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Hepatitis B virus proteins expressed by recombinant vaccinia viruses: influence of preS2 sequence on expression surface and nucleocapsid proteins in human diploid cells

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Summary. Fifteen vaccinia virus (VV) recombinants derived from VV strains Praha, LIVP and DD (i.e. Dryvax Wyeth vaccine - derived) and expressing genes for S, preS2-S or c antigens of hepatitis B virus (HBV) were tested in monkey CV-1 cells and human diploid LEP cells. The production of infectious virus was found to be alike in all the recombinants and parental viruses as well. However, several recombinants produced markedly lesser amounts of S and preS2 antigens in LEP cells than in CV-1 cells. This reduction was independent of the parental virus used. There was, however, a relationship between the production of preS2 in CV-1 cells and the production of S and preS2 antigens in LEP cells; in general, recombinants efficiently inducing preS2 antigen formation in CV-1 cells produced markedly reduced amounts of S and preS2 antigens in LEP cells. Reduction of HBV antigen production in LEP cells was not apparent in recombinants expressing only S or c antigens of HBV, and the production of c antigen by double recombinants was not influenced by simultaneous expression of preS2 and S. The various recombinants also differed in the ratio of S:preS2 antigen formation. This difference seemed to be associated with the length of the untranslated leader sequence preceding preS2 but not with the parental virus or cell type used. The titers of antibodies against S and preS2 antigens induced in mice immunized with different recombinants differed markedly. The differences in the ratio of S : preS2 antigen production in vitro were not reflected in vivo by S:preS2 antibody ratio.

Introduction

Recombinant vaccinia viruses (VV) containing genes for different forms of surface proteins of hepatitis B virus (HBV) have been constructed and their properties studied extensively [1, 2, 8, 9, 20, 27, 29]. Recombinants of VV expressing the surface protein of HBV were also prepared in our laboratory [15, 18]. In these experiments, we have observed that the amounts of S and preS2 antigens induced in human diploid LEP cells by recombinants, derived from VV Praha, were considerably lower than those produced in other cell types of non-human origin [15]. One can assume that this phenomenon, should it also occur in vivo and with other types of foreign proteins, could have a detrimental effect on the immunogenicity of recombinant viruses as human vaccines. We have therefore continued in our efforts to elucidate the influence of various recombinant constructions on the rate of expression of the heterologous proteins in different tissue culture systems.

In this study we investigated recombinants prepared from three different parental VV strains i.e. Praha, LIVP and Wyeth expressing S or preS2-S antigens of HBV. Recombinants expressing HBcAg derived from VV Praha were also used for comparison.

Materials and methods

Cells

Human embryo diploid lung cells (LEP), monkey kidney cells (CV-1) and media used for cultivation and maintenance of these cells were as described previously [15].

Plasmid vectors

Plasmids pAC2 [24], pCP10 [10], pHBV320 [25] and pHBV130 [4] were the sources of HBV sequences which were inserted into pGS20. Plasmids pAC2 and pCP10 were kindly provided by P. Tiollais (Institut Pasteur, Paris), plasmid pHBV320 by E. J. Gren (Institute of Organic synthesis, Riga) and plasmid pHBV130 by K. Murray (University of Edinburgh). Plasmid pGS20 [16] was received through the courtesy of B. Moss (National Institutes of Health, Bethesda). Plasmid pVVM (containing the HindIII M fragment of VV DNA inserted into pUC18) was prepared in our laboratory from a HindIII genomic library of VV Praha DNA. Plasmid pSN8 was prepared from pGS20 by insertion of a synthetic polylinker as described earlier for plasmid pSN11 [18]. Plasmids pSN8 and pSN11 differ only in the orientation of the inserted polylinker.

The list of recombination plasmids used is shown in Fig. 1. The preparation of recombination plasmids pM0, pM1, pM3 was described in detail previously [15, 18]. Briefly, plasmid pM0 comprises the 1.7 kb BamHI fragment of HBV-DNA-subtype *ayw* excised from the plasmid pAC2, and contains the entire preS2 and S sequence and additional 267 bp of upstream and 568 bp of downstream HBV sequences. In the plasmid pM1 the 267 upstream nucleotide pairs of pM0 were removed. Plasmid pM3 was made from pM1 by insertion of a synthetic oligonucleotide duplex upstream from the preS2 sequence; thereby the leader sequence was made 19 bp longer as compared to pM1. Plasmid pS2/15 was constructed by the insertion of 1.4 kb fragment containing HBV preS2-S sequence excised from pHBV320 [22]. Plasmid pS1 was prepared from the plasmid pHBV130 containing HBV DNA subtype

