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Transformation-Dependent Expression of Interleukin Genes Delivered by a Recombinant Parvovirus

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The prototype strain of minute virus of mice [MVM(p)] is an autonomous parvovirus with a tropism for cells expressing a neoplastically transformed phenotype. To generate gene transfer vectors for tumor-specific gene expression, human interleukin-2 (IL-2) and murine interleukin-4 (IL-4) genes were cloned under the control of the p38 late promoter of MVM(p). Upon transfection into permissive cells, the recombinant MVMIL2 or MVMIL4 DNA was excised, amplified, and, in the presence of a helper plasmid, packaged into recombinant viral particles. The recombinant viruses were able to transfer fully functional IL-2 and IL-4 genes to permissive target cells and retained the oncotropic host range properties of the parental virus. Following infection with MVMIL2, nontransformed fibroblasts of rodent (FR3T3) or human (MRC-5) origin produced minimal IL-2 compared with the high levels of IL-2 production observed in their transformed derivatives (FREJ4 and MRC-5V1).

Parvoviruses are small (20 to 25 nm in diameter), nonenveloped, icosahedral viruses containing a single-stranded (SS) DNA genome of approximately 5,000 nucleotides in length which is flanked by short, terminal hairpin structures (7). The limited coding capacity of the parvoviral genome necessitates a high degree of dependence on exogenous helper functions. Replication of the so-called autonomous parvoviruses is limited to target cells entering S phase after virus entry and is further restricted, for each parvoviral strain, to a distinct differentiated subset of actively proliferating cells. Thus, the autonomous human parvovirus B19 efficiently destroys erythroid progenitor cells (25), canine parvovirus destroys predominantly gut epithelium (38), and other parvoviral strains result in fetal death or congenital malformations by virtue of their propensity to destroy developing embryonic tissues (7, 40).

MVM(p), the prototype (fibrotropic) strain of minute virus of mice, is an autonomous parvovirus with an oncotropic host range. This virus binds to neuraminidase- and trypsin-sensitive cell surface receptors, which are fairly ubiquitously expressed on cells of both rodent and human origin, and subsequently enters the endosomal compartment via coated pits (7, 23). Following entry into cultured rodent fibroblasts, an intracellular block to MVM(p) gene expression and genome replication is frequently observed. This can be partially or completely overcome by prior malignant transformation of the target cells by the tumor virus simian virus 40 (SV40), the polyomavirus middle T gene, an activated human Harvey-*ras* oncogene, or the retroviral oncogenes v-*src* and v-*myc* (26, 27, 35). Likewise, transformation of naturally resistant human fibroblasts by gamma irradiation, by a chemical carcinogen, or by SV40 has no effect on MVM(p) uptake but leads to increased cell killing, parvoviral genome replication, and infectious virus production (5). The cell line HT1080, derived from a human fibrosarcoma, is also fully susceptible to lytic MVM(p) infection (5). Cell sensitization to MVM(p) does not correlate absolutely with neoplastic transformation, since bovine papillomavirus-transformed rat fibroblasts remained resistant to the virus (35). At the molecular level, the increased susceptibility to MVM(p) seen after fibroblast transformation correlates with increased transcription of the viral genes (6). In keeping with these in vitro observations, MVM(p) appears to be entirely nonpathogenic for adult laboratory mice (20) but can dramatically suppress the in vivo growth of Ehrlich ascites tumors (17) and of P-815 mastocytoma cells (20) when injected at a site distant from the tumor cell inoculum.

After transfer to the nucleus of a permissive target cell and synthesis of the viral cDNA strand, MVM gene expression and genome replication proceed in a coordinated fashion (for a review, see reference 7). Genome replication proceeds in three stages. Synthesis of the parental cDNA strand is followed by amplification of monomer and dimer length duplex DNA replicative forms (RFs) and finally by excision and packaging of progeny single strands. The viral termini contain all of the *cis*-acting functions necessary to direct genome replication and packaging. Transcripts arise from two overlapping transcription units driven by functional promoters at map coordinates 4 and 38. Transcripts initiating at map unit 4 encode the viral nonstructural proteins NS-1 and NS-2. NS-1 is a potently cytotoxic (4), multifunctional nuclear phosphoprotein which drives the viral DNA replication cycle (31), regulates transcription from both viral promoters (10, 11), and probably directs packaging of progeny SS DNA (8). Transcripts initiating at map unit 39 encode the MVM capsid proteins, VP-1 and VP-2, which are required for excision of progeny SS DNA viral genomes from duplex

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RF intermediates and concomitant packaging into viral particles.

Recent studies have demonstrated the impaired in vivo survival of transplantable rodent tumors induced to secrete interleukin-2 (IL-2) or IL-4 following transfer of the respective interleukin cDNAs in vitro (12, 14, 34, 39). The tumor-derived interleukins apparently stimulate host effector cells resident within the tumor in a paracrine fashion, leading to enhanced killing of both the gene-modified tumor cells and neighboring tumor cells which do not secrete the interleukin. This type of experiment has led to the suggestion that tumor cell-targeted lymphokine gene therapy might be a useful approach to the treatment of cancer. Therefore, we have generated MVM(p)-based lymphokine gene transfer vectors which should be suitable for this application. Our strategy was to replace the capsid coding sequence with lymphokine cDNAs under the control of the viral promoter at map unit 38, leaving intact the hairpin termini and the genes coding for NS-1 and NS-2. Capsid proteins were provided in *trans* to generate infectious viral particles containing the recombinant MVM genomes. It was envisaged that NS-1 protein expressed from the recombinant viral genome in susceptible (i.e., transformed) target cells would transactivate the promoter at map unit 38 (11, 30), giving rise to high-level expression of the interleukin gene. NS-1-driven replication of the recombinant genome was expected to proceed normally, and the cytotoxicity properties of NS-1 were expected to result, ultimately, in the death of infected target cells. Here we demonstrate that recombinant MVM genomes carrying cDNAs for IL-2 or IL-4 were excised from transfected plasmids, were replicated intracellularly, and (in the presence of capsid proteins) were encapsidated into infectious viral particles. The expression of interleukin genes delivered by these recombinant parvoviruses was significantly enhanced by prior transformation of the target cells.

