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Measles Virus Synthesizes Both Leaderless and Leader-Containing Polyadenylated RNAs In Vivo

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The minus-sense RNA genome of measles virus serves as a template for synthesizing plus-sense RNAs of genomic length (antigenomes) and subgenomic length [poly(A)$^+$ RNAs]. To elucidate how these different species are produced in vivo, RNA synthesized from the 3′-proximal *N* gene was characterized by Northern RNA blot and RNase protection analyses. The results showed that measles virus produced three size classes of plus-sense *N*-containing RNA species corresponding to monocistronic *N* RNA, bicistronic *NP* RNA, and antigenomes. Unlike vesicular stomatitis virus, measles virus does not produce a detectable free plus-sense leader RNA. Instead, although antigenomes invariably contain a leader sequence, monocistronic and bicistronic poly(A)$^+$ *N*-containing RNAs are synthesized either without or with a leader sequence. We cloned and characterized a full-length cDNA representing a product of the latter type of synthesis. mRNAs and antigenomes appeared sequentially and in parallel with leaderless and leader-containing RNAs. These various RNA species accumulated concurrently throughout infection. However, cycloheximide preferentially inhibited accumulation of antigenomes and leader-containing RNA but not leaderless and subgenomic RNAs late in infection, suggesting that synthesis of the former RNA species requires a late protein function or a continuous supply of structural proteins or both. These results reveal a previously undescribed mechanism for RNA synthesis in measles virus.

The genome of measles virus, a paramyxovirus, is a nonsegmented negative-strand RNA which contains at least eight genes in a 3′-to-5′ order: *N* (for nucleoprotein), *PCV* (for phosphoprotein, protein C, and protein V), *M* (for matrix protein), *F* (for fusion protein), *H* (for hemagglutinin), and *L* (for the putative polymerase) (2, 10–13, 17a, 27, 53–55, 67). A short 55-nucleotide (nt) noncoding sequence termed the leader precedes the *N* gene at the 3′ terminus of the genome (12, 25, 38). This genome organization is similar to that of many other negative-strand RNA viruses, including vesicular stomatitis virus (VSV), rabies virus, Sendai virus, and Newcastle disease virus (1, 7, 18, 21, 29, 58, 59).

During infection, a negative-strand RNA virus synthesizes three distinct classes of plus-sense RNAs from its minus-sense genome. The first class consists of monocistronic RNAs transcribed from individual cistrons, the second consists of bicistronic and polycistronic RNAs produced by readthrough transcription from more than one cistron, and the third consists of complementary copies of the entire genome called antigenomes, which serve as intermediates in genome replication (22, 27, 32, 35, 45, 54, 65, 67). On the basis of extensive in vitro and in vivo studies of the prototypic rhabdovirus VSV, models have been proposed to account for the synthesis of antigenomes and sequential transcription of mRNAs from the same RNA template. According to the stop-and-start model, transcription initiates solely at the 3′ genomic terminus and progresses toward the 5′ end by terminating and reinitiating at intergenic junctions to synthesize (i) a distinct RNA species from the leader sequence called the plus-sense leader and (ii) monocistronic mRNAs from individual cistrons (9, 28). An alternative multiple initiation site model proposes that transcription initiates not only at the 3′ terminus but also at internal intergenic regions, although initiation at any gene still requires prior transcription of the preceding sequence (61). In addition to these transcription models, a replication model has been proposed in which accumulating nucleoprotein specifically binds to nascent plus-sense leader RNA and somehow suppresses termination at the leader-*N* junction to promote readthrough synthesis and concomitant encapsidation of antigenomes (15, 41, 42).

Whether transcription and replication of other negative-strand RNA viruses, such as paramyxoviruses, can be explained by these models remains an important question. Measles virus, Sendai virus, and Newcastle disease virus also synthesize mRNAs sequentially in a 3′-to-5′ direction (21, 31, 33). In vivo, Newcastle disease virus and Sendai virus synthesize a detectable plus-sense leader RNA (40, 42), and nucleoprotein appears to be necessary for replicating genome-length RNA of Sendai virus defective interfering particles in vitro (6). However, repeated efforts to detect a distinct leader RNA species in measles virus-infected cells have failed (12, 25) and little is known about regulation of measles virus RNA synthesis.

