Cell-Type-Specific Growth Restriction of Vesicular Stomatitis Virus polR Mutants Is Linked to Defective Viral Polymerase Function[▽]

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Vesicular stomatitis virus polR mutants synthesize defective RNA replication products *in vitro* and display growth restriction in some cultured cells (J. L. Chuang, R. L. Jackson, and J. Perrault, *Virology* 229:57–67, 1997). We show here that a recombinant virus carrying the polR N protein mutation (R179H) yielded ~100-fold- and ~40-fold-lower amounts of infectious virus than the wild type in mouse L-929 and rat 3Y1 cells, respectively, but only ~3-fold less in hamster BHK cells. Virus genome accumulation was inhibited 6- to 10-fold in restricting cells, but transcription was not affected. No defect in encapsidation of replication products was detected, but virus protein accumulation was reduced two- to threefold in both restricting and nonrestricting cells. polR virus particles released from the latter were 5- to 10-fold less infectious than the wild type but showed no difference in protein composition. Phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2 α) was enhanced ~3-fold in polR versus wild-type virus-infected L-929 cells, but neither inhibition of host gene transcription nor inhibition of double-stranded RNA (dsRNA)-activated protein kinase showed significant effects on restriction. Conditioned medium studies revealed no evidence for secretion of antiviral factors from restricting cells. We conclude that the block in polR growth is due to the combined effect of reduced genome replication and lower infectivity of released virus particles and may be due to overproduction of dsRNA. An accompanying paper (D. Ostertag, T. M. Hoblitzell-Ostertag, and J. Perrault, *J. Virol.* 81:503–513, 2007) provides compelling evidence for the role of dsRNA in this unique restriction phenomenon.

Vesicular stomatitis virus (VSV) is a well-characterized member of the *Rhabdoviridae* family that serves as a model for the large group of agents known as monopartite negative-stranded RNA viruses (*Mononegavirales*). This group includes many important human pathogens such as rabies, measles, respiratory syncytial virus, and the highly lethal Ebola and Marburg agents. The VSV genome can readily be manipulated to produce useful recombinant viruses, some of which are currently being explored as vaccination vectors as well as oncolytic agents (5, 36, 38).

VSV generally replicates very efficiently in mammalian cells. Receptors are present on the surface of most if not all higher eukaryotic cells, although the nature of the receptors, possibly common phospholipids, still remains unclear (8, 11, 44). Instances of VSV growth restriction in the absence of a cellular antiviral response are rare. Such instances, however, can potentially identify critical host cell factors required for virus multiplication, and host range virus mutants are particularly useful in this regard. For example, VSV *hr1* and *hr8* mutants show defects in methylation of viral mRNA cap structures, a modification catalyzed by the virus L polymerase protein (18, 22). The existence of such mutants implies that some cell types somehow compensate for the defective viral function, although which cellular activity is responsible is unknown (21). Host

range defects can also result from virus mutations that alter the ability of the virus to counter host cell antiviral responses. For instance, wild-type (wt) VSV Indiana strains generally shut-down host cell functions rapidly under single-cycle infection conditions, which limits interferon (IFN) production, but mutants defective in host cell shutoff display strong growth restriction at low multiplicities of infection (MOI) in IFN-competent cells (1, 16, 27, 51). We document here a distinctly different instance of cell-type-specific growth restriction for a VSV mutant that displays aberrant viral RNA synthesis.

Several years ago, we reported the isolation of a unique class of VSV mutants, which we named polR because of their altered viral polymerase regulation phenotype (32). polR mutants have since proved very useful in deciphering virus polymerase function, notably, what distinguishes transcriptase from replicase activity and identifying template start sites for the two modes of synthesis (6, 9, 10, 20, 33). VSV RNA synthesis takes place entirely in the cytoplasm of infected cells and requires templates fully encapsidated by the viral nucleoprotein N (for reviews, see references 40 and 53). The first step in viral RNA synthesis is carried out by the virion-associated polymerase, a complex of L and P proteins, and entails sequential transcription of the five virus genes in the order found on the genome, 3'-N-P-M-G-L-5'. The transcription process was long thought to initiate by first copying a leader RNA complementary to the 47 nucleotides (nt) at the 3' end of the genome, but recent studies using polR1 VSV showed that the process can initiate internally at the N gene start sequence, 51 nt from the 3' end (10). Subsequent studies showed a similar phenomenon for wt virus (54). Synthesis of individual mRNAs is controlled by conserved start and polyadenylation/stop signals located at gene junctions, except in the case of the leader-N junction,

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which lacks a polyadenylation/stop signal (45). The polyadenylation signal consists of seven consecutive U residues reiteratively copied by the viral polymerase, which then releases transcripts and reinitiates synthesis at the downstream gene only two residues beyond the U stretch. Reinitiation takes place with ~60 to 70% efficiency, resulting in a decreasing gradient of transcript abundance. The viral polymerase complex also displays capping and methylase activities that modify nascent viral mRNA transcripts.

In contrast to transcription, replication of viral genomes and antigenomes requires synthesis of viral proteins and is tightly coupled to encapsidation of nascent RNA chains. The replicase necessarily initiates synthesis at the very 3' end of templates and ignores all gene start and stop sequences. Promoter sequences required for synthesis of plus-sense antigenomes, which overlap with transcription-promoting and encapsidation signals, reside within the first 50 nt at the 3' end of the genome (leader). Likewise, promoter and encapsidation signal sequences for minus-sense genome synthesis are located in the analogous 3' end region of antigenomes (complement of the trailer region at the 5' end of the genome). However, what governs the switch from transcription to replication is still poorly understood. Synthesis of an N/P assembly complex is clearly required, but something else likely signals the P/L polymerase complex to change to a replicative mode. Recent studies suggest that the N protein functions as an additional subunit of the polymerase complex when it carries out replication (37). However, in the case of polR VSV, the polymerase complex reads through the leader-N gene junction at high frequency in vitro in the absence of assembly or any additional viral protein (32). Even though this "replication-like" synthesis terminates nonspecifically within the N gene, these findings suggest that something other than encapsidation or recruitment of N protein enables the polymerase to initiate replicative synthesis.