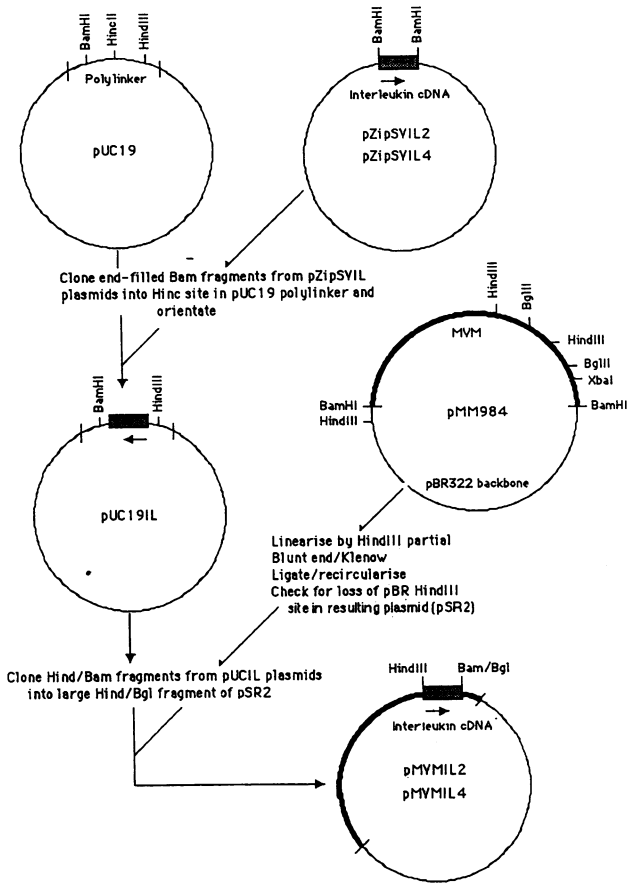

FIG. 1. Construction of plasmids pMVMIL2 and pMVMIL4. See Materials and Methods for explanation.

MATERIALS AND METHODS

Construction of plasmids pMVMIL2 and pMVMIL4. Human IL-2 and murine IL-4 cDNAs were excised as *Bam*HI fragments from pZipSVIL2 (44) and pZipSVIL4 (a gift from I. Mackay, the London Hospital, London, United Kingdom), the *Bam*HI ends were filled in with the Klenow fragment of DNA polymerase I, and the fragments were ligated into the *Hin*cII polylinker site of pUC19 (Fig. 1). Correctly orientated cDNAs were then excised as *Hin*dIII-to-*Bam*HI fragments for ligation into MVM. The gene encoding murine IL-4 was constructed by ligation of a series of synthetic oligonucleotides and does not correspond exactly with the published murine IL-4 cDNA sequence (21), although it encodes a protein with the identical amino acid sequence (23a). To delete the *Hin*dIII site in the pBR322 fragment of pMM984, the plasmid was linearized by partial digestion with *Hin*dIII and the sticky ends were filled in with the Klenow fragment of DNA polymerase I. After recircularization, minilysate restriction digests were analyzed to identify the clone pSR2 lacking the appropriate *Hin*dIII site, but with an intact right-hand MVM palindrome. Sticky (*Hin*dIII to *Bam*HI) interleukin cDNA fragments were then ligated into the large *Hin*dIII (nucleotide 2653)-to-*Bgl*II (nucleotide 4212) fragment of pSR2 to generate pMVMIL2 and pMVMIL4.

Plasmid pULB3235 was constructed as previously described (3). The plasmid was derived from pMM984 and carries a frameshift mutation at position 1884 in the NS-1 coding sequence with a premature stop codon at position 1980. The plasmid also carries a small deletion in the right-hand palindrome which renders it incapable of generating monomer and dimer virus replicative intermediates.

Cell lines. The SV40-transformed human kidney cell line NB-E (37) and its derivative NB-K were grown in minimal essential medium supplemented with 5% fetal bovine serum. IL-2-dependent CTLL-2 and HT-2 cells were grown in RPMI with 10% fetal bovine serum, 2×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, and antibiotics. Other cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. HSNLV is a variant of the HSN rat sarcoma cell line (9). MRC-5 is a finite-life human lung fibroblast strain, and MRC-5V1 is an SV40-transformed derivative of MRC-5 (19). FR3T3 is an established line of Fisher rat cells (36), and FREJ4 is an EJ-Ha-*ras*-transformed derivative of the FR3T3 line (41).

Plasmid transfections. Calcium phosphate transfections were performed as described by Gorman (16). Cultures (2×10^5 cells per 6-cm-diameter dish) were inoculated with 1 μg of plasmid pMVMIL2 or pMVMIL4 supplemented with salmon sperm DNA to a total of 10 μg. To encapsidate recombinant parvoviral genomes, helper plasmid pMM984 or pULB3235 was cotransfected at a molar ratio of 3:1 with 1 μg of pMVMIL2 or pMVMIL4 supplemented with salmon sperm DNA to a total of 10 μg. Precipitates were allowed to form for 10 min and were then added to the culture medium. After a 4-h incubation at 37°C with 5% CO_2 in the presence

of the precipitate, the plates were washed three times with phosphate-buffered saline (PBS). Plasmid DNAs for transfection were purified on CsCl gradients, extracted with CsCl-saturated butanol, ethanol precipitated, and resuspended in water.

Parvoviral infections. Cells were plated at a density of 1.3×10^5 cells per 6-cm-diameter dish. After 24 h, the cells were washed with PBS and incubated for 1 h at 37°C with 0.5 ml of test supernatant (the source of recombinant parvoviruses). The cells were then washed three times with PBS and fed with 5 ml of complete medium. The kinetics of IL-2 or IL-4 production by infected cells were assessed by removing 0.5-ml aliquots of medium (for interleukin assay) at regular intervals postinfection and replacing the volume with 0.5 ml of fresh medium. MVM(p) was titrated by infectious-center hybridization on NB-K cells (43). About 2.5×10^5 cells were plated and infected as described above. After 27 to 30 h, the plates were washed with PBS, and nitrocellulose filters (Schleicher and Schuell BA85) were applied directly to the cells. The DNA of the transferred cells was denatured by placing the filters for 2 min (two or three times) on Whatman 3MM paper saturated with 0.5 M NaOH–1.5 M NaCl and then neutralized on 1 M Tris–1.5 M NaCl–0.15 M sodium citrate in the same way. The filters were prehybridized and hybridized in 1.5 × SSPE (1× SSPE is 0.18 M NaCl, 10 mM $Na_2HPO_4 \cdot 7\ H_2O$, and 1 mM EDTA [pH 7.7]), 1% SDS, 0.5% (wt/vol) nonfat dry milk, and 0.1 mg of denatured salmon sperm DNA per ml at 65°C. ^{32}P-oligonucleotide-labelled probes (13) were prepared from the *Hin*dIII-*Bam*HI (500 bp) IL-2 fragment (Fig. 1) or the MVM VP fragment (*Hin*dIII [2653 to 3996]) for the detection of MVMIL2 and MVM(p), respectively.

