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Polioviruses containing picornavirus type 1 and/or type 2 internal ribosomal entry site elements: Genetic hybrids and the expression of a foreign gene

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ABSTRACT **A picornavirus hybrid genome was constructed in which the internal ribosomal entry site (IRES) of encephalomyocarditis virus was inserted between the 5′ nontranslated region and the open reading frame of poliovirus (PV), type 1 (Mahoney). Upon transfection into HeLa cells, the hybrid RNA replicated and yielded a derivative of PV (W1-PNENPO). The PV IRES could be removed from pPNENPO, which resulted in a hybrid picornavirus (W1-P108ENPO) in which the translation of the PV open reading frame normally promoted by the type 1 IRES of PV was promoted by the type 2 IRES of encephalomyocarditis virus. This result indicates that these elements are not likely to contain cis-acting elements necessary for PV replication or encapsidation. A foreign gene (bacterial chloramphenicol acetyltransferase, CAT) was inserted into pPNENPO cDNA between the PV and encephalomyocarditis virus IRES elements. The dicistronic RNA replicated in HeLa cells and yielded a derivative of PV (W1-DICAT) with a genome 17% longer than that of wild-type PV. CAT assays and immunoblot analyses showed that the viral RNA efficiently expressed the foreign gene in cell culture. The CAT activity diminished somewhat with each passage of the dicistronic virus, an observation which suggested that the inserted gene had a deleterious effect on viral replication. However, even after five virus passages, a significant quantity of the foreign gene was still expressed. Insertion of the open reading frame of luciferase (67 kDa) resulted in an RNA species that replicated and expressed luciferase for up to 20 hr after transfection. However, this elongated RNA was not encapsidated.**

Picornaviruses, of which poliovirus (PV) is the prototype, are small, nonenveloped viruses containing RNA genomes of plus-strand polarity (1, 2). The genetic organization of PV stands out through its simplicity (Fig. 1*A*). The protein-linked 5′ nontranslated region (NTR) is followed by a single open reading frame (ORF) that encodes a polyprotein, which is followed by a relatively short 3′ NTR and a poly(A) tail (1). The site of initiation of translation, mapped originally by sequence analyses, revealed a very long 5′ NTR harboring the internal ribosomal entry site (IRES) element common to all picornaviruses. The IRES elements, which are located downstream of the uncapped 5′ termini of the viral RNAs (3) confer both cap- and 5′-independent translation to the picornaviral ORFs (4–7). The insertion of the IRES of encephalomyocarditis virus (EMCV; a virus belonging to the genus *Cardiovirus* of *Picornaviridae*) into the single ORF of poliovirus RNA (Fig. 1*A*) between the regions encoding P1 and P2, created a viable, dicistronic poliovirus (8). This finding provided the final proof for the function of these genetic elements in an unmodified form *in vivo*.

On the basis of their nucleotide sequences and proposed secondary structures, picornavirus IRES elements have been divided into two types. Type 1 belongs to the genera *Entero-* and *Rhinovirus*, and type 2 belongs to the genera *Cardio-*, *Aphthovirus*, and, possibly, *Hepatovirus* (9–11). Indeed, except for a common Y_n-X_m-AUG motif, these elements have no sequence homology, nor do they appear to share any structural similarities (see Fig. 1*B*); yet, surprisingly, they function in an identical fashion (3). In this study, we report on the construction of a viable PV (PV; W1-PNENPO), in which the type 1 IRES of PV is immediately followed by the type 2 IRES of EMCV, lengthening the 5′ NTR of the virion RNA from 743 (wild-type virus) to 1233 nt (Fig. 1*B*). In addition, we report on the construction of a viable, hybrid picornavirus (W1-108ENPO), in which the PV IRES was replaced in its entirety by the type 2 IRES of EMCV (Fig. 1*C*). Insertion of a foreign gene (chloramphenicol acetyltransferase, CAT) between the IRES elements of the pPNENPO RNA yielded a viable, dicistronic PV (W1-DICAT) with a significantly enlarged genome (Fig. 1*D*). Insertion of the ORF of luciferase, however, produced a RNA (p-DILUC) genome that replicated but was too large to be encapsidated.