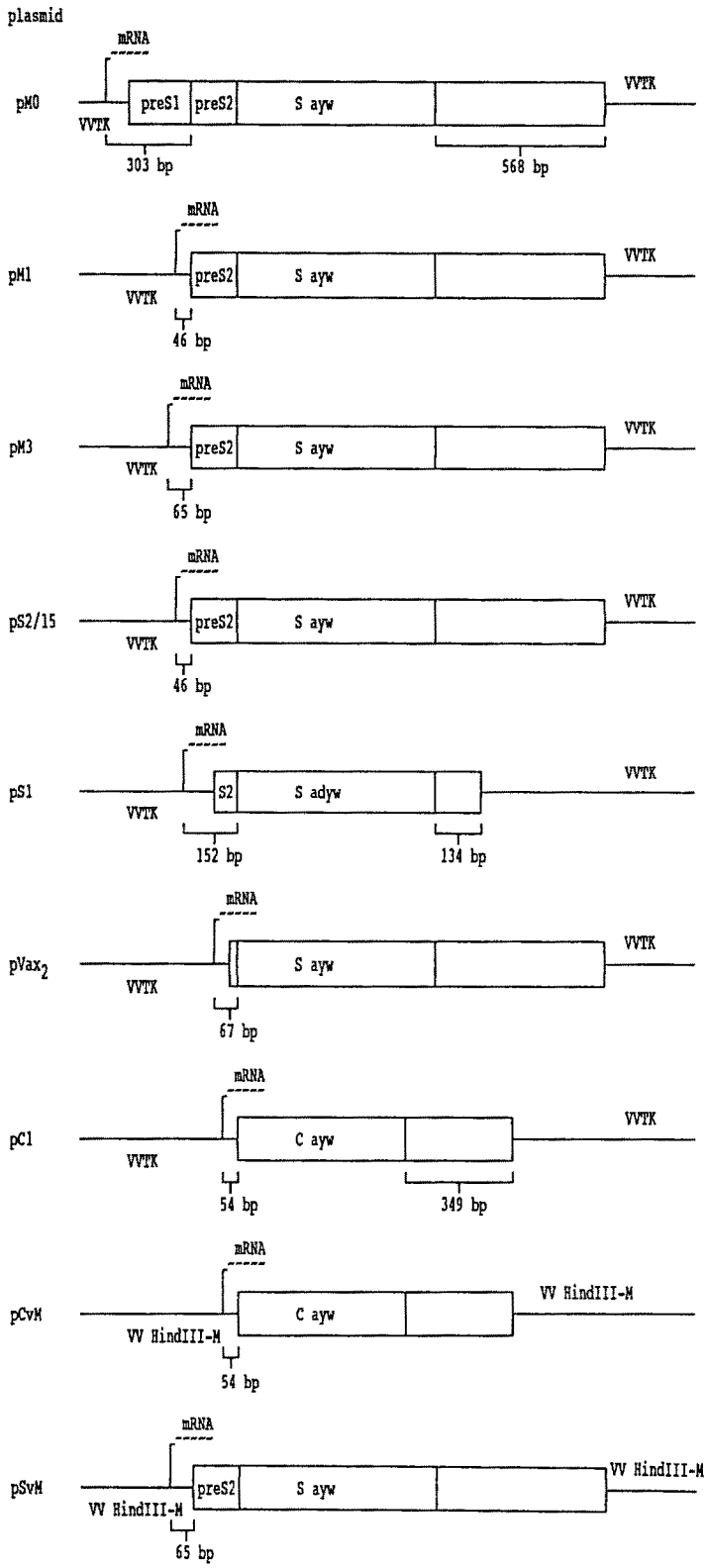

Fig. 1. Schematic representation of HBV sequences inserted into vaccinia virus genome. *PreS2* and *S* are regions of the S ORF, *C* is a region of the c ORF and open boxes represent HBV sequences downstream of the expressed ORFs. *VVTK* (thymidine kinase gene in HindIII J fragment) and VVHind III-M (HindIII M fragment) were used as insertion sites. The first translation initiation codon and its distance from the start of transcription is indicated

adyw [21] so that the 924 bp StuI fragment with HBV S gene was ligated to SmaI site of pGS20. Plasmid pVax2 contains 1.27 kb XhoI-BamHI fragment of HBV DNA-subtype *ayw* with HBV S gene, which was excised from the plasmid pHBV320 [3]. Plasmid pC1 contains 0.9 kb fragment of pCP10 with HBV-c-gene and additional 354 downstream bp inserted into plasmid pSN8. In all these plasmids HBV genes were inserted into thymidine kinase (TK) gene of VV under the control of VV 7.5 kD promoter.

