Reversible Inhibition of Bovine Parvovirus DNA Replication by Aphidicolin and L-Canavanine

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(Accepted 27 April 1984)

SUMMARY

The replication of the autonomous parvovirus, bovine parvovirus (BPV), has been studied in virus-infected cells. Gel electrophoresis was used to determine the effect of aphidicolin, a specific inhibitor of DNA polymerase α, and L-canavanine, an inhibitor of protein synthesis, on viral DNA replication. Synchronized cell cultures were infected with 32P-labelled or unlabelled BPV in the presence or absence of aphidicolin and L-canavanine. Cells were harvested at various times post-infection, and DNA was electrophoresed and blotted. When aphidicolin was added to cells at the time of infection, then removed 8 h later, BPV replicative form DNA (RF) synthesis began within 2 h after its removal. This preceded the peak of cellular DNA synthesis by 2 h, unlike an uninhibited infection, when viral RF synthesis follows the peak or S phase by 2 to 4 h. Furthermore, if aphidicolin was added at any point during the replication cycle, BPV DNA synthesis stopped. This effect was shown to be completely reversible and indicated that aphidicolin did not disrupt the replication apparatus required for viral DNA synthesis. L-Canavanine inhibited synthesis of the virus-specific proteins NP-1 and VP3 and synthesis of BPV DNA. Upon removal of L-canavanine, viral protein synthesis was detected by 30 min followed by viral DNA synthesis. These results indicate that a specific S phase function other than cellular DNA synthesis is required for initiation of BPV DNA synthesis, that DNA polymerase α plays a major role in BPV DNA replication in vivo, and that these inhibitors can be used to inhibit reversibly various stages of BPV DNA replication.

INTRODUCTION

Parvoviruses have a single-stranded DNA (ssDNA) genome of 5.0 to 5.5 kb in size (Snyder et al., 1982) with 3' and 5' terminal hairpin structures and are divided into two groups (non-defective and defective) depending upon their requirement for a helper virus during viral replication (for review, see Berns & Hauswirth, 1982). These viruses replicate in the nucleus of proliferating host cells and are cell cycle-dependent, requiring an S phase function for initiation of viral DNA synthesis (Tattersall & Ward, 1978). For the non-defective parvoviruses, several models of DNA replication have been proposed (Astell et al., 1983; Berns & Hauswirth, 1982; Rhode & Klaasen, 1982; Straus et al., 1976). The first step in the replication cycle is the conversion of the 5.0 to 5.5 kb ssDNA to the covalently closed (hairpin) parental replicative form (RF) DNA which is 10 to 11 kb (Rhode, 1974; Straus et al., 1976; Ward & Dadachanji, 1978). The second step involves amplification of the parental RF through a possible dimer intermediate of 20 to 22 kb to daughter RF molecules, which may or may not be covalently closed at one end. Progeny ssDNA genomes are synthesized from replicative intermediates by a strand displacement mechanism. The replicative intermediates have been demonstrated in virus-infected cells, although not all of the postulated replicative enzymes have been identified.

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We have investigated the replication of bovine parvovirus using aphidicolin, a specific inhibitor of DNA polymerase α (Huberman, 1981; Spadari et al., 1982), and the amino acid analogue L-canavanine. L-Canavanine inhibits viral DNA replication as well as viral protein synthesis. We have demonstrated that DNA polymerase α is an essential host enzyme in the replication process and that a specific S phase function other than cellular DNA synthesis is required for virus replication.

**METHODS**

**Cell culture, synchronization and infection.** Preparation of 32P-labelled and unlabelled BPV stocks and growth and synchronization of bovine foetal lung (BFL) cell cultures were as previously described (Robertson et al., 1984). Briefly, BFL cells were seeded into 60 mm Petri plates in the presence of 2 mM-hydroxyurea (HU). After 26 to 32 h, cells were released from synchronization and infected with BPV at a multiplicity of 20 p.f.u./cell. Aphidicolin (20 μM) or L-canavanine (5 mM) was added to the cultures with the medium after adsorption of the virus, or at the times indicated in the individual experiment. For labelling of protein samples, [35S]methionine (5 μCi/ml) was used as described previously (Lederman et al., 1983). To remove the inhibitors, cultures were washed twice with Dulbecco's phosphate buffer and fresh medium was added.

