Our data indicate that the ΔNL mutation increases Aβ production by augmenting cleavage at or very near the site of mutation on the amino side of Aβ. Thus this mutation likely increases all Aβ in a way that does not alter the specific site (or sites) of COOH-terminal cleavage. Although the βAPP_{717} mutations do not appear to increase overall Aβ production, these mutations on the COOH-side of Aβ may shift cleavage to favor generation of the longer Aβ's (42 to 43 residues long) that are specifically associated with senile plaque amyloid. As these longer Aβ's have biophysical properties that favor amyloid deposition (*25*), shifting the site of cleavage could result in amyloid deposition without increasing the overall amount of Aβ. Such a shift would not be detectable with our current gel system, and the longer forms might more readily form insoluble aggregates that would impair detection. Whatever the precise mechanism proves to be, it is likely, given the positive results obtained with the ΔNL mutation, that continued analysis of the βAPP_{717} mutations will ultimately reveal altered processing that is highly informative with regard to the mechanism through which these mutations produce AD.

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23. Each of our cell lines is polyclonal and was independently transfected using the episomal replicon CEP4β. Although polyclonal lines show less variation than monoclonal lines, it is nonetheless critically important to assess the level of expression (synthesis) of WT and mutant βAPPs before concluding that the processing of a mutant βAPP is altered in a way that increases Aβ release. Synthesis is best measured by quantitating the full-length βAPP that accumulates during a labeling interval sufficiently short that no appreciable degradation takes place. We labeled for 20 min, the shortest interval that permitted us to assess multiple lines in parallel in a way that was practical. Although some degradation may occur during this short interval, this method of assessing synthesis is nonetheless a reliable way to ensure that excess production of Aβ in lines expressing mutant βAPPs is due to altered processing and not to increased expression.
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26. To assemble CEP4β-695ΔNL, we used a βAPP_{695} template and oligonucleotides 1 and 2 to amplify by the polymerase chain reaction (PCR) a cDNA extending from base 1762 to 2119 followed by an adaptor sequence containing an Xba I restriction site [numbering is according to Kang *et al.* (*7*)]. Oligonucleotide sequences are shown below. The forward primer employed in this reaction (oligonucleotide 1) altered the WT nucleotides at positions 1785 and 1786 from G to T and A to C, respectively, changing the encoded amino acids immediately amino to $A\beta_1$ from lysine to asparagine and methionine to leucine, respectively. The Bgl II–Xba I fragment of this amplified cDNA was then used to replace the corresponding fragment of a βAPP_{695} cDNA (nucleotides −30 to 2119) in BSKS$^-$ (Stratagene). To assemble CEP4β-695ΔI, we used a βAPP_{695} template and oligonucleotides 3 and 2 to PCR amplify a cDNA extending from 1906 to 2119 followed by an adaptor sequence containing an Xba I restriction site. The forward primer used in this reaction (oligonucleotide 3) altered the WT nucleotide at position 1924 from G to A, changing the encoded valine to isoleucine. This cDNA was gel-purified and used as a template along with a βAPP_{695} template and oligonucleotides 4 and 5 to produce a ΔI cDNA extending from base 1761 to 2119 followed by an adaptor sequence containing an Xba I restriction site. The Eco RI–Xba I fragment of this amplified cDNA was then used to replace the corresponding fragment of Bluescript βAPP_{695}. Both the full-length 695ΔNL and 695ΔI sequences in Bluescript were then subcloned into Hind III–Not I sites of CEP4β. The oligonucleotides used were
(1) ACGGAGGAGATCTCTGAAGTGAATCTGGATGCAGAATTCC
(2) GAGGGCCATGCCGGCCTCTAGAGTCCAACTTCAGAGGCTGCT
(3) GTCATAGCGACAGTGATCATCATCAC
(4) GACGGAGGAGATCTCTGAAGT, and
(5) GAGGGCCATGCCGGCCTCTAGA.