MATERIALS AND METHODS

Plasmid Construction. All DNA manipulations were accomplished by using standardized procedures (12).

pT7PVXL2, a plasmid containing full-length PV1 cDNA, (Fig. 1*A*) was digested with *Kpn* I and *Msc* I restriction enzymes. A DNA fragment was synthesized by PCR encompassing bases 747–2523 of PV with primers 5′-GTGCTCAGGTTTCATCAC-3′ and 5′-GCGCGAGCTCCGGACTCTGTTGTCA-3′. The vector pBS-ECAT (7) was cleaved with *Eco*RI and *Msc* I. pSK+ (Stratagene), after cleavage with *Eco*RI and *Sac* I, was ligated to the EMCV 5′ NTR and PCR fragments to form pSKEP1.

pXL-3452 was cut with *Spe* 1, digested with T4 DNA polymerase, and cleaved with *Eco*RI. pSKEP1 was digested with *Sac* I, filled with Klenow fragment, cut with *Eco*RI, and ligated to the pXL-3452 fragment to create pXLE-1482. pT7PVXL2 and pXLE-1482 were digested with *Nhe* I and *Pvu* II and ligated together to produce pPNENPO+R.

pPNENPO+R was partially digested with *Eco*RI and filled with Klenow fragment. The expression vector that resulted, termed pPNENPO (Fig. 1*B*), contained a unique *Eco*RI site located at the very 5′ end of EMCV IRES.

The plasmid pPN6 (provided by Raul Andino, Department of Microbiology and Immunology, University of California, San Francisco) and pPNENPO were digested with *Eco*RI and

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Abbreviations: IRES, internal ribosomal entry site; EMCV, encephalomyocarditis virus; ORF, open reading frame; CAT, chloramphenicol acetyltransferase; NTR, nontranslated region; PV, poliovirus; RT, reverse transcriptase.

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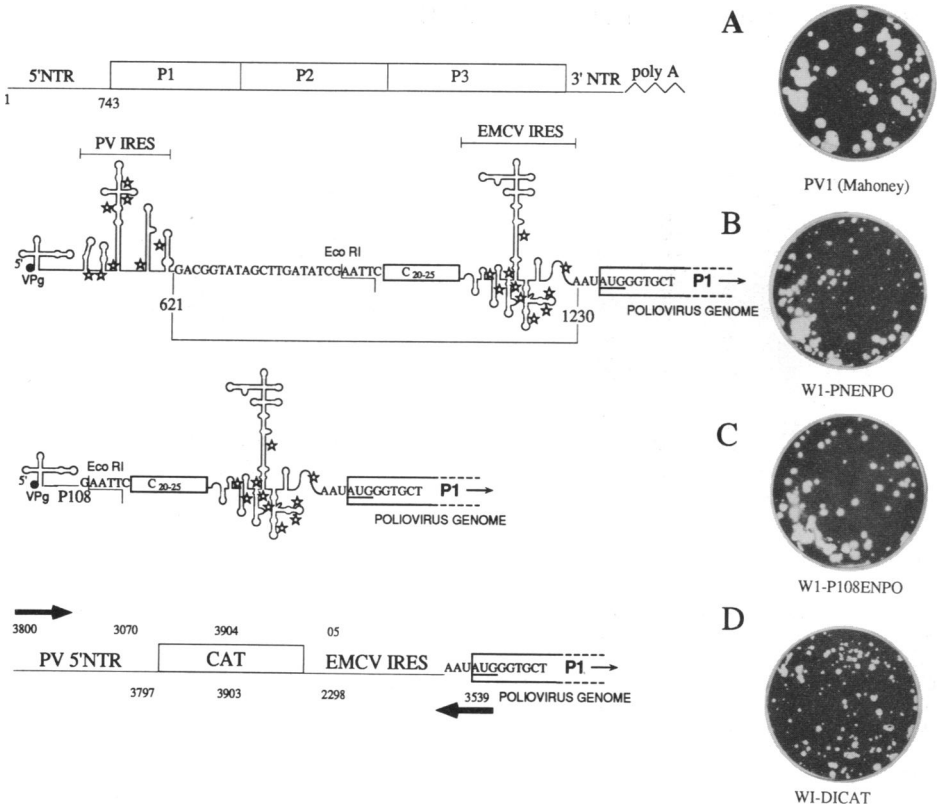

