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Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters

(recombinant vaccine/adenovirus animal model/adenovirus E3 region)

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ABSTRACT **Recombinant adenoviruses carrying the hepatitis B virus surface antigen coding sequence in the adenovirus E3 region were constructed using DNA from either adenovirus type 5 or an adenovirus type 5 E3-region deletion mutant. Both of these recombinant adenoviruses replicated as efficiently as wild-type adenovirus in all human cells tested, including the human diploid cell strain WI-38. This indicates that insertion of the hepatitis B virus surface antigen gene into the E3 region does not significantly affect viral replication. Human cells infected with these recombinant adenoviruses secreted immunoreactive hepatitis B virus surface antigen. Since a practical small animal model for human adenoviruses was lacking, a hamster model was developed to evaluate the immunogenic potential of these recombinant adenoviruses. Upon intranasal inoculation, both wild-type adenovirus and the adenovirus E3-region deletion mutant replicated in the lungs of these animals and induced an antibody response against adenovirus. Hamsters similarly immunized with the live recombinant adenoviruses produced antibody against both adenovirus and hepatitis B virus surface antigen.**

Hepatitis B is a serious worldwide disease. Approximately 200,000 people are infected with hepatitis B virus (HBV) in the United States each year. At least 10% of the population of the Far East and Africa are chronic carriers of HBV, many of whom will develop hepatocellular carcinoma (1). The global incidence of this disease could be greatly reduced with a safe, effective, and inexpensive vaccine that could be easily administered to all populations at risk. Current immunization against HBV requires intramuscular injection of HBV major surface antigen (HBsAg) purified from plasma of HBV carriers (2–4) or from recombinant yeast (5, 6). Live recombinant viral vaccines represent an attractive alternative to the present HBV vaccines. Such a vaccine could be derived from the live adenovirus vaccines already in use. The current adenovirus vaccines are safe and efficacious when administered orally as enteric-coated tablets (7, 8). They give rise to asymptomatic intestinal adenovirus infections in humans that induce immunity against adenovirus respiratory disease. These characteristics have prompted us to develop recombinant adenoviruses that direct infected cells to produce HBsAg and thus confer immunity against both adenovirus and HBV. Vaccinia virus recombinants that direct production of HBsAg in animals (9, 10) were designed with a similar strategy in mind; however, live recombinant adenovirus vaccines provide the convenience of oral administration. In this study we describe recombinant adenoviruses that carry the HBsAg-coding sequence in the adenovirus E3 region and that direct the production of immunogenic HBsAg.

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MATERIALS AND METHODS

Cells and Viruses. Cell line 293 derived from human embryonic kidney (11) was used for calcium phosphate transfection as described (12). Adenovirus type 5 (Ad5) and the recombinant adenoviruses described below were grown and titrated on 293 cells as well as on A549 cells (13) derived from human lung carcinoma. These viruses were also grown on the human diploid cell strain WI-38 (14).

Immunological Reagents. HBsAg was assayed using diagnostic RIA kits from Organon Teknika (Irving, TX) and from Abbott (North Chicago, IL). The levels of antibodies directed against HBsAg were measured using a diagnostic RIA kit (AUSAB) from Abbott. Antibody levels were converted from RIA units to milliinternational units (mIU) based on an equivalence factor of 3.5 RIA units per 1 mIU. Monoclonal antibody A5C11 against HBsAg was obtained from Centocor (Malvern, PA).

Metabolic Radiolabeling and Electrophoretic Analysis. A549 cells were metabolically radiolabeled using L-[^{35}S]cysteine at 260 μCi/ml (1 Ci = 37 GBq) during either the early phase (4.5–9 hr after infection) or the late phase (22.5–27 hr after infection) of infection with either Ad5 E3HS or Ad5 ΔE3HS. Radiolabeled HBsAg was immunoprecipitated from cell lysates or from medium with monoclonal antibody against HBsAg (A5C11), and the immunoprecipitates were resolved on NaDodSO$_4$/polyacrylamide gels and autoradiographed as described (12).

Neutralization Assay. Serum neutralization titers were determined on A549 cells using a microneutralization assay (15). The negative control was normal hamster sera.

Animal Techniques. Syrian hamsters were obtained from Charles River Breeding Laboratories. Animals, 3–4 weeks old, were anesthetized with sodium pentobarbitol prior to intranasal inoculation with virus. Infectivity of virus from lung homogenates was quantitated by plaque titration on A549 cells.

