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Use of a Bacterial Expression Vector to Map the Varicella-Zoster Virus Major Glycoprotein Gene, gC

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The genome of varicella-zoster virus (VZV) encodes at least three major glycoprotein genes. Among viral gene products, the gC gene products are the most abundant glycoproteins and induce a substantial humoral immune response (Keller et al., J. Virol. 52:293–297, 1984). We utilized two independent approaches to map the gC gene. Small fragments of randomly digested VZV DNA were inserted into a bacterial expression vector. Bacterial colonies transformed by this vector library were screened serologically for antigen expression with monoclonal antibodies to gC. Hybridization of the plasmid DNA from a gC antigen-positive clone revealed homology to the 3′ end of the VZV U_S segment. In addition, mRNA from VZV-infected cells was hybrid selected by a set of VZV DNA recombinant plasmids and translated in vitro, and polypeptide products were immunoprecipitated by convalescent zoster serum or by monoclonal antibodies to gC. This analysis revealed that the mRNA encoding a 70,000-dalton polypeptide precipitable by anti-gC antibodies mapped to the *Hin*dIII C fragment, which circumscribes the entire U_S region. We conclude that the VZV gC glycoprotein gene maps to the 3′ end of the U_S region and is expressed as a 70,000-dalton primary translational product. These results are consistent with the recently reported DNA sequence of U_S (A. J. Davison, EMBO J. 2:2203–2209, 1983). Furthermore, glycosylation appears not to be required for a predominant portion of the antigenicity of gC glycoproteins. We also report the tentative map assignments for eight other VZV primary translational products.

Varicella-zoster virus (VZV) is the causative agent of chicken pox (varicella), one of the most prevalent childhood diseases, and shingles (zoster) after reactivation of latent virus later in life. Viral infection also can cause severe complications to adults and to immunocompromised individuals. Therefore, vaccination to VZV would be desirable, and attenuated live VZV strains have been developed as vaccines (2, 3, 26). By analogy to other virus systems, VZV glycoproteins would be the focal point of the immune response mounted against the virus. In this regard, the dissection of the primary structure of VZV glycoprotein genes and their gene products would be extremely informative.

Due in part to the difficulties associated with in vitro growth of VZV, this virus has not been as well characterized at the molecular level as other herpesviruses. To circumvent this difficulty, plasmid and phage libraries of VZV DNA fragments have been prepared by several laboratories (7, 9, 25). The entire U_S region of the genome has been previously sequenced (6). In addition, several groups have characterized monoclonal antibodies to VZV glycoproteins (10,13,20). However, until recently the variable and abundant number of reported viral glycoprotein species has left unclear the actual number of major viral glycoprotein genes (11, 21, 22, 29). Furthermore, no viral gene, glycoprotein or otherwise, has been mapped yet on the VZV genome.

Recently, we described the reactivities of monoclonal antibodies to VZV (15). In toto, these antibodies recognized eight distinct glycoprotein species in infected cells and virions. However, individual monoclonal antibodies fell into one of three families of reactivity: (i) gp105 only; (ii) gp115, gp62, and gp57; or (iii) gp92, gp83, gp52, and gp45. Since the three families of polypeptides are mutually exclusive in terms of serological reactivity, we have termed the groups gA, gB, and gC, respectively. Furthermore, since antibodies to each group can neutralize viral infectivity and since the eight glycoproteins represent the major observable virion species, we have suggested that the VZV genome encodes three major glycoprotein genes (gA, gB, gC) whose polypeptide products have neutralization epitopes. Of the three glycoprotein groups, the gC family represents the predominant humoral immune-reactive and structural glycoprotein constituent of VZV virions. This predominance is reflected experimentally in the immune reactivity of convalescent zoster sera, in the predominant number of monoclonal antibodies which react with gC glycoproteins, and in the autoradiographic intensity of radiolabeled virion glycoproteins. In this communication, we have employed monoclonal antibodies to gC to precisely map the VZV gC gene on the viral DNA genome, this being the first viral gene so mapped.

MATERIALS AND METHODS

Materials. Restriction endonucleases, DNA polymerase Klenow fragment, and T4 DNA ligase were purchased from New England Biolabs, T4 DNA polymerase was from P-L Biochemicals, DNA polymerase I and DNase I were from Boehringer-Mannheim Biochemicals, proteinase K was from Calbiochem-Behring, rabbit anti-mouse immunoglobulin was from Cappel Laboratories, and Formalin-fixed staphylococcus A cells were from Bethesda Research Laboratories. The following radiochemicals were obtained from Amersham Searle: L-[^{35}S]methionine (140 Ci/mmol), ^{125}I-labeled protein A (40 mCi/mg), [α-^{32}P]dCTP (500 Ci/mmol), and [α-^{32}P]dTTP (500 Ci/mmol).

Bacterial strains. *Escherichia coli* TK1046(λ1048), a derivative of strain TK1046, was the kind gift of M. Berman (27). After transformation, cells were plated overnight at 30°C on LB plates with ampicillin and 5-bromo-4-chloro-3-indolyl-beta-D-galactosidase (XG) to detect LacZ$^+$ clones. These cells were transferred to M63 minimal lactose (0.2% [wt/vol])

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plates and grown for 2 days at 30°C. At this point, the cells were processed for serological screening, Western blot analysis, or plasmid DNA purification, as described below.

