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Antibodies Directed against a Synthetic Peptide Enable Detection of a Protein Encoded by a Vaccinia Virus Host Range Gene That Is Conserved within the *Orthopoxvirus* Genus

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A vaccinia virus gene required for multiplication in some cell lines but not in others has been previously isolated and sequenced. A synthetic peptide predicted from the nucleotide sequence and corresponding to the carboxy-terminal 18 amino acids was used to raise antibodies in rabbits, The immune serum enabled detection of a 29-kilodalton (kDa) polypeptide by either immunoprecipitation or Western immunoblot assays. Synthesis of the 29-kDa polypeptide occurred immediately after infection and lasted for about 3 h. Shutoff of its synthesis was concomitant with the appearance of a delayed early polypeptide that may be antigenically related to the 29-kDa polypeptide. Analysis of cloned segments of the genomes of other orthopoxviruses by hybridization with the vaccinia virus host range gene demonstrates that it is well conserved within this genus.

Vaccinia virus (VV) mutants deleted in the left-hand end of the viral genome have been isolated and characterized in a number of laboratories (1, 5, 7–11). The ability to isolate such mutants has shown that as much as 10% of the viral genome is not required for multiplication under in vitro conditions. Nevertheless, deletion mutants often display new phenotypic properties such as in vivo attenuation, altered host range, or a distinct cytopathic effect in tissue culture. One particular deletion mutant, which we isolated and designated VV *hr* (host range), was found to multiply only poorly or in some cases not at all in cells of human origin (1). Other cell lines, particularly rabbit RK 13 cells, have also been found to be nonpermissive, whereas chicken embryo cells, hamster BHK 21, and Mouse Ltk− cells support multiplication of the mutant. Although the VV *hr* mutant contains a deletion of approximately 18 kilobase pairs, we have obtained evidence from previous studies that loss of only a short DNA segment comprising 855 base pairs can account for the mutant phenotype (3). Nucleotide sequencing of this DNA segment and the adjacent region revealed a single open reading frame that could encode a 32.5-kilodalton (kDa) polypeptide. Furthermore, a protein product close to the expected size was detected after in vitro translation of hybrid-selected mRNA with the appropriate DNA fragment. To acquire new tools to study the product of the *hr* gene (hereafter called the *hr* protein), we have raised rabbit antibodies directed against a synthetic carboxy-terminal peptide sequence predicted from the nucleotide sequence. The antibodies have been used to study the kinetics of synthesis, protein stability, subcellular localization, and possible posttranslational processing. In connection with this analysis, we have also probed the genomes of other members of the orthopoxviruses by DNA-DNA hybridization to detect the presence of genes homologous to the VV *hr* gene.

The nucleotide sequence of the VV *hr* gene predicted a polypeptide containing 284 amino acids. An amphiphilic peptide (IISKNKELRLMYVNCVKKN) comprising 19 amino acids from the carboxy-terminal end was chosen to immunize rabbits. Animals received initial subcutaneous injections at several sites totaling 1.5 mg of peptide emulsified in Freund complete adjuvant. Two weeks later, the immunization was repeated, and another two weeks afterwards, a similar immunization was performed with Freund incomplete adjuvant. Finally, at monthly intervals the animals were boosted with 0.25 mg of peptide in incomplete adjuvant. Serum samples were taken regularly and checked for antipeptide activity by enzyme-linked immunosorbent assay. When serum was found to be positive after several booster injections, it was screened for its ability to recognize a specific viral protein by several techniques. For analysis by immunoadsorption, S100 supernatants were prepared from uninfected cells or cells infected with VV wild type or the *hr* mutant in the presence of cytosine arabinoside, since the *hr* polypeptide was expected to be an early protein. Indeed, the rabbit serum enabled the detection of a 29-kDa polypeptide in extracts from wild-type-infected cells but not from uninfected or *hr*-infected cells (Fig. 1). The specificity of the antibodies was further supported by the size of the 29-kDa polypeptide which corresponded exactly to the species previously revealed by in vitro translation of hybrid-selected mRNA (3). In addition to the 29-kDa polypeptide, the antiserum enabled the detection of another viral polypeptide of about 32 kDa in extracts from both wild-type- and *hr*-infected cells. This viral protein, although unrelated to the *hr* protein since it was encoded by the mutant virus, may share some peptide sequence with the *hr* protein. Western immunoblot analysis of S100 extracts from cells infected for 6 h in the presence of cytosine arabinoside or from uninfected cells gave essentially the same results. A 29- to 30-kDa polypeptide could be detected in wild-type-infected cells but not in mutant-infected or uninfected cells (Fig. 2). Furthermore, the same polypeptide species was also found in samples from cells infected with a recombinant virus (T1) described previously (2); T1 was derived from the *hr* mutant by inserting sequences overlapping the host range gene back into the mutant genome. The 32-kDa species that contaminated the immunoprecipitation experiment was absent in