The two VSV polR mutants we originally isolated (polR1 and polR2) show a nearly identical phenotype, and both harbor the same Arg179-to-His amino acid substitution in the N protein. The presence of this mutation in the assembled virus templates appears to be solely responsible for the observed alterations in viral RNA synthesis and ATP requirements (9, 20, 32). The properties of polR mutants suggest that the N protein component of assembled viral templates plays a role in determining polymerase function, but the mechanism involved remains unclear. Interestingly, frequent read-through of the leader-N gene in the absence of encapsidation also takes place in cells infected with the naturally occurring Z strain of Sendai virus, a member of the *Paramyxoviridae* family of monopartite negative-stranded RNA viruses, but whether this is due to an alteration in the N protein is unknown (52).

Interestingly, the VSV polR mutants display a host range phenotype for growth in some established cell lines (9). The mutants grow nearly as well as wt virus in BHK cells but show very strong growth restriction in mouse L-929 cells. What causes this host range phenotype and whether the phenomenon is linked to alterations in viral RNA synthesis has so far not been answered. We show here that polR growth restriction occurs in several established cell lines and is due solely to the Arg179-to-His mutation in the N protein. Detailed analysis of the block in virus multiplication in restricted hosts revealed a specific defect in genome replication as well as a decrease in the infectivity of released particles. Neither virus transcription

nor viral protein translation were significantly affected in restricted hosts. Several lines of evidence, including insensitivity to actinomycin D (act D) and 2-aminopurine (2-AP), indicated that growth restriction is not due to type I IFN induction or signaling. Enhanced phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2 α) was, however, evident in polR-infected L-929 cells, suggesting that polR infections produce more double-stranded RNA (dsRNA) than wt virus. These findings lead us to propose that enhanced production of viral dsRNA is responsible for the unique mode of VSV polR growth restriction.

(The work presented here formed part of a Ph.D. thesis submitted to the University of California—San Diego and San Diego State University by D. Ostertag and part of an M.S. thesis submitted to San Diego State University by T. M. Hoblitzell-Ostertag.)

MATERIALS AND METHODS

Cell culture and virus infections. BHK-21 and L-929 cells, as well as rat fibroblast 3Y1 cells (obtained from Bartholomew Sefton, Salk Institute), were grown as monolayers in Eagle's minimal essential medium (MEM) containing 7% newborn calf serum. Recombinant wt and polR VSV (see below) were grown and titers were determined by plaque assay on BHK cells as described previously (9). Infections were carried out in cell monolayers at 95 to 100% confluence in 5-cm dishes at an effective MOI of 10 PFU/cell unless otherwise stated. VSV plaque-forming efficiency is ~10-fold lower on L-929 (9) and 3Y1 cells (data not shown) than on BHK cells. L-929 and 3Y1 cells were therefore infected at an MOI of 100 PFU/cell to provide a comparable effective MOI. Unless otherwise stated, infectious virus titers in clarified infected cell supernatants ($600 \times g$, 5 min) were determined by plaque assay as described above at ~24 h postinfection (p.i.). 2-AP (Fisher Scientific) was prepared as a 0.1 M stock solution in 10 $\times$ phosphate-buffered saline by heating at 70°C for 10 min before dilution into medium at the concentrations indicated.

Generation of recombinant infectious wild-type and polR VSV mutant virus. Recombinant wt VSV (Indiana serotype) was generated from the full-length VSV cDNA plasmid (pVSVFL+) kindly provided by Jack Rose (Yale University), essentially as previously described (28), except for the inclusion of 40 μ g/ml cytosine arabinoside during the entire virus recovery procedure starting with vaccinia-T7 virus infection of BHK cells and transfection with support plasmids expressing VSV N, L, and P proteins. This modification prevented vaccinia virus production and obviated the need for supernatant filtration to remove vaccinia virus before amplifying recombinant VSV in BHK cells. Recombinant wt VSV stocks were amplified to high titers from a single plaque isolate.

To recover recombinant polR virus, the R179H polR gene mutation (9) was incorporated into the pVSVFL+ cDNA as described previously (39). In brief, the mutation was introduced by PCR mutagenesis into the XhoI/XbaI fragment of the pVSVFL+ plasmid subcloned into the pSP73 plasmid vector (Promega) using a plus-sense mutagenic primer, 5'-GCCAGAAGGTCACGATATCTT-3' (base changes underlined) and a minus-sense primer, 5'-TAGACGAGCATATGACTTGAGATAC-3'. The PCR product was then used in an extension reaction with the same plasmid using the QuikChange mutagenesis protocol (Stratagene). The XhoI/PstI fragment (containing the polR mutation) was used to replace the corresponding wt fragment in pSP73, followed by exchange of the XhoI/XbaI fragment from this plasmid for the corresponding fragment in pVSVFL+. The presence of the R179H mutation and absence of other mutations in the fragment derived from PCR in the resulting full-length VSV plasmid, denoted pVSVpolRFL+, was confirmed by sequencing and employed for recovery of recombinant polR virus as described above for recombinant wt.

Quantitation of released virus particles. Infected cell culture supernatants were clarified ($600 \times g$, 5 min), and RNA was extracted using standard proteinase K and phenol-chloroform procedures as described previously (48). Released particle genome RNA was quantified by Northern blotting as described below.

Quantitation of intracellular viral transcription and replication products. At the times indicated, RNA was extracted from cytoplasmic extracts as described previously (48). A portion of the extracts was first treated with micrococcal nuclease to digest unencapsidated RNAs for analysis of replication products or left untreated for analysis of transcription products. Northern blotting of RNA samples was carried out as before (48), except for the use of a hybridization mix

containing 8 ml of solution A (500 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ [pH 7.0], 1 mM EDTA, and 7% sodium dodecyl sulfate [SDS]), 4 ml formamide, 1 mg bovine serum albumin (BSA; Fisher Biotech Grade), and 1 mg salmon sperm DNA, brought to a total volume of 15 ml with water. Minus-sense VSV genomes were detected with a plus-sense T7 transcript encoding part of the L gene (~4 kb) generated from the pGEM-L_{ΔNstII} plasmid digested with HindIII (48). P gene transcripts were detected with a minus-sense T7 transcript (~850 bp) generated from BglII-digested pSP73-P plasmid containing the EcoRV fragment from pVSVFL+. For simultaneous probing of all VSV transcripts, a labeled DNA probe was generated by standard nick translation of the pVSVFL+ plasmid (41).