ELISA Analyses. Serum titers of anti-adenovirus antibody were evaluated by ELISA analysis using Ad5-coated microtiter plates. A standard curve was generated for each plate using serial dilutions of hyperimmune hamster antiserum. Titers were determined by relating the absorbance of test serum to the linear range of the standard curve. The reference antiserum was arbitrarily assigned to a titer of 100. All samples were tested in duplicate.

RESULTS

Construction of Recombinant Adenoviruses. The HBsAg coding sequence was inserted downstream of the Ad5 E3

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus major surface antigen; Ad2 and Ad5, adenoviruses type 2 and type 5, respectively; m.u., map unit; Ara-Cyt, cytosine arabinonucleoside; pfu, plaque-forming units; mIU, milliinternational units.

promoter at map unit (m.u.) 78.5 as shown in Fig. 1 so that it either immediately precedes, as in the recombinant adenovirus termed Ad5 E3HS, or replaces, as in the recombinant adenovirus termed Ad5 ΔE3HS, a portion of the E3 region that includes the coding sequence of the viral glycoprotein designated gp19K (19). After transfection of 293 cells with recombinant plasmid and adenovirus DNA (Fig. 1), recombinant virus plaques were isolated and identified by screening for HBsAg production using RIA as described (12). The genomic structures of AD5 ΔE3, Ad5 E3HS, and Ad5 ΔE3HS were confirmed by restriction endonuclease site analysis of DNA from plaque-purified virus (data not shown). Ad5 E3HS and Ad5 ΔE3HS were propagated in either 293 or A549 cells and subsequently titered on both 293 and A549 cells. Viral stocks had identical titers when assayed using either 293 or A549 cells. Viral yields from either cell line were similar and in several preparations ranged from 5×10^7 to 2×10^8 plaque-forming units (pfu)/ml.

Production of HBsAg in Cells Infected with Recombinant Ad5. Viral stocks of Ad5 E3HS and Ad5 ΔE3HS prepared on A549 cells were used to infect A549 cells in 75-cm^2 flasks, and the synthesis of HBsAg was monitored by RIA (Fig. 2). Examination of HBsAg accumulation in the medium during the course of infection revealed differences between Ad5 E3HS (Fig. 2*A*) and Ad5 ΔE3HS (Fig. 2*B*). Cells infected with either virus began to produce HBsAg soon after infection. This is consistent with the site of insertion of the HBsAg coding sequence downstream of the E3 promoter, which is most active in the early phase of infection, ≈2–9 hr after infection (20). Production of HBsAg by cells infected with Ad5 E3HS was unaltered by cytosine arabinonucleoside (Ara-Cyt), which specifically blocks late-phase transcription from the major late promoter by inhibiting adenovirus DNA replication (21). In contrast, production of HBsAg during the