Interleukin assays. IL-2 secretion by transfected or virus-infected cell lines was measured by the CTLL-2 cell proliferation assay (15). Aliquots (20 μl each) of neat culture supernatant and serial 10-fold dilutions were transferred to microculture plates, to which 5×10^3 CTLL-2 cells were added in a final volume of 200 μl. The cultures were incubated for 16 h at 37°C and pulsed with 0.5 μCi of [^{3}H]thymidine per well, and incorporation was measured after a further 4 h. Incorporation of [^{3}H]thymidine was used as an index of DNA synthesis. A concentration of 10 U of IL-2 per ml gives half-maximal proliferation of CTLL-2 under these conditions. IL-2 activity was expressed as units per milliliter of culture supernatant.

IL-4 secretion was measured by the HT-2 cell proliferation assay (22). Aliquots (20 μl each) of neat culture supernatant and serial 10-fold dilutions were transferred to microculture plates, to which 10^4 HT-2 cells were added in a final volume of 200 μl. The cultures were incubated for 16 h at 37°C, pulsed with 0.5 μCi of [^{3}H]thymidine per well, and incorporation was measured after a further 4 h. A concentration of 10 U of IL-4 per ml gives half-maximal proliferation of HT-2 cells under these conditions. Neutralization of the assay by monoclonal antibody 11B11 (which specifically blocks the activity of IL-4 [28]) was checked to ensure specificity for IL-4.

Parvovirus DNA replication. Excision and intracellular replication of parvoviral DNA was monitored by agarose gel and Southern analysis of low-molecular-weight (Hirt) DNA (18) from transfected or virally infected cells.

To extract low-molecular-weight DNA, cells were washed once with PBS and lysed in 1 ml of lysis buffer (10 mM EDTA, 10 mM Tris [pH 7.4], 0.6% SDS) for 10 min. Cell lysates were then incubated with proteinase K (50 μg/ml) for 1 h at 37°C, and NaCl was added to a final concentration of 1 M, followed by incubation on ice for at least 4 h. High-molecular-weight DNA and cell debris were pelleted by centrifugation (15 min, 4°C, Eppendorf Microfuge). Hirt DNA was ethanol precipitated from the supernatant fraction and resuspended in 50 to 100 μl of H_2O.

For Southern hybridization analysis, aliquots (usually one-fifth) of each Hirt extract were size fractionated on a 0.9% agarose gel, denatured, neutralized, and transferred to a GeneScreen Plus nylon filter. Blots were prehybridized and hybridized at high stringency in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulfate, 50% formamide, and 0.3 mg of denatured salmon sperm DNA per ml at 42°C with a ^{32}P-oligonucleotide-labelled (13) DNA probe, washed (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 min at room temperature, twice; 2× SSC plus 1% SDS, 30 min at 65°C, twice; 0.1× SSC, 30 min at room temperature, twice), and exposed to X-ray film.

RESULTS

Characterization of recombinant parvoviral plasmids in transient expression assays. The plasmid pMM984 (24) contains the entire MVM(p) genome (1) cloned into pBR322 and generates infectious MVM(p) virions when transfected into susceptible target cells. Plasmids pMVMIL2 and pMVMIL4 were constructed by replacing 1,562 bp of the pMM984 capsid coding sequence with cDNAs for human IL-2 or murine IL-4, thereby placing the interleukin genes under the control of the promoter at map unit 38 (Fig. 1 and Materials and Methods). The initiating methionine codons of the interleukin cDNAs were positioned to correspond approximately to the position of the deleted VP-2 start codon. The viral transcription unit encoding the nonstructural proteins NS-1 and NS-2 was left intact, as were the polyadenylation signal sequences and the palindromic sequences at both termini of the viral genome.

To determine whether pMVMIL2 could direct expression of its IL-2 gene insert, the plasmid was transfected into HSNLV rat tumor cell monolayers, and supernatants harvested at intervals from the transfected cells were analyzed for the presence of biologically active IL-2. Figure 2A shows the time course of IL-2 secretion following cell transfection and illustrates that the level of secretion was maximal during the first 72 h, diminishing rapidly thereafter. This pattern of gene expression is reminiscent of the pattern of MVM capsid gene expression seen after infection of susceptible target cells (7). Cells transfected with plasmid pMVMIL4 gave a slightly delayed time course of IL-4 secretion, compared with that seen with pMVMIL2 (Fig. 2B). The rapid decline in interleukin secretion seen in these transient transfection assays might be attributable, either singly or in combination, to instability of the transfected plasmid or to the cytotoxic effect of the NS-1 protein encoded by both pMVMIL2 and pMVMIL4.

Excision and replication of the recombinant parvoviral genome from pMVMIL2. To ensure correct excision and replication of the recombinant parvoviral genome as a linear duplex molecule following transfection of eucaryotic cells, care was taken during the construction of pMVMIL2 and pMVMIL4 to conserve the NS-1 coding region and the viral genome termini. Special vigilance was required to combat the tendency of the 206-nucleotide 5′ (right-hand) palindrome of pMM984 to acquire central, symmetrical deletions of 40 or 97 bp when propagated in *Escherichia coli* (2).

Figure 3 shows a Southern blot of low-molecular-weight (Hirt) DNA extracted from HSNLV cells at several time

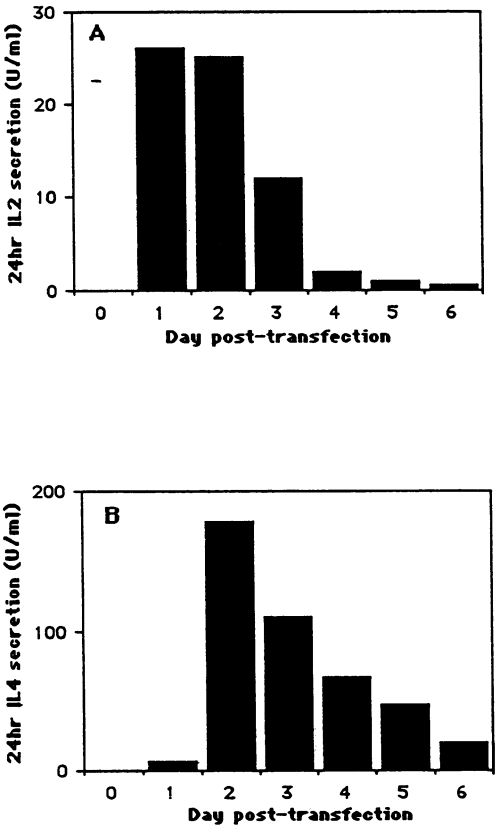

FIG. 2. Daily lymphokine secretion by HSNLV cells transfected with plasmid pMVMIL2 or pMVMIL4. Supernatants were harvested at 24-h intervals from HSNLV monolayers transfected either with pMVMIL2 (A) or with pMVMIL4 (B). Cells were washed twice in PBS, and the medium was replaced 24 h before each harvest. (A) IL-2 bioactivity in 24-h conditioned supernatants at various times posttransfection with pMVMIL2; (B) IL-4 bioactivity in 24-h conditioned supernatants at various times posttransfection with pMVMIL4.