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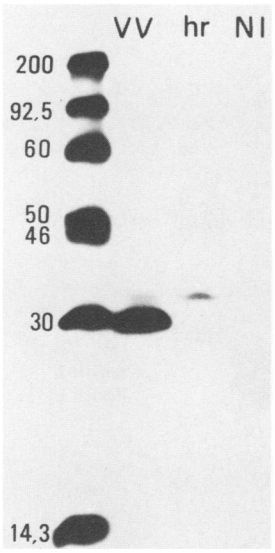

FIG. 1. Immunoadsorption of the *hr* protein with rabbit antipeptide serum. Ltk− cells were infected with 10 PFU of wild-type VV or *hr* mutant per cell, or left uninfected (NI). After 1 h of virus adsorption at room temperature, fresh Eagle minimal medium lacking unlabeled methionine but containing 50 μCi of [^{35}S]methionine per ml and cytosine arabinoside (1 mM) was added. After an additional 2.5 h, the cells were disrupted in lysis buffer containing Tris hydrochloride (10 mM, pH 7.4), NaCl (150 mM), EDTA (1 mM), Nonidet P-40 (1%), and protease inhibitors. S100 supernatants were prepared and incubated overnight at 4°C with a 100-fold dilution of rabbit antiserum and staphylococcal protein A coupled to Sepharose beads. Material bound to the beads after extensive washing with lysis buffer was analyzed on 10% polyacrylamide-sodium dodecyl sulfate gels. An autoradiograph of the dried gel including molecular weight markers is shown.

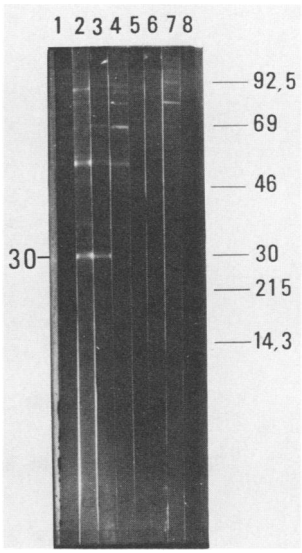

FIG. 2. Western blot detection of the *hr* protein with rabbit antipeptide serum. Ltk− cells were either left uninfected (lanes 4 and 5) or infected with wild-type VV (lanes 3 and 6), the *hr* mutant (lanes 1 and 8), or the T1 recombinant virus (lanes 2 and 7). After 6 h of infection in the presence of cytosine arabinoside (1 mM), cell extracts were prepared, separated on a 10% polyacrylamide-sodium dodecyl sulfate gel, blotted to a nitrocellulose filter, and then processed for detection of the *hr* protein with a 100-fold dilution of the immune sera (lanes 1 through 4) or the preimmune sera (lanes 5 through 8). Molecular size markers (in kilodaltons) are indicated on the right.

the Western blotting experiment, although a number of other nonspecific bands were apparent in some samples. Attempts to detect specific immunofluorescent staining with the antipeptide serum failed, suggesting either that the fixation method destroyed the antigen-binding site or that background immunofluorescence due to other viral or cellular proteins was an impediment.

The kinetics of *hr* protein synthesis was followed by immunoprecipitation of extracts from cells pulse-labeled with [^{35}S]methionine (Fig. 3). The 29-kDa polypeptide could be detected as early as the first 30-min labeling period. Maximum synthesis continued until about 3 h postinfection and declined thereafter. The contaminating 32-kDa species was mostly synthesized after 3 h postinfection. Since this experiment was conducted in the presence of cytosine arabinoside, the *hr* polypeptide appears to be an immediate-early product whose synthesis in HEp-2 cells is shut off concomitantly with the expression of delayed-early proteins. Although the *hr* polypeptide was synthesized very early in infection, it was relatively stable throughout infection (results not shown). Cells labeled during the first 2 h of infection and then chased with unlabeled methionine continued to contain the *hr* polypeptide 6 h later, and a minor amount was still detected as much as 16 h later.