Plasmid pCvM was prepared from plasmid pVVM digested with BglII and blunt-ended by Klenow fragment, by ligation to the fragment containing HBV-c gene and VV 7.5 promoter. This fragment was excised from plasmid pC1 by EcoRI and blunt-ended by Klenow fragment (Kunke et al., in press). Plasmid pSvM was constructed from plasmid pM3 which had been partially cleaved with EcoRI and blunt-ended by Klenow fragment. The isolated fragment, which contained preS2-S coding sequence and VV 7.5 promoter was ligated to plasmid pVVM cleaved by BglII and blunt-ended by Klenow fragment.

Viruses

The parental VV were (i) Praha strain used for smallpox vaccination in this country since 1892 [26], (ii) LIVP variant of Lister strain used for the smallpox vaccination in USSR and (iii) DD strain Derived from Dryvax Wyeth vaccine used for smallpox vaccination in the U.S.A.; this virus was provided to us by J. Becker (CDC, Atlanta). The list of recombinants prepared is shown in Table 1. Recombinants v137, v89, v10 containing gene coding for middle

Table 1. Characterization of recombinants

Recombinant	Plasmid	Parental virus	Fragment of HBV genome length (kb)	distance[a] (bp)	HBV gene present preS2	S	C
v137	pM0	Praha	1.68	303	+	+	−
v58	pM0	LIVP	1.68	303	+	+	−
v22	pM0	DD	1.68	303	+	+	−
v89	pM1	Praha	1.42	46	+	+	−
v7	pM1	LIVP	1.42	46	+	+	−
v10	pM3	Praha	1.42	65	+	+	−
L-preS2/15	pS2/15	LIVP	1.44	46	+	+	−
v3	pS1	Praha	0.92	152	−	+	−
L-HB32	pVax2	LIVP	1.28	67	−	+	−
v116	pVax2	LIVP	1.28	67	−	+	−
v61	pVax2	Praha	1.28	67	−	+	−
v9	pVax2	DD	1.28	67	−	+	−
v77	pC1	Praha	0.90	54	−	−	+
v17	pM3	v10	1.42	65	+	+	+
	pCvM		0.90	54			
v107	pC1	v77	0.90	54	+	+	+
	pSvM		1.42	65			

[a] Length of untranslated leader sequence preceding ATG of preS2 and S, respectively; the numbers indicate the total number of transcribed untranslated sequences, i.e. the sum of those connected to the respective HBV gene and those provided the recombination plasmid

surface protein of HBV (i.e. preS2-S coding sequence) were prepared on the basis of plasmids denoted pM0, pM1, pM3, respectively, and virus strain Praha [18]. Recombinants v58 and v7 were derived from plasmids pM0 and pM1, respectively, and LIVP strain; thus they are analogous to the recombinants v137 and v89, respectively. The recombinant denoted v22 was prepared from plasmid pM0 and DD strain; thus it parallels v137 and v58, respectively. The L-preS2/15 recombinant containing gene for middle surface protein of HBV was prepared from plasmid pS2/15 and LIVP strain. The v3 recombinant containing gene for the major HBV surface protein HBV (i.e. S coding sequence only) (serotype *adyw*) was constructed from plasmid pS1 and Praha strain. Recombinants L-HB32 [1], v116 and v61 containing gene for the major surface protein of HBV only (serotype *ayw*) were constructed from pVax2 plasmid and either LIVP or Praha strains. Recombinant v9 was prepared from the same plasmid and DD strain. Recombinant v77 was prepared from plasmid pC1 and Praha strain. Double VV recombinants v17 and v117 containing c gene as well as preS2-S gene were prepared on the basis of single recombinants v10 and v77 and recombination plasmids pCvM and pSvM, respectively (Kunke et al., in press). Recombinants were prepared by standard procedures [16, 19, 23].

Virus growth and production of HBV antigens

To compare the growth of different recombinants with the production of HBV antigens, 48 h-confluent cultures of LEP and CV-1 cells were infected at a m.o.i. of 0.1 PFU/cell. At the time of complete CPE (72 h after infection), media from two parallel cultures were centrifuged at 2 000 rpm for 20 min at 4 °C. The titres of infectious virus and HBV S and preS2 antigens in the supernatants were determined. The sediments were resuspended in the original volume of fresh medium and mixed with cells remaining in the bottles. Cell-associated VV and HBV proteins were released by three cycles of freezing and thawing. Virus titres and preS2 and S antigen content were determined after clarifying this material by low speed centrifugation (2 000 rpm/20 min at 4 °C). The remaining sediments were lysed by solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dedecyl sulphate (SDS), 1% Triton X-100, 1% sodium deoxycholate and 0.5 mM phenylmethylsulphonyl fluoride [11]. After 15 min incubation at 0 °C the lysates were clarified for 10 min in an Eppendorf centrifuge at 4 °C and tested by Western blotting (see below). The viruses were titrated in CV1 cells grown in Petri dishes using plaque assay with agar overlay. The virus titers were expressed in terms of PFU per 10^6 cells based on the cell counts in cultures at the time of infection. The two parallels were prepared and tested as independent samples.