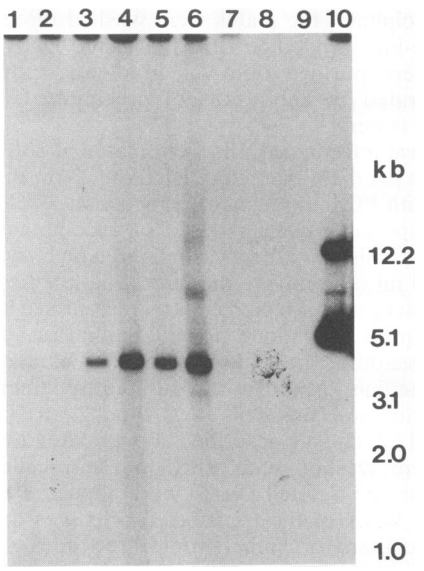

FIG. 3. Southern analysis of transfected pMVMIL2 plasmid DNA. Low-molecular-weight (Hirt) DNA was harvested (at various times posttransfection) from HSNLV monolayers transfected with pMVMIL2. One-quarter (10 μl) of each Hirt extract was electrophoresed in 0.9% agarose, transferred to a GeneScreen Plus membrane, and probed with a labelled human IL-2 cDNA (lanes 1 to 6). Shown are transfected cultures lysed after 24 h (lane 1), 48 h (lane 2), 72 h (lane 3), 96 h (lane 4), 120 h (lane 5), and 144 h (lane 6) and transfected HSNLV cells passaged (at a 1:5 dilution) after 48 h and harvested at 96 h (lane 7), 120 h (lane 8), and 144 h (lane 9). Lane 10, 1 ng of undigested pMVMIL2.

points after transfection with pMVMIL2. The blot was probed with a labelled human IL-2 cDNA which cross-hybridizes only weakly to rat IL-2 sequences and is therefore specific for the transfected pMVMIL2 DNA. The 4-kb band which appears soon after transfection is of the expected size for the monomer duplex parvoviral genome correctly excised from pMVMIL2. This band is not seen in lane 10, which was loaded with uncut pMVMIL2, indicating that it must have arisen by excision from the input plasmid.

Intracellular replication of the excised parvoviral genome is suggested both by the increasing intensity of the 4-kb monomer RF band with time (Fig. 3, lanes 1 to 6) and by the appearance of a distinct 8-kb band which represents the corresponding dimer replicative intermediate (Fig. 3, lane 6). In the autonomous parvoviruses, excision of SS progeny MVM DNA from monomer and dimer RFs is concomitant with encapsidation (32). The absence in Fig. 3 of a band corresponding to SS progeny DNA was therefore fully expected since a large segment of the MVM capsid coding sequence is missing in pMVMIL2.

To assess the cytotoxic potential of the MVMIL2 genome, cells were trypsinized and replated 48 h after transfection with pMVMIL2. Hirt DNA from these cells (Fig. 3, lanes 7 to 9) contained considerably less monomer RF DNA than corresponding Hirt supernatants from cells which were transfected in parallel but not replated (Fig. 3, lanes 4 to 6). This result suggests that at least one gene product of pMVMIL2 (probably the cytotoxic parvoviral NS-1 protein) impairs the viability of the transfected cells, thereby limiting the duration of IL-2 secretion.

Assembly of recombinant MVM virions competent for interleukin gene transfer and expression. From the preceding experiments, it was clear that plasmid pMVMIL2 could generate monomer and dimer duplex RF intermediates when transfected into susceptible target cells. To excise and encapsidate SS progeny DNA genomes from these duplex RF intermediates, MVM capsid proteins were provided in *trans* by cotransfecting pMVMIL2 with complementing plasmid pMM984 [wild-type MVM(p)] or pULB3235 (deleted helper), both of which have intact capsid genes. Plasmid pULB3235 (derived from pMM984) has a frameshift mutation in the NS-1 coding sequence and a small deletion in the right-hand terminal palindrome which prevents intracellular excision and amplification of the parvoviral genome (see Materials and Methods).

An MVM(p)-permissive, SV40-transformed human kidney cell line (NB-E) was transfected with pMVMIL2 alone or in combination with excess wild-type or deleted-helper plasmids, and conditioned medium from the transfected cells was harvested after 72 h. IL-2 was detected in conditioned medium from all pMVMIL2 primary transfectants (Fig. 4). As expected, when pMM984, which efficiently generates infectious wild-type MVM(p) virions, was transfected as a source of capsid proteins, a significant cytopathic effect in the culture was observed (data not shown).

As a definitive test for the presence of infectious parvovi-

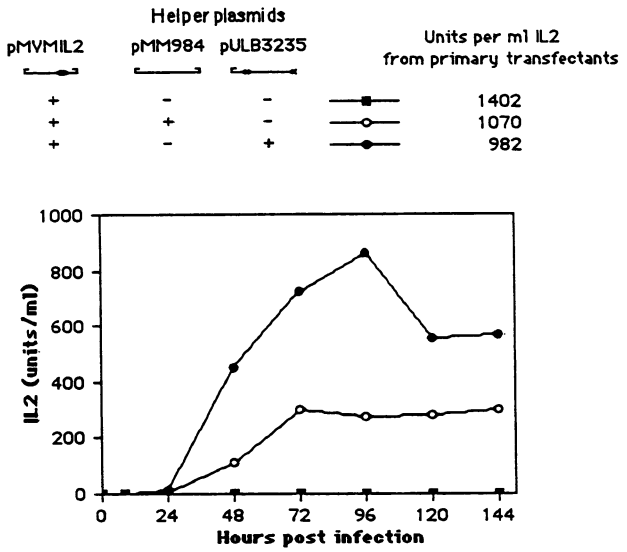

FIG. 4. IL-2 secretion by NB-K cells infected with MVMIL2 virus. NB-E cells were transfected with pMVMIL2 alone or with a threefold molar excess of helper plasmid (pMM984 or pULB3235). The concentration of IL-2 in supernatants harvested from these primary transfectants was measured after 72 h, and these 72-h-old supernatants were then used to infect NB-K cells. Accumulation of IL-2 in the medium bathing infected NB-K cells was monitored at regular intervals postinfection. The graph shows cumulative rather than daily IL-2 production.