The genomes of all members of the *Orthopoxvirus* genus have been shown to be highly conserved within a central region (6). The terminal regions, however, vary in length and restriction site distribution from one orthopoxvirus to another, and they are expected to be responsible for the variation in biological properties of the different viruses. Since the VV *hr* gene lies at a rough boundary between the conserved and variable sequences, it appeared particularly interesting to search for possible conservation of this gene. Molecularly cloned DNA fragments from variola, monkey-

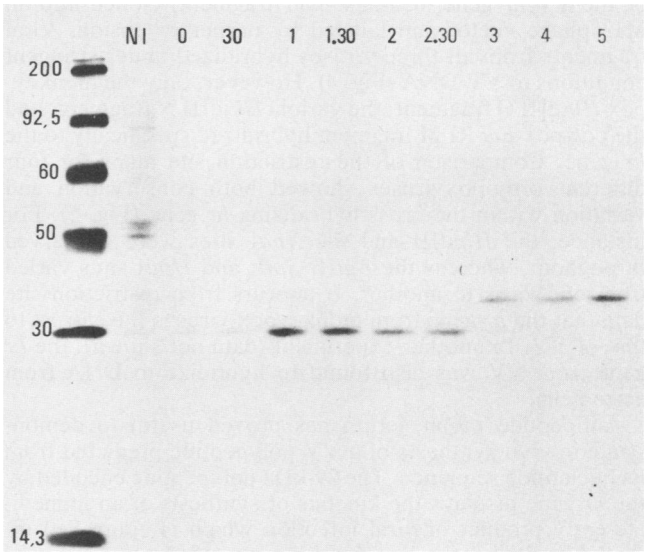

FIG. 3. Kinetics of *hr* protein synthesis. The human HEp-2 cell line was infected with 10 PFU of wild-type VV per cell in the presence of 1 mM cytosine arabinoside. At the times indicated after an initial 1-h adsorption period, the cells were pulse-labeled for 30 min with 50 μCi of [^{35}S]methionine per ml. Cell lysates were then analyzed by immunoadsorption with antipeptide serum as described in the legend to Fig. 1. NI, Not infected.

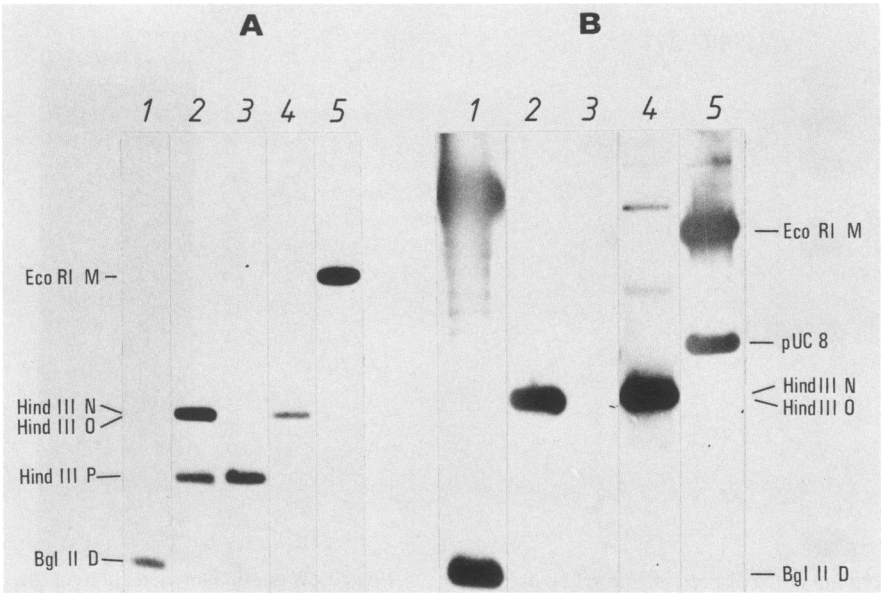

FIG. 4. Hybridization of the host range gene to cloned fragments of variola, monkeypox, and cowpox viruses. DNA from vectors containing the VV *hr* gene fragment (lane 1), the monkeypox *Hin*dIII O and P fragments (lane 2), the monkeypox *Hin*dIII P fragment (lane 3), the variola *Hin*dIII N fragment (lane 4), and the cowpox *Eco*RI M fragment (lane 5) were digested with the appropriate enzyme to dissociate vector DNA from the insert fragment. DNA was then separated on a 1% agarose gel, blotted to a nitrocellulose filter, and hybridized with either nick-translated [^{32}P]labeled VV DNA (A) or primer-extended [^{32}P]labeled M13 vector DNA containing the host range gene (B). Hybridization in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and washing in 0.1× SSC were carried out at 65°C. Note that the *hr* gene used for hybridization was contained on an M13 vector which, as expected, hybridized to itself in panel B, lane 1, and cross hybridized to the pUC8 vector used to clone the cowpox virus fragment in panel B, lane 5.