FIG. 1. Plasmid diagrams and plaque assays. (*A*) pT7PVXL2, a plasmid encoding the full-length PV1 cDNA. The open box depicts the polyprotein ORF, starting at nt 743, that is divided into domains P1, P2, and P3. (*B*) pPNENPO, showing only the 5′ NTR sequences. Stem-loop structures represent IRES elements, as indicated (structures not drawn to real relative size). Stars indicate positions of AUG triplets; the initiating AUG is underlined. The bracket between nt 621 and nt 1230 indicates the deletion in W1-PNENPO-d1. (*C*) pP108ENPO. (*D*) pDICAT. Solid arrows indicate polarity of primers used for RT/PCR, which are identified with the numbers above and below drawing. Plaques were produced by the viruses that originated from transcript RNAs of the corresponding plasmids.

/ *Pvu* II. The isolated fragments were ligated together to create pP108ENPO (Fig. 1*C*).

The chloramphenicol acetyltransferase (CAT) gene was removed from pBS-ECAT by digestion with *Bst* XI and *Ban* I, followed by Klenow treatment. The gene was then ligated into the Klenow-treated *Eco*RI site of pPNENPO, which created the expression vector pDICAT (Fig. 1*D*). The luciferase gene obtained from Peter Sarnow (Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Science Center, Denver), was digested with *Msc* I and *Nco* I, treated with Klenow fragment, and engineered into the Klenow-filled *Eco*RI site of pPNENPO, which formed plasmid pDILUC.

RESULTS

The mechanism by which highly structured IRES elements function in promoting translational initiation remains obscure. We were interested in learning whether tandemly arranged IRES elements would be disfunctional either in translation initiation or RNA replication. In addition, we wanted to determine whether a type 1 IRES element could be replaced by an entirely heterologous type 2 element. We therefore constructed first a PV with two tandemly arranged IRES elements (Fig. 1*B*) from which we subsequently deleted the PV-specific IRES element (Fig. 1*C*).

The initiation of translation of the EMCV polyprotein commences immediately at the 3′ border of its IRES (13), whereas a "spacer" region of 154 nt (3) separates the 3′ border of the PV IRES from its initiation codon. This spacer can be deleted without conferring any apparent phenotype to the mutant virus in cell culture (14). In constructing pPNENPO (Fig. 1*B*) we have partially deleted the PV spacer, fusing the 630 5′-terminal nt to a segment of the EMCV 5′ NTR (nt 260–840) containing the IRES element (6, 13). The EMCV IRES was inserted in such a way that the initiation codons of the PV and EMCV polyproteins were indentical, which presumably allowed for the proper myristoylation of VP4 (15, 16).

pPNENPO plasmid DNA was transcribed *in vitro* (17), and the product was translated in a HeLa cell-free extract (18). We obtained a wild-type translation pattern (data not shown), which indicated that the EMCV IRES element promoted the normal initiation of protein synthesis followed by the normal proteolysis of the viral polyprotein (19, 20). HeLa cell monolayers, transfected with pPNENPO RNA, displayed complete cytopathic effects 30 hr after transfection at 37°C, a delay of 10 hr in comparison with transfections with wild-type PV RNA. The progression of cytopathic effects suggested that pPNENPO RNA had replicated and spread through the cell culture by the formation of infectious viral particles (W1-PNENPO), a conclusion confirmed by subsequent plaque assay (Fig. 1*B*).