The S antigen content was determined in Sevatest ELISA MICRO I kit (Sevac, Prague), using mouse anti-S monoclonal antibody for coating microtiter plates. The amount of S antigen produced was expressed in ng/10^6 infected cells. The determinations were done by comparison with standard purified plasmatic HBsAg preparation, kindly provided to us by V. Němeček (National Institute of Health, Prague). This antigen was purified by polyethylene glycol precipitation and CsCl and sucrose gradient centrifugation; the protein content of the resulting material was determined by Lowry method.

The preS2 antigen content was measured by two ELISA techniques. Usually, the preS2 content was determined in microtitration wells coated with 5 μg of human albumin polymerized by glutaraldehyde (PHA). The antigen bound to PHA was visualized with preS2-specific monoclonal antibody F124 [5], kindly provided to us by A. Budkowska (INSERM U 131, Clamert) and peroxidase-labelled swine anti-mouse conjugate (Sevac, Prague). The content of preS2 was expressed in antigen units as determined by comparison with the laboratory standard preparation containing 128 units of preS2 antigen, one unit being defined as the highest antigen dilution giving the absorbance of 0.1 (OD 492) in the presence of

antibody excess. As reported by Chelyapov et al. [6] this technique is not sensitive enough for detecting very low amounts of preS2 antigen. Therefore, when the above test yielded negative results, the respective materials were retested using another technique. In this assay microplates were coated with rabbit immune serum containing both S and preS2 antibodies, the test preparation was added and preS2 antigen, if present, was visualized with F124 monoclonal antibody and peroxidase labelled swine anti-mouse conjugate.

The HBc antigen content was determined in plates coated with anti HBc monoclonal HBc13yF9C7 antibody, kindly provided to us by J. Škvor (Institute of Sera and Vaccines, Prague). Peroxidase labelled goat anti HBc conjugate was used for the detection of the antigen bound. The amount of HBcAg was expressed in ng/10^6 cells. The determination of the HBc antigen was made by comparing the test preparation with the standard preparation of purified *E. coli* recombinant HBcAg (obtained from Institute of Organic Synthesis, Riga).

Immunoblot analysis

Lysates of cell sediments left after repeated cycles of freezing and thawing were used in Western blot assay. Because the reactivity of HBsAg with the available rabbit anti-HBs antibody was completely abolished under reducing conditions, the samples prepared by mixing 10 µl of the lysate (see above) with the same amount of a solution containing 0.065 M Tris-HCl pH 6.8, 2% SDS and 10% glycerol were not heated before being applied to 10% SDS- polyacrylamide gel. The proteins separated by PAGE were electrophoretically transferred to BA85 nitrocellulose membrane (Schleicher & Schuell) and blots were incubated with serum from rabbits immunized with purified plasma HBsAg. This serum contained antibodies against both S and preS2 antigens. The antigen-antibody complexes were visualized with swine anti-rabbit IgG labelled with peroxidase (Sevac, Prague) and with 4-chloro-1-naphtol (Koch-Light Laboratories).

Immunization of mice

Viruses used for immunization were grown on the chorioallantoic membranes of 11-day-old chick embryos and semipurified according to Joklik [13]. The viruses were resuspended in tissue culture medium supplemented with 5% peptone, sonicated, titrated in CV-1 cells and stored at −70 °C. Before being used for immunization the viruses were again sonicated. Four-week-old non-inbred white mice (females), strain ICR, were injected intraperitoneally with 0.5 ml containing 10^7 PFU of the virus, or 0.5 ml of tissue culture medium supplemented with 5% peptone. Four weeks later, five mice from each group were bled out. The other mice were divided into two groups. One received intraperitoneally 0.5 ml of the suspension containing 93 ng of purified plasma HBsAg (prepared as described above) absorbed to alum in phosphate buffered saline, the second group received alum in phosphate saline only. As indicated elsewhere [12] this amount of plasma HBsAg was found to be inducing no or negligible S and preS2 antibody response levels in unprimed mice but it elicited significant antibody levels in mice primed with the weakly immunogenic VV-HBV v137 recombinant. Six weeks after administration of HBsAg the mice were bled out. Sera diluted 1: 10 were individually tested by ELISA for the presence of anti-VV, anti-S and anti-preS2 antibody as described previously [12].