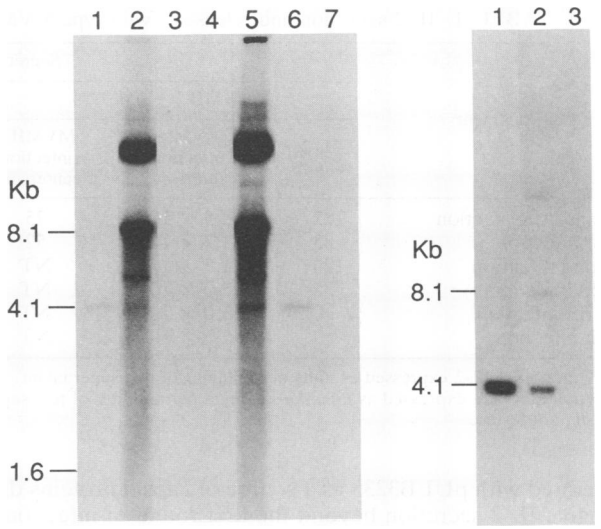

FIG. 5. Southern analysis of Hirt extracts showing parvoviral transfer of MVMIL2 and MVMIL4 genomes. Hirt DNA was prepared 72 h posttransfection or postinfection. (A) IL-2 probe for MVMIL2, 4-h exposure. Lanes: 1, 100 pg of *Bam*HI-digested pMVMIL2; 2, NB-E cells transfected with pMVMIL2 alone; 3, NB-K cells infected with supernatant from lane 2; 4, NB-K cells infected with supernatant from lane 3; 5, NB-E cells transfected with pMVMIL2 plus pULB3235; 6, NB-K cells infected with supernatant from lane 5; 7, NB-K cells infected with supernatant from lane 6. (B) IL-4 probe for MVMIL4, 6-h exposure. Lanes: 1, 100 pg of *Bam*HI-digested pMVMIL4; 2, NB-K cells infected with supernatant from NB-E which had been transfected with pMVMIL4 plus pULB3235; 3, NB-K cells infected with supernatant from lane 2.

ral particles containing SS, pMVMIL2-derived parvoviral genomes, conditioned media from the transfected cells were tested for their ability to transfer a functional IL-2 cDNA. NB-K cells were infected by exposing them for 1 h to the putative virus-containing conditioned media, which was followed by extensive washing to remove residual IL-2. The infected cells were then refed, and small aliquots of the culture medium were removed at intervals for determination of the IL-2 concentration. The accumulation of IL-2 in the culture medium of infected cells is graphically illustrated in Fig. 4. From these results, it is clear that cells transfected with a combination of pMVMIL2 and the wild-type or deleted-helper plasmid were able to generate infectious virus which efficiently transfers a functional IL-2 cDNA. Predictably, no virus in conditioned medium from cells transfected with pMVMIL2 alone was detected.

For more direct confirmation of parvovirus-mediated IL-2 and IL-4 gene transfer, Hirt DNA was extracted from NB-K cultures which had been infected with recombinant virus. Southern blots of some of these Hirt extracts are shown in Fig. 5. The IL-2 probe hybridizes to an MVMIL2-derived 4-kb monomer duplex RF band in Hirt DNA from primary transfectants (Fig. 5A, lanes 2 and 5) and from virally infected cells (Fig. 5A, lane 6). The additional bands seen in lanes 2 and 5 are due to persistence of transfected pMVMIL2 plasmid DNA. IL-2 gene transfer was not observed when helper plasmid was omitted from the primary transfection (Fig. 5A, lanes 3 and 4). The Southern blot in Fig. 5B, probed with an IL-4 probe, demonstrates efficient transfer (confirmed by IL-4 secretion) of the IL-4 gene by MVMIL4 virus generated with the deleted-helper plasmid pULB3235. Besides the 3.9-kb monomer duplex RF band, there is an 8-kb dimer RF band seen in Fig. 5B (lane 2), which further confirms successful parvoviral transfer of a replicating MVMIL4 genome.

Virus stocks generated with pMM984 as helper were highly cytopathic for infected target cells, presumably because of the relative abundance of contaminating wild-type MVM(p). In contrast, no cytopathic effect was observed with "helper-free" virus generated by using pULB3235 as helper. The absence of an observable cytopathic effect might be interpreted as evidence that viruses carrying a pMVMIL2-derived genome do not harm their target cells. However, Fig. 4 shows that the rate of IL-2 accumulation in the medium of MVMIL2-infected cells diminishes after 72 h, and most likely this is a reflection of the cytotoxic effect of the parvoviral NS-1 protein. The minimal cytopathic effect seen with helper-free virus may be due to the fact that the virus titer is low, an interpretation which is supported by semiquantitative analysis of Hirt supernatant DNA derived from the infected cultures (data not shown).

Serial parvoviral transfer of a functional interleukin cDNA. Having shown that recombinant SS MVM genomes could be displaced from plasmid-derived duplex RF DNA and encapsidated into infectious MVM particles, we next sought to determine whether the same was true for duplex RF DNA derived not from plasmid but from the SS genome delivered by a recombinant virus. To this end, recombinant virus stocks were passaged serially on NB-K cells and interleukin concentrations in the culture supernatants were measured 72 h after each round of infection.