Plaques from the initial passage of W1-PNENPO were small, although plaque-size heterogeneity was apparent (Fig. 1*B*). A one-step growth experiment showed that W1-PNENPO grew to somewhat lower titers than did wild-type PV (see Fig. 3*A*). On fifth passage, the size of most W1-PNENPO plaques were only slightly smaller (data not shown) than that of wild-type PV, an observation suggesting that deletion(s) within the elongated 5′ NTR had occurred. This result would be expected in view of the genetic plasticity of the PV genome, provided that the tandemly arranged IRES elements significantly impaired viral proliferation (21).

The genotypes of W1-PNENPO progeny were analyzed by reverse transcription (RT) PCR with primers depicted in Fig. 1*D*. After its initial passage, W1-PNENPO RNA contained both PV IRES- and EMCV IRES-specific signals (Fig. 2*A*, lanes 4 and 9, respectively), as expected, although a deletion was apparent when the analysis was done over the entire 5′ NTR (Fig. 2*A*, compare lanes 13 and 14). W-1PNENPO RNA from the fifth passage of the virus, however, no longer contained a detectable EMCV IRES-specific signal (Fig. 2*A*, lane 10). A major band was generated with primers 3800 and 3539 (Fig. 2*A*, lane 15) that, when sequenced, revealed a