**Sample preparation.** Infected cells were harvested at various times post-infection by washing the cultures twice with Dulbecco's phosphate buffer. For DNA isolation, a modified Hirt procedure was used (Robertson et al., 1984). Cells were lysed in 200 μl 10 mM-Tris-HCl pH 8-0, 10 mM-EDTA, and 0-6% SDS. The lysates were digested with proteinase K (100 μg/ml) for 8 h at 60 °C. The DNA was ethanol-precipitated, resuspended in H2O, heated to 60 °C for 10 min, and stored at 4 °C. For analysis of protein, cells were lysed with 200 μl Laemmlí's (1970) sample buffer containing 1 mM-phenylmethylsulphonyl fluoride and 2.3 units/ml aprotinin. The samples were sonicated briefly to decrease their viscosity and stored at 4 °C until use.

**Gel electrophoresis.** PAGE of the protein samples was carried out by the method of Laemmlí (1970). In some cases, 5 to 15% gradient gels were used with a 4% stacking gel. Gels were stained with Coomassie Brilliant Blue R (Lederman et al., 1983) and fluorographed using En3Hance (New England Nuclear) according to the manufacturer's instructions.

DNA samples were analysed on neutral or alkaline 1-4% agarose gels. For neutral gel electrophoresis, samples were adjusted to 10% formamide, 0-1% bromophenol blue and electrophoresed on submerged horizontal gels (8 mm thickness) with 40 mM-Tris–acetate pH 8-3, 20 mM-sodium acetate, and 2 mM-EDTA as running buffer. Electrophoresis was for 15 h at 50 V or until the dye front had migrated 10 cm. Gels were stained with ethidium bromide (5 μg/ml) and bands were detected under u.v. Alkaline gel electrophoresis was performed according to a modification of the method of Favaloro et al. (1980). Vertical 1-4% agarose gels (40 x 20 x 0-25 cm) containing 50 mM-NaCl and 2 mM-EDTA (pH 8-0) were run in 50 mM-NaOH and 2 mM-EDTA electrophoresis buffer. The gels were prerun for 30 min at 80 V before loading the samples which had been adjusted to 50 mM-NaOH, 2 mM-EDTA, 2-5% Ficoll, and 0-1% bromocresol green. Electrophoresis was for 18 h at 80 V or until the dye front had travelled 18 cm. The buffer was recirculated throughout the run and changed after 12 h. After electrophoresis, the gel was renatured in three changes of 25 mM-sodium phosphate buffer (pH 6-5) for 1 h and stained with ethidium bromide as above.

**Southern blot analysis.** Southern blots of the agarose gels were made using Gene Screen membranes according to the modification of the Southern (1975) method published in the Gene Screen instruction manual and described previously (Robertson et al., 1984). Neutral gels were denatured, neutralized, and blotted for 24 h. Ethidium bromide-stained alkaline gels were passively transferred directly to the Gene Screen membrane for 12 h. Prehybridization and hybridization were performed in the presence of 50% formamide as described by Robertson et al. (1984). All gels were probed with 3 x 10^6 d.p.m. of nick-translated BPV DNA (sp. act. 2.5 x 10^7 to 3.7 x 10^7 d.p.m./μg). All blots were exposed for autoradiography and those containing 32P-labelled BPV input were exposed before and after probing. By using the probe at this specific activity, and by electrophoresing approximately 250000 infected cells per gel lane, we should detect, at the minimum, 1 genome equivalent/cell.

