Chemical Synthesis of Poliovirus cDNA: Generation of Infectious Virus in the Absence of Natural Template

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Full-length poliovirus complementary DNA (cDNA) was synthesized by assembling oligonucleotides of plus and minus strand polarity. The synthetic poliovirus cDNA was transcribed by RNA polymerase into viral RNA, which translated and replicated in a cell-free extract, resulting in the de novo synthesis of infectious poliovirus. Experiments in tissue culture using neutralizing antibodies and CD155 receptor–specific antibodies and neurovirulence tests in *CD155* transgenic mice confirmed that the synthetic virus had biochemical and pathogenic characteristics of poliovirus. Our results show that it is possible to synthesize an infectious agent by in vitro chemical-biochemical means solely by following instructions from a written sequence.

Research on viruses is driven not only by an urgent need to understand, prevent, and cure viral disease. It is also fueled by a strong curiosity about the minute particles that we can view both as chemicals and as "living" entities. Poliovirus can be crystallized (*1*) and its empirical formula can be calculated (*2*), yet this "chemical" replicates naturally in humans with high efficiency, occasionally causing the paralyzing and lethal poliomyelitis.

Poliovirus, an enterovirus of the *Picornaviridae*, is a small, nonenveloped, icosahedral virus consisting of five different macromolecules: 60 copies each of capsid polypeptides VP1, -2, -3, and -4 and one copy of the positive-sense, single-stranded RNA genome (~7.5 kilobases in length) (Fig. 1A) (*3*). The chemical sequence (*4*, *5*), the genetic map of the genome (*4*), and the three-dimensional crystal structure of the virion (*6*) were determined 2 decades ago. Poliovirus employs one of the simplest genetic systems known for proliferation (*3*, *7*). The virus enters the cell after attaching to the cellular receptor CD155 (*8*, *9*). Immediately after the virus particle uncoats inside the cell, the genomic RNA is translated under the control of the internal ribosomal entry site (IRES) into a single polypeptide, the polyprotein (*10*, *11*). The polyprotein is then processed into functional proteins by two viral proteinases (*3*, *7*). With the aid of viral proteins, most notably the RNA-dependent RNA polymerase $3D^{pol}$ and the genome-linked protein VPg, along with cellular components, the viral RNA is transcribed into minus-strand copies that serve as templates for the synthesis of new viral genomes (plus-strand RNA). Newly synthesized plus-strand RNA can serve as messenger RNA for more protein synthesis, engage further in RNA replication, or be encapsidated by an increasing pool of capsid proteins (*7*, *12*). In suitable tissue culture cells (for example, HeLa cells), the entire replication cycle is complete in only 6 to 8 hours and yields 10^4 to 10^5 progeny virions per cell.

Here we describe the de novo chemical-biochemical synthesis of infectious poliovirus from basic chemical building blocks, independent of viral components previously formed in vivo and with the use of the known sequence as the only instruction for engineering the genome. The succession of macromolecular events in an infected cell was reproduced in a test tube containing a cell-free extract devoid of nuclei, mitochondria, and other cellular organelles and seeded with viral RNA. This result confirms that the genome sequence originally deciphered from virion RNA is correct (*4*, *5*) and demonstrates the feasibility of chemical-biochemical synthesis of an infectious agent in the absence of a natural template.

The strategy of synthesizing the genome of poliovirus type 1 (Mahoney) [PV1(M)] began with the assembly of a full-length cDNA carrying a phage T7 RNA polymerase promoter at the (left) 5′ end (Fig. 1) from three large, overlapping DNA fragments (F1, -2, and -3). Each DNA fragment was obtained by combining overlapping segments of 400 to 600 base pairs (bp). The segments were synthesized by assembling purified oligonucleotides [average length, 69 nucleotides (nt)] of plus and minus polarity with overlapping complementary sequences at their termini, and the segments were then ligated into a plasmid vector (*13*). Five to 15 clones were sequenced to identify either the correct DNA segments or the segments containing small numbers of errors that could be eliminated, either by combining the error-free portions of segments by an internal cleavage site or by standard site-directed mutagenesis (*13*). To ascertain the authenticity of the synthesized viral genome [sPV1(M)] and to distinguish it from the wild-type (*wt*) sequence of PV1(M) [*wt* PV1(M)] (*4*, *5*), we engineered nucleotide substitutions into the sPV1(M) cDNA as genetic markers (*13*).