Western blot analysis. Cytoplasmic extracts were obtained as described above for RNA extraction, except for the addition of protease inhibitors (Roche minitab protease inhibitor cocktail), and the total protein concentration was determined by the Bradford method using BSA as a standard. Unless otherwise stated, 10 µg total protein for each sample was analyzed on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Proteins were blotted overnight onto a polyvinylidene difluoride membrane (Millipore) using a Transblot apparatus (Bio-Rad) at 30-V constant voltage under the manufacturer's suggested transfer conditions. Blots shown in Fig. 1 and 3 were processed using Gibco's buffer (9.2 mM KH_2PO_4 , 30.6 mM K_2HPO_4 , 270 mM NaCl, and 0.1% Tween-20 [Sigma]) with addition of 10% BSA as a blocking agent. Blots in Fig. 4 were processed using PBST (58 mM Na_2HPO_4 , 17 mM NaH_2PO_4 , 68 mM NaCl, and 0.1% Tween-20 [Sigma]) and 5% nonfat dry milk as blocking agents. Rabbit polyclonal antibody to VSV P protein (7), mouse monoclonal antibody 23H12 to VSV M protein (a kind gift from Doug Lyles, Wake Forest University School of Medicine), mouse monoclonal antibody to VSV G (catalog no. V-5507; Sigma) were used at 1:500, 1:1,500, and 1:100,000 dilutions, respectively. Rabbit polyclonal antibodies to eIF-2α and phospho-eIF-2α (Ser51) (Cell Signaling Technologies) were used at 1:1,000 dilution. Goat anti-rabbit and goat anti-mouse alkaline phosphatase-labeled secondary antibodies (Bio-Rad) and the CSPD substrate (Boehringer Mannheim) were employed for the blots shown in Fig. 4, while horseradish peroxidase-labeled secondary antibodies (Jackson ImmunoResearch) and the ECL Plus Western detection system (Amersham Biosciences) were used for blots shown in Fig. 1 and 3. Blots were then exposed to Kodak AR film for visualization and quantitated for fluorescence using a STORM apparatus (Molecular Dynamics).

[³⁵S]methionine labeling. Pulse-labeling of virus-infected cells was carried out using 16.7 µCi/ml Trans ³⁵S label (ICN Biomedicals, Inc.) for L-929 and 3Y1 cells or 3.3 µCi/ml for BHK cells in methionine- and serum-free Dulbecco's MEM (ICN Biomedicals, Inc.). Following 30 min of labeling, cells were washed twice with saline before being chased for the indicated times with complete medium (MEM) containing 7% serum. At the times indicated, cytoplasmic extracts were recovered as described above for Western blots. For measurement of ³⁵S-labeled protein in released particles, infected cells were labeled from 1 h p.i. to harvest time ~30 h p.i. under the same conditions as above except for the addition of a 1/20 volume of complete MEM to the methionine-free medium. Supernatants from infected cells were then clarified (600 × g, 20 min) and the virus pelleted by centrifugation (100,000 × g, 1.5 h), followed by resuspension in SDS-PAGE loading buffer and analysis as above.

Conditioned media treatment of cells. Media from BHK, L-929, and 3Y1 cells were collected ~48 h after plating and supplemented with glutamine and 5% calf serum before use as "conditioned" media. Freshly plated cells (overnight growth) were then exposed to the conditioned media for ~16 h before virus infection in the continued presence of conditioned media. Infectious virus yields in infected cell culture supernatants collected at 26 h p.i. were determined as above.

RESULTS

Growth of a recombinant VSV polR mutant virus is restricted in several established cell lines. We showed previously that a single amino acid substitution in the N protein of the polR1 and polR2 VSV mutants is responsible for their altered transcriptional properties *in vitro* and that the mutants also display a host range growth defect in established cell lines under single-cycle infection conditions (9). To establish whether the same N protein mutation (Arg179 to His) is also responsible for the host range defect, we engineered this change into a recombinant VSV (see Materials and Methods). We then compared infectious virus yields of the recombinant virus, herein denoted polR, to its wt isogenic parent virus in a

number of established cell lines. Infectious titers at ~24 h p.i. were determined by plaque assay in BHK cells. Figure 1 illustrates a comparison of polR and wt yields in BHK, L-929, and rat 3Y1 cells. All infections were carried out at an equivalent MOI of 10 PFU/cell. This necessitated using 100 PFU/cell of virus for which titers were determined on BHK for L-929 and 3Y1 infections to compensate for the ~10-fold-lower plaque-forming efficiency of wt VSV in these cell types (9). As observed earlier with polR1 and polR2 isolates, the recombinant polR virus yielded ~3-fold-lower amounts of infectious virus than wt in BHK cells, i.e., ~3,000 versus ~9,000 PFU/cell (Fig. 1A, left panel) and ~100-fold-lower amounts in L-929 cells (Fig. 1B, left panel). Note that wt virus yields were ~15-fold lower in L-929 cells (520 PFU/cell) than in BHK cells, in agreement with previous studies (47), but polR virus growth was clearly restricted to a much greater extent (~5 PFU/cell). PFU/cell yields for both viruses varied to some extent in several independent experiments, as expected, but polR consistently produced 100-fold-lower amounts than the wt in L-929 cells when assayed in parallel. The effect of various MOIs on virus growth in BHK and L-929 cells was also examined. wt virus yields in BHK and L-929 cells, as well as polR yields in BHK cells, varied little as a function of MOI (10, 1.0, or 0.1 PFU/cell). However, polR yields in L-929 cells decreased a further 10-fold at an MOI of 1.0 or 0.1 compared to 10 (data not shown). These results leave no doubt that the polR host range growth defect is indeed caused by the Arg179-to-His substitution in the N protein. Further studies showed that the recombinant polR displayed the alterations in viral RNA synthesis characteristic of the polR1 and polR2 viruses (39).

We reported earlier that polR1 and polR2 show modest growth restriction in MDBK and Vero cells (5- to 10-fold) and little, if any (<3-fold), in HeLa, CHO, and CV1 cells (10). We tested additional cell lines here for recombinant polR restriction. Both rat 3Y1 (Fig. 1C, left panel) and mouse BALB/3T3 cells showed strong restriction (~30- to 40-fold in several independent experiments), while HeLa, A549, and RC-60 displayed none (data not shown). The polR host range defect thus involves a unique subset of cell types insofar as established cell lines are concerned.

polR virus-restrictive cell lines release virus particles of low infectivity. Previous studies have demonstrated that wt VSV growth restriction in the Raji lymphoblastoid cell line is due at least in part to production of noninfectious virus particles (34). We tested whether this also pertained to polR virus restriction. Quantitation of released virus particles in the medium of infected cells for which determined titers are shown in Fig. 1 was determined by Northern blot analysis of particle genomic RNA (right panels). Amounts released in wt infections were arbitrarily given a value of 100. polR-infected BHK cells yielded roughly half as many released particles as the wt, in rough agreement with the threefold-lower PFU yield (Fig. 1A). For L-929 and 3Y1 cells, however, released polR virus particles were reduced only ~9-fold and ~6.5-fold compared to the wt, respectively (Fig. 1B and 1C). polR virus particles released from restricted cells therefore showed substantially lower infectivity than those from the wt. The resulting particle/PFU ratios were ~12-fold lower for L-929 cells and ~5-fold lower for 3Y1 cells. Overall restriction therefore involves both a decrease in total virus particle release and reduced particle

