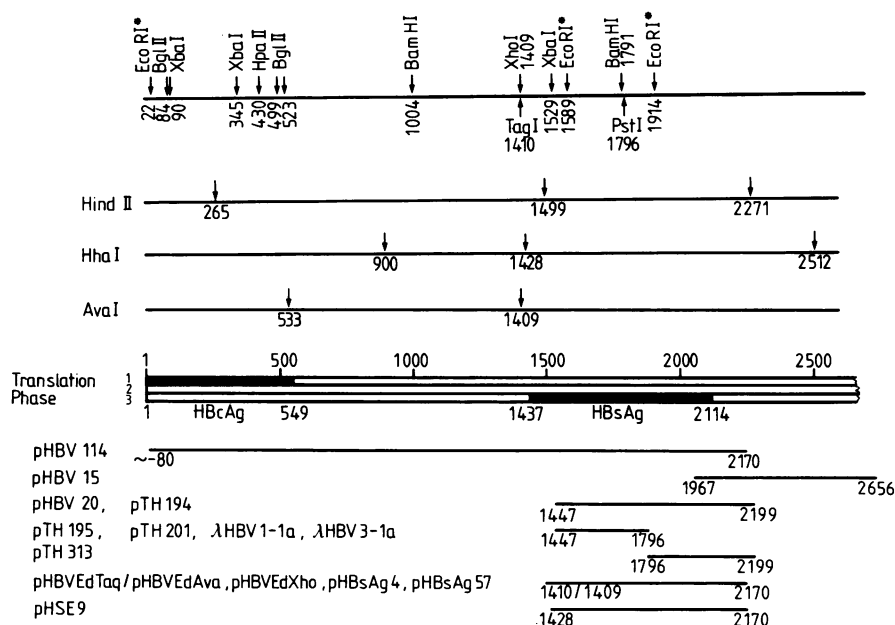


FIG. 1. Location of HBcAg and HBsAg genes and recognition targets for various restriction endonucleases on the HBV DNA molecule (2, 7). The horizontal lines in the lower part of the figure represent the HBV DNA content of the recombinant DNA molecules. The numbers refer to nucleotide positions in the HBV DNA sequence in which the first nucleotide of the HBcAg gene is designated no. 1 (7). The nucleotide sequence of this HBV DNA variant (serotype *adyw*) has now been completed and additional restriction targets are located as follows: *Ava* I, 2745; *Bam*HI, 2682; *Hha* I, 2689, 2720, 2806, and 3083; *Hind*II, 2964; *Hpa* II, 2791 and 2852 (H. Will and N. M. Gough, personal communication). The first nucleotide of a restriction target sequence is used to define these positions in the HBV sequence.

