Utilization of DNA Recombination for the Two-Step Replacement of Growth Factor Sequences in the Vaccinia Virus Genome

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An efficient procedure for the generation of sequence-specific alterations of the vaccinia virus genome was demonstrated. Homologous DNA recombination within cells infected with vaccinia virus was used for the deletion or replacement of promoter sequences of the viral growth factor gene by a procedure comparable to transplacement in *Saccharomyces cerevisiae*. This DNA replacement procedure can potentially be used to generate any sequence alteration within the vaccinia virus genome. Deletion of growth factor promoter sequences resulted in a dramatic reduction in growth factor gene transcription and protein synthesis. Replacement of growth factor promoter sequences with promoter sequences of the strong constitutive 40-kDa gene resulted in an increase in gene transcription and protein synthesis and an altered temporal pattern of expression. Virus containing mutations in the growth factor gene demonstrated different plaque morphologies on cell culture monolayers.

Vaccinia virus is one of the most complex of animal viruses and was the first virus purified and characterized in chemical, serological, and biochemical studies (for reviews, see references 11, 26, and 32). The 187-kb genome of vaccinia virus encodes more than 200 viral polypeptides, and approximately half of these are found in purified virions. Vaccinia virus replicates efficiently within the cytoplasm of most mammalian cells and requires only limited host cell involvement. Viral polypeptides involved in mRNA biogenesis, DNA replication, membrane biosynthesis, virion assembly, and viral dissemination have been identified and studied. Genes encoding some of these polypeptides have been mapped within the viral genome by a combination of molecular biological and biochemical approaches (26). The further analysis of these functions has been hindered by an inability to manipulate essential viral sequences.

Until recently, a procedure that would allow the site-directed alteration of any sequences in the viral genome has not been available (15, 36). Homologous DNA recombination involving the appropriate flanking viral sequences and genetic markers has been used to insert exogenous sequences into nonessential regions of the viral genome (16, 21, 27, 28, 33, 34). These techniques, however, are practically limited to the creation of genetic null mutations in genes which are not required for viral growth in tissue culture.

Presented in this report is an efficient procedure for the generation of specific sequence alterations of the vaccinia virus genome. This procedure, referred to as transplacement in *Saccharomyces cerevisiae*, involves the formation and condensation of duplicated sequences within the genome (31, 36). A selectable genetic marker is only transiently incorporated into the viral genome and therefore allows for the generation of progeny genomes that ultimately contain

only the desired sequence alterations. Using this procedure, promoter sequences of the vaccinia virus growth factor genes were either deleted or replaced with a strong constitutive promoter element. Potentially, any sequences within the viral genome can be altered by this procedure.

MATERIALS AND METHODS

Cells and viruses. The African green monkey kidney cell line BSC40 was obtained from R. Condit and the thymidine kinase (TK)-deficient human cell line Hu143tk⁻ was obtained from T. Sieck (2, 4, 29). The Swiss 3T3 cell line was obtained from the American Type Culture Collection (41). All three of these cell lines were cultured as monolayers at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% fetal calf serum unless stated otherwise.

Vaccinia virus strain WR was obtained from J. Kates. The TK gene (*tk*)-deleted vaccinia virus WRΔ417 was selected by its ability to replicate in the presence of 5-bromodeoxyuridine (BUDR) and was generated as previously described (36). Both virus were plaque purified three times before use. Viral titers were determined on the above cell lines after amplification on monolayers of BSC40 cells at a multiplicity of less than or equal to 0.1 PFU per cell.

Escherichia coli HB101 was used for the replication of pUC18- and pAG3-derived plasmids, and *E. coli* TG1 was used for the replication of M13-derived plasmids (3, 22).

Plasmid substrates for recombination. Procedures for the enzymatic manipulation, amplification, and purification of DNA were used as described by Maniatis et al. (23). Plasmid pAG3 was derived from pUC18 as previously described (36). A 2.0-kb *Bam*HI-to-*Kpn*I fragment containing the viral growth factor gene was isolated from the *Hind*III C fragment of the vaccinia virus (strain WR) genome and was cloned into pAG3 linearized with *Bam*HI and *Kpn*I (Fig. 1A). The resulting plasmid was digested with *Bam*HI and ligated to a 702-bp *Bam*HI fragment containing the viral *tk* gene. This plasmid contains the growth factor and *tk* genes oriented in the same direction. *Ssp*I partial digestion of this plasmid and insertion of a *Pst*I linker created a unique *Pst*I restriction

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site. A new *SphI* site was then created by oligonucleotide-directed mutagenesis of these DNA sequences in an M13 vector (Amersham) as described by Taylor et al. (39, 40).