FIG. 1. VSV wt and VSV polR growth in BHK, L-929, and 3Y1 cells. All infections were carried out at an effective MOI of 10 PFU/cell. PFU yields in the medium of infected cells (panels A, B, and C, left side) were determined by plaque assay on BHK cells at ~24 h p.i. Amounts of released virus particles in the medium (panels A, B, and C, right side) were determined by Northern blotting for virus genomes (gel bands shown above graphs). Particle/PFU ratios were calculated relative to the wt. (D) SDS-PAGE of released virus particles from infected cells. The top panel shows an autoradiograph of [³⁵S]methionine-labeled proteins blotted onto membranes, and the bottom panel shows the same blot probed with antibodies to P and G proteins. Samples in each lane correspond to virus released from an approximately equal number of infected cells.

infectivity. Additional experiments also showed that wt virus particles released from L-929 and 3Y1 cells were two- to four-fold less infectious than wt virus released from BHK cells (data not shown), but this host-mediated effect on wt virus was always significantly less than the differences seen between wt and polR virus particles released from restricted hosts.

polR and wt virus particles released from restricted host cells have identical protein compositions. We next tested whether the lower infectivity of polR virus particles released from restricted host cells might be attributable to a change in viral protein composition. Virus proteins were labeled by incubating infected cells with [³⁵S]methionine from 1 h p.i. to harvest time at 30 h p.i., and the proteins from released virus particles from equivalent numbers of cells were separated by SDS-PAGE and transferred onto membranes. The top panel in Fig. 1D shows an autoradiograph of the blotted proteins. All five labeled VSV proteins were present in wt and polR particles released from BHK, L-929, and 3Y1 cells. No significant difference in the relative ratios of viral proteins in polR versus wt virus was observed for any of the samples. Notably, G protein from wt and polR particles released from L-929 and 3Y1 cells migrated slightly faster than that found in particles from BHK cells. A more diffuse

band migrating faster than G was also present in all particles and was more prominent in virus released from L-929 and 3Y1 cells. Although these results suggest G protein processing differences between the host cells, it is nonetheless clear that wt and polR particles originating from the same cell type show identical protein composition.

The same blot was also probed with antibodies to P and G proteins (Fig. 1D, lower panel). All particles showed similar G-to-P protein ratios regardless of their origin. Note also that the radiolabeled diffuse band migrating ahead of G protein also failed to react with the anti-G antibody. This species may therefore be unrelated to G protein or represent a species lacking the reactive C-terminal epitope. In any case, the Western blot results show that the lower infectivity of polR virus particles is not due to a deficiency in antibody-reactive G protein.

polR virus genome replication but not transcription is reduced in restricted infections. To further characterize polR restriction, we examined intracellular effects on viral RNA synthesis. Cytoplasmic extracts were prepared at various times p.i., and a portion of each extract was first treated with micrococcal nuclease before RNA extraction to probe for replication products (encapsidation provides protection from nuclease),

FIG. 2. Intracellular viral RNA synthesis in wt- and polR-infected BHK (A), L-929 (B), and 3Y1 (C) cells. Accumulation of minus-sense viral genomes and plus-sense P transcripts was determined by Northern blotting. Bar graphs reflect minus-genome accumulation (left panels) and P transcript accumulation (right panels). (D) Encapsidation of genomes and antigenomes in wt- and polR-infected L-929 cells. Cytoplasmic extracts were either treated with micrococcal nuclease to digest unencapsidated RNA (replication products) (+) or left untreated (–) before RNA extraction and Northern blot analysis. The ratio of wt signals from treated and untreated samples at 14 h p.i. was arbitrarily set to 100% encapsidation. Note that longer exposure times were used for antigenomes, since these are much less abundant than genomes.

while the untreated portion was used to probe for transcription products. Minus-sense genome products were detected by Northern blot analysis with a plus-sense L gene riboprobe, whereas transcription products were monitored with a minus-sense P gene riboprobe. Note that kinetics of infectious VSV production and cytopathic effect are slower in L-929 and 3Y1 cells than in BHK cells (not shown). The latest time points shown in Fig. 2A, B, and C reflect the time of maximum viral RNA accumulation for each cell type. polR accumulated twofold more genomes than the wt in BHK cells and nearly fourfold more P gene transcripts (Fig. 2A), despite a roughly threefold-lower yield of infectious virus. In contrast, polR synthesized fewer genomes than the wt in L-929 cells (fourfold less at 8 h and sixfold less at 12 h), while P transcript accumulation remained roughly similar to that of the wt (Fig. 2B). Additional experiments showed that the specific deficit in genome accumulation in polR-infected L-929 cells was similar when sampled at 14, 18, and 24 h p.i. (data not shown).

polR infection of restricted 3Y1 cells showed a defect very similar to that of L-929 cells with genome replication almost

4-fold lower than that of the wt at 4 h p.i. and 10-fold lower at 8 h and 12 h, with no significant differences in P transcript accumulation (Fig. 2C). These results demonstrate that polR transcription, at least as far as the P gene is concerned, is not inhibited in restricted host cells, but genome replication clearly is. Moreover, inhibition of genome replication appeared to be roughly equivalent to the decrease in particle release, from which we infer that packaging of the genome for export as virus particles does not play a significant role in polR restriction. Note, however, that L-929 and BHK cells accumulated roughly similar amounts of intracellular genomes and transcripts but L-929 cells were ~10-fold less efficient than BHK cells at packaging intracellular genomes into released virus particles, accounting at least in part for lower wt virus yields (data not shown). Packaging efficiency of accumulated genomes may thus be a critical step in determining overall yields of infectious VSV in different cell types.