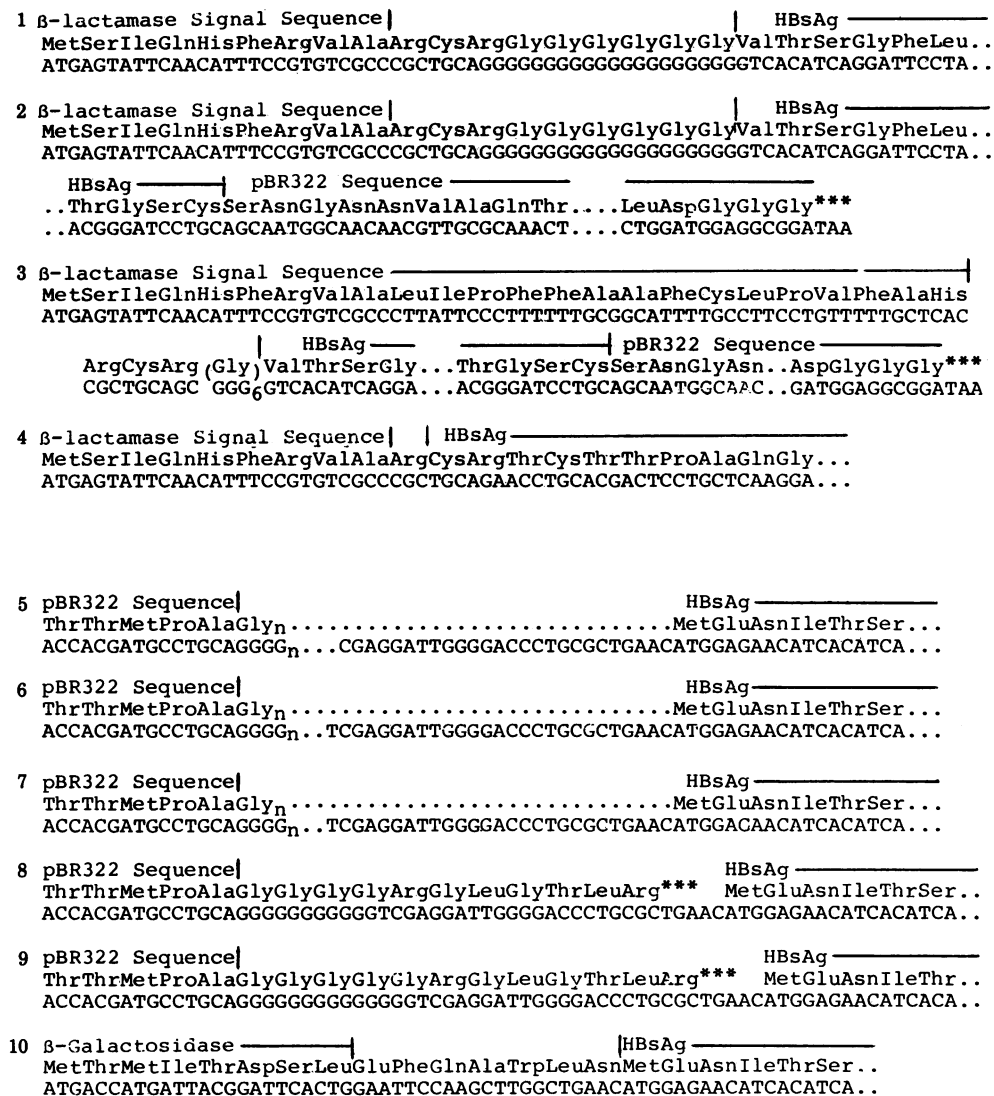


FIG. 2. Nucleotide sequences at the junctions with the HBsAg gene. See Table 1 for details of construction. The three asterisks denote a translational termination codon. The dots indicate that some amino acids or nucleotides have been omitted from the sequence to conserve space.

produced in minicells (20–22) were examined by electrophoresis and radioautography; in another, rabbits were inoculated with bacterial extracts and their sera subsequently were assayed for anti-HBsAg.

Electrophoretic analysis was complicated by the presence of *E. coli* polypeptides that comigrated with the HBsAg derivatives (results are therefore not shown) but the bioassay method gave definitive results.

Immunological Activity of HBsAg Preparations Induced by the Recombinants. Extracts from bacteria that gave an indication of HBsAg production were injected into rabbits and serum samples were assayed for anti-HBsAg in a double-antibody radioimmunoprecipitation system (18). Several of the crude bacterial preparations induced antibodies that reacted specifically with ¹²⁵I-HBsAg (Fig. 3A), and this was found (at similar efficiency) with HBsAg derivatives fused to part of the β -lactamase sequence as well as with those arising from reinitiation of translation after the TGA triplet that lies just before the HBsAg gene (Fig. 2) in a manner resembling expression of HBcAg from pHBV139 or pHBV139a (7). It is particularly interesting that anti-HBsAg was induced by extracts from cells carrying pTH195 (or induced lysogens of λ HBV1-1a) or pTH313 which contain segments of the HBsAg gene roughly equivalent to the NH₂-

terminal and COOH-terminal halves, respectively, of the polypeptide. However, extracts from cells carrying pTH201, which furnishes an HBsAg derivative similar to that from pTH195 but including a larger segment of β -lactamase (Fig. 2), was apparently not immunogenic.