Results

Production of infectious virus and preS2 and S antigens in LEP and CV-1 cells

The production of infectious virus and the expression of HBV antigens in human diploid cells (LEP) and monkey kidney cells (CV-1) infected with different VV-HBV recombinants were determined. The virus yields were found to be similar for all recombinants studied and they did not differ markedly from the parental viruses. The virus yields tended to be somewhat higher (by 0.1 to 1.2 $\log_{10}$) in human LEP cells than in CV-1 cells (not shown).

The amounts of S antigen released from LEP and CV-1 cells infected with the recombinant viruses is shown in Table 2. It can be seen that the recombinants differed not only by the total amounts of S antigen produced but also by the ratio of the CV-1: LEP antigen harvests. The reduction of HBsAg production in LEP cells is well evident in v137, v89 and v10 recombinants derived from Praha virus; this reduction was observed in both fluid-phase and cell-phase preparations but was somewhat more marked in the former than the latter materials (not shown). The reduction was less apparent in recombinants v58 and v7 which were derived from LIVP virus. In the remaining two recombinants L-HB32 and L-preS2/15 derived from LIVP strain, the reduction was negligible. The amounts of preS2 antigen released from the infected LEP cells were also reduced (Table 3) but the differences between Praha-derived and LIVP-derived recombinant were less marked. When using polymerized human albumin, no preS2 was detected not only in L-HB32, which is free of preS2 sequence but also in L/preS2/15, which possesses preS2 sequence. However, in agreement with the previous experiments [7] the preS2 antigen was detected in cultures infected with the later virus, when this virus preparation was monitored in plates coated with rabbit polyclonal anti-HBsAg antibody and preS2 monoclonal antibody was used for visualization (see Materials and methods); the ratios of titers in

Table 2. Amount of S antigen produced by different recombinants in LEP and CV-1 cells

Recombinant	Amount of S antigen[a]		Ratio
	LEP cells	CV-1 cells	CV-1/LEP
P-v137	179	940	5.2
P-v89	249	1052	4.2
P-v10	315	1319	4.2
L-v58	298	854	2.9
L-v7	883	1644	1.9
L-HB32	636	762	1.2
L-preS2/15	1307	1775	1.4

[a] ng per 10^6 cells, in each experiment determinations based on two independently prepared samples. P, L-recombinants derived from Praha or LIVP viruses, respectively

Table 3. Amount of preS2 antigen produced by different recombinants in LEP and CV-1 cells

Recombinant	Amount of preS2 antigen[a]		Ratio
	LEP cells	CV-1 cells	CV-1/LEP
P-v137	110	323	2.9
P-v89	57	210	3.7
P-v10	69	204	3.0
L-v58	162	296	2.5
L-v7	108	241	2.2
L-HB32	< 15	< 15	—
L-preS2/15	< 15	< 15	—

[a] units per 10^6 cells, based on two independent preparations; binding to polymerised human albumin was used for the detection of preS2 antigen

CV-1 and LEP cells were 1.5 and 0.9 for fluid-phase and cell-phase preS2, respectively (not shown).

Tables 2 and 3 present results of a representative experiment in which all the recombinants were tested in parallel. In repeated tests, in which various combinations of the recombinants were examined, very similar data were obtained. Although there was some variation in the total amounts of S and preS2 antigens produced, the differences in CV-1/LEP ratios did not change substantially.