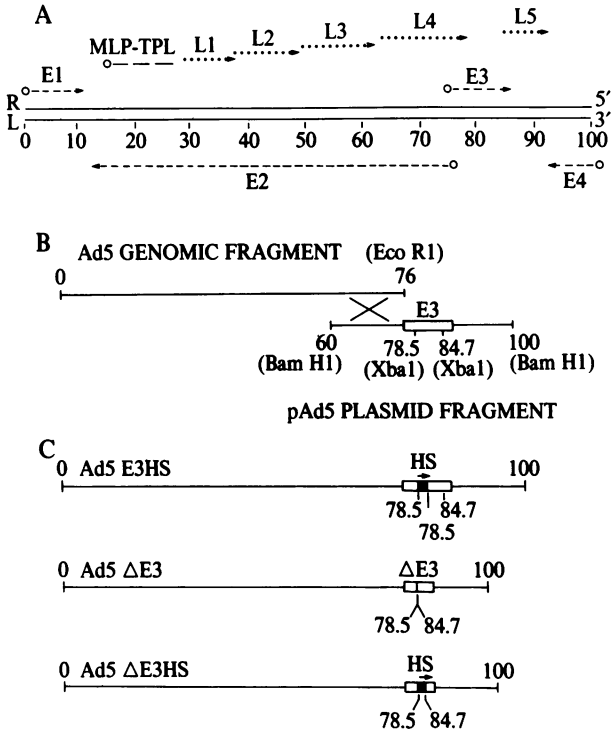

FIG. 1. Construction of recombinant adenoviruses. (*A*) Simplified diagram of the transcript map of Ad2 (16); each m.u. is ≈360 base pairs. Open circles, promoters. Dashed lines, families of transcripts from promoters active early in infection; dotted lines, families of transcripts from the major late promoter (MLP) [all of which are spliced to the tripartite leader (TPL)]. (*B*) Recombinant adenoviruses were constructed by transfecting into cultured 293 cells the large *Eco*RI fragment of Ad5 genomic DNA and a partially overlapping fragment of cloned Ad5 DNA from a plasmid (pAd5). Recombination occurs *in vivo* between the homologous overlaps, generating full-length viruses (17). (*C*) Ad5 DNA from m.u. 60 to m.u. 100 cloned as described (18) was altered to create the new plasmids pAd5 E3HS, pAd5 ΔE3, and pAd5 ΔE3HS that were subsequently used to generate the corresponding recombinant adenoviruses Ad5 E3HS, Ad5 ΔE3, and Ad5 ΔE3HS. In Ad5 E3HS, the HBsAg coding sequence (HS) was inserted at the *Xba* I site at m.u. 78.5. In Ad5 ΔE3 the *Xba* I fragment between m.u. 78.5 and m.u. 84.7 that includes most of the E3 region was excised. In Ad5 ΔE3HS, this *Xba* I fragment was replaced by the HBsAg coding sequence.

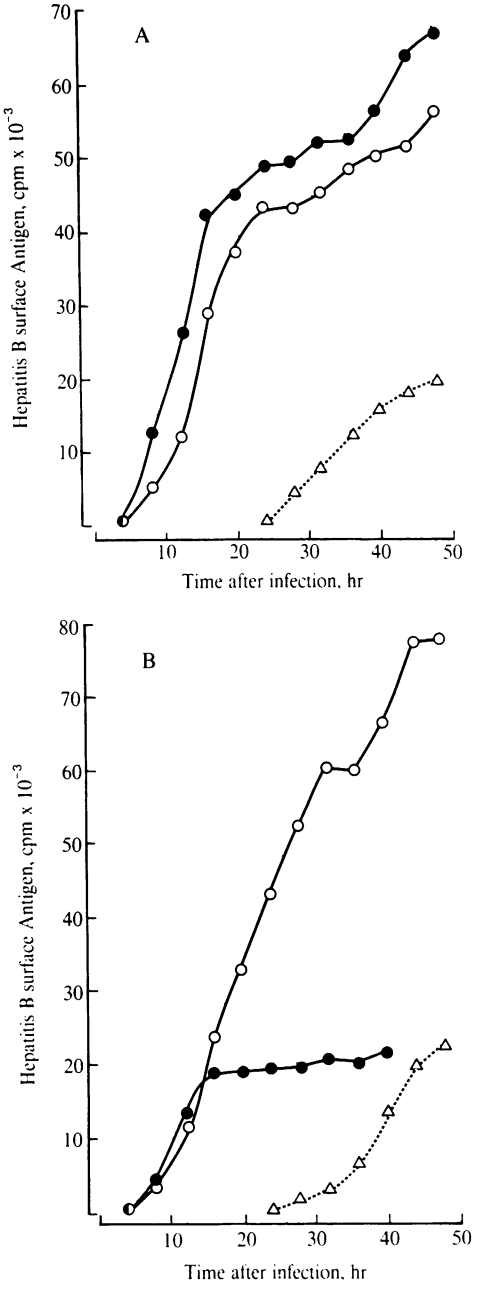

FIG. 2. Time course of production of HBsAg by A549 cells infected with Ad5 E3HS (*A*) or with Ad5 ΔE3HS (*B*) and the effect of Ara-Cyt. Flasks containing 1.5×10^7 A549 cells were infected with 7.5×10^7 pfu of either Ad5 E3HS or Ad5 ΔE3HS (○). Duplicate flasks with Ara-Cyt at 20 μg/ml added (21) were infected with either Ad5 E3HS or Ad5 ΔE3HS (●). The medium containing Ara-Cyt was removed 12 hr after infection from one set of duplicate flasks and was replaced with medium containing cytidine at 20 μg/ml (△). Samples (50 μl) of culture medium were withdrawn at the times indicated, and the accumulated HBsAg was estimated by RIA.

late phase of infection, ≈12–48 hr after infection, by cells infected with Ad5 ΔE3HS was reversibly abolished by Ara-Cyt. The total accumulation of HBsAg in the medium 60 hr after infection in the absence of Ara-Cyt was 1.5 μg per 10^6 cells infected with Ad5 E3HS and 2.8 μg per 10^6 cells infected with Ad5 ΔE3HS. WI-38 cells and 293 cells infected with these viruses produced similar levels of HBsAg (data not shown).