Antigenomes are much less abundant than genomes and mRNA transcripts in VSV-infected cells, but antigenomes were nonetheless detectable with the P transcript riboprobe

(Fig. 2A, B, and C, right panels). Although quantitation of these bands, which were more readily visible on longer exposures, was less precise because of low signal intensities, antigenome accumulation appeared to be inhibited to roughly the same extent as the genome at 8 and 12 h p.i. for L-929 cells and at 12 h p.i. for 3Y1 cells. Note that polR genome inhibition was clearly less severe at early times in L-929 cells (Fig. 2B, 4 h) and even more so for antigenomes (data not shown). Nonetheless, several independent experiments showed that antigenome inhibition always approached that of genomes at time points beyond 8 to 9 h in L-929 cells. These results suggest that replication inhibition is less prominent early in infection, especially for antigenomes.

Since the transcription analysis in Fig. 2 measured changes in the P gene mRNA only, it was important to determine whether other virus transcripts behaved similarly and whether polR infections produced abnormal transcripts. For this purpose, we employed a nick-translated plasmid probe containing the full-length VSV genome. Both wt- and polR-infected BHK and L-929 cells generated a very similar array of transcripts that included all VSV mRNAs (data not shown). Importantly, no heterogeneous size leader-N gene read-through transcripts migrating below the M/P band were evident, even though these are readily observable when transcription is carried out with the polR virion-associated polymerase *in vitro* (9). Quantitation of individual bands revealed a small increase (30 to 35%) in the ratio of L and M/P transcripts relative to N in polR- versus wt-infected cells. The significance of this minor change in transcript ratios is unclear, but it may be a result of altered termination properties of the viral polymerase evident *in vitro*. In any case, we conclude from these results that polR restriction does not involve significant changes in viral mRNA transcription despite a substantial decrease in genome replication.

polR virus restriction does not block genome or antigenome encapsidation. Since the replication product analysis used above measured encapsidated products only, we next considered the possibility that the polR N protein mutation might cause a cell-type-specific defect in encapsidation. To test this, we again measured accumulation of replication products in L-929 cells but, in this case, both before and after micrococcal nuclease treatment of cytoplasmic extracts (Fig. 2D). A lower recovery of either genome or antigenome RNAs from samples pretreated with nuclease would thus indicate a lack of encapsidation. To facilitate comparison, the ratio of the Northern blot signals (treated to untreated) obtained for wt virus at 14 h p.i. was arbitrarily set to represent 100% encapsidation. Note that, although antigenomes are present in much smaller amounts than genomes in infected cells, probe-specific activities and exposure time were adjusted to provide comparable signal intensities. Genomes from either wt- or polR-infected cells showed no significant difference in encapsidation at all times p.i. (Fig. 2D, left panels). Likewise, polR antigenomes also displayed no consistent difference from their wt counterpart, although the encapsidation values obtained here varied somewhat more, which we attribute to higher background to signal ratios (Fig. 2D, right panels). Similar results using a slightly different method were obtained at 9 and 12 h p.i. (not shown). As in Fig. 2B, polR antigenome accumulation was inhibited to the same extent as that of the genome, confirming that the defect in replication applies equally to both strands.

FIG. 3. (A) Autoradiograph of SDS-PAGE analysis of [35 S]methionine-labeled proteins from wt- and polR-infected BHK and L-929 cells. Cells were pulse-labeled for 30 min at 3.5 h p.i. (BHK) or 7.5 h p.i. (L-929), followed by a 0.5-h or 4-h chase. (B) The infected cell samples from panel A were analyzed on a second SDS-PAGE gel by Western blotting with a polyclonal antibody to P protein and a monoclonal antibody to G protein.

No evidence for accumulation of smaller size replication products in either nuclease-treated or untreated samples was found (data not shown). These results thus provide strong evidence that polR restriction does not involve an encapsidation defect.

polR virus restriction is not due to a block in overall virus protein synthesis or failure to shut off host cell protein synthesis. We next probed for possible defects in virus protein expression in polR virus-infected cells. wt- and polR-infected cells were pulse-labeled with [35 S]methionine for 30 min at 3.5 h (BHK cells) or 7.5 h (L-929 cells) p.i., followed either by a 30-min chase or a 4-h chase in cold medium and analysis by SDS-PAGE (Fig. 3A). Bands corresponding to all five VSV proteins (L, G, N, P, and M) were detected following the 30-min chase with wt and polR virus in both cell types (Fig. 3A, lanes 2, 3, 8, and 9). polR virus, however, accumulated two- to fourfold less labeled virus protein than wt virus in both BHK and L-929 cells. The ratio of viral proteins was, however, similar for both viruses. The rate of viral protein synthesis was thus somewhat reduced in polR-infected cells compared to wt, at least at the time points examined, but this deficit is clearly not linked to restriction. L, G, and M viral protein band intensities of wt virus in BHK cells clearly decreased after a 4-h chase (Fig. 3A, lane 5 versus 2), presumably due to incorporation of labeled proteins into released virus particles. A similar loss of labeled L, G, and M proteins may also have occurred for polR in BHK cells to some extent, but this was not evident because of low band intensity and high background (Fig. 3A, lane 6

versus 3). No decrease of the more readily visible N and P proteins was observed for either virus in BHK cells following the chase, presumably because these proteins are synthesized in large excesses. Very little change in band intensity of any wt or polR viral protein was evident following the chase in L-929 cells (lanes 11 versus 8 and 12 versus 9), which we attribute to a substantially lower release of viral particles. Importantly, shutoff of host cell protein synthesis, as judged from mock-infected samples (lanes 1, 4, 7, and 10) versus virus-infected samples, was more prominent in L-929 than in BHK cells, but polR-induced shutoff was comparable to that of the wt in either cell type.

To confirm and extend the pulse-labeling results, the [35 S]methionine-labeled extracts obtained after both chase periods were probed by Western blotting with a polyclonal antibody to the VSV P protein and a monoclonal antibody directed to the C terminus of the VSV G protein (Fig. 3B). In BHK cells, polR accumulated ~ 2 -fold less P protein than the wt at 4 h but almost the same amount as the wt at 7.5 h p.i. G protein in polR-infected BHK cells accumulated to $\sim 40\%$ of the level seen with the wt at 7.5 h, but little or none was present at 4 h. P protein accumulation in polR-infected L-929 cells was nearly equal to that of the wt at 8 h p.i. but ~ 3 -fold lower at 11.5 h p.i. Unexpectedly, G protein accumulation in polR-infected L-929 cells was considerably less than the wt (~ 20 -fold) at either 8 or 11.5 h p.i., despite the presence of [35 S]methionine-labeled G protein bands in the same samples (Fig. 3A). The absence of antibody-reactive G protein in polR-infected L-929 but not BHK cells was confirmed in independent experiments. The reason for this remains unclear, but these findings suggest that polR virus infection in restricted cells somehow causes a defect or delay in G protein processing that impairs reactivity to the C-terminal antibody. Note also that G protein accumulated as a single band in L-929 cells and as a doublet band in BHK cells, which could also reflect host cell differences in G processing.