We have shown previously that poliovirus cDNA carrying a phage T7 promoter for the phage RNA polymerase can be transcribed with T7 RNA polymerase into highly infectious RNA (*14*). Accordingly, the sPV1(M) cDNA and *wt* PV1(M) cDNA were transcribed (*13*) and were found to yield transcript RNAs of the same length as virion RNA (*15*). De novo synthesis of poliovirus from transcript RNA of *wt* PV1(M) cDNA in a cell-free extract of uninfected HeLa cells has been previously described by Molla *et al.* (*2*). Therefore, the incubation of transcript RNA from sPV1(M) cDNA in cytoplasmic extracts of uninfected HeLa cells should result in the generation of poliovirus. To examine this possibility, transcript RNA derived from sPV1(M) cDNA was incubated with a cytoplasmic extract of HeLa S3 cells, and the synthesis of virus-specific proteins and infectious viruses were monitored. The products of sPV1(M) cDNA–derived RNA translation and proteolytic processing were the same as those obtained with *wt* PV1(M) RNA (Fig. 2), an observation suggesting that the open reading frame (ORF) of the sPV1(M)-specific RNA is intact. We then tested for the presence of infectious virus particles in the cell-free incubation mixture by adding aliquots of the incubation mixture to monolayers of HeLa cells. After 48 hours, plaques appeared [0.5 to 1 × 10^5 plaque-forming units (PFU) per μg of transcript RNA in 50 μl of reaction] whose heterogeneous morphology was characteristic of those produced by authentic poliovirus (Fig. 3). All together, these results indicate that the input synthetic RNA was translated and replicated in the cell-free extract and that newly synthesized RNA was encapsidated into newly synthesized coat proteins, resulting in the de novo synthesis of infectious poliovirus.

Experiments were then carried out to confirm that the infectious material isolated from the cell-free extract was indeed sPV1(M), as designated by the oligonucleotide sequence. Restriction enzyme digestion of the reverse transcriptase–polymerase chain reaction (RT-PCR) product of the viral RNA recovered from sPV1(M)-infected HeLa cells revealed the presence of all engineered markers (fig. S1, lanes 1 and 2).

We also tested the effects of the poliovirus

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receptor–specific monoclonal antibody (Mab) D171 and type-specific hyperimmune sera on plaque formation by sPV1(M) (Table 1). Mab D171 has been shown to completely block infection of all three serotypes by specifically binding to CD155, the cellular receptor of poliovirus (*8*, *9*, *16*). The treatment of HeLa cells with Mab D171 before the addition of sPV1(M) completely abolished plaque formation (Table 1). Similarly, no plaques were observed when sPV1(M) was incubated with poliovirus type 1–specific rabbit hyperimmune serum [anti-PV1(M)]. Neutralization of the synthetic virus was type-specific because hyperimmune serum to poliovirus type 2 (Lansing) [PV2(L)] did not inhibit plaque formation (Table 1). These results were in full agreement with those obtained with *wt* PV1(M) (Table 1). They imply that the de novo poliovirus particles synthesized in the cell-free extract were serotype 1, requiring the authentic poliovirus receptor for infection.

The sPV1(M) virus was assayed to determine whether it expresses a neurovirulent phenotype in mice transgenic for the human poliovirus receptor [CD155 tg mice strain ICR.PVR.tg I (*17*)]. When injected with *wt* poliovirus strains, these animals develop a neurological disease indistinguishable, clinically and histologically, from primate poliomyelitis (*17*–*19*). Intracerebral injection of sPV1(M) caused flaccid paralysis or death in CD155 tg