To better understand the nature of measles virus RNA synthesis, we cloned and characterized a full-length cDNA representing a poly(A)$^+$ *NP* bicistronic RNA which contained a complete leader sequence. Using this clone as a probe, we examined the pattern, kinetics, and protein requirement of plus-sense RNA synthesis in vivo. The results suggest that plus-sense RNAs are synthesized by a mechanism which is distinct from the current models developed for VSV.

MATERIALS AND METHODS

Cells and viruses. The Edmonston strain of measles virus (kindly provided by C. Miller, University of Southern California School of Medicine) was plaque purified, passaged, assayed in African green monkey kidney (CV-1) cells, and subsequently adapted to HeLa cells (American Type Culture Collection, Rockville, Md.). The Mudd-Summers strain of VSV (Indiana serotype; kindly provided by J. Holland,

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University of California at San Diego) was similarly grown in CV-1 or HeLa cells. CV-1 cells were maintained at 37°C in Eagle minimal essential medium containing 10% fetal bovine serum, and HeLa cells were maintained in the same medium supplemented with nonessential amino acids. Infected cells were maintained in the appropriate medium containing 2% fetal bovine serum. Virus stocks were prepared essentially as previously described (62–64).

Plasmids. pcD-LDR was isolated from an expressible cDNA library generated with poly(A)$^+$ RNA obtained from measles virus-infected CV-1 cells in the Okayama-Berg vector, which contains the simian virus 40 early promoter and polyadenylation signal (49, 66). After transformation of *Escherichia coli* X1776 with a sample from this library, *N* gene-specific cDNAs were identified by colony hybridization by using a ^{32}P-labeled *Pst*I-*Kpn*I 5′ DNA fragment obtained from pcD-N7, a full-length cDNA clone of the measles virus *N* gene (66). By restriction enzyme analysis of positive *N* clones, pcD-LDR was identified by the presence of the *Hgi*A1 site within the leader sequence (12). The structure of this clone was confirmed through sequencing of M13 subclones by the dideoxynucleotide-induced chain termination method (56; Fig. 1b).

Time course experiments. Cultures of CV-1 cells were infected with measles virus at a multiplicity of infection of 0.1. Total RNA was harvested every 2 to 4 h postinfection (p.i.) and analyzed as described below. To examine the effects of cycloheximide on RNA accumulation, separate parallel cultures were replenished with medium with or without 10 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml at 0, 8, 16, or 24 h p.i. At 32 h p.i., RNA was isolated from all of the cultures as described below. In pulse-labeling experiments with [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.), 10 μg of cycloheximide per ml reduced protein synthesis to 20 to 30% of the control values, while 100 μg of cycloheximide per ml reduced protein synthesis to 5% of the control values. However, the two concentrations of cycloheximide had similar effects on viral RNA synthesis and 10 μg of cycloheximide per ml did not visibly alter cell morphology within the duration of the experiment.

RNA isolation. For most experiments, total RNA was prepared from measles virus-infected cells displaying 80 to 100% cytopathic effects or at specified times by use of 4 M guanidine thiocyanate (GSCN; 20). Briefly, cell lysates in 4 M GSCN were centrifuged through a CsCl cushion (1.72 g/ml) at 110,000 × *g* for 16 to 72 h at 20°C in a Beckman SW50.1 rotor as described previously (30). RNA pellets were dried, dissolved in 300 μl of TSE (10 mM Tris chloride [pH 7.5], 1 mM EDTA, 0.2% sodium dodecyl sulfate [SDS]), and precipitated with 0.3 M sodium acetate and 3 volumes of ethanol at −70°C. Poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography (5). In two experiments (see Fig. 3 and 4), total RNA was also prepared by the phenol-SDS method (4, 39) or the LiCl-urea method (47).

Specific size classes of viral RNAs were isolated by electrophoresis in a denaturing 1% agarose gel. Positions of monocistronic, bicistronic, and antigenomic RNA species were identified by Northern blot hybridization of parallel samples (see below), and RNA was electroeluted from gel slices into dialysis bags containing 0.5× running buffer and ethanol precipitated.