Table 1 shows that serial transfer of a functional IL-2 cDNA was observed when the original MVMIL2 virus stock had been generated with the wild-type MVM helper plasmid and therefore contained significant numbers of wild-type MVM virions. In contrast, helper-free stocks of MVMIL2

TABLE 1. IL-2 secretion and release of wild-type MVM(p) during serial passage of MVMIL2 virus stocks in NB-K cells

Cell treatment	Secretion and release with the following input plasmids:							
	pMVMIL2 + pMM984			pMVMIL2 + pULB3235			pMVMIL2	
	IL-2 (U/ml)[a]	MVM(p) (infectious centers/ml)[b]	MVMIL2 (infectious centers/ml)	IL-2 (U/ml)[a]	MVM(p) (infectious centers/ml)[b]	MVMIL2 (infectious centers/ml)	IL-2 (U/ml)[a]	MVM(p) (infectious centers/ml)[b]
Primary transfection	122	2.5×10^4	75	108	5	26	50	0
First infection	85	1.3×10^6	52	89	7	0	0	0
Second infection	126	1.3×10^6	NT[c]	0	1.6×10^3	NT	0	NT
Third infection	35	5.0×10^5	NT	0	6.0×10^4	NT	0	NT
Fourth infection	0.2	3.0×10^4	NT	0	1.6×10^6	NT	0	NT
Fifth infection	0	5.5×10^4	NT	0	1.3×10^6	NT	0	NT

[a] Secretion of IL-2 expressed as units per milliliter of test supernatant.
[b] Virus titers are expressed as infectious centers per milliliter of test supernatant.
[c] NT, not tested.

generated with pULB3235 as a source of capsid proteins did not give IL-2 secretion beyond the first round of infection.

This is in agreement with the titers of MVMIL2 and wild-type MVM(p), detected in supernatants by means of a sensitive infectious-center hybridization assay. With wild-type MVM as helper, the MVM titer rose to a peak after the second round of infection, falling off rapidly thereafter, and this was associated with a greatly diminished cytopathic effect of the virus stock on the fourth and fifth rounds of infection (from massive cell death to minimal cell detachment). IL-2 secretion by the infected target cells also fell off rapidly after the third round of infection, suggesting the presence of selective pressures which prevented amplification both of wild-type MVM and of the MVMIL2 recombinants. Ethidium-stained agarose gel electrophoretic profiles of Hirt DNA extracted from these cultures (Fig. 6) revealed the presence of partially deleted (4.5-kb monomer RF) MVM genomes, which first appeared on the second round of infection and were progressively amplified thereafter at the expense of wild-type MVM (5-kb monomer RF) genome replication. Similar bands which appeared during serial high-multiplicity passage of the closely related H-1 virus were identified as defective interfering H-1 genomes (29). Heterogeneous DI genomes of H-1 virus, modestly deleted by up to 10 to 12%, are produced by serial propagation at high multiplicities of infection and interfere with capsid protein synthesis and replication of wild type H-1 virus (29).

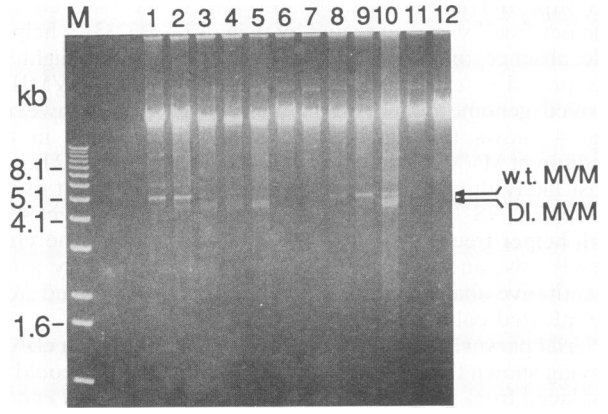

FIG. 6. Ethidium-stained agarose gel electrophoretic profiles of Hirt extracts during serial passage of MVMIL2 parvoviral stocks in NB-K cells. Hirt DNA was extracted 72 h postinfection and fractionated by electrophoresis in a 0.9% agarose gel. See Table 1 for corresponding concentrations of IL-2 and infectious titers of wild-type (w.t.) MVM. Lane M, 1-kb DNA ladder size markers; lanes 1 to 5, first to fifth infection, respectively, after initial cotransfection of pMVMIL2 with pMM984 (wild-type MVM) helper; lanes 6 to 10, first to fifth infections, respectively, after initial cotransfection of pMVMIL2 with deleted helper pULB3235; lanes 11 and 12, first and second infections, respectively, after initial cotransfection of pMVMIL2 with no helper. DI.MVM, putative defective interfering parvoviral genome (monomer RF intermediate).

As discussed previously, helper-free MVMIL2 virus stocks generated with the plasmid pULB3235 as helper gave rise to little cytopathic effect, and wild-type MVM replicative intermediates were not detected in Hirt DNA prepared from cultures infected by these virus stocks. However, the viral titers listed in Table 1 show very low-level contamination of the initial helper-free virus stock with wild-type MVM, which was rapidly amplified during serial infections (also Fig. 6, lanes 8 to 10). Complete MVM genomes must, therefore, have arisen by recombination between the cotransfected plasmids pMVMIL2 and pULB3235.

IL-2 secretion by transformed and nontransformed target cells following parvoviral transfer of an IL-2 cDNA. MVM(p) was chosen as a vehicle for interleukin gene transfer purely on the basis of its specificity for human and rodent fibroblasts expressing a transformed phenotype. It was therefore of considerable importance to determine whether this specificity was maintained by the recombinant parvoviral vectors we had generated.

Finite-life-span human fibroblasts (MRC-5) and their SV40-transformed derivatives (MRC-5V1) were infected with MVMIL2 virus, and the accumulation of IL-2 in the culture supernatants was monitored at regular intervals thereafter. IL-2 secretion by the SV40-transformed cells was greatly increased relative to their nontransformed counterparts (Fig. 7). Equally, IL-2 secretion was undetectable in MVMIL2-infected rat FR3T3 fibroblasts but greatly increased in their EJ-Harvey-*ras* oncogene-transformed derivatives (FREJ4). These data convincingly demonstrate that the MVM(p)-derived vectors described in this study retain the desired host range properties of the parental virus.