The resulting plasmid, pGFTK, was used to create two plasmids containing different growth factor gene promoter alterations: (i) plasmid pΔGFTK contained a deletion of the putative growth factor gene promoter; and (ii) plasmid p40GFTK contained a replacement of the putative promoter sequences with the strong constitutive 40-kDa gene promoter (Fig. 1B). To generate these plasmids, pGFTK was first linearized by *SphI* partial digestion and then digested with *PstI*. Plasmid pΔGFTK was created by the insertion of a *PstI* linker before plasmid recircularization. Plasmid p40GFTK was created by ligation of the linearized pGFTK to the 161-bp *PstI*-to-*SmaI* fragment of pAbT4544 which contains the 40-kDa gene promoter sequences flanked by M13 polylinker sequences. Each of these plasmids contains a new *PstI* restriction site not found within the wild-type viral sequences (Fig. 1). These *PstI* sites were used to distinguish altered from wild-type viral sequences. Sequence analysis of double-stranded DNA plasmids was conducted by using the dideoxy sequencing method of Chen and Seeborg (10, 38).

DNA transfection and recombination in vaccinia virus-infected cells. The human TK-deficient cell line Hu143tk⁻ was used in DNA transfection experiments as described previously (36). For the selection of TK-positive recombinant virus, cells were pretreated for 4 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and methotrexate (MTX) solution (5 μM MTX, 15 μM thymidine, 50 μM adenosine, 50 μM guanosine, 100 μM glycine) (8, 12, 36).

The two-step replacement procedure involved the selection of virus twice in MTX and once in BUdR solutions. Recombinant virus were first plated on monolayers of Hu143tk⁻ cells in growth medium and MTX solution. These plates were scraped 24 h postinfection, and the resulting virus were replated on fresh monolayers of Hu143tk⁻ cells under agar containing growth medium and MTX solution. To ensure that viral genomes contained tk-positive duplicated structures or were derived from those that contained tk-positive duplicated structures, we subsequently picked and pooled 10 viral plaques. The resulting virus were then replated on monolayers of Hu143tk⁻ cells and grown under agar containing growth medium and BUdR solution. From these plates, two sets of 15 individual viral plaques were picked and separately amplified.

Isolation and analysis of viral DNA. Dishes (60 mm) containing confluent monolayers of Hu143tk⁻ cells were infected with vaccinia virus and incubated for 48 h at 37°C. Infected monolayers were subsequently scraped off the plates, placed in 15-ml Corning tubes, and pelleted for 10 min at 4°C in a clinical centrifuge at maximum speed. Growth medium was then removed by aspiration. Pellets were resuspended in 1 ml of Dulbecco's modified Eagle's medium, and 300-μl aliquots were removed and placed in Eppendorf tubes for DNA purification. The remaining 700 μl of sample was stored at -70°C. Viral cores and DNA from viral cores were purified from virus-infected cells by a procedure described previously (14). The resulting DNA samples were resuspended in 20 μl of 10 mM Tris-HCl (pH 8.0)-1 mM EDTA. Aliquots of 1 to 2 μl were digested with restriction endonucleases in a volume of 20 μl before gel electrophoresis. The structures of recombinant viral genomes were determined by DNA hybridization analysis (35). Viral DNA fragments containing recombinant growth factor sequences were

cloned, isolated by colony hybridization (18), and sequenced (10, 38). All autoradiographs were exposed at -70°C with Kodak X-Omat film and Cronex intensifying screens.

Isolation and analysis of RNA in virus-infected cells. Monolayers containing 2×10^7 BSC40 cells on 15-cm dishes were first infected with plaque-purified virus at a multiplicity of 10 PFU per cell and then incubated at 37°C for various times. At the appropriate times, monolayers of virus-infected cells were washed twice with phosphate-buffered saline (PBS) and lysed with 2 ml of guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 0.5% sodium lauroyl sarcosinate, 25 mM sodium citrate, adjusted to pH 7.0 with citric acid and passed through a 0.45-μm-pore-size filter) containing 0.1 M 2-mercaptoethanol. Lysates were subsequently passed five times through a 23-gauge needle and frozen at -70°C until used. RNA was purified from lysates by CsCl centrifugation as described previously (17). The resulting RNA samples were resuspended in 20 μl of sterile water, and their concentrations were determined by using a spectrophotometer.