Analysis of RNA. (i) Northern (RNA) blot hybridization. RNA was electrophoresed in denaturing 1% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose membranes, and hybridized with a ^{32}P-labeled minus- or plus-sense *N*-specific riboprobe transcribed in vitro by T7 or SP6 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) from the *Xho*I-*Xba*I fragment of pcD-N7 in a pGEM vector (Promega Biotec, Madison, Wis.) (43, 46). After hybridization, filter papers were treated with 10 μg of RNase A (Boehringer Mannheim) per ml for 15 min at room temperature to remove nonspecifically hybridized probe.

(ii) RNase protection assay. The 290-nt 5′ *Bam*HI fragment of pcD-LDR was inserted into the *Bam*HI site of pTZ-18R (Pharmacia, Piscataway, N.J.). A ^{32}P-labeled 354-nt minus-sense leader-*N* riboprobe was transcribed in vitro by T7 RNA polymerase as previously described (46; Fig. 1c and d). An analogous VSV leader-*N* riboprobe was prepared by inserting the *Pst*I fragment of pJS77 (containing approximately 360 nt starting from the 3′ end of the VSV genome) into the *Pst*I site of pTZ18R (pJS77 was kindly provided by J. Keene, Duke University Medical Center; 60), followed by in vitro transcription. Riboprobes were precipitated twice with ethanol in 2 M ammonium acetate and suspended in 50 μl of hybridization buffer {80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.7], 0.4 M NaCl, 1 mM EDTA}. A molar excess of the riboprobe (5 × 10^5 cpm) was added in a volume of 1 μl to RNA samples dissolved in 29 μl of hybridization buffer, denatured for 5 min at 85°C, and incubated for more than 12 h at 30 or 55°C as specified in the figure legends. Samples were digested with 40 μg of RNase A per ml and 2 μg of RNase T_1 (Boehringer Mannheim) per ml in 300 μl of 0.3 M NaCl–10 mM Tris chloride (pH 7.5)–5 mM EDTA for 60 min at 30°C, and reactions were terminated by addition of 50 μg of proteinase K and 20 μl of 10% SDS for 15 min at 37°C or as otherwise specified (see the legends to Fig. 3 and 4). After phenol extraction and ethanol precipitation with carrier tRNA, RNA samples were dissolved in sample buffer containing 80% formamide and separated on 8 M urea–6% polyacrylamide sequencing gels.

Synthetic leader DNA and plus-sense leader RNA. Two 60-nt leader oligonucleotides, 5′-CTAGACCAAACAAAGTTGGGTAAGGATAGTTCAATCAATGATCATCTTCTAGTGCACTTG-3′ and 5′-GATCCAAGTGCACTAGAAGATGATCATTGATTGAACTATCCTTACCCAACTTTGTTTGGT-3′, which contained the complementary measles virus leader sequence (underlined) were synthesized with a Biosearch 8600 DNA synthesizer.

To establish the melting temperature (T_m), one oligonucleotide was 5′ end labeled with [γ-^{32}P]ATP (Dupont, NEN) by T4 polynucleotide kinase (New England BioLabs, Beverly, Mass.), mixed with a 50-fold molar excess of the complementary oligonucleotide, heated to 85°C for 5 min, and annealed at 15°C overnight in 40 mM PIPES (pH 6.4)–0.4 M NaCl–1 mM EDTA–80% formamide. The T_m was determined by incubating samples of hybrids at increasing temperatures and resolving the double- and single-strand oligonucleotides by electrophoresis at 4°C in a nondenaturing 5% polyacrylamide gel, followed by autoradiography.

To generate plus-sense synthetic measles virus leader RNA, the annealed oligonucleotides were inserted into pTZ19R at the *Bam*HI and *Xba*I sites. In vitro transcription by T7 RNA polymerase from this construct linearized at the *Xba*I site produced an 87-nt RNA containing the leader sequence flanked by 31 nt of 5′ and 1 nt of 3′ vector sequences.