Recombinant DNA. pORF2 was a gift from M. Berman (27). A library of recombinant VZV plasmids, consisting of 14 *Eco*RI clones in pACYC184 and 15 *Hin*dIII clones in pBR322, was provided by R. Hyman (9). For preparation of VZV inserts, an equimolar pool of the 29 recombinant VZV plasmid DNAs was digested at a DNA concentration of 250 μg/ml with 12 ng of DNase I per ml in 20 mM Tris (pH 7.5)–1.5 mM $MnCl_2$–100 μg of bovine serum albumin per ml for 30 min at 25°C (1). DNA fragments in the size range of 0.3 to 1.5 kilobase pairs (kbp) in length were purified by agarose gel electrophoresis, electroelution, and Whatman DE23 ion-exchange chromatography. T4 DNA polymerase was used to blunt end the DNA fragments. pORF2 DNA was digested with *Sma*I, and the linearized vector fragment was purified by preparative agarose gel electrophoresis, as described above. The vector and insert DNA fragments were ligated at a 1:2 molar ratio and used to transform *E. coli* TK1046(λ1048).

To test the fidelity of blunt-end restriction by *Sma*I, linearized pORF2 DNA was self-ligated and used to transform *E. coli*. Of ca. 2,000 transformants, only 2 (0.1%) were $LacZ^+$. One of these clones, which we term pORF2B (provided by P. Kniskern), was used in our experiments as a constitutive $LacZ^+$ $insert^-$ control.

For fine structural analysis, the insert from the gC-expressing pORF2 clone was excised with *Bam*HI and cloned into pBR322. DNA sequence analysis of this clone was performed by the procedure of Maxam and Gilbert (18).

Serological screening. The procedure we used to screen bacterial colonies for expression of VZV antigens was a modification of a previously published procedure (14), as suggested by J. Ravetch (personal communication). Colonies were lifted onto nitrocellulose filters (Schleicher & Schuell Inc.) and suspended for 15 min in saturated chloroform vapor. All incubations of the filters were at room temperature. Filters were incubated in 10 mM Tris (pH 7.5)–150 mM NaCl (TN) with 0.1% sodium dodecyl sulfate (SDS) for 60 min with shaking, washed for 15 min in TN, incubated for 15 min in TN with 2 μg of DNase I per ml, and washed two times for 15 min in TN. Further incubations of filters utilized quench buffer, consisting of phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.2% SDS, 0.02% sodium azide, and 3% bovine serum albumin (Sigma catalog no. 7030). Boiled *E. coli* was made by growing *E. coli* to saturation, boiling for 10 min, pelleting, washing two to three times in PBS, and suspending in a 1/50 volume of PBS for use at 1:200. Preincubations of filters were performed for 2 h each in quench buffer plus boiled *E. coli*. Incubations of filters with primary antibody (a pool of eight monoclonal antibodies, termed C1 to C8 [15] to VZV gC, each at 1:500), secondary antibody (rabbit anti-mouse immunoglobulin at 1:500), and ^{125}I-labeled protein A (0.25 μCi/ml) were performed for 2 h in quench buffer plus boiled *E. coli*. Filters were washed six times for 20 min in quench buffer.

VZV-infected (strain KMcC) (19) and uninfected MRC-5 cell lysates were prepared as previously described (15) to a final concentration equivalent of 2×10^4 cells per μl. For use as internal standards for the serological screenings, undiluted lysates or serially fivefold-diluted lysates in PBS were dotted in 2-μl volumes onto nitrocellulose filters, air dried, and processed as described above.

Western blots. *E. coli* TK1046(λ1048) $LacZ^+$ colonies were scraped from minimal lactose plates into SDS-polyacrylamide gel sample buffer, boiled, electrophoresed, transferred to nitrocellulose, and reacted with antibodies as previously described (15). Rabbit anti-beta-galactosidase antiserum (a gift from V. Larson) was prepared to *E. coli* beta-galactosidase (Sigma catalog no. G-6008). The source of other ascites fluids, convalescent zoster serum, and normal human serum has been previously described (15).

Southern blots. Plasmid DNA from *E. coli* TK1046(λ1048) colonies transformed by pORF2-VZVgC1 (see below) or by pORF2B was purified by the alkaline lysis technique (17). The KMcC strain of VZV was the source of virion DNA, which was purified after nucleocapsid isolation (24). Probe DNA was nick translated in the presence of [α-^{32}P]dCTP and [α-^{32}P]dTTP (17).

DNA samples were digested with restriction endonucleases, resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with radiolabeled DNA probes under stringent conditions (17).

Hybrid selection of mRNA. The guanidinium isothiocyanate technique (4) was employed to isolate cytoplasmic RNA from VZV-infected or uninfected MRC-5 cells. Plasmids from the VZV recombinant library were used to hybrid select mRNA by standard procedures (5).

In vitro translation of mRNA and immunoprecipitation analysis. RNA was translated in vitro in rabbit reticulocyte lysates (Bethesda Research Laboratories) according to the recommendations of the supplier. In vitro translational products were immunoprecipitated, electrophoresed, and autoradiographed as previously described (15).