pox, and cowpox viruses were chosen for study on the basis of their known physical localization (6). Plasmids containing the appropriate fragments were digested with a restriction enzyme to separate them from vector DNA, run on agarose gels, and blotted to nitrocellulose filters. The radioactive probes used were either VV DNA labeled by nick translation or the VV *hr* gene (855-base-pair fragment) cloned into an M13 phage vector and labeled by primer extension. Viral fragments from all three viruses hybridized under stringent conditions to VV DNA (Fig. 4). However, only the monkeypox *Hin*dIII O fragment, the variola *Hin*dIII N fragment, and the cowpox *Eco*RI M fragment hybridized specifically to the *hr* gene. Comparison of the restriction site maps for four different orthopoxviruses showed both conservation and variation within the cross-hybridizing *hr* gene (Fig. 5). For instance, the *Hin*dIII and the *Xba*I sites were conserved throughout, whereas the *Bgl*II, *Sal*I, and *Hpa*I sites varied from one virus to another. It appears from restriction site data that the *hr* gene from monkeypox virus is the closest to that of VV. In another experiment (data not shown), the *hr* gene from VV was also found to hybridize to DNA from ectromelia.

Antipeptide rabbit serum has proved useful to demonstrate in vivo synthesis of a VV polypeptide predicted from its nucleotide sequence. The 29-kDa polypeptide encoded by the *hr* gene displays the kinetics of synthesis of an immediate-early product of viral infection which is controlled directly or indirectly by delayed-early products, since its shutoff occurs in the absence of DNA synthesis. Thus, the *hr* protein belongs to the same class of early proteins as the virally encoded thymidine kinase, judging by the similar kinetics of the latter in L cells (4). The identity of the molecular weight of the polypeptide synthesized in vivo to that of the in vitro-translated product suggests that is does not undergo extensive posttranslational modifications. Attempts to label the protein with phosphate showed in particular that it is not an in vivo substrate for a protein kinase. Immunoprecipitation of the *hr* protein after fractionation of cell lysates into various subcellular components did not allow us to demonstrate any clear-cut distribution into one compartment (data not shown). Although the 29-kDa polypeptide is required for multiplication of VV in some cell types, the basis of this property is unknown. The observa-

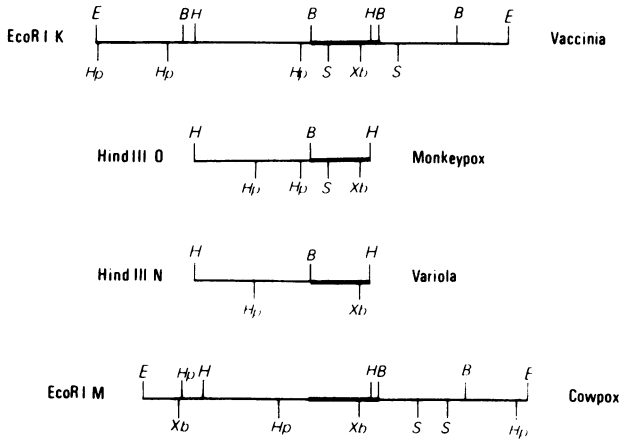

FIG. 5. Restriction site maps of cross-hybridizing orthopoxvirus fragments containing the *hr* gene. The identity of the fragment used for the analysis is indicated in the left-hand margin. The thick line corresponds to the *hr* gene in the VV fragment or to the cross-hybridizing material in DNA fragments from the other orthopoxviruses. Restriction enzymes: E, *Eco*RI; Hp, *Hpa*I; B, *Bgl*II; H, *Hin*dIII; S, *Sal*I; Xb, *Xba*I.

tion that cells infected with a mutant lacking the *hr* gene failed to maintain both viral and cellular protein synthesis suggests that the 29-kDa polypeptide is in some manner involved in the protein-synthesizing machinery of infected cells; however, no in vitro assay to demonstrate this property has yet been devised. When such an assay is available, the antiserum directed against the 29-kDa polypeptide should become particularly useful. A gene product identical or very similar to the 29-kDa polypeptide no doubt also has an important biological role in all orthopoxviruses, since we have detected cross hybridization with similarly located sequences within variola, monkeypox, cowpox, and ectromelia viruses. Furthermore, the antipeptide serum could immunoprecipitate an early 29-kDa polypeptide from cells infected with cowpox virus, although not from ectromelia-infected cells (results not shown). Thus, the full extent of variation in the sequence encoding the 29-kDa polypeptide, already apparent in the restriction site maps, must await complete sequence comparisons.

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