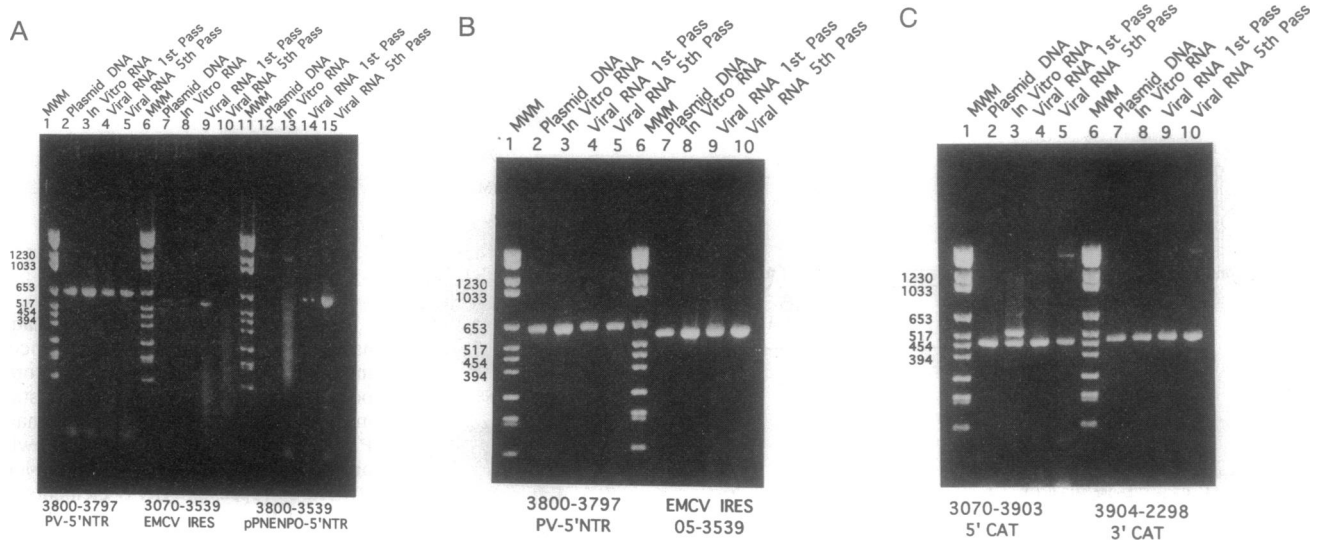

FIG. 2. RT/PCR analysis of W1-PNENPO (*A*) and W1-DICAT viral RNAs (*B* and *C*) isolated from infected cells. The RT reactions with avian myeloblastosis RT (Promega) were incubated at 55°C for 1 hr with 5% dimethyl sulfoxide that used either viral RNA (first and fifth passage) or *in vitro* transcribed RNA as templates. These cDNAs and the corresponding plasmid DNAs were used as templates for PCR reactions with *Taq* polymerase (Boehringer Mannheim). The oligonucleotides were allowed to anneal for 30 sec at 60°C. The oligonucleotides used were as follows: 3800 (5'-TTAAAACAGCTCTGGGGTTGTACCCACCCCAG-3'), 3539 (5'-GCGCCCACTTTCTGTGATGAAACC-3'), 3070 (5'-TGGCTGCTTATGGTGAC-3'), 3797 (5'-CCAATTCGCTTTATGATAACAATCTGTG-3'), 2298 (5'-GACAAACGCACACCGGCC-3'), 05 (5'-GGCCGGTGTGCGTTTGTC-3'), 3904 (5'-CCACGACGATTTCCGGCAG-3'), and 3903 (5'-CTGCCGAAATCGTCGTGG-3'). The extra band in *C*, lane 3, has not been identified. MWM, molecular weight markers.

deletion in the viral RNA from nt 621 to 1230 (see the bracket in Fig. 1*B*). This result showed that the variant virus (W1-PNENPO-d1) had lost the entire heterologous IRES element. The alternative product, a PV that contained the EMCV IRES alone, but lacked the PV IRES, was not detected.

To determine whether a type 2 IRES itself was at all compatible with PV proliferation, a PV genome was constructed in which the entire PV IRES was replaced by the EMCV IRES (pP108ENPO; Fig. 1*C*). *In vitro* translation of pP108ENPO RNA yielded a wild-type translation and processing pattern, as expected (data not shown). Transfection of HeLa cells with pP108ENPO RNA produced complete cytopathic effect after 26 hr, and subsequent plaque assays revealed a medium plaque phenotype (Fig. 1*C*). A one-step growth experiment indicated that W1-P108ENPO replicated with comparable efficiency to PV type 1 (Maloney) [PV1(M)] (Fig. 3*A*). RT/PCR experiments of pP108ENPO RNA with primers 3800 and 3539 (see Fig. 1*D*) yielded single DNA bands after the first and fifth passages of the virus (data not shown). Sequence analysis of the RT/PCR product (fifth passage) indicated that the viral RNA contained the 5' NTR as originally constructed. We conclude that the EMCV IRES can replace the PV IRES in the hybrid virus W1-P108ENPO. To our knowledge, W1-P108ENPO is the only picornavirus in which ≈8% of the genome has been reported to be exchanged with a genetic element of a picornavirus belonging to a different genus.

We have inserted the ORF of a foreign gene (CAT), into the 5' NTR of pPNENPO, which generated the dicistronic construct pDICAT (Fig. 1*D*). *In vitro* translation of pDICAT transcript RNA yielded, apart from the PV polypeptides, a band that migrated to the expected position of the CAT protein (data not shown). Transfection of HeLa cells with this RNA produced complete cytopathic effects after 45 hr. The lysate contained virus that expressed plaques of various sizes (Fig. 1*D*). W1-DICAT clearly expressed the CAT polypeptide, the enzymatic activity increasing temporally with the virus titer in a one-step growth experiment (Figs. 3*A* and 4*A*). Moreover, CAT-specific antigen was readily apparent 9 hr after infection by immunoblot analysis (Fig. 5). Densitometric scanning of lanes 1 and 3 (Fig. 5) indicated that ≈5 ng of CAT antigen was present in 120 μg of soluble protein extract derived from 2×10^5 infected cells.