RESULTS

Cloning a full-length poly(A)$^+$ bicistronic *NP* RNA which contains a leader sequence. The 5′ terminus of monocistronic

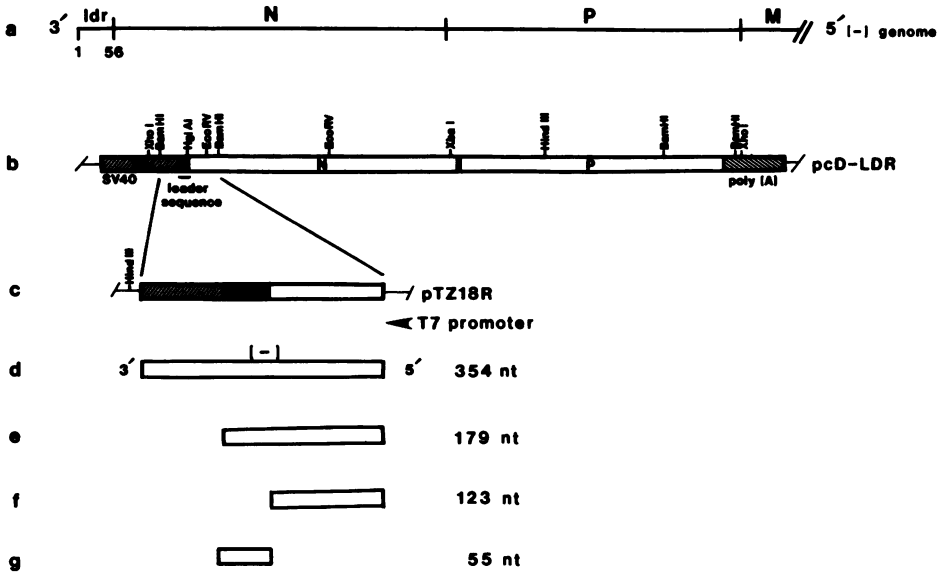

FIG. 1. Structure of a leader (ldr)-containing bicistronic cDNA clone and rationale for the RNase protection assay. (a) 3'-Proximal region of the measles virus genome showing the leader and the *N* and *P* cistrons. (b) Structure of pcD-LDR. Clone pcD-LDR contains the entire leader sequence (filled box), the *N* and *P* genes (open boxes), and an approximately 125-nt-long poly(A) tail. Measles virus-specific sequences are flanked by a vector-derived simian virus 40 early gene promoter and polyadenylation signal (hatched boxes). (c) Subclone of pcD-LDR for generating the leader-*N* riboprobe. The *Bam*HI fragment from the 5' end of pcD-LDR containing the 55-nt leader and 123 nt of the *N* gene was inserted into pTZ18R in an orientation opposite to transcription directed by the T7 promoter. In vitro transcription from this plasmid linearized at the *Hin*dIII site generated a 354-nt minus-sense leader-*N* riboprobe (d). Leader-containing and leaderless RNAs protected 179- and 123-nt regions of this riboprobe from RNase digestion (e and f, respectively). A free plus-sense leader RNA was predicted to protect a 55-nt fragment (g).

mRNA encoding the first (*N*) gene of measles virus has been mapped to a position 56 nt (25) or 57 nt (12) from the 3' terminus of the genome, but a distinct plus-sense leader RNA species transcribed from the preceding 55 or 56 nt has not been detected (12, 25). To address whether the leader sequence is ever included in an RNA species other than genomes and antigenomes, we searched for leader-containing clones in a library rich in measles virus-specific full-length cDNAs (66) and successfully identified one such clone, called pcD-LDR (Fig. 1).

Sequence analysis revealed that this cDNA represents a bicistronic RNA species which initiated at the extreme 3' terminus of the genome and copied through both the *N* and *P* genes without interruption before correctly terminating at the end of the *P* gene by polyadenylation (Fig. 1b). The leader sequence of pcD-LDR, including the 5'-CTT-3' sequence complementary to the trinucleotide found in all intergenic regions, is 55 nt long and corresponds exactly to the sequence reported by Crowley et al. (25). Isolation of a cDNA representing a leader-containing poly(A)$^+$ RNA species raised interesting questions about the mechanism of measles virus transcription.

Leader-containing RNAs present in both total and poly(A)$^+$ RNA preparations. To establish whether leader-containing RNA species are regularly synthesized in vivo and to determine the relative abundancies of such RNAs, we prepared a minus-sense riboprobe which contained the entire leader, the intergenic CTT sequence, and the first 123 nt of the *N* gene from a subclone of pcD-LDR (Fig. 1d and c). This probe was used in an RNase protection assay to quantitate the proportion of *N*-containing RNAs with or without the leader sequence in both total and poly(A)$^+$ RNA preparations from measles virus-infected cells (Fig. 2; Materials and Methods).