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Sequence and Genome Organization of a Human Small Round-Structured (Norwalk-Like) Virus

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Small round-structured viruses (SRSVs), also known as Norwalk or Norwalk-like viruses, are the major worldwide cause of acute, epidemic nonbacterial gastroenteritis in humans. These viruses, which contain a single-stranded RNA genome, have remained refractory to molecular characterization because of the small amounts of virus in clinical samples and the absence of an animal model and an in vitro culture system. The complete genomic nucleotide sequence of an SRSV, Southampton virus, was determined. The 7696-nucleotide RNA genome encodes three open reading frames whose sequence and organization strongly support proposals that SRSVs are members of the Caliciviridae.

The term "epidemic winter vomiting disease" was first used by Zahorsky in 1929 (*1*) to describe a syndrome in humans now globally recognized as acute nonbacterial gastroenteritis (*2*). The prototypical pathogen, Norwalk virus, was identified by American workers after an outbreak of acute nonbacterial gastroenteritis in an elementary school in Norwalk, Ohio (*3*). Morphologically indistinguishable viruses have since been described in clinically similar outbreaks and have been termed Hawaii (*4*, *5*), Snow Mountain (*6*, *7*), and Taunton agents (*8*, *9*) after the geographical locations in which they were first found. Our electron microscopy (EM) studies, performed with human convalescent sera (*10*), of virus isolates collected from laboratory-confirmed outbreaks have established at least four serotypes in the United Kingdom and the United States. We designate the Norwalk and Hawaii viruses as serotypes 1 and 2, respectively, because of their historical precedence; the prototype strains for serotypes 3 and 4 are the Snow Mountain and Taunton viruses. Others also recognize at least four serotypes (*11*). Since February 1991, serotype 3 has been the most preva-

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lent serotype, causing outbreaks of acute nonbacterial gastroenteritis in England and Wales (*12*).

The inability to propagate this group of viruses in vitro has severely hampered the development of diagnostic reagents. As a result, EM remains the most widely used technique for identification and diagnosis. The amorphous nature of the Norwalk-like viruses permits their differentiation from other small round viruses such as astroviruses, parvoviruses, and the classical caliciviruses and has resulted in an interim scheme for their classification (*13*). This scheme has entailed the introduction of the term "small round-structured virus" (SRSV) within the United Kingdom and elsewhere to differentiate this group of viruses from other small round fecal viruses. Comparative EM studies have suggested that this virus has some morphological similarities to caliciviruses (8). The caliciviruses are 35- to 40-nm particles with a characteristic cupped morphology, possessing a single-stranded RNA genome and a major structural protein of 60 to 71 kD (*14*). Like the caliciviruses, Norwalk virus has a single-stranded RNA genome (*15, 16*) and a single major capsid protein with a molecular weight of 59,000 (*17*).

The objective of our work here was to determine the complete nucleotide sequence of a recent SRSV isolate. A stool sample that contained characteristic SRSV particles (Fig. 1) was collected from a 2-year-old child with acute diarrhea during a recent family outbreak of acute gastroenteritis in Southampton, United Kingdom (*18*). The virus, hereafter designated Southampton virus, was shown to be serotype 3 by immuno-EM, and this sample was the sole source of viral RNA for cDNA synthesis and DNA sequence determination (*19, 20*).

The genome of Southampton virus consists of 7696 nucleotides, which does not include a 3′ polyadenylate [poly(A)] tail. The GenBank accession number for the cDNA sequence is L07418. The 5′ end of the RNA contains an untranslated leader sequence of