**RESULTS**

**Aphidicolin synchronization of RF DNA synthesis**

It has been shown that in synchronized cell cultures, parvovirus DNA replication does not begin until mid to late S phase after the peak of cellular DNA replication (Parris & Bates, 1976;
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Fig. 1. Aphidicolin synchronization of BPV RF DNA production. Cells were released from HU and infected with $^{32}$P-labelled BPV (a, b, d, e) or unlabelled virus (c) in the presence of 20μM-aphidicolin. After 8 h, aphidicolin was removed and cells were harvested at the times (h) indicated (after removal of aphidicolin). BPV DNA synthesis was monitored by neutral (a, b, c) and alkaline (d, e) gel electrophoresis. (a) Unprobed blot of the neutral gel using $^{32}$P-labelled input virus, 48 h autoradiographic exposure. (b) As (a), but after probing with $^{32}$P-labelled nick-translated BPV DNA, 4 h exposure. (c) Probed blot of a BPV infection using unlabelled virus at 1 to 5 p.f.u./cell, 48 h exposure. (d) Unprobed blot of the alkaline gel using $^{32}$P-labelled input virus, 48 h exposure. (e) As (d), but after probing, 4 h exposure. The positions of BPV dimer-length DNA (dm, 22 kb), dsDNA (ds, 11 kb), ssDNA (ss, 5.5 kb), monomer unit-length DNA (m, 5.5 kb), and the covalently closed hairpin duplex (d, 11 kb) are noted. Other BPV-specific DNA species are indicated by size. Sizes were determined by comparison with restriction fragments of lambda phage DNA.

Robertson et al., 1984; Wolter et al., 1980). We have also shown that this stage of BPV DNA replication is sensitive to aphidicolin (Robertson et al., 1984). To determine the effect on BPV DNA synthesis of delaying cellular S phase, synchronized BFL cells were infected with $^{32}$P-labelled or unlabelled BPV in the presence of aphidicolin. After 8 h, the cells were released from the aphidicolin block, and viral and cellular DNA synthesis were monitored. By analysis of $[^{3}H]$thymidine incorporation (data not shown), it was determined that the cells passed through a normal S phase with the peak of DNA synthesis occurring 4 h after removal of aphidicolin (Robertson et al., 1984).

BPV RF DNA synthesis was analysed during this period. To follow the fate of infecting genomes specifically, $^{32}$P-labelled input virus was used. On the unprobed blot of the neutral gel, BPV input ssDNA (5.5 kb) was converted to RF DNA (dsDNA, 11 kb) within 2 h after the removal of aphidicolin (Fig. 1a) or 2 h before the peak of cellular DNA synthesis, unlike an uninhibited infection where viral RF synthesis follows the peak of S phase by 2 to 4 h (Parris & Bates, 1976; Robertson et al., 1984). This conversion began by 0.5 h post-release and was completed between 1 and 2 h post-release (data not shown). Analysis of the probed blot indicated that amplification of dsDNA (daughter RF synthesis) began between 2 and 4 h post-release (Fig. 1b). In addition, the other BPV DNA species (dimer, 4.5 kb and 2.1 kb) began to appear between 2 and 4 h post-release.
Fig. 2. Aphidicolin inhibition of BPV DNA synthesis. Synchronized BFL cells were released from HU, infected with BPV, and harvested at various times (a, b). (c) Aphidicolin was added after infection at the times (h) indicated and samples were harvested at 4 h after addition of aphidicolin (lanes a), 8 h post-addition (lanes b), and 28 h post-infection (lanes c). Each gel was exposed for autoradiography as follows: (a) 12 h; (b) 6 h; (c) lanes 8a to 10c, 15 h and lanes 11a to 18c, 12 h. The positions of dimer-length DNA (dm), dsDNA (ds) and ssDNA (ss) are indicated in the marker lane (M).

Alkaline gel analysis of this experiment revealed that, as seen on the unprobed blot (Fig. 1 d), the major BPV DNA species present was of monomer unit-length (5-5 kb). Thus, $^{32}$P-labelled input virus was not immediately converted to a detectable, fully linear, covalently closed hairpin molecule (11 kb) (Fig. 1d). Furthermore, on the probed blot (Fig. 1e), species greater than monomer unit-length were not seen until 4 h post-release or concomitantly with the appearance of daughter RF synthesis on neutral gels (Fig. 1b). Also a doublet consisting of 2-2 and 2-0 kb species was observed from 4 h post-release (Fig. 1e). The intensities of these bands suggest that they may have arisen from the 4-5 kb band seen on neutral gels at comparable times (Fig. 1b). Likewise, the 2-1 kb band observed on the neutral gel (Fig. 1b) beginning at 4 h post-release may be the same species as the 1-0 and 0-8 kb bands seen on the alkaline gel (Fig. 1e).