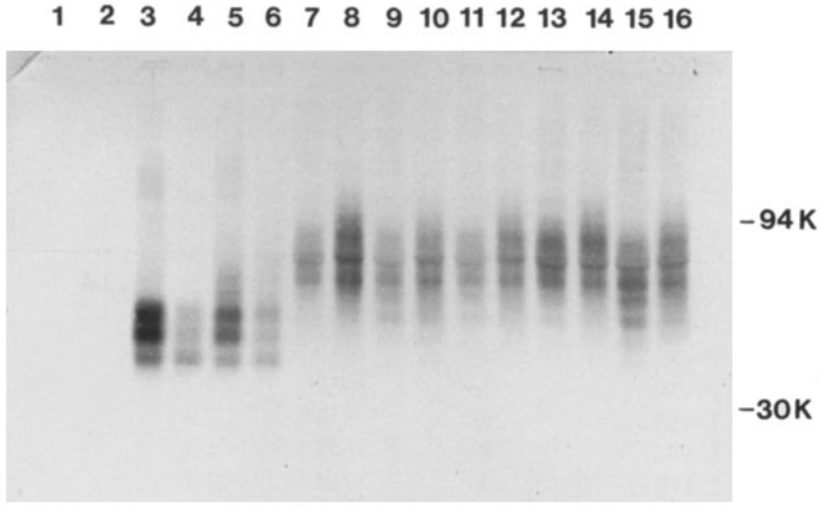

Fig. 2. Western blot analysis of HBsAg expression by different recombinants in LEP and CV-1 cells. LEP cells (odd numbers), CV-1 cells (even numbers). Parental VV-Praha (*1, 2*), L-HB32 (*3, 4*), L-PreS2/15 (*5, 6*), v137 (*7, 8*), v89 (*9, 10*), v10 (*11, 12*), v58 (*13, 14*), v7 (*15, 16*). The samples were prepared under non-reducing conditions

Immunoblots presenting both S and preS2 antigens remaining cell-associated after repeated cycles of freezing and thawing are shown in Fig. 2. The incomplete solubilization of protein products (see Materials and methods), their partial degradation and different level of glycosylation could be responsible for the presence of multiple bands reactive with antibodies against HBsAg. The differences in the amounts of antigens produced in LEP and CV-1 cells were similar to those determined by ELISA except the recombinants L-HB32 and L-preS2/15, in the case of which markedly more HBsAg remained associated with the LEP than with the CV-1 cells. Thus it seems that the somewhat less efficient release of the small S antigen from LEP cells infected with these two viruses may be rather due to a stronger association of this product with the cell structure than to the reduction of its overall production in these cells.

Association of S antigen production in LEP cells with recombinant construction

The results shown in Tables 2 and 3 suggested that the reduction of HBV antigen production in LEP cells might somehow be associated with the parental virus used. To examine this and to rule out that some uncontrolled experimental factors were involved in the construction and/or selection of the recombinants tested, new recombinants from plasmid pVax2 and parental viruses Praha, LIVP and DD (v61, v116, v9, respectively), were prepared in parallel and tested simultaneously with recombinants L-HB32, v3, v22 and v137. The reasons for including these recombinants were as follows. LHB32 was used as a reference virus. Recombinant v3, prepared from plasmid pS1 and VV Praha, was included to examine the possible effect of the plasmids used as the source of HBV sequences. Both these viruses were expressing S antigen only. The newly prepared v22 (derived from DD virus) and the reference recombinant v137 (derived from

Table 4. Production of infectious virus and S antigen by recombinants v61, v116, v9, v3, v22 and v137 in LEP and CV-1 cells

Recombinant	Virus titer[a]		S antigen amount[b]		Ratio
	LEP cells	CV-1 cells	LEP cells	CV-1 cells	CV-1/LEP titers
P-v61	5.7	5.5	647	880	1.4
L-v116	5.9	5.3	761	940	1.2
D-v9	6.1	6.0	621	1126	1.8
L-HB32	5.8	5.2	830	776	0.9
P-v3	6.4	5.9	937	1353	1.4
D-v22	6.1	6.0	117	901	7.7
P-v137	5.8	5.7	106	1239	11.7

P, L, D -recombinants derived from Praha, LIVP, or DD strain, respectively

[a] Log_{10} PFU per 10^6 cells

[b] ng per 10^6 cells

Table 5. Production of c, S and preS2 antigens in LEP and CV-1 cells by recombinants expressing either c, preS2 or both c and preS2-S antigens

Virus	Amount of antigens						Ratio		
	LEP cells			CV-1 cells			CV-1/LEP		
	c[a]	S[a]	preS2[b]	c	S	preS2	c	S	preS2
Praha	<8	<8	<15	<8	<8	<15	—	—	—
P-v77	202	<8	<15	223	<8	<15	1.1	—	—
P-v10	<8	372	93	<8	1391	253	—	3.7	2.7
P-v107	190	421	111	214	1616	271	1.1	3.8	2.4
P-v17	198	423	102	168	1306	249	0.9	3.1	2.4

[a] ng per 10^6 cells
[b] units per 10^6 cells

Praha virus) were used as representatives of recombinants expressing both preS2 and S. In this experiment the total amounts of S antigen produced in LEP and CV-1 cultures were determined in the whole culture extracts prepared by three cycles of freezing and thawing and low speed centrifugation. The results are shown in Table 4. It can be seen that significant reduction of S antigen production in LEP cells was only found in recombinants v22 and v137 expressing both preS2 and S antigens. On the other hand, no reduction of S antigen production in LEP cells was observed in recombinants expressing S antigen only, and there were no marked differences in this respect among recombinants derived from the various VV strains. Similar results were obtained in repeated experiments. The data suggested that the reduction of S antigen production in LEP cells was dependent on neither the parental VV strain nor the plasmid used for recombinant construction, but that it was associated with the presence and expression of the preS2 coding sequences.