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ORF1
   1 MALGLIGRTTPEPTGTAGPPPKQQRDRPPRTQEEVQYGMGWSDRPIDQNVKSWEELDTTVKEEILDNHKEWFDAGGLGPCTMPPTYERVKDDSPPGEQVK
 101 WSARDGVNIGVERLTTVSGPEWNLCPLPPIDLRNMEPASEPTIGDMIEFYEGHIYHYSIYIGQGKTVGVHSPQAAFSVARVTIQPIAAWWRVCYIPQPKH
 201 RLSYDQLKELENEPWPYAAITNNCFEFCCQVMNLEDTWLQRRLVTSGRFHHPTQSWSQQTPEFQQDSKLELVRDAILAAVNGLVSQPFKNFLGKLKPLNV
 301 LNILSNCDWTFMGVVEMVILLLELFGVFWNPPDVSNFIASLLPDFHLQGPEDLARDLVPVILGGIGLAIGFTRDKVTKVMKSAVDGLRAATQLGQYGLEI
 401 FSLLKKYFFGGDQTERTLKGIEAAVIDMEVLSSTSVTQLVRDKQAAKAYMNILDNEEEKARKLSAKNADPHVISSTNALISRISMARSALAKAQAEMTSR
 501 MRPVVIMMCGPPGIGKTKAAEHLAKRLANEIRPGGKVGLVPREAVDHWDGYHGEEVMLWDDYGMTKILDDCNKLQAIADSAPLTLNCDRIENKGMQFVSD
 601 AIVITTNAPGPAPVDFVNLGPVCRRVDFLVYCSAPEVEQIRRVSPGDTSALKDCFKLDFSHLKMELAPQGGFDNQGNTPFGKGTMKPTTINRLLIQAVAL
 701 TMERQDEFQLQGKMYDFDDDRVSAFTTMARDNGLGILSMAGLGKKLRGVTTMEGLKNALKGYKISACTIKWQAKVYSLESDGNSVNIKEERNILTQQQQS
 801 VCTASVALTRLRAARAVAYASCIQSAITSILQIAGSALVVNRAVKRMFGTRTATLSLEGPPREHKCRVHMAKAAGKGPIGHDDVVEKYGLCETEEDEEVA
 901 HTEIPSATMEGKNKGKNKKGRGRRNNYNAFSRRGLNDEEYEEYKKIREEKGGNYSIQEYLEDRQRYEEELAEVQAGGDGGIGETEMEIRHRVFYKSKSRK
1001 HHQEERRQLGLVTGSDIRKRKPIDWTPPKSAWADDEREVDYNEKISFEAPPTLWSRVTKFGSGWGFWVSPTVFITTTHVIPTSAKEFFGEPLTSIAIHRA
1101 GEFTLFRFSKKIRPDLTGMILEEGCPEGTVCSVLIKRDSGELLPLAVRMGAIASMRIQGRLVHGQSGMLLTGANAKGMDLGTIPGDCGAPYVYKRANDWV
1201 VCGVHAAATKSGNTVVCAVQASEGETTLEGGDKGHYAGHEIIKHGCGPALSTKTKFWKSSPEPLPPGVYEPAYLGGRDPRVTVGPSLQQVLRDQLKPFAE
1301 PRGRMPEPGLLEAAVETVTSSLEQVMDTPVPWSYSDACQSLDKTTSSGFPYHRRKNDDWNGTTFVRELGEQAAHANNMYEQAKSMKPMYTGALKDELVKP
1401 EKVYQKVKKRLLWGADLGTVVRAARAFGPFCDAIKSHTIKLPIKVGMNSIEDGPLIYAEHSKYKYHFDADYTAWDSTQNRQIMTESFSIMCRLTASPELA
1501 SVVAQDLLAPSEMDVGDYVIRVKEGLPSGFPCTSQVNSINHWLITLCALSEVTGLSPDVIQSMSYFSFYGDDEIVSTDIEFDPAKLTQVLREYGLRPTRP
1601 DKSEGPIIVRKSVDGLVFLRRTISRDAAGFQGRLDRASIERQIYWTRGPNHSDPFETLVPHQQRKVQLISLLGEASLHGEKFYRKISSKVIQEIKTGGLE
1701 MYVPGWQAMFRWMRFHDLGLWTGDRNLLPEFVNDDGV*
ORF2
   1                                                MMMASKDAPQSADGASGAGQLVPEVNTADPLPMEPVAGPTTAVATAGQVNMIDPWIVNNFVQSPQGEF
  69 TISPNNTPGDILFDLQLGPHLNPFLSHLSQMYNGWVGNMRVRILLAGNAFSAGKIIVCCVPPGFTSSSLTIAQATLFPHVIADVRTLEPIEMPLEDVRNV
 169 LYHTNDNQPTMRLVCMLYTPLRTGGGSGNSDSFVVAGRVLTAPSSDFSFLFLVPPTIEQKTRAFTVPNIPLQTLSNSRFPSLIQGMILSPDASQVVQFQN
 269 GRCLIDGQLLGTTPATSGQLFRVRGKINQGARTLNLTEVDGKPFMAFDSPAPVGFPDFGKCDWHMRISKTPNNTGSGDPMRSVSVQTNVQGFVPHLGSIQ
 369 FDEVFNHPTGDYIGTIEWISQPSTPPGTDINLWEIPDYGSSLSQAANLAPPVFPPGFGEALVYFVSAFPGPNNRSAPNDVPCLLPQEYITHFVSEQAPTM
 469 GDAALLHYVDPDTNRNLGEFKLYPGGYLTCVPNGVGAGPQQLPLNGVFLFVSWVSRFYQLKPVGTASTARGRLGVRRI*
ORF3
   1                                                                                MAQAIIGAIAASAAGSALGAGI
  23 QAGAEAALQSQRYQQDLALQRNTFEHDKDMLSYQVQASNALLAKNLNTRYSMLIAGGLSSADASRAVAGAPVTRLIDWNGTRVAAPRSSATTLRSGGFMA
 123 VPMPVQPKSKTPQSSGFSNPAYDMSTVSSRTSSWVQSQNSLRSVSPFHRQALQTVWVTPPGSTSSSSVSSTPYGVFNTDRMPLFANLRR*
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Fig. 2. The deduced amino acid sequences encoded by the three major ORFs of Southampton virus RNA (*28*). The GenBank accession number for the cDNA nucleotide sequence is L07418. The first codon of ORF1 occurs at nt 146. The putative helicase (GPPGIGKT) and RNA polymerase (GLPSG and YGDD) motifs are underlined. The overlapping reading frames ORF2 and ORF3 begin at nt 5343 and 6983, respectively. Asterisks denote positions of terminator codons. Sequence analyses were performed with DNASTAR software (*22*).