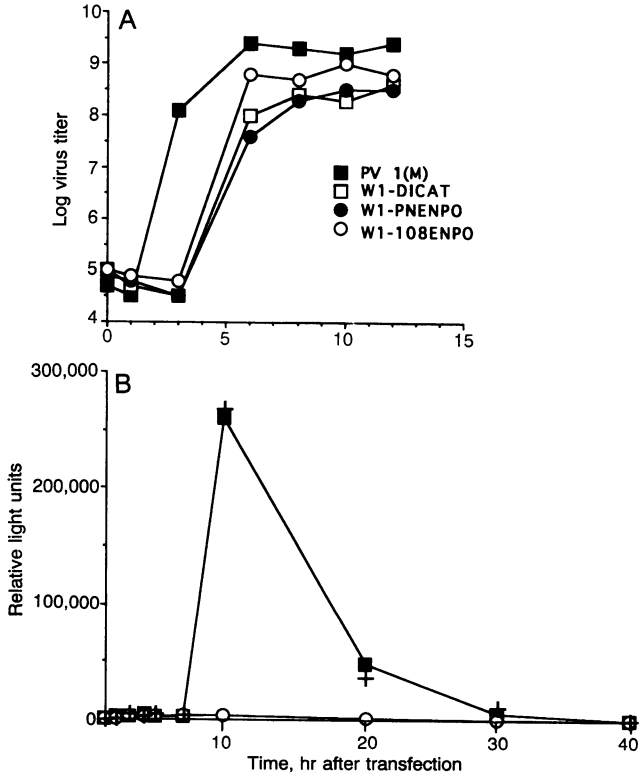

FIG. 3. (*A*) One-step growth curves of PV 1 (Mahoney), W1-PNENPO, W1-P108ENPO, and W1-DICAT. HeLa cell (35 mm; R19) plates (grown to confluence) were infected with 10^5 plaque-forming units. The infections were allowed to progress for different periods before being harvested. The resulting lysates were titered by plaque assay. (*B*) Luciferase assays were done at the indicated time points after transfection. ■, Without guanidine hydrochloride; ○, with 2 mM guanidine hydrochloride added immediately after transfection; +, 2 mM guanidine hydrochloride added to cell lysates immediately before luciferase assays.