Characterization of the HBsAg Produced in Cells Infected with Recombinant Ad5. Electrophoretic analysis of metabolically radiolabeled immunoprecipitates from A549 cells infected with either Ad5 E3HS or Ad5 ΔE3HS (Fig. 3) revealed immunoreactive material with the approximate molecular weights of the unglycosylated (p24) and glycosylated (gp27) forms of HBsAg (22). There was no apparent difference between the molecular weight of HBsAg produced in the early phase of infection and that produced in the late phase. HBsAg immunoprecipitated from lysates of infected cells (Fig. 3*A*) had the same apparent molecular weight as HBsAg secreted into the medium (Fig. 3*B*), although the bands that represented glycosylated polypeptide from medium appeared to be slightly broader than their counterparts from lysates. The relative amounts of HBsAg recovered from cells radiolabeled in the early versus the late phase of infection with Ad5 E3HS or Ad5 ΔE3HS, as shown by comparison of lanes 3 and 4 with lanes 6 and 7 in Fig. 3*A*, corresponded well with the relative rates of accumulation of HBsAg in the medium at these times as estimated by RIA (Fig. 2), and is consistent with the observed patterns of sensitivity to Ara-Cyt (Fig. 2). Although in the late phase of infection cells infected with Ad5 ΔE3HS produced more HBsAg than cells infected with Ad5 E3HS, the total amount of HBsAg produced by cells infected with Ad5 ΔE3HS is only about two-fold greater than that produced by cells infected with Ad5 E3HS.

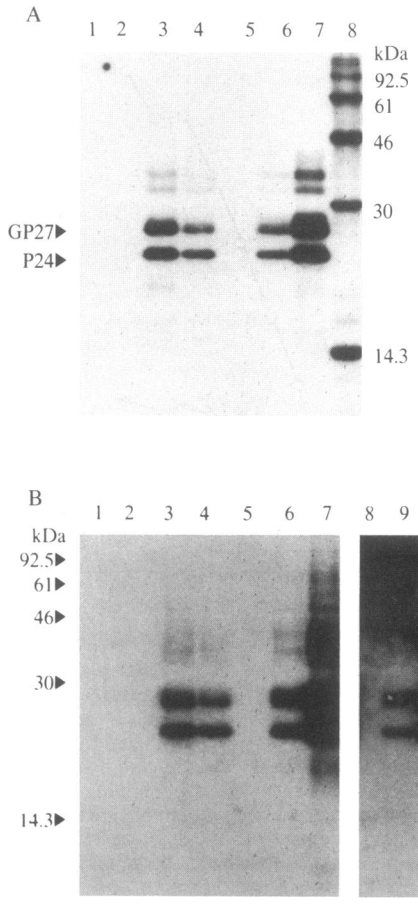

FIG. 3. Electrophoretic analysis of HBsAg immunoprecipitated from A549 cells infected with recombinant adenoviruses. Radiolabeled HBsAg was prepared and analyzed. (*A*) Immunoprecipitates of lysed cells. Lanes: 1, uninfected; 2, early-phase Ad5; 3, early-phase Ad5 E3HS; 4, early-phase Ad5 ΔE3HS; 5, late-phase Ad5; 6, late-phase Ad5 E3HS; 7, late-phase Ad5 ΔE3HS; 8, molecular weight markers. (*B*) Immunoprecipitates of medium. Lanes: 1, uninfected; 2, early-phase Ad5; 3, early-phase Ad5 E3HS; 4, early-phase Ad5 ΔE3HS; 5, late-phase Ad5; 6, late-phase Ad5 E3HS; 7, late-phase Ad5 ΔE3HS. Samples of medium (5 μl) were also analyzed without immunoprecipitation. Lanes: 8, uninfected cells; 9, late-phase Ad5 E3HS; 10, late-phase Ad5 ΔE3HS. The size and location of the protein markers are given.