Total RNA from infected CV-1 cells protected two major bands approximately 123 and 179 nt long, whereas RNA from uninfected cells did not (Fig. 2, lanes d and e, respectively). The more abundant 123-nt fragment represents leaderless *N*-containing RNA with a 5' end corresponding to nt 56 (Fig. 1f). The less abundant 179-nt fragment represents *N*-containing RNA which initiated at the extreme 3' terminus of the genome (nt 1; Fig. 1e). Since poly(A) selection did not eliminate the 179-nt fragment (Fig. 2, lane c), a leader sequence must be present in at least some poly(A)$^+$ RNAs. Fragments of identical lengths were also protected with RNA from infected HeLa cells but not with RNA from uninfected HeLa cells by using the leader-*N* riboprobe (Fig. 2, lanes f and g, respectively) and in S1 nuclease analysis by using a single-strand leader-*N* DNA probe (data not shown). Quantitation by densitometry revealed that about 5% of total and 2% of poly(A)$^+$ *N*-containing RNA contained a leader sequence.

A protected 55-nt fragment corresponding to free plus-sense leader RNA (Fig. 1g) was not observed in this experiment (Fig. 2, lanes d and f). The faint series of fragments smaller than 123 nt represents nonspecific digestion products, since these species were also observed with poly(A)$^+$ and gel-purified RNAs (Fig. 2, lane c; see Fig. 5).

Measles virus leader sequence is not transcribed into a detectable distinct RNA species. The predicted T_m of a 55-base-pair hybrid corresponding to the measles virus leader sequence is 28.2°C in 80% formamide, but the actual T_m for short hybrids might deviate from calculated values (17, 44). To test more definitively for the presence of a

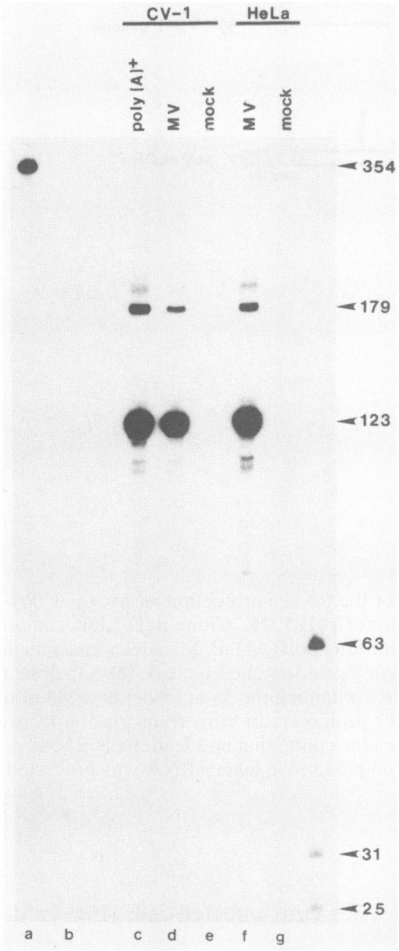

FIG. 2. RNase protection analysis of measles virus (MV) RNAs. A 2.5-μg sample of total RNA purified by the GSCN method from measles virus-infected CV-1 or HeLa cells (lanes d and f, respectively) or mock-infected CV-1 or HeLa cells (lanes e and g, respectively) or 0.5 μg of poly(A)$^+$ RNA from measles virus-infected CV-1 cells (lane c) was annealed with the minus-sense leader-*N* riboprobe (Fig. 1d) at 55°C and digested with RNases A and T_1 at 30°C. Protected fragments were resolved in a 6% sequencing gel along with the undigested or RNase-digested leader-*N* riboprobe (lanes a and b, respectively). Lengths of size markers and protected fragments are shown in nucleotides.