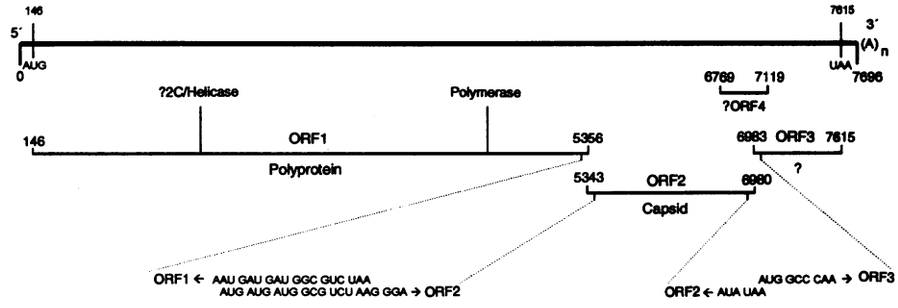

Fig. 3. Genome organization of Southampton virus. The nucleotide coordinates and the putative translation products of the ORFs are indicated. The intergenic regions showing the nucleotide sequences of the overlapping ORFs are also shown. Question marks indicate unknown or uncertain function; $(A)_n$, polyadenylate tail.

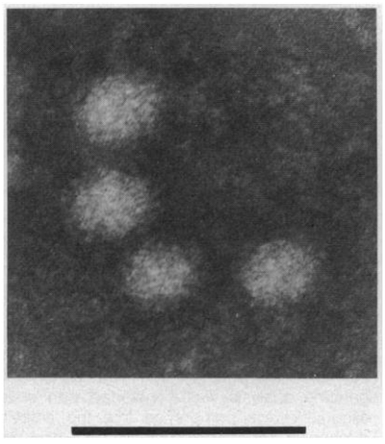

Fig. 1. Electron micrograph of SRSV serotype 3 particles. The virus was negatively stained for 2 min in 0.5% phosphotungstic acid (pH 6.0) and visualized with a Hitachi H7000 transmission electron microscope. The scale bar represents 100 nm.