DISCUSSION

It has been suggested that tumor cell-targeted delivery and expression of certain lymphokine genes might cause regression of an established tumor (33). Here we report the development of a viral vector suitable for this application. MVM(p) is a fibrotropic, S-phase-dependent autonomous

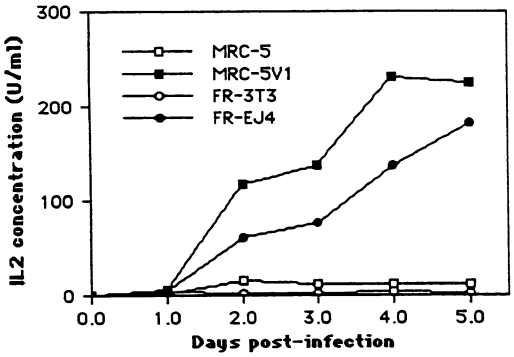

FIG. 7. IL-2 secretion by MVMIL2-infected fibroblasts: effect of target cell transformation. MVMIL2 virus-containing supernatants were harvested from NB-E monolayers cotransfected with pMVMIL2 plus helper plasmid (pMM984 or pULB3235). Target cells were infected with 0.5 ml of viral supernatant generated by using wild-type or deleted helper, and accumulation of IL-2 in the medium was monitored daily thereafter. Choice of helper plasmid did not significantly affect the results, and for clarity, the data for wild-type and deleted helper-generated virus have been averaged.

parvovirus which is weakly pathogenic in its natural host (42). The intracellular block to MVM(p) gene expression frequently seen in normal fibroblasts of rodent or human origin is often reversed following malignant transformation of the same cells. This unusual specificity of MVM(p) made it a suitable candidate for the development of gene transfer vectors giving tumor-specific expression of nonviral genes.

In this study, starting with an infectious molecular clone of MVM(p), we have removed 1,558 nucleotides of capsid coding sequence downstream of the viral promoter at map unit 38 and replaced them with cDNAs for human IL-2 or murine IL-4. The genome termini (which are required in *cis* for intracellular amplification of viral RF DNA), the viral transcription unit encoding nonstructural proteins NS-1 and NS-2, and the viral polyadenylation signal sequences were retained intact. Transfection of mammalian cells with the recombinant parvoviral plasmids (pMVMIL2 and pMVMIL4) was followed by transient secretion of the appropriate interleukins. The recombinant MVM genomes were correctly excised from input plasmid, and subsequent intracellular amplification of the corresponding duplex monomer and dimer RF DNAs was observed. To displace and encapsidate SS progeny DNA genomes from these duplex RF intermediates, capsid proteins were provided in *trans* by replication-defective (pULB3235) or wild-type (pMM984) MVM plasmids. In this way, it proved possible to generate recombinant parvoviruses which could transfer functional IL-2 or IL-4 cDNAs to MVM-susceptible target cells, inducing transient, high-level secretion of the appropriate biologically active interleukin. Parvovirus-mediated IL-2 secretion was greatly enhanced in oncogene-transformed rat and human fibroblasts, compared with their nontransformed counterparts, indicating that these MVM(p)-derived vectors retain the desired host range properties of the parental virus.

The transience of interleukin secretion seen following transfer of the MVMIL2 and MVMIL4 genomes (whether delivered by plasmid transfection or viral infection) can be attributed to the cytotoxic properties of the viral NS-1 protein. NS-1 is a multifunctional nuclear phosphoprotein which displays a potent cytotoxic activity (4), the purpose of which may be to mediate the timely lysis of parvovirus-infected target cells. This cytotoxicity property of NS-1 makes it a potential candidate for gene therapy of cancer in its own right, although it suffers from the disadvantage that its killing effect is limited to cells actually expressing the protein. Combined delivery of NS-1 and interleukin genes may thus have much greater therapeutic potential than delivery of either gene alone. NS-1 positively regulates transcription from the viral promoter at map unit 38 (11) and is required for amplification of duplex monomer and dimer RFs (31). Inclusion of NS-1 in our vectors therefore not only allows amplification of the recombinant parvoviral genome (and hence copy number of the interleukin cDNA) in infected target cells, but also transactivates p38-driven interleukin gene expression.

In order to test these vectors in vivo, high-titer stocks are necessary. It is currently unclear why the MVMIL2 titer is 2 orders of magnitude lower than that of MVM(p) after transfection. We tried to amplify MVMIL2 by serial infections of permissive cells with culture supernatants. However, the titers of both wild-type MVM and MVMIL2 decreased during these serial passages, and this was associated with progressive amplification of a 4.5-kb monomer RF band in Hirt extracts. Similar bands which appeared during serial high multiplicity passage of H-1 virus (a closely related parvovirus) were identified as defective interfering H-1 genomes (29). These genomes were characterized by tandemly repeated sequences immediately inboard of the right-hand palindrome and short deletions between map units 80 and 90 or between map units 32 and 44. One possibility to enhance the titer of MVM recombinant viruses would be to use packaging cells stably transfected with MVM capsid-coding plasmid constructs.

The parvoviral vectors described in this study represent an important step in the development of vectors suitable for in vivo gene delivery to tumors. As to the choice of gene to be delivered, there are several possibilities besides interleukin genes. Locally secreted chemotactic peptides, tumor-specific monoclonal antibodies, and monoclonal antibody-toxin fusion proteins are some of the alternative gene products with therapeutic potential.

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REFERENCES

1. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57**:656–669.
2. **Boissy, R., and C. R. Astell.** 1985. An Escherichia coli recBC sbcB recF host permits the deletion-resistant-propagation of plasmid clones containing the 5'-terminal palindrome of minute virus of mice. Gene **35**:179–185.
3. **Brandenburger, A., D. Legendre, B. Avalosse, and J. Rommelaere.** 1990. NS-1 and NS-2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. Virology **174**: 576–584.
4. **Caillet-Fauquet, P., M. Perros, A. Brandenburger, P. Spegelaere, and J. Rommelaere.** 1990. Programmed killing of