The above results indicate that polR restriction is not due to an overall deficit in viral protein accumulation or to a defect in host cell protein shutoff. Although polR showed a modest two- to threefold reduction in accumulated viral protein, this took place in both BHK and L-929 cells. Moreover, earlier studies have shown that VSV proteins accumulate in excess in BHK cells relative to the amount released in virions (34) and must therefore accumulate in even greater excess in L-929 cells, since the latter release even fewer virus particles. However, since polR mRNA accumulation was comparable to that of the wt in L-929 cells and somewhat higher in BHK cells (Fig. 2), translation of these mRNAs likely takes place somewhat less efficiently than the wt in both types of host cells.

polR virus infection leads to enhanced eIF-2 α phosphorylation in L-929 cells. As noted above, polR mRNAs appear to be translated less efficiently than their wt counterparts in infected cells. One possible explanation for this deficiency is down regulation of protein synthesis, which commonly occurs as a result of eIF-2 α phosphorylation by the host cell dsRNA-activated protein kinase (PKR). Phosphorylated eIF-2 α sequesters eIF-2B, which prevents further initiation of protein synthesis. PKR activation is known to play a major role in type I IFN-mediated inhibition of VSV multiplication (3, 50). The Western blot results in Fig. 4 do in fact show that polR infection causes more extensive eIF-2 α phosphorylation than the wt

FIG. 4. Effects of wt and polR infection on host cell eIF-2 α phosphorylation. Cytoplasmic extracts from infected BHK (A) and L-929 (B) cells were analyzed by Western blotting. Duplicate blots were used for probing total eIF-2 α versus eIF-2 α phosphorylated on Ser51 (eIF-2 α -P). A nonspecific band labeled X also reacted with anti-eIF-2 α and served as a control for loading. A third blot from the same L-929 extracts was used for probing VSV P and M proteins. A nonspecific band just above P protein is also visible. UN, uninfected.

in L-929 cells. Cytoplasmic extracts from both BHK cells (Fig. 4A) and L-929 cells (Fig. 4B) contained roughly comparable amounts of total eIF-2 α , which remained unchanged following virus infection. BHK cells showed no evidence of virus-induced eIF-2 α phosphorylation with either wt or polR even at 9 h p.i. (Fig. 4A). These results differ only slightly from recently published work reporting that eIF-2 α phosphorylation does not occur until 8 h p.i. of BHK cells with wt VSV (12). In L-929 cells, however, eIF-2 α phosphorylation was evident starting at 8 h p.i., but notably, this phosphorylation was nearly threefold higher for polR than the wt at both at 8 h and 12 h p.i. and subsequently decreased to near wt levels at 24 h p.i. (Fig. 4B). We infer from these results that polR virus most likely activates cellular PKR more effectively than the wt in L-929 cells.

To examine whether the timing of PKR activation might be responsible for reduced viral protein accumulation in polR-infected L-929 cells, we probed the same L-929 cell extracts for the presence of viral P and M proteins (Fig. 4B, lower panels). As before, polR-infected cells accumulated somewhat less protein than the wt by 8 h p.i. (~ 2 -fold less), but very little further increase, if any, took place beyond this time for either wt or polR. Clearly, most of the viral protein had already accumulated by the time substantial eIF-2 α phosphorylation occurred. Although PKR activation might conceivably be responsible for the modest decrease in polR virus protein accumulation in L-929 cells, this clearly cannot be the cause for the similar reduction occurring in BHK cells (Fig. 3). polR virus restriction is therefore not due to a PKR-mediated effect on viral

FIG. 5. Effect of 10 mM 2-AP and 5 μ g/ml act D on wt and polR growth in BHK, L-929, and 3Y1 cells. The inhibitors were added at the start of virus adsorption and maintained throughout the course of infection. Titers were determined ~24 h p.i. for BHK cells and ~30 h p.i. for L-929 and 3Y1 cells. +, act D present; -, act D absent.

protein translation. These results, however, do not rule out that PKR activation is involved in polR restriction independently of eIF-2 α phosphorylation and its effects on translation.

polR growth restriction does not require PKR activation. To test the possibility that PKR activation might nonetheless play a role in polR restriction, we made use of a well-established inhibitor of PKR activity, 2-AP. This ATP analog has been used extensively in the past to probe the role of PKR in signaling in a number of host cells, including L-929 (24, 31, 56). Figure 5 shows the effects of 10 mM 2-AP on infectious virus yields from wt- and polR-infected BHK, L-929, and 3Y1 cells. wt virus growth was only slightly inhibited by 2-AP in BHK and L-929 cells (2-fold or less), but 3Y1 cells appeared to be somewhat more sensitive to this inhibitor, showing an ~5-fold reduction in wt titer (Fig. 5A). polR yields were reduced ~3-fold by 2-AP in BHK and 3Y1 cells but increased ~6-fold in L-929 cells (4 PFU to 23 PFU/cell). This increase in yield in L-929 cells, however, was still well below wt yields in the presence of 2-AP (111 PFU/cell). No such increase in polR yields from L-929 cells was observed with 5 mM 2-AP (not shown). Higher concentrations of 2-AP (30 mM) blocked virus growth almost completely in all three cell types (data not shown). Although these results suggest that PKR activation might play a minor role in polR restriction in L-929 cells, this is clearly not the case for 3Y1 cells.

polR growth restriction does not depend on induction of cellular gene transcription. Even though single-cycle infections with wt VSV Indiana produce very little type I IFN because of the accompanying shutdown of host cell functions (2), transcriptional activation of the IFN- β gene and other primary response antiviral cellular genes nonetheless takes place in at least some cell types (51). To test whether polR restriction required host gene induction, we determined the effects of act D on wt and polR virus yields in BHK, L-929, and 3Y1 cells. Previous studies have shown that as little as 0.5 μ g/ml act D leads to a 99% reduction in total cellular synthesis in L-929 cells (23). To ensure complete shutdown of host gene transcription, we employed a 10-fold-higher dose of act D (5 μ g/ml). As expected, wt virus yields in all three cell types were

FIG. 6. Effect of conditioned media on wt and polR growth in BHK, L-929, and 3Y1 cells. Conditioned media were obtained from uninfected cells 48 h postplating. Freshly plated cells were exposed for 16 h to conditioned media of each cell type before virus infection as before. Titers were determined at ~30 h p.i.