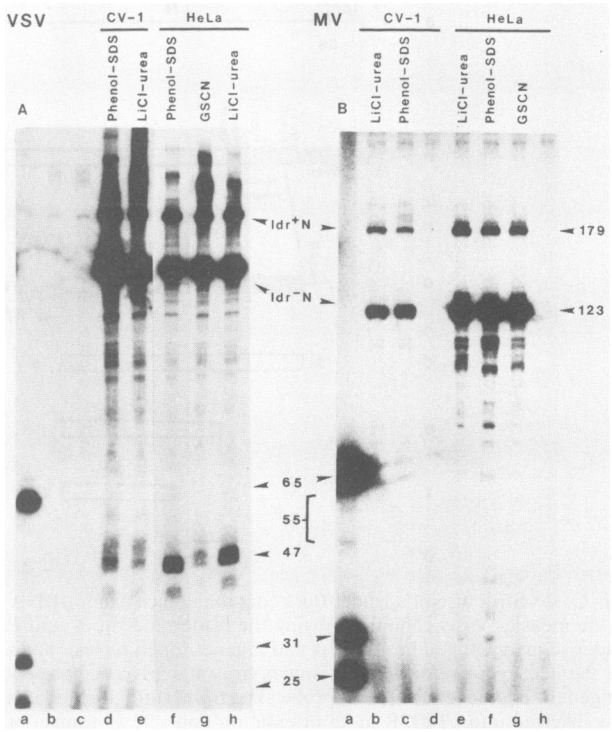

FIG. 3. RNase protection assay detects a distinct plus-sense leader (ldr) RNA in VSV-infected cells but not in measles virus (MV)-infected cells. (A) A 10-μg sample of RNA obtained from mock-infected HeLa cells (lane b) or CV-1 cells (lane c) or VSV-infected CV-1 cells (lanes d and e) or HeLa cells (lanes f to h) purified by the phenol-SDS (lanes d and f), LiCl-urea (lanes e and h), or GSCN (lane g) method was annealed with a VSV-specific leader-*N* riboprobe at 30°C, digested with RNases A and T_1 at 15°C, and separated on a 6% sequencing gel. (B) A 10-μg sample of RNA from mock-infected CV-1 cells (lane d) or HeLa cells (lane h) or from measles virus-infected CV-1 cells (lanes b and c) or HeLa cells (lanes e to g) purified by the LiCl-urea (lanes b and e), phenol-SDS (lanes c and f), or GSCN (lane g) method was annealed with the minus-sense leader-*N* riboprobe and analyzed as described for panel A. The lengths of size markers (lanes a) and protected fragments are indicated in nucleotides.

putative free leader RNA species, we synthesized two single-strand DNAs which contained 55 nt of complementary sequences corresponding to the leader region (plus a total of 5 extra nt at the termini for cloning purposes) and empirically determined that the T_m of the double-strand hybrid formed by these DNAs was 33°C (see Materials and Methods). Since an RNA-RNA hybrid is more stable than a comparable DNA-DNA hybrid, a putative free leader RNA should stably anneal with the minus-sense leader-*N* riboprobe at temperatures below 33°C (17).

Using a hybridization temperature of 30°C, we searched for a free leader RNA species in measles virus-infected cells. As a control, we performed identical analyses with parallel cell cultures infected with VSV by using an analogous minus-sense leader-*N* riboprobe derived from a VSV cDNA subclone (see Materials and Methods). To minimize potential loss of a putative free leader RNA due to technical reasons, three different approaches were used to isolate RNA: the phenol-SDS method predominantly used in many VSV studies (14, 39); the LiCl-urea method, which has also been shown to recover small RNA species (47); and the GSCN method, which maximizes RNA yield while minimizing RNA degradation (20). With these approaches, we observed a protected 47-nt RNA band corresponding to free plus-sense leader RNA in VSV-infected CV-1 cells (Fig. 3A, lanes d and e) or HeLa cells (Fig. 3A, lanes f to h) but no 55-nt RNA fragment corresponding to a free leader RNA in measles virus-infected CV-1 cells (Fig. 3B, lanes b and c) or HeLa cells (Fig. 3B, lanes e to g). Interestingly, the levels of the VSV free leader relative to those of the other RNA species were consistently lower in CV-1 cells.