Further analysis demonstrated that the time of conversion of ssDNA to RF was dependent upon the multiplicity of infection (Fig. 1c). If cells were infected with 1 to 5 p.f.u./cell instead of 20 to 25 p.f.u./cell, the time of conversion to RF dsDNA was delayed to 4 h post-release. This effect was not due to an inability to detect earlier conversion since, on a 48 h exposure of the autoradiogram, viral ssDNA could be seen. If aphidicolin was removed prior to 8 h after infection, the time of conversion (i.e. 10 h post-infection) was not altered (data not shown). That is, RF synthesis never occurred earlier than 7 to 8 h after infection, regardless of the presence or absence of aphidicolin during 0 to 8 h post-infection. These data indicate that the completion of cellular DNA synthesis is not required for viral RF synthesis but that some other S phase-specific function is.

**Inhibition of BPV DNA replication by aphidicolin**

Cells were released from synchrony, infected with BPV, and aphidicolin was added at 1 and 4 h, hourly from 6 to 14 h, and at 16 and 18 h post-infection. Cells were harvested at various times after the addition of aphidicolin and at 28 h. After electrophoresis, gels were blotted, and the membranes were probed with nick-translated BPV DNA. A comparison was made between DNA species present at different times during the normal infection (Fig. 2a, b) and between DNA species present in aphidicolin-treated samples (Fig. 2c). Early in the normal infection (4 to 9 h, Fig. 2a), low levels of dsDNA were detected. An increase in dsDNA (11 kb) was first seen at 10 h and continued to accumulate up to 28 h post-infection (Fig. 2a). After aphidicolin was added, the amount of virus-specific DNA did not increase (Fig. 2c) as it did during a normal infection (Fig. 2a, b). Not only was the synthesis of dsDNA and ssDNA inhibited by aphidicolin, but also the synthesis of dimer, 4-5 kb and 2-1 kb species. When aphidicolin was added before progeny DNA was seen (i.e. up to 18 h in this experiment), the production of progeny genomes was inhibited. These results indicate that if aphidicolin was added any time during the normal infection cycle, BPV DNA synthesis was blocked.
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Fig. 3. Reversibility of aphidicolin inhibition of BPV DNA synthesis. BFL cells were infected as described in Methods. At the times indicated after infection, aphidicolin was added and a sample was harvested (lanes a). Four h after the addition of aphidicolin, one sample was released from the aphidicolin block (lanes d) and another was harvested (lanes b). Four h later, both the released sample (lanes d) and an unreleased sample (lanes c) were harvested. The positions of dimer-length (dm), dsDNA (ds) and ssDNA (ss) are indicated.

Fig. 4. L-Canavanine inhibition of BPV DNA synthesis. (a) Cells released from HU and infected with BPV in the presence (lanes 1, 3, 5, 7) or absence (lanes 2, 4, 6, 8) of L-canavanine. Cells were harvested at 10 h (lanes 1 and 2), 12 h (lanes 3 and 4), 14 h (lanes 5 and 6) and 16 h post-infection (lanes 7 and 8). (b) Reversibility of L-canavanine inhibition. At 16 h, infected cells were released from L-canavanine inhibition and samples were harvested at 15 min (lane 1), 30 min (lane 2), 1 h (lane 3) and 2 h (lane 4). The positions of dimer-length DNA (dm), dsDNA (ds) and ssDNA (ss) are indicated.

Reversibility of aphidicolin inhibition

To determine whether aphidicolin inhibition of BPV DNA replication was reversible, cells were infected with BPV and treated with aphidicolin early in infection; then, aphidicolin was removed and progression of viral DNA synthesis monitored. In Fig. 3, aphidicolin was added at 8, 10 or 12 h post-infection. In each case the sample in lane a was harvested immediately, that in lane b was harvested after 4 h of aphidicolin treatment, that in lane c was harvested after 8 h of aphidicolin treatment, and that in lane d was treated with aphidicolin for 4 h and then released for 4 h before harvesting. BPV DNA replication did not proceed further in the presence of aphidicolin (Fig. 3, compare lanes a with b and c for each time of aphidicolin addition). When aphidicolin was removed after a 4 h incubation, BPV DNA synthesis resumed (Fig. 3, lanes d). Furthermore, in each case where BPV DNA synthesis was interrupted by aphidicolin, the BPV DNA species observed (dimer, ds, ss, 4.5 kb, 2.1 kb) were those seen during a normal infection cycle (Fig. 2a). This indicated that, as far as could be determined by gel analysis, DNA replication was proceeding normally. It also demonstrated that addition and removal of aphidicolin could be used to stop and start BPV DNA replication.