RESULTS

We utilized an open reading frame (ORF) expression vector to attempt to physically map the VZV gC gene. Our rationale was to express in bacteria VZV gC antigens detectable by monoclonal antibodies to gC. Small fragments of randomly digested VZV DNA would be inserted into the expression vector. Such recombinant plasmids would be introduced into bacteria that would permit detection of ORF-positive inserts. After screening for antigen production with monoclonal antibodies, the plasmid DNA from a gC antigen-positive clone would be employed as a probe for VZV genomic DNA and would specify the gC gene map location.

Design of expression vectors producing VZV antigens. The method we used to construct a VZV genomic library of recombinant expression vectors is depicted in Fig. 1. The pORF2 vector contains the promoter, regulatory region, and 5′ coding sequence (initial 33 amino acids) from the *E. coli ompF* gene. Adjacent to this region is a linker containing several restriction endonuclease recognition sites, including that of the blunt-end cutter *Sma*I. This is followed by most of the *lacZ* gene, which encodes the enzymatically active carboxy terminus of beta-galactosidase and retains enzymatic activity after the amino-terminal fusion of foreign polypeptides. The pORF2 vector is phenotypically $LacZ^-$, since the *lacZ* gene sequences are out of frame with respect to the 5′ *ompF* sequences. To produce a random set of VZV genomic fragments, a dual library of VZV plasmids (*Eco*RI as well as *Hin*dIII clones) constituting ca. 99% of the VZV genome was digested with DNase I in the presence of Mn^{2+} to produce double-stranded scissions. A dilution of DNase I was used which produced fragment lengths in the range of 0.1 to 2.5 kbp. DNA sized 0.3 to 1.5 kbp in length, which constituted ca. 60% of this pool, was purified by preparative agarose gel electrophoresis, and the termini of these fragments were made blunt ended by T4 DNA polymerase. We focused on this size range as one which statistically was

small enough to contain a reasonable percentage of ORFs and large enough to potentially encode serological epitopes. It should be noted that ca. 30% of the DNA content of the VZV insert pool was plasmid in origin; however, the use of serological screening renders these sequences functionally inert in the library. The blunt-ended VZV insert pool was ligated to *Sma*I-digested pORF2 DNA, and the recombinant plasmids were used to transform *E. coli* TK1046(λ1048). Any insert of length 3n+1 which is an ORF is capable of restoring the *lacZ* gene to the correct reading frame and producing the LacZ$^+$ phenotype. In theory, independent of the presence of stop codons, 1 of 18 recombinant clones would express a VZV ORF and would be LacZ$^+$ (phenotypically identifiable with the indicator substrate XG).

Characterization of a gC antigen-positive bacterial clone. After transformation of TK1046(λ1048) cells by the recombinant plasmids, ca. 2% of the colonies were LacZ$^+$. These colonies were replated onto minimal lactose plates and grown at 30°C for 48 h. The colonies were transferred to nitrocellulose filters and screened serologically for gC antigen expression with a pool of eight monoclonal antibodies to VZV gC. From among 2,000 LacZ$^+$ colonies screened, 1 demonstrated a consistently strong reactivity in the serological screen, and all further analysis was performed on this clone, called pORF2-VZVgC1. The reactivity of this clone in the gC serological screen is demonstrated in Fig. 2A. Note that when tested for comparative reactivity to VZV-infected and uninfected MRC-5 cell lysates, the pool of antibodies to gC demonstrated an approximately 100-fold preference in reactivity to the infected cell lysate (Fig. 2B).

To characterize the polypeptide product(s) encoded by the recombinant clone, Western blot analysis was performed upon pORF2-VZVgCl-containing bacteria grown on agar plates. Six of eight monoclonal antibodies to gC detected several polypeptide bands unique to this clone. A representative profile utilizing one of these antibodies is shown in Fig. 3A. There were three major species of molecular weights 35,000 (35K), 21K, and ca. 160K. Of these, only the latter