Northern (RNA) blot analysis of viral RNA. Total cellular RNA was suspended in a buffer containing formamide, formaldehyde, and MOPS (morpholinepropanesulfonic acid) and denatured at 70°C for 5 min. Samples were loaded on a 1% (wt/vol) agarose gel containing 2.2 M formaldehyde and MOPS. Electrophoresis was conducted at 50 V for 18 h. RNA was transferred and cross-linked to Hybond filters according to conditions specified by the manufacturer (Amersham). The filters were incubated for 4 h at 42°C in prehybridization solution (750 mM NaCl, 150 mM Tris [pH 8.0], 1 mM EDTA, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate [SDS], 0.1% sodium PP_i, 100 μg of tRNA per ml). Filters were subsequently incubated for 18 h at 42°C in fresh prehybridization solution containing 100 ng of oligonucleotide, labeled at the 5' end with ³²P and containing vaccinia virus growth factor sequences. Filters were then washed at 50°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed for 48 h at -70°C to Kodak X-Omat film with Cronex intensifying screens.

Primer extension of viral RNAs. A 23-base oligonucleotide containing vaccinia virus growth factor sequences was labeled at its 5' end by using polynucleotide kinase and [γ -³²P]ATP (6,000 Ci/mmol; Du Pont). Labeled oligonucleotide was purified by passage over a NENSORB-20 column (Du Pont) and subsequently used as a primer for DNA synthesis. About 5×10^6 cpm of labeled oligonucleotide were combined with 15 μg of total cellular RNA, denatured for 2 min at 90°C, and quickly chilled on ice for 2 min. Primer annealing was accomplished by adding NaCl to a concentration of 150 mM, heating to 68°C for 10 min, and cooling at room temperature for 15 min. Nucleic acids were precipitated by adding NaCl to a concentration of 650 mM and 2 volumes of ethanol and incubating at -70°C for 18 h. Primer extension was conducted with avian myeloblastosis virus reverse transcriptase according to conditions specified by the manufacturer (Promega bioTech). Elongated products were separated by electrophoresis on a 6% polyacrylamide-7 M urea gel. A Sanger dideoxy-chain termination sequence ladder with the same oligonucleotide as a primer was run next to each extension reaction to locate the site of transcript initiation. Polyacrylamide gels were fixed in 10% methanol-10% acetic acid, dried, and exposed for 48 h at -70°C to Kodak X-Omat film with Cronex intensifying screens.

Quantitation of intracellular vaccinia virus growth factor by immunoprecipitation. Anti-peptide antibodies directed against the vaccinia virus growth factor gene were kindly

provided by Wen Chang and Sheu Loc Hu (9). Monolayers of 2×10^7 cells on 15-cm dishes were first infected at a multiplicity of 5 to 30 PFU per cell and incubated at 37°C. After 30 min of incubation, the viral inoculum was removed, monolayers were rinsed in PBS, and cysteine-free or complete medium was added to the plates. This time is arbitrarily designated as the starting point of infection. At 30 min postinfection, medium was removed from plates containing cysteine-free medium and replaced with cysteine-free medium containing 200 μ Ci of [35 S]cysteine. After two more hours of incubation at 37°C, these monolayers were scraped, pelleted for 5 min at maximum speed in a clinical centrifuge, and frozen at -70°C. These samples represent early protein synthesis. For late protein synthesis, medium was removed from plates containing complete medium at 8 h postinfection and replaced with cysteine-free medium. After 30 min of incubation at 37°C, medium was removed from plates containing cysteine-free medium and replaced with cysteine-free medium containing 200 μ Ci of [35 S]cysteine. After two more hours of incubation at 37°C, these monolayers were scraped, pelleted for 5 min at maximum speed in a clinical centrifuge, and frozen at -70°C. Cell pellets were subsequently lysed in radioimmunoprecipitation assay lysis buffer and immunoprecipitated in radioimmunoprecipitation assay lysis buffer with antibody and protein A-Sepharose (CL4-B; Pharmacia) as described by Harlow and Lane (19). Immunoprecipitated samples were resuspended in Laemmli sample buffer and run on an SDS-15% polyacrylamide gel at 20 mA constant current for 4 to 5 h. Gels were fixed for 30 min in 30% methanol-10% glacial acetic acid-0.1% Coomassie blue and destained for 30 min in 10% methanol-5% glacial acetic acid. Gels were enhanced for 30 min in En³Hance (New England Nuclear) and rinsed in water twice for 30 min prior to drying. Dried gels were exposed at room temperature.