human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J. **9**:2989–2995.
5. **Cornelis, J. J., P. Becquart, N. Duponchel, N. Salomé, B. L. Avalosse, M. Namba, and J. Rommelaere.** 1988. Transformation of human fibroblasts by ionizing radiation, a chemical carcinogen, or simian virus 40 correlates with an increase in susceptibility to the autonomous parvovirus H-1 and minute virus of mice. J. Virol. **62**:1679–1686.
6. **Cornelis, J. J., Y. Q. Chen, N. Spruyt, N. Duponchel, S. F. Cotmore, P. Tattersall, and J. Rommelaere.** 1990. Susceptibility of human cells to killing by the parvovirus H-1 and minute virus of mice correlates with viral transcription. J. Virol. **64**:2537–2544.
7. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**:91–169.
8. **Cotmore, S. F., and P. Tattersall.** 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. J. Virol. **63**:3902–3911.
9. **Currie, G. A., and J. O. Gage.** 1973. Influence of tumour growth on the evolution of cytotoxic lymphoid cells in rats bearing a spontaneously metastasizing syngenetic fibrosarcoma. Br. J. Cancer **28**:136–146.
10. **Doerig, C., B. Hirt, J. P. Antonietti, and P. Beard.** 1990. Nonstructural protein of parvovirus B19 and minute virus of mice controls transcription. J. Virol. **64**:387–396.
11. **Doerig, C., B. Hirt, B. Beard, and J. P. Antonietti.** 1988. Minute virus of mice non-structural protein NS-1 is necessary and sufficient for trans-activation of the viral P39 promoter. J. Gen. Virol. **69**:2563–2573.
12. **Fearon, E., D. M. Pardoll, T. Itaya, P. Golumbek, H. I. Levitsky, J. W. Simons, H. Karasuyama, B. Vogelstein, and P. Forst.** 1990. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell **60**: 397–403.
13. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
14. **Gansbacher, B., K. Zier, B. Daniels, K. Cronin, R. Bannerji, and E. Gilboa.** 1990. Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. J. Exp. Med. **172**:1217–1224.
15. **Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith.** 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. **120**:2027–2032.
16. **Gorman, C.** 1985. High efficiency gene transfer into mammalian cells, p. 143–165. *In* D. M. Glover (ed.), DNA cloning, a practical approach, vol. 2. IRL Press, Oxford.
17. **Guetta, E., Y. Graziani, and J. Tal.** 1986. Suppression of Ehrlich ascites tumors in mice by minute virus of mice. JNCI **76**:1177–1180.
18. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:363–369.
19. **Huschtscha, L. I., and R. Holliday.** 1983. Limited and unlimited growth of SV40-transformed cells from human diploid MRC-5 fibroblasts. J. Cell Sci. **63**:77–99.
20. **Kimsey, P. B., H. D. Engers, B. Hirt, and C. V. Jongeneel.** 1986. Pathogenicity of fibroblast- and lymphocyte-specific variants of minute virus of mice. J. Virol. **59**:8–13.
21. **Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann, D. Rennick, N. Roehm, C. Smith, A. Zlotnik, and K. Arai.** 1986. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activity. Proc. Natl. Acad. Sci. USA **83**:2061–2065.
22. **Lichtman, A. H., E. A. Kurt-Jones, and A. K. Abbas.** 1987. B-cell stimulatory factor 1 and not interleukin 2 is the autocrine growth factor for some helper T lymphocytes. Proc. Natl. Acad. Sci. USA **84**:824–827.
23. **Linser, P., H. Bruning, and R. W. Armentrout.** 1977. Specific binding sites for a parvovirus minute virus of mice, on cultured mouse cells. J. Virol. **24**:211–221.
23a. **Mackay, Ian.** Personal communication.
24. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infective molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47**:227–232.
25. **Mortimer, P. P., R. K. Humphries, J. G. Moore, R. H. Purcell, and N. S. Young.** 1983. A human parvovirus-like virus inhibits haematopoietic colony formation in vitro. Nature (London) **302**:426–429.
26. **Mousset, S., J. Cornelis, N. Spruyt, and J. Rommelaere.** 1986. Transformation of established murine fibroblasts with an activated cellular Harvey-ras oncogene or the polyoma virus middle T gene increases cell permissiveness to parvovirus minute virus of mice. Biochimie **68**:951–955.
27. **Mousset, S., and J. Rommelaere.** 1982. Minute virus of mice inhibits cell transformation by simian virus 40. Nature (London) **300**:537–539.
28. **Ohara, J., and W. E. Paul.** 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. Nature (London) **315**:333–336.
29. **Rhode, S. L., III.** 1978. Defective interfering particles of parvovirus H-1. J. Virol. **27**:347–356.
30. **Rhode, S. L., III.** 1985. *trans*-Activation of parvovirus P_{38} promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
31. **Rhode, S. L., III.** 1989. Both excision and replication of cloned autonomous parvovirus DNA require the NS1 (rep) protein. J. Virol. **63**:4249–4256.
32. **Richards, R., P. Linser, and R. W. Armentrout.** 1977. Kinetics of assembly of a parvovirus, minute virus of mice, in synchronized rat brain cells. J. Virol. **22**:778–793.
33. **Russell, S. J.** 1990. Lymphokine gene therapy for cancer. Immunol. Today **11**:196–200.
34. **Russell, S. J., S. A. Eccles, C. Flemming, C. A. Johnson, and M. K. L. Collins.** 1991. Decreased tumorigenicity of a transplantable rat sarcoma following transfer and expression of an IL-2 cDNA. Int. J. Cancer **47**:244–251.
35. **Salomé, N., B. van Hille, N. Duponchel, G. Meneguzzi, F. Cuzin, J. Rommelaere, and J. J. Cornelis.** 1990. Sensitization of transformed rat cells to parvovirus MVMp is restricted to specific oncogenes. Oncogene **5**:123–130.
36. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721–728.
37. **Shein, H. M., and J. F. Enders.** 1962. Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. Proc. Soc. Exp. Biol. Med. **109**:495–500.
38. **Siegl, G.** 1984. Biology and pathogenicity of autonomous parvoviruses, p. 363–388. *In* K. I. Berns (ed.), The parvoviruses. Plenum Press, New York.
39. **Tepper, R. I., P. K. Pattengale, and P. Leder.** 1989. Murine interleukin 4 displays potent anti-tumor activity in vivo. Cell **57**:503–512.
40. **Toolan, H. W.** 1972. The parvoviruses. Prog. Exp. Tumor Res. **16**:410–425.
41. **van Hille, B., N. Duponchel, N. Salomé, N. Spruyt, S. F. Cotmore, J. J. Cornelis, and J. Rommelaere.** 1989. Limitations to the expression of parvoviral nonstructural proteins may determine the extent of sensitization of EJ ras-transformed rat fibroblasts to minute virus of mice. Virology **171**:89–97.
42. **Ward, D. C., and T. Tattersall.** 1982. Minute virus of mice, p. 313–332. *In* H. L. Foster, J. D. Small, and J. G. Fox (ed.), The mouse in biomedical research, vol. 2. Academic Press, New York.
43. **Yakobson, B., T. A. Hrynko, M. J. Peak, and E. Winocour.** 1989. Replication of adeno-associated virus in cells irradiated with UV light at 254 nm. J. Virol. **63**:1023–1030.
44. **Yamada, G., Y. Kitamura, H. Sonoda, H. Harada, S. Taki, R. C. Mulligan, H. Osawa, T. Diamantstein, S. Yokoyama, and T. Taniguchi.** 1987. Retroviral expression of the human IL-2 gene in a murine T cell line results in cell growth autonomy and tumorigenicity. EMBO J. **6**:2705–2709.