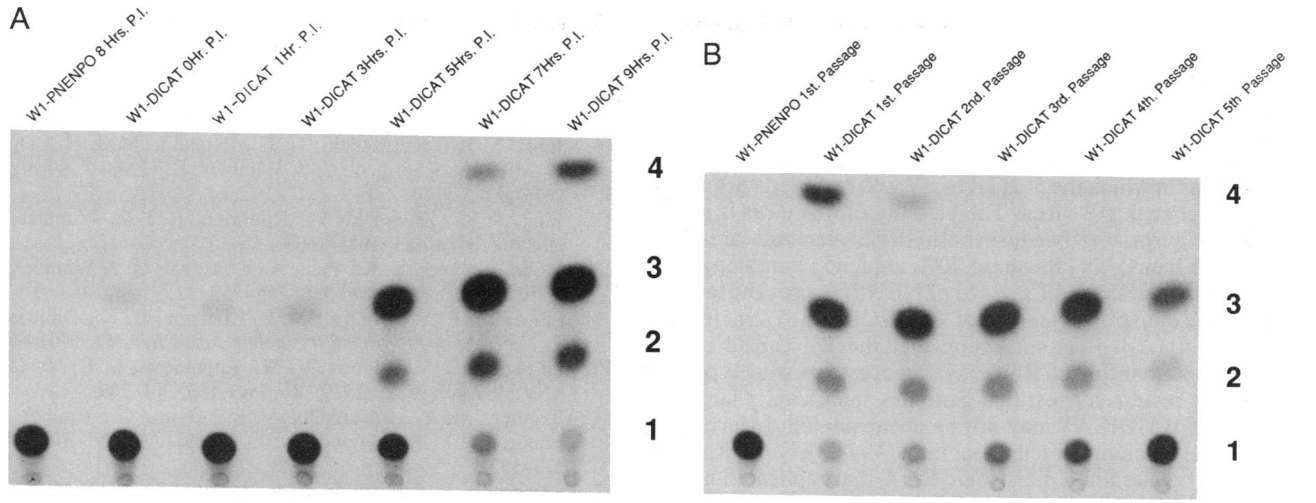

FIG. 4. (*A*) CAT assays of W1-PNENPO- and W1-DICAT-infected R19 HeLa cells by using standard procedures (12). (*B*) W1-DICAT from the initial viral passage was titered and used to infect fresh R19 HeLa cells at a multiplicity of infection of 10. This process was repeated for a total of five passages before CAT assays were done.

When W1-DICAT was serially passaged, the CAT activity expressed in HeLa cells decreased with each passage (Fig. 4*B*). Concomitant with this loss was an increase in the average plaque size of the passaged virus, although there were always small plaques, as found in the initial viral passage (data not shown). These results suggested that either the CAT ORF had mutated to inactivate the enzyme, or that some portion, if not all, of the inserted gene in W1-DICAT had been deleted. RT/PCR analyses showed that, in addition to the PV 5′ NTR (Fig. 2*B*, lanes 4 and 5), the heterologous IRES was also retained, even after five viral passages (Fig. 2*B*, lanes 9 and 10). The CAT ORF, on the other hand, was less stable during W1-DICAT passage than either of the IRES elements. Probing of the CAT-specific RNA from virus passaged five times yielded, in addition to the expected full-length band, several shorter bands that, in different experiments, appeared weaker than the expected full-length band (Fig. 2*C*, lanes 5 and 10). Sequence analysis of the full-length band showed, however, that those viruses that retained the CAT gene also maintained the wild-type CAT sequence. The decrease in active CAT enzyme expression, therefore, was probably not from the introduction of deleterious point mutations within the foreign gene sequence but more likely was from random deletions within the dicistronic viral genome.

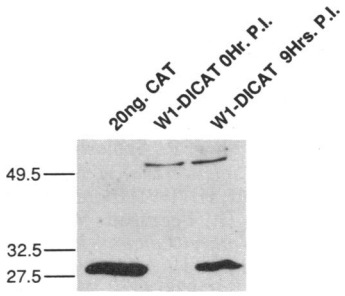

FIG. 5. W1-DICAT infection was allowed to progress for 9 hr postinfection (P.I.) as described in text. One hundred and twenty micrograms of soluble protein derived from the infected lysates and 20 ng of purified CAT protein (Sigma) were probed with anti-CAT antiserum (Accurate Chemicals) and a horseradish peroxidase-conjugated secondary antibody (Amersham) before being subjected to the enhanced chemiluminescence immunoblotting detection system (Amersham) and subsequent densitometric scanning by an LKB UltroScan densitometer.

We then tested whether an ORF significantly larger than CAT could be inserted into pPNENPO. We chose luciferase (67 kDa), an enzyme that was easily tested for expression (22). Accordingly, a plasmid (pDILUC) was constructed, and its transcripts were transfected into HeLa cells. Cell extracts derived from the transfected cells were tested for luciferase activity at different time points after transfection. Luciferase activity was detectable up to 20 hr after transfection, at which point the activity diminished (Fig. 3*B*, solid squares). To determine whether the luciferase signal originated from expression of the transfected dicistronic RNA introduced into the cells or if the signal was derived from replicated RNA, we added guanidine hydrochloride at a 2 mM concentration immediately after transfection. Although the drug had no effect on the luciferase assay itself (Fig. 3*B*, crosses), drug addition to the cell monolayers, immediately after transfection, reduced the signal >100-fold (Fig. 3*B*, open circles). Guanidine hydrochloride at a concentration of 2 mM completely inhibits PV RNA replication without apparent effect on cellular metabolism (39; and references therein). We conclude that pDILUC RNA replicated in HeLa cells. However, PV recovered 42 hr after transfection did not produce luciferase activity in infected HeLa cells, a result suggesting that the dicistronic genome was not encapsidated.