L-Canavanine inhibition of BPV DNA synthesis

It has been shown for the defective parvovirus, adeno-associated virus (AAV), that L-canavanine inhibits progeny DNA synthesis and assembly of virus particles by inhibiting viral protein synthesis (Buller & Rose, 1978; Myers & Carter, 1980, 1981). We wanted to define the effect of L-canavanine on BPV DNA replication and the reversibility of its action.

BFL cells were infected with BPV in the presence or absence of 5 mM-L-canavanine. Comparison between a normal infection (Fig. 4a, lanes 2, 4, 6, 8) and one in which L-canavanine was present (Fig. 4a, lanes 1, 3, 5, 7) showed that L-canavanine inhibited BPV DNA replication. At 10, 12 and 14 h (Fig. 4a, lanes 1, 3, 5), the only detectable species of BPV DNA was in the size range of input ssDNA. At 16 h (Fig. 4a, lane 7), some accumulation of dsDNA was apparent. This may have occurred as a consequence of the metabolism of L-canavanine by the cells. Alternatively, the synthesis of RF DNA may continue in the presence of L-canavanine, as is the case for AAV RF DNA synthesis (Myers & Carter, 1981). The synthesis of other BPV DNA species (ssDNA, 4.5 kb and 2.1 kb) was inhibited to a greater extent than was dsDNA.
To determine whether the inhibition of BPV DNA synthesis by L-canavanine was reversible, cells were infected and incubated with 5 mM-L-canavanine for 16 h. The drug was then removed from the cells, and samples were collected for DNA and protein analysis at 15 min, 30 min, 1 h and 2 h post-release. From 15 min to 1 h after removal of L-canavanine an increase in the amounts of dimer, ds- and ssDNA species was detected (Fig. 4b, lanes 1 to 3) when compared to cells continuously in the presence of L-canavanine for 16 h (Fig. 4a, lane 7). However, by 2 h after removal of L-canavanine, the synthesis of all the species approached that seen in a normal infection except the 4.5 and 2.1 kb bands which were under-represented (Fig. 4b, lane 4).

Analysis of the proteins present on Coomassie Brilliant Blue-stained polyacrylamide gels (Fig. 5a) demonstrated that synthesis of the most abundant capsid protein, VP3 (62K mol. wt.) was blocked by L-canavanine, as was synthesis of the smaller non-structural protein, NP-1 (28K) (Lederman et al., 1984). When [35S]methionine was added at release from L-canavanine, label incorporated into NP-1 and VP3 was detectable by 30 min post-release and was continuing to increase by 1 h post-release (Fig. 5b, lanes 4 and 6). These data indicate that L-canavanine inhibited the synthesis of BPV DNA and the viral proteins NP-1 and VP3. Furthermore, this inhibition was reversible.

DISCUSSION

We have investigated the effects of aphidicolin and L-canavanine on BPV DNA replication in virus-infected cells. Both inhibited viral DNA synthesis and upon removal of the inhibitors DNA synthesis resumed. All of the BPV DNA species (dimer-length dsDNA, dsDNA and ssDNA) seen in a normal infection were synthesized after release from the inhibition, as well as 4.5 kb and 2.1 kb species. These latter two species may be by-products of normal replication derived from replicative intermediates, may represent strong stop signals on partially completed molecules or may represent the replication of specific defective genomes.

The rapid resumption of DNA synthesis after removal of aphidicolin (Fig. 3) indicates that aphidicolin does not disrupt the replication machinery responsible for viral DNA synthesis. This is consistent with a previous observation of reversibility of aphidicolin inhibition of cellular DNA synthesis (Pedrali-Noy et al., 1980). When aphidicolin was removed 8 h after infection, BPV input ssDNA was converted to the double-stranded RF DNA before the peak of cellular DNA synthesis was reached (Fig. 1). This observation indicates that S phase-specific events other than cellular DNA synthesis are required for the initiation of viral DNA synthesis. Hardt
et al. (1983) reached a similar conclusion for the replication of minute virus of mice (MVM), based on gradient analysis of aphidicolin inhibition of viral DNA synthesis. Since the initiation of BPV DNA synthesis is dependent upon the multiplicity of infection and since BPV DNA and proteins are known to associate with the nuclear matrix of infected cells (L. Briggs et al., unpublished results), at least a part of the S phase requirement may involve an association with the nuclear matrix.