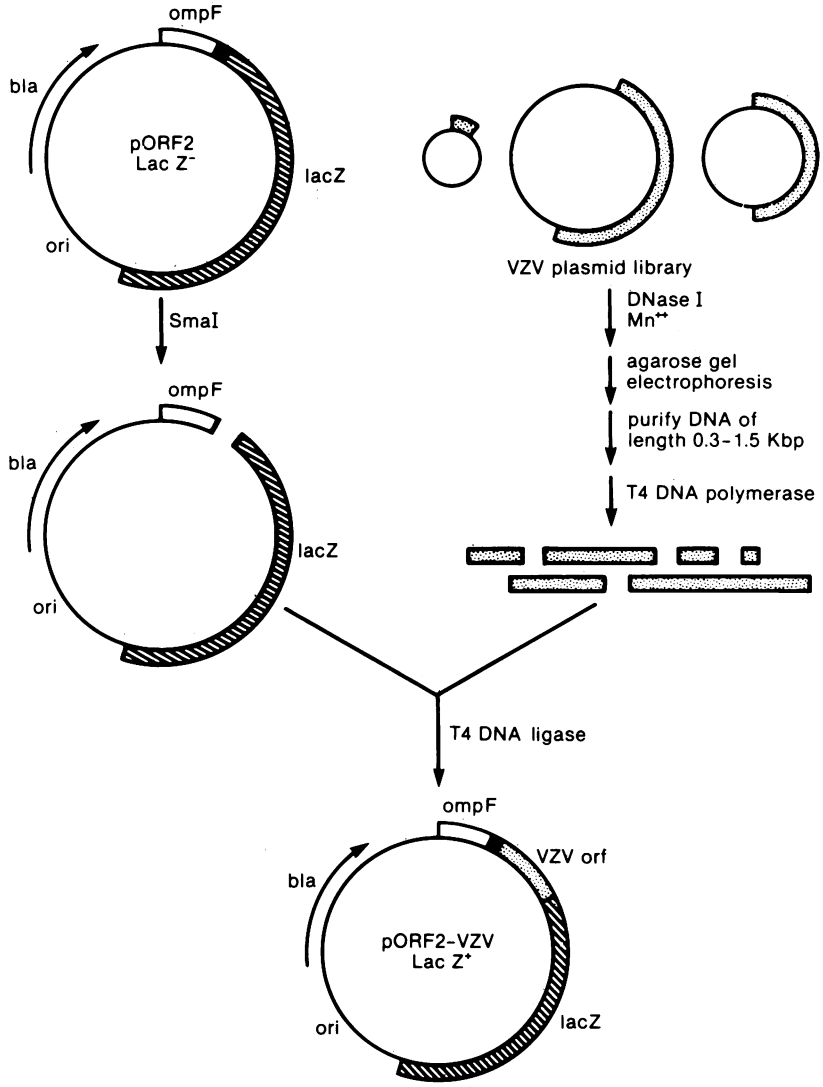

FIG. 1. Strategy for the construction of a VZV plasmid DNA expression library. The schematized DNA sequences are derived from the following sources: (i) pBR322 (thin line), including the origin of replication (ori) and beta-lactamase gene (bla); (ii) *ompF* gene promoter, regulatory region, and 5′ coding sequence (□); (iii) synthetic linker with restriction endonuclease sites (■); (iv) *lacZ* gene complete coding sequence missing the first eight amino acids (▨); (v) VZV genomic sequences (▩).

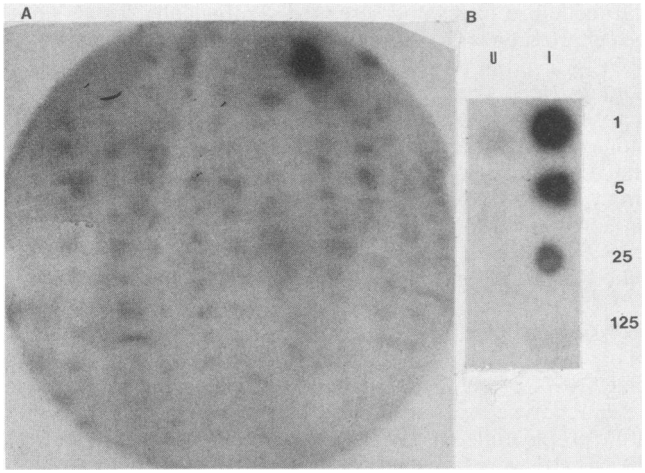

FIG. 2. Serological screening of reactive polypeptides by monoclonal antibodies to VZV gC. Filters were incubated successively with a pool of monoclonal antibodies to VZV gC, rabbit anti-mouse immunoglobulin, and ^{125}I-labeled protein A. (A) Bacterial colonies containing pORF2-recombinant plasmids were transferred to nitrocellulose filters disrupted by chloroform and treated with SDS and DNase I. (B) The indicated reciprocal dilutions of VZV-infected (I) and uninfected (U) MRC-5 cell lysates were dotted onto nitrocellulose filters and air dried.

polypeptide was reactive with mouse anti-beta-galactosidase antibodies (Fig. 3B). Interestingly, 8 of 11 convalescent zoster sera tested recognized the 35K gC band, suggesting that nonglycosylated gC epitopes are immunogenic in humans. A representative profile utilizing one of these sera is shown in Fig. 3C; note the reactivity of the 35K band in lane 2. None of the polypeptides in lane 2 of Fig. 3A were reactive with other non-gC monoclonal antibodies, other ascites fluids, or normal human serum (data not shown). Three independent preparations of pORF2-VZVgC1 lysates possessed similarly sized polypeptides reactive with anti-gC monoclonal antibodies (data not shown).

The pORF2-VZVgC1 DNA was purified from bacteria grown on agar plates. The size of the insert could not be calculated directly by *Sma*I digestion, since this site in pORF2 was destroyed as a consequence of the cloning procedure. However, the *Sma*I site in the linker is circumscribed by two *Bam*HI sites within five nucleotides. *Bam*HI digestion of pORF2-VZVgC1 DNA revealed an insert of ca. 0.9 kbp (data not shown). An ORF of this length would specify ca. 35K of gC polypeptide coding information. This size is consistent with the 160K *ompF*-ORF(VZV)-beta-galactosidase tribrid protein, as encoded by the pORF2-VZVgC1 vector, relative to the 120K *ompF*-beta-galactosidase hybrid protein encoded by the in-frame pORF2B vector. In addition, this size is consistent with the 35K species reactive with monoclonal antibodies. This polypeptide probably represents a breakdown product of the 160K species, since mouse anti-beta-galactosidase antibodies recognize a 120K species in these cells (Fig. 3B, lane 2).