Fig. 4. Comparison of Southampton virus protein sequences with partial sequences of Norwalk virus proteins. Identical amino acid residues (*28*) are denoted as dots. (**A**) Alignment with the immunogenic region of Norwalk virus (*16*). (**B**) Alignment with the putative RNA-dependent RNA polymerase of Norwalk virus (*15*). Asterisks mark the conserved RNA polymerase motifs.

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A        680       690       700       710       720       730
QGGFDNQGNTPFGKGTMKPTTINRLLIQAVALTMERQDEFQLQGKMYDFDDDRVSAFTTMARDNGLGIL
..............V.....................PT....T...A...R...A....LI

740       750       760       770       780       790
SMAGLGKKLRGVTTMEGLKNALKGYKISACTIKWQAKVYSLESDGNSVNIKEERNIL  Southampton
...S......S...I.......S.....K.S.Q..SR..II....A..Q...DKQA.  Norwalk

B 1470      1480      1490      1500      1510      1520      1530
HFDADYTAWDSTQNRQIMTESFSIMCRLTASPELASVVAQDLLAPSEMDVGDYVIRVKEGLPSGFPCTSQ
.........................S.........E.....................................
                                                          *****

 1540      1550      1560      1570      1580      1590
VNSINHWLITLCALSEVTGLSPDVIQSMSYFSFYGDDEIVSTDIEFDPAKLTQVLRE  Southampton
.......I.........A.......V.....................D....R...I.K.  Norwalk
                    ****
```

145 nucleotides (*21*), and the 3′ end contains 81 untranslated nucleotides preceding the poly(A) tail. Computer analysis (*22*) revealed three open reading frames (ORFs) (Fig. 2), each with the consensus translation initiator sequence RNNAUGG (*23*). A fourth potential ORF with a weaker translation initiator sequence (AUUAUGU) is located in frame 1 and overlaps ORF2 and ORF3 (Fig. 3). ORF1 starts at the first AUG codon [nucleotide (nt) 146] and codes for a 1737–amino acid polypeptide. Such large ORFs are characteristic of picornavirus polyproteins and have recently been found in the genomes of feline calicivirus (FCV) and rabbit hemorrhagic disease virus (RHDV) (*24, 25*).

Comparison of the Southampton virus polyprotein sequence with the two published partial amino acid sequences of Norwalk virus revealed 79% similarity to an immunogenic region covering amino acids 669 to 794 (Fig. 4A) and 93% similarity to a region coding for the RNA-dependent RNA polymerase (Fig. 4B). Similarity to the translated sequences of FCV (*24*) and RHDV (*25*) was limited to the polymerase region of the polyprotein and was approximately 31% and 27%, respectively. Amino acids 510 to 517 contain a nucleotide binding motif [GXXGXGK(S or T), where G is Gly, K is Lys, S is Ser, T is Thr, and X is any amino acid], which is typical of the picornavirus-encoded helicase P2C; this motif occurs in an almost identical position in the RHDV polyprotein (*25*).

We assigned ORF2 to the major capsid protein on the basis of its limited similarity to the capsid proteins of FCV (31%, amino acid residues 15 through 223) and RHDV (30%, amino acid residues 4 through 224). There are three potential initiator codons in ORF2, with the third most closely resembling the consensus (*23*). There is a 17-nt overlap between the terminator sequence of ORF1 and the first predicted codon of ORF2 (Fig. 3). A 1-nt overlap occurs at the junction of ORF2 and ORF3, a situation analogous to the 4-nt overlap found at a similar position in the FCV genome. ORF3 is of unknown function but has counterpart ORFs in the FCV and RHDV genomes. It shows limited similarity to FCV ORF3 (24%, amino acid residues 80 through 124) and is approximately double its size. ORF4 does not appear to have a counterpart in either the FCV or RHDV genomes. A similarly sized ORF (369 nt) that overlaps ORFs 1 and 2 has been identified in the 7.5-kb RNA genome of human hepatitis E virus (HEV), another virus found in stool samples. Like the two characterized animal caliciviruses (*24, 25*), HEV encodes a large ORF and a capsid protein (*26*); however, the taxonomic position of HEV remains unresolved.