only minimally affected by act D (Fig. 5B). More importantly, polR virus yields were likewise slightly reduced in the presence of this inhibitor. These results clearly show that host gene induction is not required for polR growth restriction.

polR restriction is not due to constitutive release of antiviral factors from restricted host cells. Lastly, we tested the possibility that restricted cells constitutively release antiviral factors that show preferential activity against polR VSV. As a source of putative released factors, we collected conditioned media from BHK, L-929, and 3Y1 cells 48 h following plating. We then exposed freshly plated cells to each of these conditioned media for ~16 h, at which time cells were infected with wt or polR virus in the continued presence of the conditioned media (Fig. 6). wt virus yields from BHK cells exposed to any of the conditioned were minimally affected (≤ 2 -fold), while wt yields from L-929 and 3Y1 cells were slightly reduced (3- to 4-fold) (Fig. 6A). polR yields from BHK cells decreased ~6-fold in the presence of conditioned medium from L-929 cells but were not significantly affected by conditioned media from either BHK or 3Y1 cells (Fig. 6B). None of the conditioned media showed significant effects on polR yields from L-929 and 3Y1 cells. We conclude from these results that constitutive release of antiviral factors does not play a major role in polR restriction, since conditioned medium from 3Y1 cells had no greater effect than that from BHK cells on wt or polR yields in any of the cells. L-929 conditioned medium had a moderate inhibitory effect on polR growth in BHK cells but also showed a similar effect on wt virus growth in L-929 and 3Y1 cells. It is worth noting here that L-929 cell conditioned medium is known to contain macrophage colony-stimulating factor (49), but whether this could conceivably be responsible for the effects seen here is unclear.

DISCUSSION

Virus host range mutants clearly provide a useful tool for understanding virus-host cell interactions. Our earlier characterizations of the VSV polR viral RNA synthesis alterations revealed telling aspects of viral polymerase regulation but left unanswered the relationship between these changes and the mutant virus host range phenotype. Our aim here was to shed

light on the causes of polR VSV growth restriction. We first established beyond doubt that the single amino acid substitution responsible for the altered polR RNA synthesis phenotype (N protein Arg179 to His) is also the cause of the host range defect. The mutation was engineered into a recombinant virus, and this virus displayed growth properties essentially identical to the previously characterized polR1 and polR2 mutant viruses, yielding only slightly less infectious virus in BHK cells than the wt (~ 3 -fold) and at least 100-fold less in mouse L-929 cells. We tested several additional cell lines for recombinant polR virus growth and found two, rat 3Y1 fibroblasts and mouse BALB/3T3 cells that also showed strong restriction (~ 40 -fold). Even though these restricted hosts are all of rodent origin, our previous findings showed that polR restriction is not species specific. For example, Vero cells but not CV1 cells display modest (5- to 10-fold) polR growth restriction (9), even though both cell types are derived from African green monkeys.

VSV host range mutants have been described previously (22, 29, 35, 47), but none show a phenotype similar to polR. Only a few such mutants have been characterized extensively. The VSV *hr1* and *hr8* mutants are restricted in a number of cell lines, especially of human origin, and show a defect in viral mRNA cap methylation catalyzed by the L polymerase protein (18, 22, 47). VSV *tdCE* mutants are restricted in chicken embryo cells at high temperature but not in BHK cells. Mutations affecting L protein conformation are thought to be responsible for the *tdCE* host range (35). While rare, wt VSV also shows growth restriction in established cell lines such as the human B-lymphoblastoid Raji cells (34) and the RC-60 rabbit corneal cells (19). These wt virus restrictions involve late steps in viral multiplication, and the mechanisms responsible remain unknown. wt VSV New Jersey serotype was also reported to be restricted in rat 3Y1 cells at the level of transcription early in infection (43), but we found no such restriction for the wt VSV Indiana strain used in our studies.

We demonstrated here that VSV polR restriction does not involve early virus functions. polR genome transcription was comparable to that of the wt in L-929 and 3Y1 cells and was even somewhat enhanced in the permissive BHK cells. Transcript ratios were similar to wt virus. polR genome and antigenome replication on the other hand were reduced 6- to 10-fold in the restricted hosts. This specific effect on replication bears some resemblance to the earlier report of wt VSV restriction in RC-60 cells (19). In our hands, however, wt VSV restriction in RC-60 cells was minimal and inconsistent (unpublished). Interestingly, some temperature-sensitive mutants of VSV isolated from persistent infections display the converse phenotype, i.e., reduced mRNA synthesis while maintaining high levels of genome synthesis (17). Together, these phenotypes indicate that VSV transcription and replication are not necessarily coupled to each other in infected cells and that either process can be substantially inhibited without affecting the other.

VSV genome replication is normally tightly coupled to encapsidation, and replication products accumulate only as RNP structures. VSV polR deviates from this rule because, in addition to mRNAs, it produces large amounts of unencapsidated, leader-N gene read-through transcripts in vitro. We found no evidence here for substantial accumulation of these

heterogeneous size transcripts (~ 300 to 500 nt) in polR-infected permissive or nonpermissive cells. However, these aberrant transcripts are detectable in vivo when using an end-labeled genome probe (unpublished). It is not clear why these accumulate less readily in vivo than in vitro for polR VSV. In the case of the paramyxovirus Sendai Z strain, accumulation of these transcripts in infected cells is readily observed (52).

polR VSV also synthesizes twofold less leader RNA than N mRNA in vitro in contrast to wt, which generates equal amounts (10). This finding provided the first compelling evidence that the VSV transcription process can initiate internally at the N gene. We could not address here whether polR also synthesizes more leader RNA than wt in infected cells, as this small RNA is degraded rapidly in the cytoplasm. Likewise, the changes in ATP concentration requirements displayed for polR transcription in vitro could not easily be addressed in infected cells. Nonetheless, it is somewhat surprising to find that the prominent alterations in polR polymerase behavior observed in vitro resulted in relatively little change in overall virus RNA synthesis in the permissive BHK host cell.

Specific inhibition of polR genome replication in restricted cells could well have resulted from a deficit in viral protein synthesis, as VSV genome replication depends on a source of newly synthesized N protein for encapsidation of progeny templates. But polR viral protein accumulation was reduced only two- to threefold compared to wt, and this moderate impairment occurred in both L-929 and BHK cells, thus ruling out this phenomenon as an explanation for the block in replication. It also seemed possible that the N protein mutation somehow disturbed encapsidation of progeny in a cell-type-specific manner, but nuclease protection assay of polR intracellular genome and antigenome replication products in L-929 cells showed no difference from wt. The polR N protein thus appears to function normally in restricted cells as far as transcription and assembly of functional templates is concerned.