Materials. Enzymes, including restriction endonucleases, were purchased from Bethesda Research Laboratories, Inc., New England Biolabs, Inc., and Boehringer Mannheim Biochemicals and used under conditions specified by the manufacturer. Oligonucleotide-directed mutagenesis was conducted with materials supplied by Amersham. DNA sequencing was conducted with materials supplied by U.S. Biochemical Corp. Proteinase K, MTX, BUdR, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), thymidine, adenosine, and guanosine were supplied by Sigma Chemical Co. Formamide, chloroform, and guanidinium thiocyanate were purchased from Fluka Chemical Co. Radioisotopes and scintillation fluids were purchased from New England Nuclear Corp. Nitrocellulose filters and filter manifolds were purchased from Schleicher & Schuell, Inc.

RESULTS

The vaccinia virus growth factor gene was chosen as the target for site-directed alteration. Virus null for growth factor have been generated and shown to be viable but attenuated in laboratory animals (6, 7). Growth factor gene products are synthesized early in the viral cycle and are mitogenic for cells that express epidermal growth factor receptors (37, 42, 45). The growth factor gene has been cloned and sequenced and is diploid owing to its location within the inverted terminal repeats of the viral genome (44).

Construction of plasmids containing growth factor promoter alterations. Two sequence-specific alterations within the growth factor promoter were transferred to the viral genome by homologous DNA recombination. Plasmid pGFTK which contains the entire vaccinia virus growth

factor gene and contiguous upstream sequences, in addition to the intact viral *tk* gene, was used to generate plasmids containing the promoter alterations (Fig. 1A). Plasmid p Δ GFTK contains a 121-bp deletion of growth factor promoter sequences, and plasmid p40GFTK contains 204 bp of the 40-kDa gene promoter in place of these deleted growth factor promoter sequences (Fig. 1B) (30). To distinguish viral genomes containing these altered promoter sequences from wild type, we included a new *Pst*I restriction site within each of the altered promoter elements (Fig. 1). The viral sequences flanking the promoter alterations were used to direct homologous recombination (Fig. 2). The vaccinia virus *tk* gene, located outside of the homologous viral sequences, was used as the genetic marker to select for the products of single-crossover events (Fig. 2).

Generation and identification of viral genomes containing growth factor promoter alterations. Viral genomes containing the promoter deletion and the promoter replacement were generated through two steps of recombination involving the pGFTK plasmids and *tk*-deleted virus (Fig. 2). The procedure used to select for the products of recombination in each step involved (i) MTX selection for the insertion of plasmid into the viral genome by single-crossover events; followed by (ii) BUdR selection for the excision of plasmids and the ensuing generation of viral genomes containing a single copy of the virus- or plasmid-derived sequences (Fig. 2).

To analyze the products of recombination, DNA was purified from an aliquot of total virus produced in each selection step, digested with *Pst*I, fractionated by gel electrophoresis, and hybridized to growth factor sequences (Fig. 3). The first two lanes of Fig. 3 contain DNA from wild-type (WR) and *tk*-deleted (WR Δ 417) virus. Both lanes show bands of 12.3 and 16.4 kbp corresponding to the two wild-type copies of the growth factor gene located within the left and right genomic termini, respectively. In addition to the two wild-type bands, some of the remaining lanes demonstrate hybridizing bands of 4.8, 6.9, and 9.1 kbp. These smaller three bands correspond to genomic termini containing altered growth factor promoter sequences. Three additional bands corresponding to the predicted duplicated structures were only faintly visible on greatly overexposed blots (Fig. 3). This result is consistent with the duplicated structures being highly unstable (36).

Plaque isolation was used to purify mutants deleted for the growth factor promoter (p Δ GFTK) and those in which the growth factor promoter was replaced with the 40-kDa promoter (p40GFTK). Individual viral plaques isolated after the second round of BUdR selection and analyzed by hybridization for the promoter alterations demonstrated that mutant virus is represented at a frequency of approximately 15 to 20% (Fig. 4). Theoretically, this value would approach 50% if positive selection absolutely excluded nonrecombinant virus and mutant virus were not at a growth disadvantage. The greater intensity of bands corresponding to wild-type virus probably represents nonrecombinant virus surviving MTX selection through complementation by TK-positive recombinants (Fig. 3).