The complete nucleotide sequence of Southampton virus, together with comparative biophysical and biochemical studies recently reviewed by Greenberg and Matsui (*27*), strongly suggest that human SRSVs are members of the Caliciviridae. The similarity in genome organization between Southampton virus and the animal caliciviruses is clear, although their ORF sizes and frameshift positions do not match precisely. Indeed, the RHDV genome appears to encode the capsid sequence within the large polyprotein ORF, whereas the FCV genome contains a stop codon and frameshift between the polyprotein and capsid gene. The Southampton virus genomic organization more closely resembles the arrangement seen in FCV, although the capsid gene appears to be smaller and ORF3 double in size. The high degree of similarity at the amino acid level between Southampton virus (serotype 3) and the partial sequences available for Norwalk virus (serotype 1) further confirms the relatedness of these two viruses.

The sequence and genome organization of SRSVs shows that the virus encodes three ORFs and supports the proposal that it is a member of the Calciviridae. The availability of such nucleotide sequence information will make it possible to develop highly sensitive diagnostic assays and will allow comprehensive molecular epidemiological and phylogenetic studies on this important group of human pathogens.

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18. The family outbreak occurred on 3 July 1991 with the 2-year-old child as the index case. Illness started with vomiting (six episodes) and elevated temperature (38°C) over a 6-hour period. The index case had only one diarrheal episode, which occurred 8 hours after the onset of vomiting; this sample was frozen at −70°C within 1 hour of collection. Both parents simultaneously exhibited symptoms of fever, nausea, vomiting, and explosive diarrhea, which started 22 hours after the first vomiting episode of the index case and lasted for 36 hours. The outbreak source was not identified.
19. Viral RNA was isolated directly from 0.5 g of stool sample (estimated to contain 10^6 virus particles per gram) by extraction with RNAzol B (Biogenesis, Bournemouth, United Kingdom). The RNA was purified from the aqueous phase by adsorption onto silica particles (RNAid, BIO 101, Vista, CA). We produced single-stranded cDNA with ribonuclease (RNase) H^- (minus indicates mutant) Moloney murine leukemia virus reverse transcriptase (Superscript, Gibco BRL, Paisley, United Kingdom) by priming total stool RNA with oligo$(dT)_{20}$, random hexamers, or specific primers. RNA-DNA hybrids were treated with RNase H and purified by Sephacryl S-400 chromatography before polymerase chain reaction (PCR) amplification. Amplification (Perkin-Elmer Cetus Cycler model 9600) of segments of viral cDNA for sequencing was achieved with Taq or Pfu polymerases and oligo$(dT)_{20}$ and specific primers. Extension times of 3 to 5 min were used to favor amplification of relatively long (2- to 4-kb) cDNA molecules. Successful amplification of 3.1-kb and 2.4-kb DNA fragments was achieved with the following primers: NOR3 (5′-TTGATGCAGATTATACAG-3′) (nt 4545 through 4562) versus oligo$(dT)_{20}$ and NOR9 (5′-GCTACGCAGTGTCACCAC-3′) (nt 2380 through 2397) versus SV1 (5′-ACTCTGTCATGATTTGCCTA-3′) (nt 4601 through 4582). The 3.1-kb PCR product that extended from the RNA polymerase gene to the polyadenylated 3′ terminus and the 2.4-kb PCR product that extended from the RNA polymerase gene to the upstream immunogenic region were digested with the restriction endonucleases Hae III, Rsa I, and Alu I and cloned into M13mp8 to generate a series of libraries for sequencing. Direct Taq polymerase sequencing was then performed on both strands of the PCR-amplified cDNA pool. Initially, sequencing was performed manually with Sequenase (U.S. Biochemical Corp., Cleveland, OH) and [α-^{35}S]deoxyadenosine triphosphate; the later stages of the work were completed with Taq DyeDeoxy terminator cycle sequencing on an automated sequenator (Applied Biosystems 373A).