Cells infected with paroviruses usually generate defective genomes which may be encapsidated into particles of lower density. These probably result from spontaneous deletion mutations giving rise to DNA containing less than one genome equivalent per molecule. Two types of defective genomes have been described for MVM (Faust & Ward, 1979). Type I consists of a family of ssDNA molecules with various sizes of deletions from the internal portion of the molecules. These were preferentially replicated during serial undiluted passage, suggesting that they are replicated at a rate greater than unit-length genomes (Faust & Ward, 1979). Subgenomic molecules (less than 5.5 kb) are present in viral DNA preparations following low multiplicity infections with plaque-purified BPV. At high multiplicities of infection (20 to 25 p.f.u./cell), such DNA molecules can be observed in the DNA prepared from cells following the virus adsorption period (Fig. 1 a, 0 h). At lower multiplicities of infection (1 to 5 p.f.u./cell), the heterogeneity of the input viral DNA was less apparent (Fig. 1 c, 0 h).

Two DNA species in addition to those presumably involved in normal replication are seen on neutral gels of DNA from BPV-infected cells. These are 4.5 kb and 2.1 kb. These two bands appear to yield doublets when the DNA is electrophoresed under alkaline conditions. The 4.5 kb species gives rise to 2.2 and 2.0 kb bands, while the 2.1 kb species yields 1.0 and 0.8 kb bands. These accumulate at a greater rate than does the monomer-length progeny genome. This observation is consistent with previous results demonstrating preferential replication of MVM and H-1 defective genomes (Faust & Ward, 1979; Rhode, 1978) during high multiplicity infections. Since the synthesis of these subgenomic species was inhibited by aphidicolin (Fig. 2), it is apparent that DNA polymerase α is required for their replication, as is the case for the genome-length molecules (Pritchard et al., 1981; Robertson et al., 1984).

L-Canavanine, an arginine analogue, inhibited the synthesis of BPV-specific proteins when added to virus-infected cells (Fig. 5). The production of the predominant capsid protein, VP3, and the non-structural protein, NP-1, was inhibited to a similar extent. In these experiments the concentrations of VP1 and VP2, minor capsid proteins, were too low to determine their sensitivity to L-canavanine inhibition and its reversal. Synthesis of BPV DNA including subgenomic molecules was also inhibited by L-canavanine (Fig. 4). Upon removal of L-canavanine, specific incorporation of [35S]methionine into viral proteins was detected within 30 min (Fig. 5) whereas appreciable DNA synthesis resumed between 1 and 2 h after removal of the inhibitor (Fig. 4). It has been shown for the defective parovirus, AAV, and the non-defective parovirus, H-1, that production of progeny genomes is dependent on viral protein synthesis (Myers & Carter, 1980, 1981; Rhode, 1976). Like AAV, BPV possesses a non-structural protein of comparable molecular weight (24K for AAV, 28K for BPV) which is found in high concentration in infected cells (Lederman et al., 1984) and its synthesis is inhibited by L-canavanine. Also, like AAV, synthesis of the major BPV capsid protein was inhibited by L-canavanine while cellular protein synthesis remained largely unaffected (Fig. 5; Buller & Rose, 1978). The data reported here for BPV are consistent with the hypothesis that the replication of BPV DNA beyond the RF stage is dependent upon the synthesis of one or more viral proteins.

We have demonstrated that aphidicolin will arrest BPV DNA replication when added to infected cells at various times. This includes addition of aphidicolin late in infection following release from L-canavanine inhibition (data not shown) demonstrating a role for DNA polymerase α in the final stages of BPV DNA replication leading to progeny DNA production. This approach will allow synchronization of the steps in BPV DNA replication in vivo, providing the basis for a detailed examination of these events.

We thank Dr Matthew Suffness, Developmental Therapeutics Program, Chemotherapy, National Cancer Institute, for the gift of aphidicolin. This research was supported by grant PCM-8021787 from the National Science Foundation.
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(Received 22 November 1983)