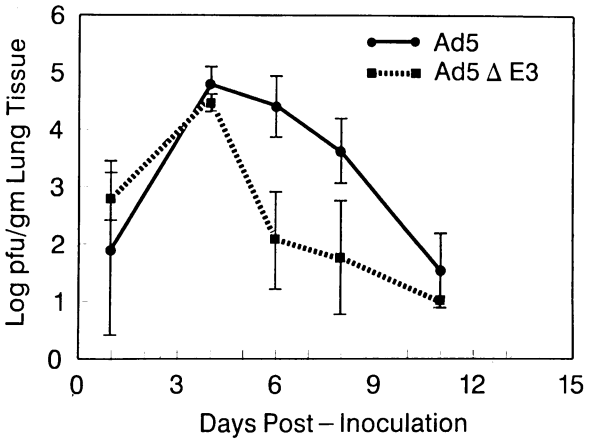

FIG. 4. Replication of Ad5 and Ad5 ΔE3 in hamster lungs. One-month-old hamsters were inoculated intranasally with 10^7 pfu of Ad5 (●) or of Ad5 ΔE3 (■). Viral titers (pfu/gm of lung tissue) were determined from four animals for each virus at each time point and are expressed as the geometric mean (± SD).

Replication of Ad5 and Ad5 ΔE3 in Hamsters. A small animal model was developed to evaluate the immune response to these recombinant Ad5 viruses. Ad5 has been shown to replicate in the lower respiratory tract of hamsters following intranasal inoculation (R. N. Hjorth, personal communication). We here confirm the above finding and further show that Ad5 ΔE3 also replicates in the lungs of these animals (Fig. 4). Titers of infectious virus recovered from lungs of animals infected with either Ad5 or Ad5 ΔE3 peaked at the same level, but subsequently titers of Ad5 ΔE3 fell more rapidly than the titers of Ad5. The antibody responses

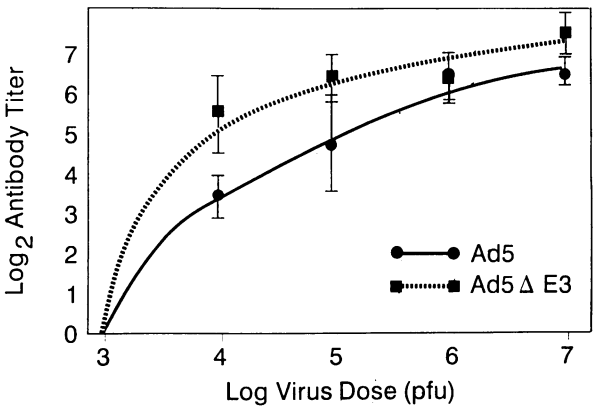

FIG. 5. Seroresponses to Ad5 in hamsters inoculated with various doses of Ad5 and Ad5 ΔE3. One-month-old hamsters were inoculated intranasally over a range of doses (10^3–10^7 pfu per animal) with Ad5 (●) or Ad5 ΔE3 (■), and seroresponses were determined by ELISA analysis 28 days later. Antibody titers represent geometric means (± SD) from four animals at each dose for each virus for all test groups except groups receiving Ad5 ΔE3 at doses of 10^7 pfu and 10^5 pfu, which consisted of three animals per group.

Table 1. Seroconversion of hamsters to HBsAg following immunization with Ad5 E3HS and Ad5 ΔE3HS

Virus	Dose, pfu	Anti-HBsAg response: Positive animals/total animals inoculated, no./no.	Anti-HBsAg response: Titer, mIU
Ad5 E3HS	10^8	7/10	126 ± 89
	10^7	4/10	119 ± 121
	10^6	1/10	46
	10^5	0/10	0
	10^4	0/10	0
	UV (10^8)*	0/10	0
Ad5 ΔE3HS	10^8	10/10	75 ± 50
	10^7	5/10	48 ± 39
	10^6	0/10	0
	10^5	1/10	21
	10^4	0/10	0
	UV (10^8)*	0/10	0
Ad5	10^7	0/10	0

One-month-old hamsters were challenged intranasally with virus purified by CsCl isopycnic density gradient centrifugation and subsequently shown to be free of HBsAg contamination by RIA. The anti-HBsAg titer is reported as mean ± SD of positive sera.
*Inocula corresponding to 10^8 pfu per dose were irradiated with ultraviolet radiation to reduce infectivity to $\approx 10^2$ pfu per dose.

against adenovirus in hamsters inoculated with Ad5 or Ad5 ΔE3 are presented as a function of virus dose in Fig. 5. Elevated antibody titers were observed in animals receiving a minimum dose of 10^4 pfu of either virus.