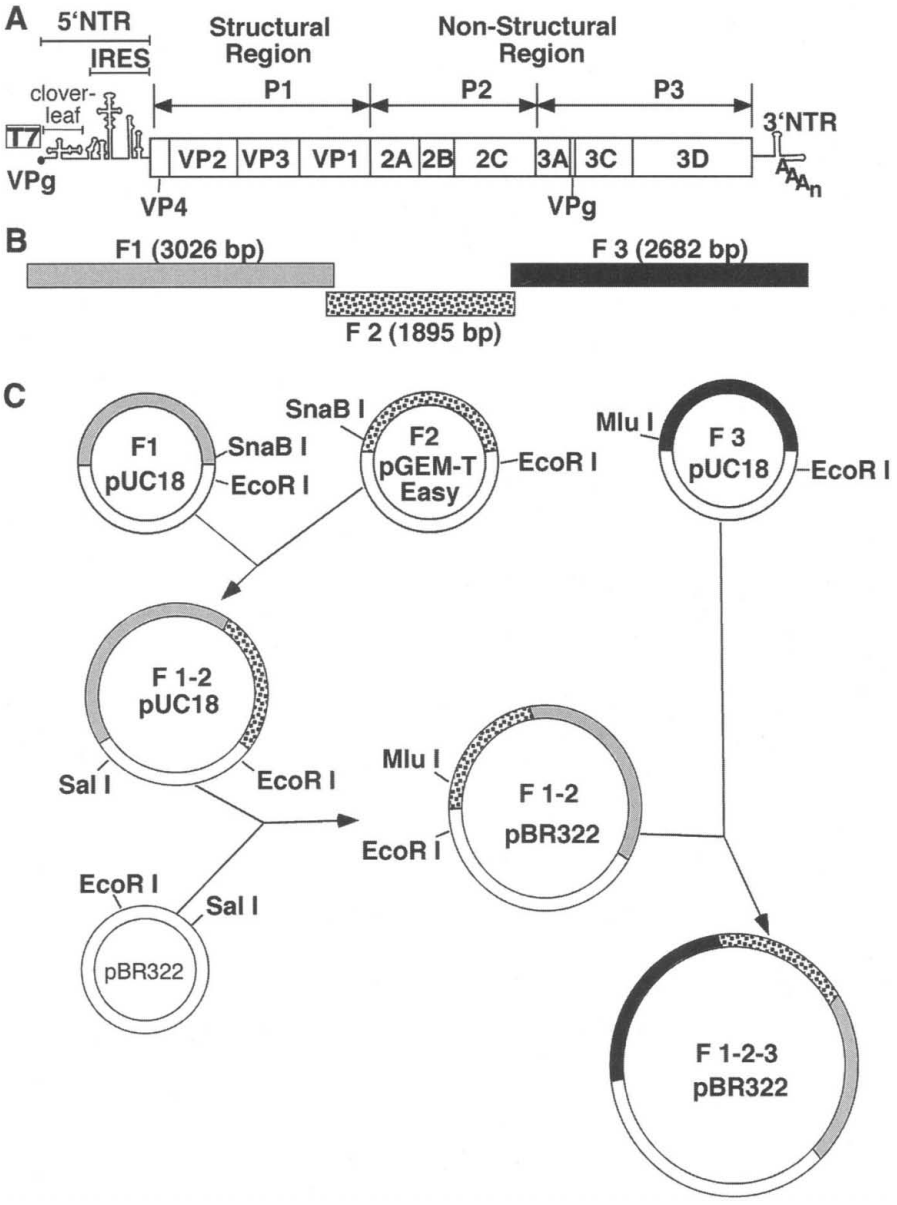

Fig. 1. Genomic structure of PV1(M) and strategy for the synthesis of its full-length cDNA. (**A**) The positive-stranded RNA of poliovirus is shown with VPg at the 5′ end of the NTR. In the cDNA, VPg is replaced by the T7 RNA polymerase promoter. The polyprotein contains one structural (P1) and two nonstructural (P2 and P3) domains. The 3′ NTR contains a heteropolymeric region and is polyadenylated (shown as AAA_n). (**B**) PV1(M) cDNA carrying a T7 RNA polymerase promoter at the 5′ NTR end was subdivided into three large fragments for the synthesis of full-length sPV1(M) cDNA. The sizes of the fragments (in bp) are depicted above or below each rectangle that represents the respective fragment. The genome sequence encoded by each fragment was described in (*13*). (**C**) The three DNA fragments were synthesized as described in the text. The DNA fragments were assembled stepwise via common unique restriction endonuclease cleavage sites to yield full-length sPV(M) cDNA (F1-2-3 pBR322). The sequence of sPV(M) cDNA was confirmed by automated sequence analyses.

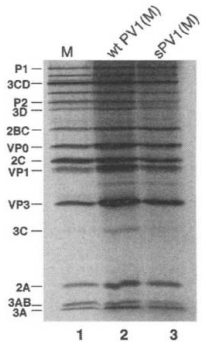

Fig. 2. Products of in vitro translation and proteolytic processing of poliovirus RNAs in a HeLa cell-free extract. Transcript RNA derived from sPVM cDNA (*13*) and virion RNA derived from *wt* PV1(M) were translated and analyzed as described in (*13*). Lane 1, *wt* PV1(M) marker (M) displaying a lysate of [^{35}S] methionine-labeled poliovirus-infected HeLa extract; lane 2, virion RNA derived from *wt* PV1(M); lane 3, RNA derived from sPV1(M) cDNA. Bands correspond with the segments in Fig. 1A. VPO, 2BC, 3AB, and 3CD are precursor polypeptides.

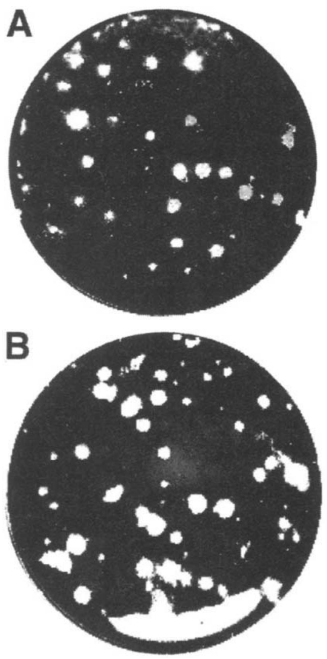

Fig. 3. Plaque phenotypes of polioviruses generated in the HeLa cell-free extract. De novo synthesis of poliovirus from transcript RNA in cell-free extract of uninfected HeLa cells was done as described in (*13*). (**A**) Plaque phenotype of virus derived from transcript sPV1(M) RNA. (**B**) Plaque phenotype of virus derived from virion *wt* PV1(M) RNA.