The antibody response to inoculation with the bacterial extracts was compared with that elicited by purified human HBsAg particles in a competitive binding assay. A commercial rabbit anti-HBsAg antiserum at a dilution of 1:3200 gave maximal binding equivalent to that of the sera under examination at a dilution of 1:10. The competing effect of purified, unlabeled HBsAg (serotype *adyw*) on the precipitation of ¹²⁵I-HBsAg by the antisera is shown in Fig. 3B. The inhibition curves for the commercial anti-HBsAg antiserum and antiserum to one of the bacterial extracts (i.e., from the induced lysogen, λ HBV1-1a) are very similar as are the concentrations of unlabeled antigen required for 50% inhibition of the two antisera. With the antiserum raised against another bacterial extract (from cells harboring pTH194), the linear portion of the inhibition curve has a similar slope, but the displacement of the curve indicates a lower binding affinity for HBsAg. Collectively, these results confirm that the HBsAg derivatives in the bacterial extracts induce specific antibody formation in animals.

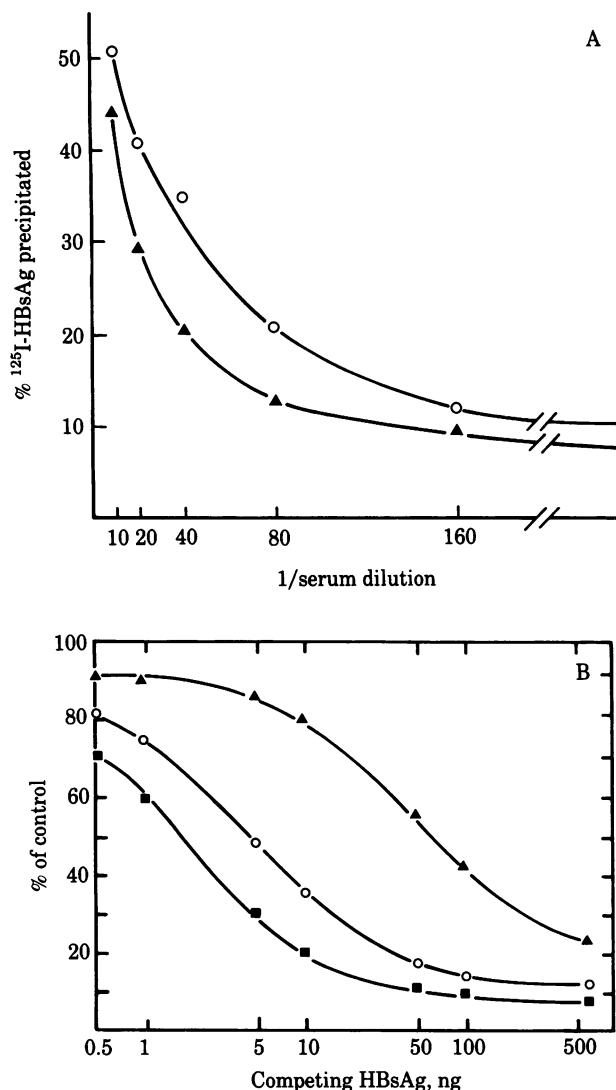


FIG. 3. (A) Titration of rabbit antisera against bacterial extracts λ HBV1-1a (○) and pTH194 (▲) by double-antibody radioimmuno-precipitation (17) with ^{125}I -HBsAg (Subtype *adyw*). (B) Competitive inhibition assays with increasing amounts of purified unlabeled HBsAg used to block the reaction between ^{125}I -HBsAg and rabbit antisera. ○, Anti- λ HBV1-1a; ▲, anti-pTH194; ■, anti-HBsAg. The anti-HBsAg antiserum was diluted 1:3200; the other two were diluted 1:10 so that, in each case, 45–50% of the labeled antigen was precipitated at zero competition.