Induction of Antibody to HBsAg in Hamsters. Hamsters were inoculated with Ad5 E3HS or Ad5 ΔE3HS and evaluated for production of antibody to HBsAg 1 month later. Table 1 shows that by 33 days after inoculation with 10^8 pfu of virus, 7 out of 10 hamsters inoculated with Ad5 E3HS and 10 out of 10 hamsters inoculated with Ad5 ΔE3HS had produced significant levels of antibody to HBsAg. Test animals inoculated with equivalent dosages of either virus that had been inactivated with ultraviolet light failed to produce a detectable antibody response to HBsAg. There was no major difference in the minimum dose of either Ad5 E3HS or Ad5 ΔE3HS required to induce antibody to HBsAg. Similar antibody titers against HBsAg were attained with equal doses of either virus.

Table 2 shows a detailed analysis of hamster seroresponses to both Ad5 and HBsAg following inoculation with 10^7 pfu of recombinant adenovirus on days 0 and 33. By 33 days after inoculation, five out of eight animals inoculated with Ad5 E3HS and four out of eight animals inoculated with Ad5 ΔE3HS had seroconverted to HBsAg. One additional animal per group had seroconverted to HBsAg when tested at day 65 (animals 4 and 16). A wide range of antibody levels against HBsAg were induced in these hamsters. Again, no consistent differences in antibody titers against HBsAg were observed between groups immunized with the two different recombinant viruses. By day 33, all animals inoculated with either of the recombinant adenoviruses had produced antibodies against Ad5 as assessed by ELISA and neutralization analyses.

Table 2. Anti-HBsAg and anti-Ad5 responses in hamsters inoculated with Ad5 E3HS and Ad5 ΔE3HS

Virus	Animal number	Anti-HBsAg, mIU: 33 days	Anti-HBsAg, mIU: 65 days	Anti-Ad5 at 33 days: ELISA†	Anti-Ad5 at 33 days: Neutralization, titer‡
Ad5 E3HS	1	93*	411*	+	160
	2	59*	502*	+	80
	3	41*	26*	+	80
	4	4	32*	+	160
	5	59*	160*	+	80
	6	2	0	+	80
	7	2	0	+	160
	8	11*	186*	+	80
Ad5 ΔE3HS	9	50*	160*	+	160
	10	2	2	+	80
	11	21*	21*	+	80
	12	2	0	+	320
	13	162*	675*	+	80
	14	2	2	+	40
	15	26*	46*	+	20
	16	2	26*	+	80
Negative control	17	0	0	−	20

One-month-old hamsters were inoculated intranasally on days 0 and 33 with crude viral lysate containing 10^7 pfu recombinant virus per dose.
*Positive sera (≤10 mIU).
†+, ELISA reading of >0.15 absorbancy units at a 1:800 dilution of serum; −, reading of <0.15 at a 1:50 serum dilution.
‡Neutralization titer is the greatest reciprocal serum dilution that prevents visible cytopathic effect.

DISCUSSION

Our results demonstrate the potential utility of recombinant adenoviruses as live oral vaccines. We describe here recombinant adenoviruses that can replicate in human cells and that direct the production of immunogenic HBsAg. Ad5 E3HS and Ad5 ΔE3HS were constructed as autonomously replicating adenoviruses to enhance HBsAg production through successive rounds of replication after administration. In addition, the recombinant viruses described here can be propagated as pure isolates in a cell strain appropriate for vaccine production such as WI-38. Although replication-defective recombinant adenoviruses have been useful in demonstrating the feasibility of producing immunoreactive HBsAg particles in infected cells (12, 23), their propagation requires nondefective helper virus or transformed cell lines that complement their defect (11); this limits their potential as live oral vaccines. These defective viruses do illustrate the importance of utilizing adenovirus control elements such as the major late promoter and tripartite leader to produce hybrid adenovirus-HBsAg mRNA that is efficiently translated to produce HBsAg. In contrast, a recombinant adenovirus that contains most of the HBV genome is not replication defective but cells infected with this virus do not produce HBsAg (24). Cells infected with this recombinant virus produce abundant HBV mRNA from HBV promoters, but barely detectable levels of HBsAg are expressed. This poor utilization of HBV mRNA is consistent with the complex post-transcriptional controls observed in adenovirus infected cells (for reviews, see refs. 16 and 25).