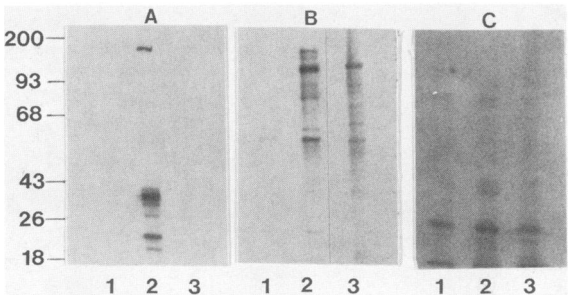

FIG. 3. Western blot analysis of bacterial polypeptides. Lysates of bacterial colonies were electrophoresed, transferred to nitrocellulose, and reacted with the following antibodies: A, monoclonal antibody C5 to VZV gC; B, rabbit anti-beta-galactosidase antiserum; C, convalescent zoster serum. TK1046(λ1048) colonies were untransformed (lane 1), transformed by pORF2-VZVgC1 (lane 2), or transformed by pORF2B (lane 3). Myosin (200K), phosphorylase *b* (93K), bovine serum albumin (68K), ovalbumin (43K), chymotrypsinogen (26K), and beta-lactoglobulin (18K) are the molecular weight markers.

Lysates prepared from pORF2-VZVgC1 bacteria after overnight growth in minimal lactose liquid media contained negligible levels of gC antigens (<1% of those shown in Fig. 3A, lane 2). Furthermore, after such growth, pORF2-VZVgC1 DNA had undergone extensive rearrangement, as judged by restriction endonuclease analysis (data not shown). These observations suggest that the production of antigen from pORF2-VZVgC1 is unstable in TK1046(λ1048) cells during growth in liquid media and suggest that the instability in tribrid protein production was manifest at the DNA level.

Genetic mapping of the VZV gC gene by means of pORF2-VZVgC1 DNA homology. Hybridization of pORF2-VZVgC1 DNA to restriction endonuclease fragments of VZV genomic DNA should specify uniquely the map location of the gC glycoprotein gene. Purified VZV viral DNA was digested with each of six restriction endonucleases whose sites had been mapped previously on the viral genome (7–9, 25). Figure 4 demonstrates a Southern blot analysis of these restriction digests after hybridization to a pORF2-VZVgC1 DNA probe. The pattern of hybridization of the VZV gC ORF to viral DNA restriction fragments uniquely specifies homology to the U_S region of VZV (arrow, Fig. 5A). This was deduced from the map locations of the unambiguously assigned *Pst*I A and *Sac*I G fragments. The *Eco*RI-A and -F inversion isomers (9, 25) map to U_S. In addition, other hybridizing fragments containing the complete U_S sequence could be assigned from among other closely migrating bands: (i) *Hin*dIII-C (compared with -D), (ii) *Pvu*II-A (compared with -B), and (iii) *Xho*I-D (compared with -C and -E).

Finer structural analysis of the hybridizing sequences was performed on the VZV pVHC (HC = *Hin*dIII-C) clone, which circumscribes U_S and contains substantial portions of both IR_S and TR_S. A restriction map of this clone was constructed (Fig. 5B). The location of many of these restriction sites was confirmed by the recently published sequence of U_S (6). Restriction fragments of pVHC were subjected to Southern blot analysis by hybridization to a pORF2-VZVgC1 probe. The extent of homology of these restriction fragments to the expression vector probe is highlighted schematically (arrow, Fig. 5B). These data localize the region of homology to a segment of ca. 1 kbp in U_S.

The recently published complete nucleotide sequence of U_S contains four ORFs of coding potential >80 amino acids. The longest of these, which spans the U_S-TR_S junction, would specify a 70K or 74K polypeptide (there are two in-frame ATG codons). Our Southern blot hybridization analysis of pVHC places the homology to the ORF in pORF2-VZVgC1 completely within the 74K-70K ORF defined by Davison's U_S sequence between map positions 0.925 and 0.942 (Fig. 5C).

To analyze the homology further, the pORF2-VZVgC1 ORF was excised from the vector by *Bam*HI digestion and cloned into pBR322. Restriction digestion of the pBR322 subclone revealed the presence of *Nru*I, *Nco*I, *Cla*I, *Bgl*III, and *Bst*EII sites at locations predicted from the published U_S sequence (arrow, Fig. 5C). For precise alignment, the DNA sequence for ca. 60 nucleotides at each terminus of the insert was determined after radiolabeling with the DNA polymerase Klenow fragment and *Bgl*II digestion. The insert terminal sequences aligned perfectly with the published U_S sequence and demonstrated that the bacterial ORF spanned amino acids 38 to 337 in the 70K VZV ORF (624 amino acids).