Two isolates of pure mutant virus containing the 40-kDa promoter were obtained in the first round of plaque purification (Fig. 4, lower panel, lanes 5 and 14). An additional round of plaque purification of the virus shown in lane 7, upper panel, was required to obtain a pure stock of the mutant promoter-deleted virus. Both mutants were subsequently plaque purified an additional three rounds and amplified prior to further analysis. The simplest explanation for the banding pattern observed with recombinants 5 and 7

FIG. 1. (A) Construction of plasmids containing growth factor promoter alterations. Plasmids were derived from the *Hind*III B and C fragments of the vaccinia virus (strain WR) genome as described in Materials and Methods. (B) Growth factor promoter alterations. Wild-type growth factor promoter sequences were either deleted or replaced by promoter sequences of a constitutively expressed viral gene as described in Materials and Methods. Symbols: white bar, viral sequences; dark bar with arrowhead, viral growth factor coding sequences; speckled square, viral growth factor gene promoter sequences; ring, altered promoter sequences containing a unique *Pst*I site; thin line, plasmid sequences; shaded bar, *tk* gene.

of the upper panel and recombinant 6 of the lower panel of Fig. 4 is that the plaques giving rise to these virus contain a mixture of wild-type and altered genomes.

DNA sequence analysis of growth factor promoter alterations. To demonstrate that the genomic alterations identified were in fact those engineered on the pGFTK plasmids, viral DNA containing growth factor sequences was subcloned into pUC vectors and sequenced. As shown in Fig. 5, the sequences derived from these plasmids were identical to those from the pΔGFTK and p40GFTK plasmids originally used for transfection.

Taken together, the results presented demonstrate a procedure for the efficient replacement of specific sequences within the vaccinia virus genome. These specific viral sequences were shown to be accurately replaced by cloned DNA fragments containing altered sequences through homologous DNA recombination.

Analysis of growth factor gene expression in mutant virus. Mutant virus containing alterations within the growth factor gene promoter were generated to study the effect of modulating gene expression on the lytic cycle of vaccinia virus. Thus, alterations in gene transcription and translation first had to be characterized in each mutant virus.

To study the timing and level of growth factor transcription in each of the mutants, we isolated total cellular RNA from infected cells at 1.5 and 8 h postinfection. This RNA was fractionated by gel electrophoresis and subjected to RNA-DNA hybridization analysis with growth factor sequences. RNA isolated from wild-type virus WRΔ47

("wt"), growth factor promoter-deleted virus ("Δ"), and growth factor promoter replacement virus ("40") is shown in Fig. 6. Under the conditions used, DNA replication and late viral gene expression are expected to initiate at approximately 2 h postinfection (26). Growth factor transcripts were present both early and late in the infectious cycle in cells infected with the wild-type and 40-kDa promoter replacement virus but absent in cells infected with the promoter-deleted virus (Fig. 6). Significantly, more growth factor transcripts were observed early than late in the infectious cycle with the wild-type promoter. Since the wild-type growth factor gene is only expressed early in the infectious cycle, the discrete transcripts seen at 8 h postinfection probably represent the remnants of stable early transcripts (Fig. 6, wt, 8 h) (26). In contrast, large amounts of transcripts were observed early and late in the infectious cycle with the strong 40-kDa promoter and correspond to the constitutive expression of the growth factor gene. The heterogeneous size of the late transcripts is characteristic of transcripts generated after DNA replication within the infectious cycle (26).

To demonstrate that these growth factor transcripts were produced by the 40-kDa promoter, we conducted primer extension analysis on the RNA (Fig. 7). Primer extension of growth factor RNAs generated by the wild-type and 40-kDa promoters early in infection demonstrated that transcription initiates at the correct sets of nucleotides located within each promoter element (Fig. 7). Furthermore, as would be predicted for this constitutive promoter element, transcripts

FIG. 2. Replacement of viral growth factor promoter sequences by the two-step procedure. A circular plasmid is constructed which contains altered growth factor gene promoter sequences within homologous sequences and the viral *tk* gene outside of homologous sequences. Single-crossover events between homologous sequences on this plasmid and the *tk*-deleted WRΔ417 virus genome result in the insertion of the entire plasmid into the genome at the crossover point. The recombinant structure produced contains a duplication of the homologous sequences separated by plasmid sequences and the viral *tk* gene. One copy of the duplicated sequences is wild type, while the other contains the altered promoter sequences. Virus containing this duplicated structure are selected for in the presence of MTX. A second single-crossover event between duplicated sequences results in the excision of the entire plasmid containing the viral *tk* gene and the ensuing generation of a viral genome containing a single copy of either the wild-type or altered growth factor promoter sequences. Virus containing either of these resulting structures are selected for in the presence of BUdR. Shown in this figure is a series of single-crossover events that result in the replacement of viral growth factor gene promoter sequences with altered promoter sequences. Series of single-crossover events can also result in the regeneration of the starting viral structures. Symbols: white bar, viral sequences; dark bar with arrowhead, viral growth factor-coding sequences; speckled square, viral growth factor gene promoter sequences; ring, altered promoter sequences containing a unique *Pst*I site; thin line, plasmid sequences; shaded bar, *tk* gene.