Ad5 E3HS and Ad5 ΔE3HS are not defective because only the nonessential E3 region (18, 26–28) is altered. The HBV DNA segment inserted into Ad5 contains only the HBsAg coding sequence and does not include an HBV promoter. The expression of HBsAg in cells infected by Ad5 E3HS and Ad5 ΔE3HS is, therefore, dependent on transcription from an Ad5 promoter to produce hybrid Ad5-HBsAg mRNA. The production of HBsAg soon after infection with either Ad5 E3HS or Ad5 ΔE3HS is consistent with the position of insertion downstream of the E3 promoter. The significant difference in the rate of production of HBsAg during the later phase of infection with Ad5 E3HS versus Ad5 ΔE3HS may be due to the large E3 deletion in Ad5 ΔE3HS, which removes all of the

RNA splice sites downstream of the HBsAg coding sequence in the E3 region.

During the late phase of infection, transcripts that originate at the major late promoter proceed through the E3 region to the L5 region (Fig. 1). Most of the E3 region is subsequently removed during RNA splicing, but mRNA that originates at the major late promoter and includes E3 region coding sequences is produced at low abundance (29). It has been reported (29) that deletion of a downstream splice site from the E3 region can increase the relative abundance of this mRNA by altering the RNA splicing pattern. The Ara-Cyt sensitivity of late-phase HBsAg expression from Ad5 ΔE3HS suggests that this expression derives from transcripts originating at the major late promoter. An increase in the relative abundance of late-phase mRNA that retains the HBsAg coding sequence after RNA splicing would be consistent with the reported effect of deleting downstream splice sites from the E3 region (29).

Human adenoviruses have a highly restricted host range, which makes development of a useful animal model for adenoviral disease difficult. The best small animal model for human adenoviral disease to date is the cotton rat model (15). This model suffers, however, from the limited commercial availability of these animals. We thus sought a more readily available small animal model to evaluate recombinant human adenoviruses. Initial studies confirmed that Ad5 replicated in hamster lungs and induced a strong anti-adenovirus humoral immune response. We further demonstrate that Ad5 ΔE3 replicates in hamster lungs to peak titers comparable to those attained by wild-type Ad5. Thus, the Ad5 E3 region, which is known to be nonessential for virus replication in tissue culture (18, 26–28), is here shown to be nonessential for virus replication *in vivo*.

The E3 region of adenovirus type 2 (Ad2) encodes a glycoprotein, designated as gp19K, that blocks expression of class I major histocompatibility antigens on Ad2-infected cell surfaces (30, 31). Since viral antigens are recognized by cytolytic T lymphocytes only in the context of class I major histocompatibility antigens, it has been suggested that gp19K enables Ad2-infected cells to evade immune surveillance (32). It would thus be predicted that adenoviruses that express gp19K would show enhanced virus replication *in vivo* relative to adenoviruses that lack this glycoprotein. The relevance of gp19K expression to the replication of Ad5 and Ad5 ΔE3 in hamsters is not clear, as the role of cytolytic T lymphocytes in the hamster immune response to Ad5 infection is unknown. Nevertheless, our observation that Ad5 persists at elevated titers in hamster lungs relative to Ad5 ΔE3 is consistent with the proposed function of gp19K.

Inoculation of hamsters with relatively high doses of Ad5 E3HS or Ad5 ΔE3HS resulted in the induction of anti-HBsAg antibodies to high levels in a large proportion of the animals tested. It is relevant to note that 50% of the 1-month-old hamsters immunized with a single dose (0.1–1.0 μg) of Merck HBV vaccine seroconvert to HBsAg (data not shown). Hamsters thus resemble guinea pigs in their relatively high immunoresponsiveness to HBsAg (33).

Some animals receiving high doses of adenovirus recombinants mounted a humoral immune response to adenovirus antigens but not to HBsAg. The reasons for this are not clear at present, but may be related to the following factors: the large excess of adenovirus proteins relative to HBsAg produced (data not shown), the young age (3–4 weeks) and consequent immunological immaturity of the animals used, and the lack of genetic uniformity among the outbred hamsters employed in this study.

Both recombinant adenoviruses were equally efficient in seroconverting hamsters to HBsAg, and they induced comparable levels of anti-HBsAg antibodies in positive animals. On the whole, these data indicate a good prospect for developing recombinant adenovirus vaccines that will effectively immunize humans against HBV.