To extend the present observation to another protein we constructed and subsequently tested double recombinants expressing simultaneously c and preS2-S proteins of HBV in LEP and CV-1 cells. Single recombinants expressing either c antigen only (v77) or preS2-S antigen only (v10) were tested in parallel. Again, whole culture extracts were used. The results are shown in Table 5. It can be seen that the production of c antigen by either the single or double recombinants was not influenced by the cell type used. On the other hand, both preS2 and S antigen productions were less efficient in LEP than CV-1 cells, irrespective of the use of single or double recombinants. This indicated that the presence and expression of the preS2 coding sequence did not influence c antigen production.

Differences in relative production of S and pre-S2 antigens by different recombinants

When the S: preS2 (ng: units) ratios were compared (Table 6), the recombinants could be divided into two groups. The ratios in the case of P-v89 and L-v7

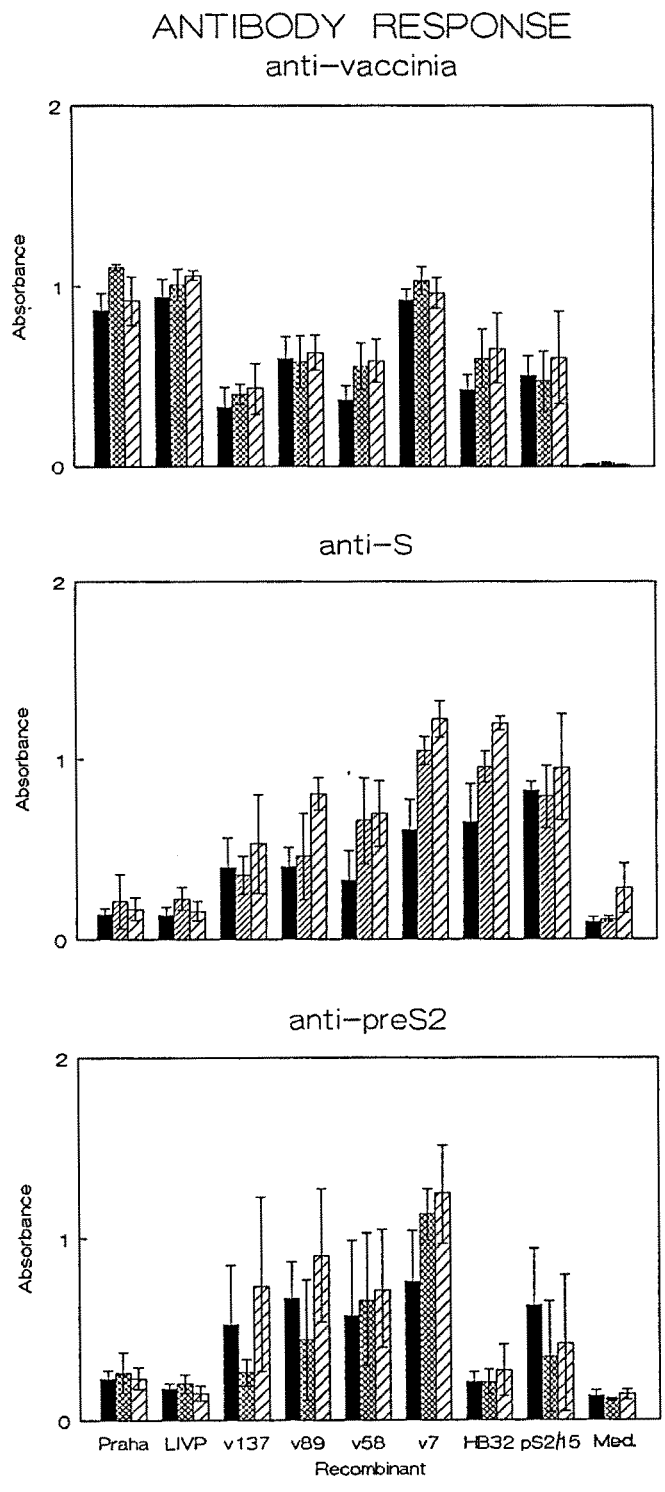

Fig. 3. Antibody response of mice inoculated with the parental VVs and their recombinants to VV, S and preS2 antigens. Each column represents arithmetical mean of absorbance values as detected in sera of five individual mice inoculated i.p. with 10^7 PFU of different viruses (■ four weeks after virus inoculation, ▩ six weeks after alum administration, ▨ six weeks after administration of alum-adsorbed 93 ng of plasma HBsAg; the interval between virus and plasma HBsAg administration was four weeks). Bars indicate the confidence intervals at 0.05 level