Hybrid selection and mapping of VZV gC mRNA. The homology mapping data described above, in conjunction with the DNA sequence analysis, predict that the primary translational product of the VZV gC gene is a 70K-74K polypeptide. To ascertain this prediction and to confirm independently the map location of the VZV gC gene, we performed hybrid selection analysis of VZV mRNA. Total infected cellular mRNA was hybrid selected by plasmids in the VZV *Hin*dIII library, translated in vitro in rabbit reticulocyte lysates, and immunoprecipitated with human convalescent zoster serum or with a monoclonal antibody to VZV gC (Fig. 6). The immunoprecipitation analyses demonstrate that the serum, which had been shown previously to possess strong reactivity to VZV gC (15), strongly recognized a 70K polypeptide whose mRNA is hybrid selected by pVHC DNA (Fig. 6A, lane 3). Note that the autoradiographic exposure of this lane is eightfold less than all the other hybrid selection lanes. This 70K species was the most prominent polypeptide immunoprecipitated from the in vitro translational products of unselected VZV mRNA (Fig. 6A, lane 12) but was absent from the in vitro translational products of unselected uninfected MRC-5 cellular mRNA (Fig. 6A, lane 13). The 70K polypeptide can also be immunoprecipitated by zoster serum, but not normal human serum, after hybrid selection by pVHC, pVEA, and pVEE, but not by the DNA of other *Hin*dIII and *Eco*RI clones (Fig. 6B, lanes 1 and 2). The pVEA and pVEE clones abut at a common *Eco*RI site in U_S which bisects the 74K-70K ORF (Fig. 5C). Furthermore, an anti-gC monoclonal antibody, but not an unrelated monoclonal antibody, immunoprecipitated the 70K polypeptide after hybrid selection of VZV mRNA by pVHC, pVEA, and pVEE, but not by the DNA of other *Hin*dIII and *Eco*RI clones (Fig. 6B, lanes 3 and 4). It is noteworthy that seven of the monoclonal antibodies to gC were able to immunoprecipitate this 70K nonglycosylated primary translational product (data not shown). From these analyses, we conclude that gC is derived from a 70K primary translational product which maps to the *Hin*dIII C fragment circumscribing U_S. Moreover, these data are consistent with the mapping data utilizing pORF2-VZVgC1 which identify the gC gene as the 74K-70K ORF spanning the U_S-TR_S junction.

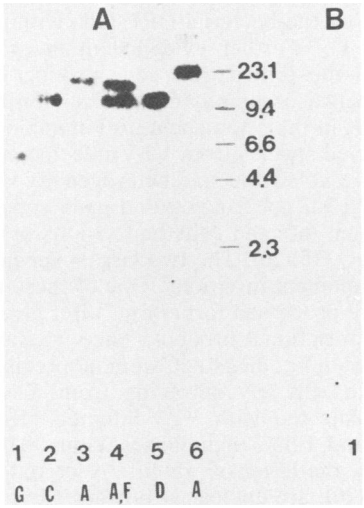

FIG. 4. Southern blot analysis of restriction endonuclease-digested VZV viral DNA. Purified viral DNA (2 μg) was digested, electrophoresed, and blotted. (A) Hybridization to ^{32}P-labeled pORF2-VZVgC1 DNA probe. Enzymes used for digestion were *Sac*I (lane 1), *Hin*dIII (lane 2), *Pst*I (lane 3), *Eco*RI (lane 4), *Xho*I (lane 5), and *Pvu*II (lane 6). Based upon previously published restriction endonuclease cleavage maps of VZV genomic DNA, tentative assignments for each hybridizing restriction endonuclease fragment (as noted by the dots) have been noted beneath each lane number. (B) Hybridization to ^{32}P-labeled pORF2B DNA probe. Lane 1: purified viral DNA (2 μg) digested with *Eco*RI. Molecular weight markers (in kbp) are *Hin*dIII digestion products of lambda DNA.

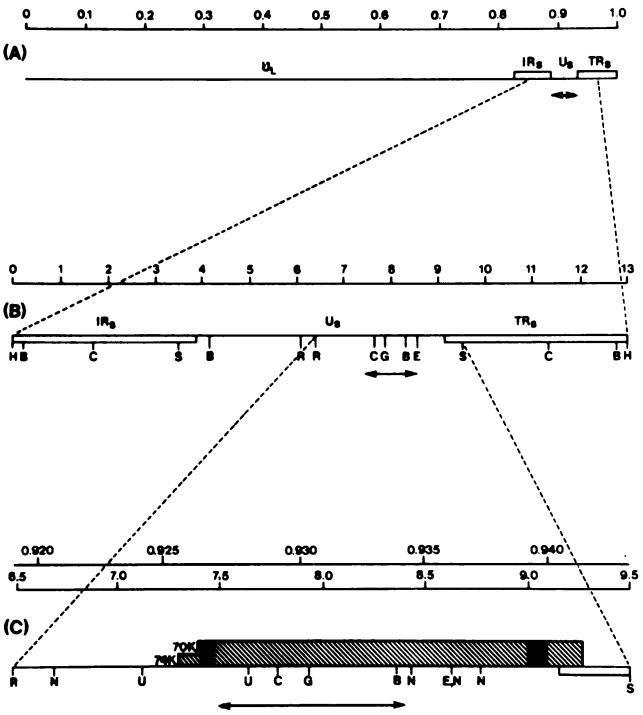

FIG. 5. Summary of the hybridization pattern of pORF2-VZVgC1 DNA to the U_S region of the VZV genome. (A) Gross structural regions (U_L, IR_S, U_S, TR_S) of the VZV genome are aligned with a scale of fractional genomic length. The arrow beneath the map highlights the region of homology to pORF2-VZVgC1 DNA defined by the analysis in Fig. 4. This region is expanded into (B). Restriction endonuclease sites were mapped onto pVHC, the *Hin*dIII C fragment. Distances on the map of the clone are marked in kbp. The arrow beneath the map highlights the region of homology of restriction endonuclease fragments of pVHC to the pORF2-VZVgC1 probe. This region is expanded into (C). The location of the 74K-70K ORF (6) between 7.3 and 9.3 kbp (0.925 to 0.942) is highlighted by a hatched box. Two large hydrophobic protein domains imputed from the DNA sequence of this ORF are demarcated by the solid box. The arrow beneath the map defines the location of the pORF2-VZVgC1 ORF on the 74K-70K ORF as determined by DNA sequence analysis. Restriction endonuclease cleavage sites are: B, *Bst*EII; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Nde*I; R, *Eco*RI; S, *Sac*I; U, *Nru*I.