Table 1. Construction of hybrid plasmid and phage genomes

Line Fig. 2	Recombinant in plasmid or phage	Donor plasmid	Site cleaved*	Vector	Anti-HBs induction†
1	pTH194	pHBV20	<i>Pst</i> I	pKT234	1/1
2	pTH195	pHBV20	<i>Pst</i> I	pKT234	2/4
3	pTH201	pHBV20	<i>Pst</i> I	pKT279	0/2
4	pTH313	pHBV20	<i>Pst</i> I	pKT234	3/4
	λ HBV1-1a	pTH195	<i>Eco</i> RI	λ NM989	2/2
	λ HBV3-1a	pTH201	<i>Eco</i> RI	λ NM989	0/1
5	pHBVEdTaq	pHBV114	<i>Taq</i> I	pBR322	
6	pHBVEdAva	pHBV114	<i>Ava</i> I	pBR322	
7	pHBVEdXho	pHBV114	<i>Xho</i> I	pBR322	
8	pHBsAg4	pHBV114	<i>Xho/Hpa</i> II	pBR322	0/1
9	pHBsAg57	pHBV114	<i>Xho/Hpa</i> II	pBR322	1/2
10	pHSE9	pHBV114	<i>Hha</i> I	pEXlac205	

Recombinants pHBVEdTaq, pHBVEdAva, and pHBVEdXho refer to populations of which some give a fused polypeptide containing HBsAg and some, like pHBsAg4 and pHBsAg57, give polypeptides resulting from the translational stop and restart signals.

* Recombinants pTH194, pTH195, pTH201, pTH313, λ HBV1-1a, and λ HBV3-1a were made by ligation of the cleaved vector and donor molecules; pHSE9 was made by ligation of the component DNA molecules together with a synthetic oligonucleotide linker (*Eco*RI); the others were made by hybridizing donor fragments carrying 3' oligo(dC) tails to the restricted vector carrying 3' oligo(dG) tails.

† Number of anti-HBsAg-positive sera/number of rabbits inoculated.

Specificity of the Induced Antibodies. The antibody response could be directed against the group- or subtype-specific determinants of HBsAg. The cloned HBV DNA used in these experiments was derived from Dane particles isolated from an unusual plasma, from a single individual, which exhibited the complex serotype *adyw*. It therefore was of particular interest to measure the blocking effect of purified HBsAg of *ad* or *ay* subtype in the inhibition assay system. Antiserum raised against the λ HBV1-1a extract was blocked more effectively by the *ad* antigen than by an equal quantity of the *ay* antigen; the converse was observed with antiserum against the pTH194 extract (Table 2). The antigen extracted from cells infected with λ HBV1-1a therefore induced antibodies with activity against group determinant *a* and subtype determinant *d*; that from cells carrying pTH194 induced anti-*a* and anti-*y* activity. In contrast, extracts from cells carrying pTH313 induced an antibody response directed against group determinant *a* but not discriminating between the subtypes.

DISCUSSION

Some of the reconstructed plasmids direct the synthesis of HBsAg derivatives that react poorly if at all with antibodies to

Table 2. Type specificities of the rabbit antisera

Bacterial extract	^{125}I -HBsAg subtype	^{125}I -HBsAg bound in presence of 10 ng unlabeled HBsAg		Relative blocking (%) by 10 ng unlabeled HBsAg	
		Subtype <i>ad</i>	Subtype <i>ay</i>	Subtype <i>ad</i>	Subtype <i>ay</i>
λ HBV1-1a	<i>ad</i>	26	83	74	17
	<i>ay</i>	60	51	40	49
pTH194	<i>ad</i>	78	76	22	24
	<i>ay</i>	95	69	5	31
pTH313	<i>ad</i>	18	15	82	85
	<i>ay</i>	11	11	89	89

Results are expressed as the percentage of a control value obtained in reactions lacking any blocking antigen. Duplicate experiments with independently purified preparations of both labeled and unlabeled HBsAg of each subtype gave the same results.

the native surface antigen but which nevertheless induce specific antibody formation in rabbits, although at a low titer. The positive responses *in vivo* were only observed at relatively long periods after inoculation (and booster injections), but an extended study to optimize the immunization schedule has yet to be completed. Although the antibody titer is low when measured against native HBsAg, it is quite possible that the titer against the immunogen used (i.e., the bacterial HBsAg derivative) could be much higher, but this assay is more complex.