20. To obtain sequence information for PCR amplification of the 5′ terminus of the Southampton virus genome, we used two approaches. A random-primed cDNA library of 3×10^5 recombinants from whole stool total RNA was constructed in λGEM2 (Promega, Southampton, United Kingdom). A single recombinant selected from this library carried a 2.6-kb cDNA insert that stretched from the upstream immunogenic sequence toward the 5′ terminus of the RNA (nt 21 to 2543). Subsequent sequence analysis revealed that this recombinant was truncated by 20 nt from the authentic 5′ terminus of the genome. To define the 5′ terminus of the Southampton virus genome, we used the procedure of M. A. Hofmann and D. A. Brian [*PCR Methods Appl.* 1, 43 (1991)]. Briefly, single-stranded linear cDNA was concatenated with T4 RNA ligase, which joined the 5′ terminus to the 3′ end of the primer used for the initiation of cDNA synthesis. A nested set of internal primers was then used to amplify the region generated by ligation of the 5′ and 3′ termini. Sequence analysis of the ligated junction within this fragment defined the sequence of the 5′ terminus of the genome. A primer complementary to this 5′ terminal sequence was synthesized and used to complete the PCR amplification, which allowed direct nucleotide sequencing of the 5′ terminal 2.2 kb of the Southampton virus genome. The genome analysis was concluded with specific oligonucleotide primers so that the entire sequence of Southampton virus cDNA was determined for both strands by direct PCR sequencing.
21. Computer analysis of the 5′ region of the Southampton viral RNA with the FOLD program [M. Zuker, *Nucleic Acids Res.* 9, 133 (1981)] predicted extensive secondary structures surrounding the putative AUG initiation codon of the polyprotein. This codon is preceded by 21 un-

paired nucleotides which in turn are preceded by four stem-loop structures. The AUG initiator codon itself also overlaps part of a predicted stem-loop structure.
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28. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Activation of the Sphingomyelin Signaling Pathway in Intact EL4 Cells and in a Cell-Free System by IL-1β

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The mechanism of interleukin-1 (IL-1) signaling is unknown. Tumor necrosis factor–α uses a signal transduction pathway that involves sphingomyelin hydrolysis to ceramide and stimulation of a ceramide-activated protein kinase. In intact EL4 thymoma cells, IL-1β similarly stimulated a rapid decrease of sphingomyelin and an elevation of ceramide, and enhanced ceramide-activated protein kinase activity. This cascade was also activated by IL-1β in a cell-free system, demonstrating tight coupling to the receptor. Exogenous sphingomyelinase, but not phospholipases A_2, C, or D, in combination with phorbol ester replaced IL-1β to stimulate IL-2 secretion. Thus, IL-1β signals through the sphingomyelin pathway.