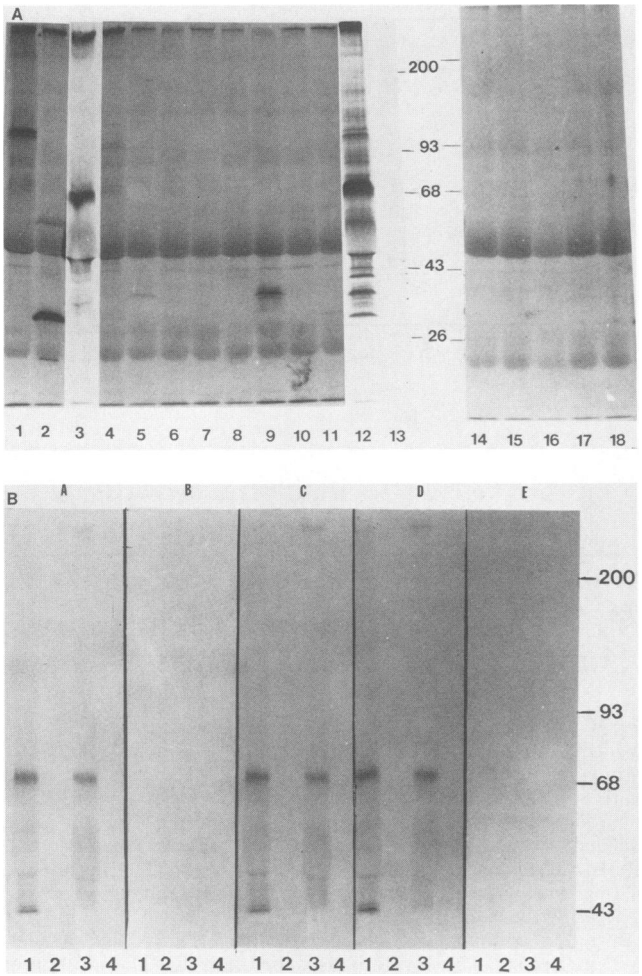

FIG. 6. Electrophoretic analysis of immunoprecipitations of in vitro translational products. (A) RNA samples were translated in vitro and immunoprecipitated with human convalescent zoster serum. Lanes 1 to 11 and 14 to 18 represent the products of infected cellular RNAs hybrid selected by the following plasmid DNAs: 1, pVHA (*Hin*dIII A fragment); 2, pVHB; 3, pVHC; 4, pVHD; 5, pVHE; 6, pVHF; 7, pVHG; 8, pVHH; 9, pVHI; 10, pVHK; 11, pVHL; 14, pVHM; 15, pVHO; 16, pVHP; 17, pVHQ; 18, pBR322. Lanes 12 and 13 represent the translational products of unselected VZV-infected MRC-5 cellular RNA and unselected uninfected MRC-5 cellular RNA, respectively. Note that the autoradiographic exposures of lanes 3, 12, and 13 are eightfold less than those of the other lanes in this figure. (B) RNA samples were translated in vitro and immunoprecipitated with convalescent human zoster serum (lane 1), normal human serum (lane 2), monoclonal antibody C5 against VZV gC (lane 3), or monoclonal antibody B1 against VZV gB (lane 4) after hybrid selection by the following plasmid DNAs: A, pVHC; B, pVHK; C, pVEA; D, pVEE; E, pVEI.

Map assignments of other VZV polypeptides. Convalescent zoster serum contains antibodies reactive with many VZV polypeptides in infected cells (12, 15, 22, 29). One such serum can immunoprecipitate ca. 12 major in vitro translational products of unselected VZV mRNA (Fig. 6A, lane 12). Other than the 70K gC translational product, eight other primary translational products could be mapped to various *Hin*dIII fragments on the basis of hybrid selection analysis. These are: (i) 120K to *Hin*dIII-A, (ii) 115K to *Hin*dIII-A, (iii) 88K to *Hin*dIII-D, (iv) 57K to *Hin*dIII-B, (v) 40K to *Hin*dIII-H, (vi) 35K to *Hin*dIII-E, (vii) 35K to *Hin*dIII-I, and (viii) 30K to *Hin*dIII-B.

DISCUSSION

The mapping of VZV genes on the viral genome had been hampered until recently by the lack of availability of critical nucleic acid and serological reagents. Classically, mapping studies in several viral systems have combined the use of monoclonal antibodies to specific viral polypeptides with recombinant plasmids carrying fragments of the viral genome. One of the most widely used such combinations has been the hybrid selection of viral RNA to recombinant plasmid DNA, in vitro translation of selected RNA, and immunoprecipitation of translational products with monoclonal antibodies. Numerous examples of such mapping studies abound in the herpes simplex and vaccinia virus systems (5, 16). In the past three years, several laboratories have reported the isolation of recombinant VZV genomic plasmids and monoclonal antibodies to VZV glycoproteins. These reagents have laid the foundation for the genetic mapping of VZV genes, of which the glycoprotein gene reported in this manuscript is the first to be mapped. In addition, we report the tentative map assignments of eight other primary translational products to various *Hin*dIII fragments.