DISCUSSION

The mechanism by which IRES sequences promote cap-independent translation is unknown. Both type 1 and type 2 IRES elements, although dissimilar in primary sequence and higher order structure, bind to similar sets of cellular trans-acting factors, such as the nuclear polypyrimidine tract-binding protein (23) and the La autoantigen (24). The key question, however, as to how the initiating AUG is selected in IRES-mediated initiation of translation has not been answered. The 5′ NTR of W1-PNENPO is 1233 nt long and contains a total of 18 AUG triplets (Fig. 1*B*), of which two are in a most favorable nucleotide context for initiation of translation (25). Apparently, the tandemly arranged IRES elements, each of which forms extensive stem-and-loop structures, neither block the initiation of translation nor RNA replication. The elongated 5′ NTR does, however, impair viral replication, and selective pressure leads to the heterologous IRES being rapidly removed either by loop-out deletion or nonhomologous recombination (21).

The generation of W1-P108ENPO, which grows to almost wild-type levels, allows us to conclude that the PV IRES is

dispensable in W1-PNENPO replication. Thus, PV can proliferate when its type 1 IRES is replaced by a type 2 IRES lacking sequence homology. This observation suggests that the PV IRES is not likely to contain cis-acting signals essential for RNA replication or encapsidation. Semler and his colleagues (26, 27) have previously constructed a hybrid picornavirus in which the 5′ NTR of PV was replaced with the 5′ NTR of coxackie B virus. The viability of this virus is not completely surprising because both IRES elements are of type 1, and their 5′ NTRs share 70% sequence homology.

The addition of the CAT gene to pPNENPO raises the total length of the dicistronic genome of W1-DICAT to 8725 nt [not counting poly(A)]. It is surprising that the PV capsid can package this quantity of RNA (17% more than wild type). Preliminary results suggest that genomes significantly larger than that of W1-DICAT may not be encapsidated. Insertion of the gene encoding luciferase into W1-PNENPO RNA (pDILUC) resulted in a dicistronic RNA that replicated and expressed the foreign gene. The pDILUC full-length genome, however, that was 31% longer than wild-type RNA, was not encapsidated.

Expression systems based on viral vectors have been widely used for the introduction of polypeptides into animal cells. The animal virus vector of choice for the expression of foreign genes, of virtually any size and in large quantities, is vaccina virus (28). In addition, recombinant retrovirus and adenovirus vectors have been developed that can be stably transduced into many cell types (29, 30) to express a variety of gene products. PV replicons in which the gene segment coding for the capsid region has been deleted and replaced with foreign gene have also been described (31, 32), but in only one case could the replicon be encapsidated, in trans, by the capsid proteins of helper PV (32). Finally, alphavirus replicons have been developed to express foreign genes (33, 34), but they also require helper virus for encapsidation. These systems contrast with W1-DICAT, which, to our knowledge, is the only known replication and encapsidation competent, lytic, animal RNA virus that expresses a foreign gene.

The ability of attenuated poliovirus to infect and replicate in the human gut and to induce a strong primary mucosal and humoral immune response makes it an ideal vector for the expression of foreign genes in this tissue. In an attempt to use these properties, antigenic hybrid viruses have been constructed, in which heterologous antigenic sites were engineered into the PV capsid (35–38). However, these PV derivatives have not kept their promise as possible vaccine candidates: only a small foreign antigenic determinant was tolerated, and the viral growth properties and/or antigen presentations were poor. We believe that the inclusion of an entire gene or a large segment thereof, translated from an independent cistron in the PV genome, is a promising alternative to the antigenic hybrid viruses.

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