were initiated more proximal to the translational start site late in infection (30). In the 40-kDa gene promoter, the TAAATG motif, which is common among vaccinia virus late transcriptional start sites, is actually TAAATAC (Fig. 7, 40KGF, 8-h RNA) (30).

As predicted, the analysis of growth factor transcripts demonstrated that the deletion of growth factor promoter sequences results in an elimination of the transcription of the growth factor gene, while the replacement of wild-type early promoter sequences with the strong constitutive promoter of the 40-kDa gene results in an increase in the transcription of the growth factor gene both early and late in the infectious cycle.

Analysis of growth factor protein synthesis in mutant virus. To demonstrate that alterations in the transcription of the growth factor gene correlate to alterations in the level of gene product, proteins synthesized both early and late in

virus-infected cells were labeled with [³⁵S]cysteine and subjected to immunoprecipitation with antibody directed against growth factor (9). As shown in Fig. 8, growth factor protein was detected in cells infected with wild-type virus WRΔ417 (wt) and the promoter replacement virus (40kGF) but not at all in the promoter-deleted virus (Δ) or mock-infected cells. The 14- to 29-kDa polypeptides observed with wild-type and promoter replacement virus were those predicted by Chang et al. (9). The band intensities observed were consistent with the promoter replacement virus producing more growth factor protein than wild-type virus. Collectively, these results demonstrate that the deletion of growth factor promoter sequences eliminates growth factor production and that the replacement of promoter sequences with a strong constitutive promoter element greatly increases the amount of growth factor protein synthesized.

Analysis of plaque morphology in mutant virus. To initiate

FIG. 3. Molecular analysis of viral recombinants selected for growth factor gene promoter alterations. Viral DNA was purified and analyzed as described in Materials and Methods. Viral DNA was digested with *Pst*I, subjected to gel electrophoresis (0.7% agarose), transferred to nitrocellulose filters, and hybridized with the 2.0-kbp *Bam*HI-to-*Kpn*I fragment from plasmid pGFTK. Each lane contains DNA from the following virus: TK-positive wild-type virus WR (lane 1); parental TK-negative virus WRΔ417 (lane 2); first round, MTX-selected virus (lanes 3 to 6); second round, MTX-selected virus (lanes 7 to 10); first round, BUdR-selected virus (lanes 11 to 14); second round, BUdR-selected virus (lanes 15 to 18). The first two lanes in each set of recombinant viral DNAs are from transfections with plasmid pΔGFTK, and the last two are from transfections with plasmid p40GFTK. The figure is a composite made from a single gel in which lanes 3 to 10 were exposed four times longer than the other lanes, which were exposed for 4 h.

the biological characterization, we used these mutant virus to assess whether altered growth factor synthesis had effects on viral plaque morphology. BSC40 cells were maintained as either quiescent or growing monolayers in culture medium containing either 0.2 or 10% fetal calf serum, respectively. With quiescent cells, each mutant virus produced plaques that were smaller than those of the wild-type virus. Virus containing the promoter deletion produced the smallest plaques, while mutant virus constitutively expressing the growth factor gene produced plaques intermediate in size between those of wild-type and the promoter-deleted virus (Fig. 9). With growing cells, wild-type and promoter-deleted virus produced plaques of equal size, while surprisingly, virus constitutively overexpressing the growth factor produced smaller plaques.

These results are consistent with the hypothesis that the viral growth factor induces proliferation in neighboring cells to accommodate impending viral replication but that certain cell types become unresponsive to growth factors when present in excessive amounts (6, 7, 20, 37, 42, 43). Hence, the effect of constitutively overexpressing the viral growth factor is an inhibition of plaque growth and is most pronounced in the presence of high levels of serum growth factors. The effect of abolishing viral growth factor expression is appropriately seen only when serum growth factors are limiting.