The reasons for the poor interaction of the various HBsAg polypeptides with native anti-HBsAg antiserum are not clear, but one possibility is that the polypeptides produced in *E. coli* are not glycosylated and therefore may be less effective than the native form of the antigen in crossreactions with antibodies, although Neurath *et al.* (24) claim that the nonglycosylated form is more effective in stimulation of antibody synthesis even though it has a shorter half-life in plasma. Another is that membrane or other components from the host cell could be involved in the correct assembly of native HBsAg and these are lacking in the bacterial system.

A more likely possibility is that the physical state of the antigen, including its mode of folding or degree of aggregation or association, is appreciably different in the bacterial extracts from that in which it occurs naturally. In this context, it is relevant to recall the results of Shih and Gerin (25) who examined several fractions of HBsAg after treatment with NaDodSO₄ and found that the monomeric polypeptide differed from its aggregated forms in that it did not react with antibodies to the native antigen but it did induce antibody formation at a low efficiency when injected into guinea pigs. Similar observations have been made by Melnick and his collaborators with chimpanzees (9). Thus, other derivatives of HBsAg produced from bacteria carrying the HBsAg gene in different configurations may elicit higher antibody titers and exhibit better crossreactivity with specific antibodies.

Charnay *et al.* (26) recently described recombinant phage derivatives in which part of the HBsAg gene (cleaved at the *Xba* I site, nucleotide 1629 in Fig. 1) was fused to the β -galactosidase gene via the *Eco*RI site near its distal end. The resulting fused polypeptide (M_r 138,000) comprised almost the entire β -galactosidase polypeptide followed by an HBsAg sequence lacking the first 29 amino acid residues of its normal sequence. This polypeptide reacted poorly in radioimmunoassays but could be precipitated with anti-HBsAg; its structure differs considerably from the structures described here, which carry little extraneous amino acid sequence, and it is interesting to note this difference in properties between the two types, although the immunogenicity of this derivative was not described.

The experiments described here provide a good example of the importance of an *in vivo* assay to detect the synthesis of an immunologically active polypeptide that may be missed by dependence upon more convenient methods, such as the radioimmunoassay, which are well suited to the rapid screening of large numbers of samples. They thus highlight the possibility of screening for antigen production on relatively large numbers of isolates by inoculating mice, instead of rabbits as in this instance, with crude cell extracts. For screening on a larger scale, mixtures of several samples could clearly be used initially, with individual colonies from groups being examined subsequently.

A point of further interest is the variation in specificity observed in the antibodies raised against extracts containing different parts of the HBsAg fused in each case to the first 12 residues of β -lactamase via six glycine residues. The NH₂-terminal part of the polypeptide (residues 4–121, expressed from pTH195) and the largest polypeptide made (residues 4–226, expressed from pTH194) gave antibodies that reacted differ-

entially with HBsAg of the *ad* and *ay* subtypes, respectively; the COOH-terminal region (residues 121–226, expressed from pTH313) was directed only against the group determinant *a*. Because these three new recombinants were all derived from a single cloned DNA fragment from Dane particles from one individual, the complex and unusual serotype *adyw* may be the manifestation of a rare HBV variant, or perhaps recombination between two different virions at some earlier stage of the patient's history, rather than the direct result of infection with two different viral populations.

In an earlier publication (7) the synthesis of immunologically active HBcAg by *E. coli* was described. This demonstrated the feasibility of viral vaccine production via *E. coli* fermentation products. The results given here show that immunologically active HBsAg may be made by similar means, thus advancing the development of a vaccine against HBV (and attendant disease) based upon a preparation that is incapable of infecting its recipients with serum hepatitis because it is made from material lacking the genetic capacity to produce the virus.

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