Hydrolyis of sphingomyelin to ceramide at the plasma membrane by a neutral sphingomyelinase may initiate a cascade that functions in signaling (*1*–*6*). Ceramide may stimulate a Ser-Thr kinase termed ceramide-activated protein kinase to transduce the signal (*2*–*4*). Ceramide-activated protein kinase is membrane-bound, Mg^{2+}-dependent, and defined by its capacity to phosphorylate a synthetic peptide (amino acids 663 to 681) derived from the amino acid sequence surrounding Thr^{669} of the epidermal growth factor receptor (EGFR). Ceramide-activated protein kinase may be a member of an emerging family of proline-directed Ser-Thr kinases that includes the extracellular signal-regulated (also referred to as mitogen-activated) and $p34^{cdc2}$ kinases (*7*). Substrates for these kinases contain the minimal recognition sequence, X-Ser/Thr-Pro-X, in which the phosphorylated site is flanked on its COOH-terminus by a proline residue and X can be any amino acid.

Tumor necrosis factor (TNF)–α may use the sphingomyelin pathway for signaling (*3*, *4*, 6). TNF stimulates this pathway early during HL-60 cell differentiation into monocytes, and synthetic ceramide analogs bypass receptor activation and directly induce differentiation (*4*–6). This cascade can be reconstituted in a cell-free system comprised of extracts of HL-60 cells, which demonstrates tight coupling of this pathway to the TNF receptor (*4*). The present studies were performed because of numerous reports that TNF and IL-1 stimulate a common set of events in diverse biologic systems (*8*).

The murine thymoma EL4 cell line is a well-defined IL-1–responsive cell line that expresses functional IL-1 receptors (*9*, *10*). Upon stimulation with IL-1, these cells up-regulate the IL-2 receptor and secrete IL-2 (*10*). Initial studies were designed to investigate the effects of IL-1β on cellular sphingomyelin content. Cells grown in Dulbecco's modified Eagle's (DME)–Ham's F-12 medium containing 10% horse serum and [^{3}H]choline (1 μCi/ml) were resuspended in the same medium at 10 × 10^6 cells per milliliter and stimulated with IL-1β. Under these conditions, IL-1β induced time- and concentration-dependent sphingomyelin hydrolysis (Fig. 1, A and B). A maximally effective concentration of IL-1β (40 ng/ml) induced a detectable reduction by 2 min in sphingomyelin content from a baseline of 800 ± 14 pmol per 10^6 cells (mean ± SEM) to 648 ± 16 pmol per 10^6 cells ($P < 0.005$) (*11*) at 30 min. Concentrations of IL-1β of 0.01 ng/ml were effective, with a maximal effect at 10 ng/ml [effective dose (ED_{50}) ~ 2 ng/ml (Fig. 1B)]. A similar reduction in sphingomyelin content after IL-1 stimulation was determined by direct measurement of phosphorus content (*12*). In contrast, the content of phosphatidylcholine, the other major choline-containing phospholipid, was unchanged.

Under the same conditions, IL-1β in-

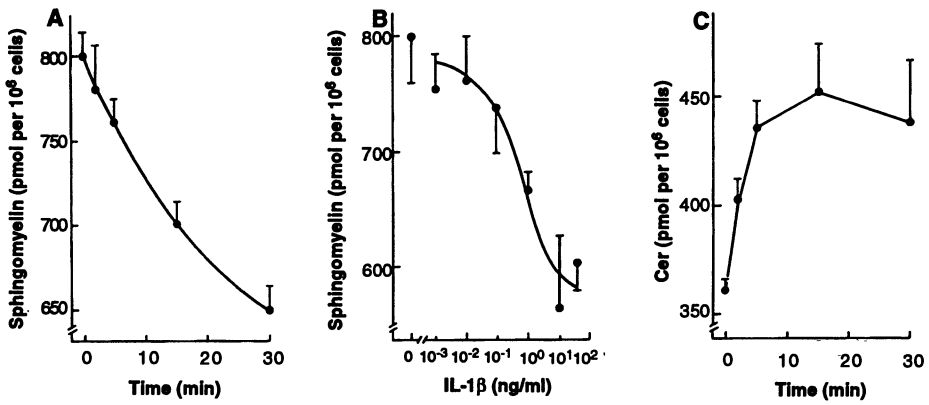

Fig. 1. IL-1β effects on sphingomyelin and ceramide in EL4 cells. (**A** and **B**) Sphingomyelin time course (A) and dose response (B). (**C**) Ceramide time course. Cells were grown to growth arrest (1 to 1.5 × 10^6 cells per milliliter) in DME–Ham's F12 medium (1:1, v/v) containing 10% horse serum. For sphingomyelin measurements, [^{3}H]choline (1 μCi/ml) was added 48 hours before an experiment. The use of [^{3}H]choline as a measure of sphingomyelin content was validated by simultaneous phospholipid phosphorus measurements (*12*). On the day of an experiment, cells were resuspended in the same medium at 10 × 10^6 cells per milliliter and stimulated with IL-1β (40 ng/ml) for the indicated times (A and C) or for 30 min with increasing concentrations of IL-1β (B). After extraction of lipids, sphingomyelin was resolved by TLC (*30*). Ceramide was quantified enzymatically with the use of the *Escherichia coli* diacylglycerol kinase reaction as described (*31*). Each value represents the mean ± SEM of triplicate determinations from four experiments in (A), one representative of four similar studies performed in triplicate in (B), and triplicate determinations from ten experiments in (C).

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