DISCUSSION

The results presented in this report demonstrate a procedure for the efficient replacement of vaccinia virus sequences with exogenous altered sequences. Circular plasmids containing the altered sequences are inserted into and

FIG. 4. Molecular analysis of individual viral recombinants selected for growth factor gene promoter alterations. Viral DNA was purified and analyzed as described in Materials and Methods. Viral DNA was digested with *Pst*I, subjected to gel electrophoresis (0.7% agarose), transferred to nitrocellulose filters, and hybridized with the 2.0-kbp *Bam*HI-to-*Kpn*I fragment from plasmid pGFTK. Top and bottom panels contain DNA from 15 individual virus that formed plaques in the presence of BUdR and originated from a transfection with plasmids pΔGFTK and p40GFTK, respectively. Hybridizing bands from the parental virus WRΔ417 comigrated with those in the first lanes of each panel (not shown). This figure was made from a single gel exposed for 4 h.

subsequently excised out of the viral genome by homologous DNA recombination. The resulting viral genomes contain either the original or altered sequences and lack superfluous plasmid or marker DNAs. The high frequency of homologous insertion and excision events that occurs within virus-infected cells will facilitate a precise dissection of the vaccinia virus genome by this DNA replacement procedure. Furthermore, the extensive information available on the infectious cycle, viral polypeptides, and the viral genome makes vaccinia virus highly suited for such a genetic analysis.

The two-step DNA replacement or transplacement procedure was originally developed over a decade ago with *Saccharomyces cerevisiae* and was demonstrated more recently with *Aspergillus nidulans* (25, 31). For a variety of reasons, however, transplacement has not been used extensively in these fungi. The low frequency of plasmid excision events, the occurrence of multiple plasmid insertions, and difficulties associated with negative selection procedures require the replica plating of large numbers of colonies to identify the desired recombinants (1, 5). Transplacement is also less efficient than procedures involving high-frequency transformation and positive selection for simple plasmid insertions. In contrast, the high frequency of homologous plasmid insertion and excision events within the vaccinia virus genome, the great instability of multiple plasmid insertions, and the availability of positive and negative selection make vaccinia virus better suited for transplacement (36).

The transplacement procedures presented in this work and that by Falkner and Moss (15) are essentially the same and are based on the initial work of Spyropoulos et al. (36). The greatest difference between these two procedures is that selection was used in both steps of recombination in this work (i.e., positive and negative), whereas selection was only used in the first step of recombination by Falkner and

FIG. 5. DNA sequence comparison between growth factor promoter-altered plasmids and altered viral genomes. Altered vaccinia virus growth factor sequences on the plasmids used for the initial transfection experiment (denoted plasmid) and those cloned from the wild-type and resulting viral genomes (denoted viral) were subjected to DNA sequence analysis by the dideoxy termination method of Chen and Seeberg (10, 38). Both DNA strands were sequenced by using primers for DNA synthesis that annealed to both the template and coding strands. Shown are sequences generated using the primer that annealed to the coding strand within growth factor-coding sequences. Figure headings describe the derivation of the original plasmids for transfection. The 40-kDa gene promoter construct was derived from the promoter deletion, which was derived from wild type by the removal of the sequences labeled WT promoter. Both of the altered growth factor sequences contain a new *Pst*I restriction site to facilitate the identification of virus containing altered genomes by DNA hybridization analysis. Translational initiation codons are denoted by ATG. Owing to the direction of the sequencing ladder, ATG corresponds to CAT in the figure. This figure is from a single gel exposed for 2 days at ambient temperature without enhancement.

Moss (15). Negative selection may not prove to be as important as positive selection owing to the great instability of duplicated structures within virus-infected cells (15, 36). Ultimately, the best procedure will probably be one that employs a positive selectable marker which best excludes nonrecombinant virus in the first step of the selection process.

As demonstrated in this report, transplacement can be used to alter sequences, like those of the growth factor gene,

which are duplicated within the inverted terminal repeats of the viral genome. Although the mechanism is unclear, previous work demonstrating the rapid generation of mirror-image deletions within both termini suggests that other diploid genes are as easy to alter as the viral growth factor gene (24). In addition, transplacement can potentially be used to generate sequence-specific alterations anywhere within the vaccinia virus genome. Clearly, whether virus containing a transplacement-generated alteration will be

FIG. 6. Northern (RNA) blot analysis of total cellular RNA. Total cellular RNA was isolated at the indicated times postinfection from monolayers of BSC40 cells infected with virus at a multiplicity of 10 PFU per cell, size fractionated by electrophoresis on a formaldehyde-agarose gel, and transferred to a filter as described in Materials and Methods. Filter hybridization was conducted with a 5'-end-labeled oligonucleotide containing 23 bases of the vaccinia virus growth factor gene. Filter washes and exposures were conducted as described in Materials and Methods. Abbreviations: wt, WRΔ417 virus which contains the wild-type growth factor promoter; Δ, virus deleted for growth factor promoter sequences; 40, virus containing the 40-kDa gene promoter in place of growth factor promoter sequences.