Previously, we have presented evidence that the VZV genome encodes three major glycoprotein genes whose polypeptide products have neutralization epitopes (15). By means of two independent approaches, we have utilized recombinant plasmids and monoclonal antibodies to gC to genetically map gC to the 3′ end of the U_S region of the VZV genome. Our data suggest that gC is expressed as a 70K primary translational product. In this regard, Davison's sequence analysis of U_S is noteworthy in that the homology region defined by the bacterial ORF falls within a 74K-70K ORF in VZV U_S. Further evidence in favor of the 70K polypeptide as the primary gC gene product comes from pulse-chase analysis, whereby monoclonal antibodies to gC (94K, 83K, 55K in their nomenclature) immunoprecipitate a 70K pulse-labeled species from VZV-infected cells (20). We and others have noted the size heterogeneity of mature gC polypeptides; gp92, gp83, gp52, and gp45 can be immunoprecipitated from infected cells and virions by monoclonal antibodies to gC (15, 20). The two largest species, gp92 and gp83, are predominant in virions. One of these might represent the mature processed form of gC after glycosylation of the primary translational product, since we have observed that monoclonal antibodies to gC immunoprecipitate heterogenous 85K to 90K glycoproteins from *Xenopus laevis* oocytes microinjected with VZV-infected MRC-5 cellular RNA (Lowe and Ellis, unpublished data). The origins of gp52 and gp45, the latter of which is secreted from virally infected cells (20), are unclear at present.

Our studies underscore the utility of ORF expression vectors for the mapping of viral genes. The primary requirement for this type of experiment is a monoclonal antibody reactive with polypeptides immobilized on nitrocellulose. Antibodies can be prescreened for this purpose by means of the dot blot assay of the type we used which utilizes lysates of virally infected and uninfected cells. In the case of glycoprotein genes, a monoclonal antibody is required which is capable of recognizing a nonglycosylated polypeptide. Although either viral DNA or a recombinant plasmid library can be utilized as the starting material for ORFs, an appropriate DNA size range must be selected after DNase I

titration to isolate a reasonable percentage of ORFs as well as serological epitopes. The vector pORF2 system which we have utilized was useful for our mapping studies in that it allowed for the selection of ORF-containing plasmids. The drawback of this system is the apparent instability of protein expression, so that large-scale isolation and analysis of expressed polypeptides are infeasible. This is apparently due to the rearrangement of plasmid DNA, probably resulting from selective pressure against expression and subsequent insertion of the signal sequence containing tribrid proteins into the bacterial membrane (23). However, at least in the case of the gC tribrid protein, the expression of the ORF is of sufficient stability and amount such that monoclonal antibodies can be utilized for screening purposes. We recently have succeeded in attaining more stable and higher level expression of the gC-beta-galactosidase fusion protein adjacent to the inducible λ P_L promoter (unpublished data).

Several of our observations have addressed the role of glycosylation in the biology of gC polypeptides. Firstly, 8 of 11 convalescent zoster sera recognize the nonglycosylated gC polypeptide fragment expressed in bacteria. Secondly, seven of eight monoclonal antibodies to gC can immunoprecipitate the 70K primary in vitro translational product of the gC gene. Thirdly, six of eight monoclonal antibodies to gC can recognize the nonglycosylated bacterial tribrid protein. This means that glycosylation of gC polypeptides may not be required for a substantial portion of the immunogenicity of the gene product. A related observation has been made with the gD glycoprotein of herpes simplex virus type 1 (28). Not only was the nonglycosylated gD polypeptide produced in bacteria found to be antigenic, the polypeptide also was found to be capable of inducing the production of neutralizing antibodies.

The gC mapping experiments described here represent an encouraging beginning toward the development of a subunit vaccine against VZV. On the one hand, it is likely that VZV gA and gB antigenic determinants in a subunit vaccine might contribute to protective immunity, since both glycoproteins can induce the production of neutralizing monoclonal antibodies (13, 15; C. Edson, submitted for publication). On the other hand, the fact that gC polypeptides represent the most immunogenic glycoprotein constituents of the virion and that monoclonal antibodies to gC neutralize infectivity in the presence of complement raises the possibility that the presence of gC antigenic determinants in a subunit vaccine would be necessary and sufficient for conferring protective immunity to a vaccinee. Therefore, given the conclusion that glycosylation apparently is not required for a substantial portion of the antigenicity of gC polypeptides, a nonglycosylated gC polypeptide produced in *E. coli* or *Saccharomyces cerevisiae* should be effective in inducing neutralizing antibodies and in providing protective efficacy against VZV to vaccinees. We note that the pORF2-VZVgC1 ORF, which constitutes ca. 45% of the complete gC ORF in VZV U_S, may encode that peptide portion with the bulk of antigenicity contained by the complete gene product, since six of eight monoclonal antibodies do react with the tribrid protein. Our mapping data position the pORF2-VZVgC1 ORF peptide between the two large hydrophobic domains imputed from Davison's DNA sequence data (6), which potentially represent a signal sequence and membrane-spanning sequence characteristic of membrane glycoproteins. On that basis, the ORF peptide, which constitutes 300 of 515 (58%) of the domain between the two hydrophobic segments, would be expected to be exposed on the exterior of the VZV virion membrane.

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