Table 1. Biological characterization of sPV1(M). Plaque reduction assay in the presence (+) and absence (−) of antibodies as described in (*13*). Anti-PV1(M) and anti-PV2(L) are neutralizing polyclonal antibodies specific for types 1 and 2 poliovirus, respectively. Neuropathogenicity of sPV1(M) and *wt* PV1(M) was assayed in hPVR-tg mice as described in (*13*). PLD_{50} is defined as the amount of virus that caused paralysis or death in 50% of the inoculated mice.

Virus	PFU						PLD_{50} (log_{10} PFU)
	Mab D171		Anti-PV1(M)		Anti-PV2(L)		
	−	+	−	+	−	+	
sPV1(M)	83	0	91	0	88	92	6.2
wt PV1(M)	89	0	86	0	90	87	2.0

mice, resembling the disease produced by *wt* PV1(M) (*13*). However, a larger inoculum of sPV1(M) than PV1(M) was necessary to paralyze or kill the animals (Table 1). The increase in the magnitude of attenuation was unexpected, because all nucleotide substitutions introduced into sPV1(M) resulted in silent mutations in the ORF, except for the newly created Xma I and Stu I sites in the 5′ nontranslated region (NTR) and 2B region, respectively. These latter changes had been shown previously to have no influence on viral replication in tissue culture (*20*, *21*). However, the silent mutations that we introduced into the poliovirus genome may exert a strong influence on pathogenesis by hitherto unknown mechanisms.

The presence or absence of genetic markers in the inoculated virus and the virus isolated from the spinal cords of paralyzed mice was confirmed by amplification of the viral RNA by RT-PCR and restriction enzyme analysis. Our results show that the viruses isolated from the spinal cords of paralyzed mice resembled the inoculated virus (fig. S1). Our data also confirm that the synthetic virus was the causative agent of the flaccid paralysis observed in the sPV1(M)-infected mice.

The chemical synthesis of the viral genome, combined with de novo cell-free synthesis, has yielded a synthetic virus with biochemical and pathogenic characteristics of poliovirus. In 1828, when Wöhler synthesized urea, the theory of vitalism was shattered (*22*). If the ability to replicate is an attribute of life, then poliovirus is a chemical [$C_{332,652}H_{492,388}N_{98,245}O_{131,196}P_{7501}S_{2340}$, see (*2*)] with a life cycle.

As a result of the World Health Organization's vaccination campaign to eradicate poliovirus (*23*), the global population is better protected against poliomyelitis than ever before. Any threat from bioterrorism will arise only if mass vaccination stops (*23*) and herd immunity against poliomyelitis is lost. There is no doubt that technical advances will permit the rapid synthesis of the poliovirus genome, given access to sophisticated resources. The potential for virus synthesis is an important additional factor for consideration in designing the closing strategies of the poliovirus eradication campaign.

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Fig. S1
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MAP Kinase Phosphatase As a Locus of Flexibility in a Mitogen-Activated Protein Kinase Signaling Network

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Intracellular signaling networks receive and process information to control cellular machines. The mitogen-activated protein kinase (MAPK) 1,2/protein kinase C (PKC) system is one such network that regulates many cellular machines, including the cell cycle machinery and autocrine/paracrine factor synthesizing machinery. We used a combination of computational analysis and experiments in mouse NIH-3T3 fibroblasts to understand the design principles of this controller network. We find that the growth factor–stimulated signaling network containing MAPK 1, 2/PKC can operate with one (monostable) or two (bistable) stable states. At low concentrations of MAPK phosphatase, the system exhibits bistable behavior, such that brief stimulus results in sustained MAPK activation. The MAPK-induced increase in the amounts of MAPK phosphatase eliminates the prolonged response capability and moves the network to a monostable state, in which it behaves as a proportional response system responding acutely to stimulus. Thus, the MAPK 1, 2/PKC controller network is flexibly designed, and MAPK phosphatase may be critical for this flexible response.

Intracellular signaling pathways communicate extracellular information to modulate cellular functions in response to external stimuli. Signaling pathways function not only to transmit information but also to process the information as it is being transmitted. Such processing occurs because signaling pathways interact with one another to form networks (*1*–*3*). The processing occurs both through summation of inputs and through the temporal characteristics of pathways. For instance, the MAPK cascade communicates signals from growth factors that bind to receptor tyrosine kinases to the transcriptional