FIG. 7. Primer extension analysis of total cellular RNA. Total cellular RNA was isolated at the indicated times postinfection and annealed to a 5'-end-labeled 23-base oligonucleotide containing vaccinia virus growth factor sequences, as described in Materials and Methods. DNA fragments generated by primer extension with avian myeloblastosis virus reverse transcriptase were separated by electrophoresis on a polyacrylamide-urea gel. A Sanger dideoxy-chain termination sequence ladder with the same oligonucleotide as a primer was run next to each extension reaction to locate the site of transcript initiation. The polyacrylamide gel was fixed and exposed as described in Materials and Methods. Abbreviations: wt, WRΔ417 virus which contains the wild-type growth factor promoter; 40kGF, virus containing the 40-kDa gene promoter in place of growth factor promoter sequences.

FIG. 8. Immunoprecipitation of protein extracts from virus-infected cells. Monolayers of cells were infected with vaccinia virus, labeled with [³⁵S]cysteine at the indicated times, and immunoprecipitated with antibody to growth factor peptide sequences (9) as described in Materials and Methods. As a control to test the specificity of immunoprecipitations, antibody-specific peptide was added to antibody prior to the addition of supernatant in the lanes labeled +. Abbreviations: wt, WRΔ417 virus which contains the wild-type growth factor promoter; Δ, virus deleted for growth factor promoter sequences; 40kGF, virus containing the 40-kDa gene promoter in place of growth factor promoter sequences.

viable or nonviable depends on the size of the alteration, the juxtaposition of sequences, and the location of the alteration within the viral genome. When the alteration occurs within essential sequences, mutant virus can be isolated only if the alteration is nonlethal or conditionally lethal. By monitoring mutant virus production, transplacement can potentially be used to map essential sequences within the viral genome. The alterations generated by transplacement could be as large as several kilobase pairs or as small as a single base pair. Large alterations within regulatory sequences could be generated to modulate the timing and level of gene expression. As was demonstrated with the viral growth factor gene, replacement of its early promoter sequences with a strong constitutive promoter element was found to be deleterious to the infectious cycle. Modulating the expression of other viral genes could improve or decrease the efficiency of the infectious cycle; within the animal host, it could also alter virulence and spread. Large alterations within coding sequences could potentially include the insertion, deletion, and replacement of functional domains, generating fusion proteins or proteins with altered specificity. Viral enzymes involved in mRNA biogenesis and DNA replication, as well as envelope and core structural proteins, could be the targets of such alterations.

Transplacement could also be used to generate small alterations. Small alterations within coding sequences could include base-pair insertions and deletions to generate frame-shift mutations and base-pair substitutions to alter the amino acid composition of a protein, alter protein folding, or alter protein phosphorylation and glycosylation. Structural proteins within the viral envelope and core may be good candidates for such alterations in the study of viral virulence. Base-pair substitutions in coding sequences could be made to generate conditional lethal mutations within specific viral genes and thereby replace antiquated random mutagenesis procedures (13).

In summary, the DNA replacement procedure demonstrated in this report will facilitate the molecular dissection of the vaccinia virus genome in a manner previously unob-

FIG. 9. Plaque morphology of growth factor promoter-altered virus. Monolayers of BSC40 cells on 60-mm dishes were infected with 20 to 200 PFU of virus and incubated for 48 h in Dulbecco's modified Eagle's medium containing either 0.2 or 10% fetal calf serum (FCS). Monolayers were subsequently stained with crystal violet (0.1% in 30% ethanol) and photographed. Abbreviations: wt, WRΔ417 virus containing the wild-type growth factor promoter; 40kGF, virus containing the 40-kDa gene promoter in place of growth factor promoter sequences; ΔGF, virus deleted for growth factor promoter sequences; Abt 70, NYCBH strain of vaccinia virus containing the *E. coli lacZ* gene inserted into and disrupting the growth factor gene.

tainable. The nature of DNA recombination in virus-infected cells sets vaccinia virus apart from other organisms in which transplacement has been demonstrated. By combining the wealth of information available on vaccinia virus with this DNA replacement procedure, the structure and function of viral polypeptides can be understood. Host-virus interactions can be illustrated, and entire biochemical pathways can be described on a molecular level.

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