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1. Tiollais, P., Pourcel, C. & Dejean, A. (1985) *Nature (London)* **317**, 489–495.
2. Purcell, R. H. & Gerin, J. L. (1975) *Am. J. Med. Sci.* **270**, 395–399.
3. Hilleman, M. R., Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U. & Lampson, G. P. (1975) *Am. J. Med. Sci.* **270**, 401–404.
4. Maupas, P., Goudeau, A., Coursaget, P., Drucker, J. & Bagros, P. (1976) *Lancet* **i**, 1367–1370.
5. Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G. & Hall, B. D. (1982) *Nature (London)* **298**, 347–350.
6. Scolnick, E. M., McLean, A. A., West, D. J., McAleer, W. J., Miller, W. J. & Buynak, E. B. (1984) *J. Am. Med. Assoc.* **251**, 2812–2815.
7. Couch, R. B., Chanock, R. M., Cate, T. R., Lang, D. J., Knight, V. & Huebner, R. J. (1963) *Am. Rev. Respir. Dis.* **88**, 394–403.
8. Top, F. H., Jr., Buescher, E. L., Bancroft, W. H. & Russell, P. K. (1971) *J. Infect. Dis.* **124**, 155–160.
9. Moss, B., Smith, G. L., Gerin, J. L. & Purcell, R. H. (1984) *Nature (London)* **311**, 67–69.
10. Paoletti, E., Lipinskas, B. R., Samsonoff, C., Mercer, S. & Panicali, D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 193–197.
11. Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977) *J. Gen. Virol.* **36**, 59–72.
12. Davis, A. R., Kostek, B., Mason, B. B., Hsiao, C. L., Morin, J., Dheer, S. K. & Hung, P. P. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7560–7564.
13. Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W. & Todaro, G. (1976) *Int. J. Cancer* **17**, 62–70.
14. Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell Res.* **25**, 585–621.
15. Pacini, D. L., Dubovi, E. J. & Clyde, W. A., Jr. (1984) *J. Infect. Dis.* **150**, 92–97.
16. Flint, S. J. (1986) *Adv. Virus Res.* **31**, 169–228.
17. Gluzman, Y., Reichel, H. & Solnick, D. (1982) in *Eukaryotic Viral Vectors*, ed. Gluzman, Y. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 187–192.
18. Berkner, K. L. & Sharp, P. A. (1983) *Nucleic Acids Res.* **11**, 6003–6020.
19. Cladaras, C. & Wold, W. S. M. (1985) *Virology* **140**, 28–43.
20. Nevins, J. R., Ginsberg, H. S., Blanchard, J. M., Wilson, M. C. & Darnell, J. E. (1979) *J. Virol.* **32**, 727–733.
21. Young, C. S. H., Cachianes, G., Munz, P. & Silverstein, S. (1984) *J. Gen. Virol.* **51**, 571–577.
22. Peterson, D. L. (1981) *J. Biol. Chem.* **256**, 6975–6983.
23. Ballay, A., Levrero, M., Buendia, M., Tiollais, P. & Perricaudet, M. (1985) *EMBO J.* **4**, 3861–3865.
24. Saito, I., Oya, Y., Yamamoto, K., Yuasa, T. & Shimojo, H. (1985) *J. Virol.* **54**, 711–719.
25. Kozak, M. (1986) *Adv. Virus Res.* **31**, 229–292.
26. Kelly, T. J. & Lewis, A. M. (1973) *J. Virol.* **12**, 643–652.
27. Flint, S. J., Wewerka-Lutz, Y., Levine, A. S., Sambrook, J. & Sharp, P. A. (1975) *J. Virol.* **16**, 662–673.
28. Jones, N. & Shenk, T. (1978) *Cell* **13**, 181–188.
29. Bhat, B. M. & Wold, W. S. M. (1986) *J. Virol.* **60**, 54–63.
30. Andersson, M., Paabo, S., Nilsson, T. & Peterson, P. A. (1985) *Cell* **43**, 215–222.
31. Burgert, H. & Kvist, S. (1985) *Cell* **41**, 987–997.
32. Severinsson, L., Martens, I. & Peterson, P. A. (1986) *J. Immunol.* **137**, 1003–1009.
33. Gerety, R. J., Tabor, E., Purcell, R. H. & Tyeryar, F. J. (1979) *J. Infect. Dis.* **140**, 642–648.