Table 6. Ratio of S and preS2 antigens induced by different recombinants in LEP and CV-1 cells

Recombinant	S/preS2 ratio[a] LEPcells	CV-1 cells
P-v137	1.6	2.9
L-v58	1.8	2.9
P-v89	4.4	5.0
L-v7	8.2	6.8

[a] Data from Tables 2 and 3 were used for determination of S/preS2 ratio (ng:units)

derived from the same plasmid (pM1) but from different VV strains, were considerably higher in both LEP and CV/1 cells than the corresponding figures for P-v137 and L-v58 which had been derived from another plasmid (pM0). Similar data were obtained in repeated tests. Thus, the S/preS2 ratio does not seem to be related to the parental virus but to the plasmid construct used, in this particular situation to the length of the untranslated leader sequence (see Materials and methods and Table 1). This effect was somewhat more obvious in the case of fluid-phase than cell-phase preparations (not shown).

Immunogenicity of selected recombinants in mice

The capabilities of selected recombinants to induce antibodies against VV, S and preS2 antigens in mice were compared. As indicated in Fig. 3, at the virus dose inoculated all recombinants except v7 induced lower levels (as reflected by O.D. values) of anti-VV antibodies than their parental viruses. There were differences in the levels of S and preS2 antibody and in the kinetics of antibody development elicited by individual recombinants. The production of antibodies against S and preS2 by LIVP-derived v58 and v7 recombinants continued to increase after the four week period following the virus administration. In animals immunized with these viruses, the booster effects of the plasma-derived HBsAg were negligible. Anti-S antibody induced by Praha-derived v137 and by LIVP-derived L-preS2/15 recombinant, tended to persist following the four-week postinfection period and only slight booster effect of the plasma- derived antigen were observed in the respective animals. However, the booster effect was quite apparent in the case of pre-S2 antibody in mice immunised with v137 and v89 recombinants. It is noteworthy that the S/preS2 antibody ratios did not reflect the differences in the production of these antigens in vitro (see Table 6).

Discussion

Should VV recombinants be used as human vaccines in the future, an efficient production of the respective foreign antigens by these viruses in human cells will

certainly be a necessary precondition. It is therefore important to find out why the production of some HBV antigens by VV recombinants has been markedly lower in human cells than in monkey, rabbit or rat cells [15]. In an attempt to elucidate the point we prepared and tested a new set of VV-HBV recombinants. The first collection of data suggested that the phenomenon was associated with the parental virus used. However, subsequent comparative experiments with the recombinants prepared from three different VV strains did not confirm this suspicion. These experiments also revealed that the reduction of S and preS2 production in LEP cells were associated with an efficient production of preS2. Recombinants expressing only S protein and the recombinant expressing only minute amounts of preS2 (L-preS2/15) did not reveal the reduction in LEP cells. This effect was HBV surface protein-specific, because c protein production in LEP was not influenced by simultaneous production of preS2-S, as it was revealed by testing double recombinants expressing both c and preS2-S. One can speculate that LEP cells contain an undefined factor, absent in CV-1 cells, which, if activated, by preS2 protein, is responsible for the low production of the preS2-S protein. At present it is unclear which level of gene expression control was affected. It is possible that either the low efficiency of transcription of the preS2-S transcription unit in LEP cells or the relative instability of the preS2-S mRNA (e.g. due to improper processing) in these cells were involved; however, the control at the translational level could also be involved. It is also possible that cell specific factors involved in the pos-translational modification of the preS2-S protein resulting in different efficiency of assembly, transportation and secretion were responsible for the observed differences. Experiments are under way to get more information.

The present results seem to confirm and extend the previously reported association of the length of the untranslated leader sequence preceding preS2-ATG with the relative production of S and preS2 as reflected by the S/preS2 ratio [18]. Similarly as in the in vitro model of Kozak [14] the length of the 5′ noncoding sequence could influence the initiation of translation and the resulting proportion of preS2 could influence the secretion of the product. The complexity of factors associated with the secretion of different forms of HBsAg was discussed by Standring et al. [28] and by McLachlan et al. [71]. It is noteworthy that in our experiments the differential in vitro production of the S and preS2 antigens was not associated with the ratio of S and preS2 antibodies in mice infected with the respective recombinants. This may reflect either a different relative production of the two antigens in mouse cells or a different processing and immunogenicity of the respective antigens in vivo, or both.

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