The substantial block in polR genome replication in restricted cells cannot on its own explain the 40- to 100-fold reduction in infectious virus production. Virus particle assembly and release appeared to be unaffected, since the amounts of wt and polR virus particles released from restricted and nonrestricted host cells correlated roughly with their respective levels of intracellular genome accumulation (6- to 10-fold less than the wt in the case of polR in restricted cells). polR virus particles released from restricted cells, however, were 5- to 10-fold less infectious than wt virus particles released from the same cells. The replication deficit, in concert with the reduced particle infectivity, thus accounted for essentially all of the observed restriction in overall polR PFU yields. Interestingly, low doses of IFN also led to release of VSV particles of low infectivity from L-929 cells, and this defect was attributed to a deficiency in G and M proteins (14, 30). In the polR case here, however, virus particles released from restricted cells showed no obvious protein composition differences from the wt. A similar loss of particle infectivity with no apparent change in protein composition was also reported in the case of wt VSV restriction in Raji cells (34). What causes the loss of virus particle infectivity in either instance remains unclear and deserves further study. Curiously, G protein accumulating in polR- but not wt-infected L-929 cells failed to react with the C terminus-specific G antibody despite normal reactivity in re-

leased virions. Perhaps subtle differences in G protein processing between restricted and nonrestricted host cells are somehow responsible for reduced polR particle infectivity, but why this would occur only with G protein produced in polR-infected restricted cells is difficult to imagine.

Current evidence suggests that most if not all virus infections generate small amounts of dsRNA as a by-product of multiplication, which can potentially lead to cellular PKR activation and eIF-2 α phosphorylation (42). This phosphorylation down regulates protein translation by functionally sequestering the GTP exchange factor eIF-2B required for maintaining eIF-2 α activity. This mechanism is in fact thought to be responsible for blocking VSV multiplication following upregulation of PKR levels by type I IFN signaling (3, 15, 50). We showed here that eIF-2 α phosphorylation is enhanced about threefold in polR-infected L-929 cells relative to the wt, although this phosphorylation occurred too late in infection (~8 h p.i.) to significantly reduce viral protein accumulation. No eIF-2 α phosphorylation was evident in wt- or polR-infected BHK cells even at 9 h p.i., despite the presence of comparable amounts of total eIF-2 α . The latter result is in general agreement with a recent study showing a similar lack of eIF-2 α phosphorylation in wt VSV-infected BHK cells until very late in infection (12). Since shutdown of host mRNA translation observed here in L-929 cells was essentially complete by 8 h p.i. for both wt and polR, the virus-induced shutoff in this host must occur through a mechanism other than eIF-2 α phosphorylation, as postulated before for BHK cells (12).

Enhanced eIF-2 α phosphorylation in polR-infected L-929 cells, even if not responsible for polR restriction, suggests that the mutant virus produces more intracellular dsRNA, or a highly structured RNA capable of activating PKR, than wt virus. In addition to modifying eIF-2 α , PKR also plays a role in several cellular signaling pathways (4, 55). Our results here, however, show that inhibiting PKR activity with 2-AP had no effect on polR restriction in 3Y1 cells and stimulated polR yields only slightly in L-929 cells. PKR activation is therefore not required for restriction. The only other well-established antiviral effector protein directly activated by dsRNA, namely 2', 5'-oligoadenylate synthetase (42), is also clearly not required for polR restriction, since activation in this case would lead to viral mRNA degradation via RNase L.

Production of dsRNA during viral infection also serves as the immediate trigger of a host cell antiviral transcriptional response by binding either to the membrane-associated Toll-like receptor 3 and/or to cytoplasmic RNA helicases, two of which have so far been identified, retinoic acid-inducible gene I protein and melanocyte differentiation-associated gene 5 protein (reviewed in references 25 and 46). Our findings here, however, rule out a requirement for host gene induction in polR restriction, since neither wt nor polR virus yields were significantly affected by high concentrations of act D. These results argue very strongly against the possibility that polR restriction is due to an enhanced host antiviral transcriptional response, which then functions in an autocrine manner to block virus growth. As noted above, polR VSV was as effective as the wt in shutting down host protein synthesis in L-929 cells, making translation of induced type I IFN mRNA or other signaling molecule mRNA unlikely. Moreover, type I IFN induction in L-929 cells is inhibited by 2-AP (13, 31).

Lastly, even though L-929 cells do not constitutively produce

type I IFN (26), we entertained the possibility that restricted cells might nonetheless constitutively release very small amounts of these cytokines or some unknown antiviral factor, which somehow restricts polR but not wt virus growth. Conditioned media from restricted cells, however, showed no evidence of secreted antiviral factors acting preferentially on polR virus. Moreover, addition of type I IFN antibodies to the medium of L-929 cells had no effect on polR restriction (data not shown). This evidence against type I IFN involvement is also consistent with the lack of restriction in IFN-competent cells such as HeLa and A549 and moderate restriction in IFN-defective Vero cells (9). In addition, wt and polR virus showed inhibitory dose response curves identical to those of type I IFN pretreatment in HeLa cells (data not shown). Taken together, these observations make a compelling case against a role for a classical type I IFN response in polR restriction.

What then is responsible for the specific block in polR viral genome replication and particle infectivity in restricted cells? As mentioned above, the mutated polR N protein appears to function normally in restricted cells. However, it is conceivable that the viral N protein must interact with a host cofactor for replication but not transcription, perhaps as part of the recently proposed VSV replicase complex (37). The mutated polR N protein could then be viewed as interacting effectively with the host cofactor from permissive cells but not that found in restricted cells. However, the existence of polR revertant viruses that retain the N protein mutation while losing the restricted growth phenotype (9) is difficult to reconcile with this hypothesis. Furthermore, it is not clear why a defective interaction of polR N protein with a host factor would lead to a decrease in particle infectivity. Alternatively, the enhanced eIF-2 α phosphorylation observed in polR-infected L-929 cells suggests increased dsRNA production and an explanation based on a cell-type-specific antiviral response. We therefore hypothesize that polR activates a constitutive antiviral effector(s) pathway that responds to enhanced dsRNA production and does not require de novo transcription of cellular genes. The accompanying paper (31a) establishes without a doubt that enhanced dsRNA production in polR infections is solely responsible for triggering this unconventional cellular antiviral response.

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