Synthetic free measles virus leader RNA recovered and detected in reconstruction experiments. The apparent absence of a free plus-sense leader RNA in measles virus-infected cells was inconsistent with the current models of VSV RNA synthesis. To show that a putative free measles virus leader RNA could be detected if present and to ascertain the sensitivity of the above-described assay, we performed reconstruction experiments with an 87-nt RNA species containing the plus-sense leader sequence of measles virus

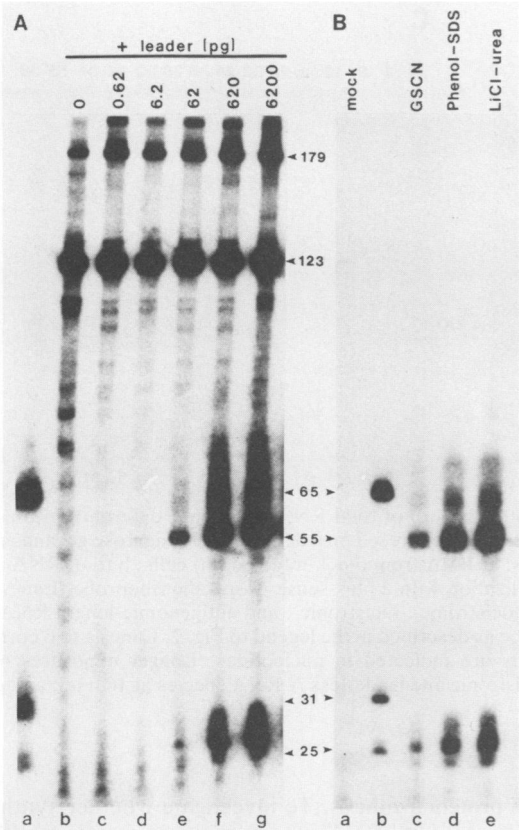

FIG. 4. Recovery and detection of in vitro-generated plus-sense measles virus leader RNA. (A) Plus-sense RNA containing the measles virus leader sequence was generated in vitro by T7 RNA polymerase as described in Materials and Methods. A 5-μg sample of total RNA from measles virus-infected CV-1 cells was mixed with none (lane b), 0.62 pg (lane c), 6.2 pg (lane d), 62 pg (lane e), 620 pg (lane f), or 6.2 ng (lane g) of this artificial leader RNA, and the resulting samples were annealed with the leader-*N* riboprobe and analyzed as described in the legend to Fig. 3. (B) In vitro-generated leader RNA (1.4 μg) was mixed with uninfected HeLa cell lysates containing approximately 5.5 mg of total RNA. (This was equivalent to the expected molar amount of monocistronic *N* RNA in the same number of measles virus-infected HeLa cells.) RNA was purified by the GSCN (lane c), phenol-SDS (lane d), or LiCl-urea (lane e) method and analyzed as described above. The lengths of size markers (panel A, lane a; panel B, lane b) and protected fragments are shown in nucleotides.

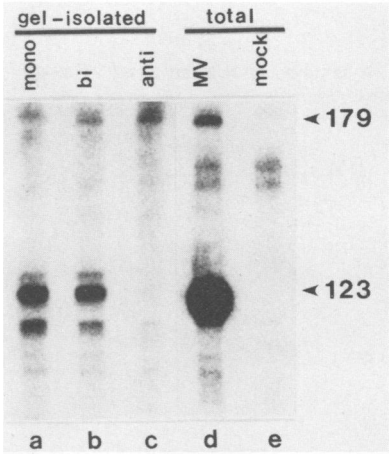

FIG. 5. RNase protection analysis of gel-isolated RNA species. A 150-μg sample of total RNA from measles virus (MV)-infected CV-1 cells was electrophoresed in a denaturing 1% agarose gel. Monocistronic, bicistronic, and antigenomic RNAs were electroeluted from gel slices, and one-third of the recovered RNA was analyzed by an RNase protection assay as described in the legend to Fig. 2. Lanes: a to c, fragments protected by gel-isolated monocistronic *N*, bicistronic *NP*, and antigenomic RNAs, respectively; d and e, fragments protected by total RNA extracted from measles virus-infected or mock-infected CV-1 cells, respectively. Fragment lengths are indicated in nucleotides.

synthesized in vitro by a T7 RNA polymerase reaction (see Materials and Methods). Increasing amounts of this artificial leader RNA were mixed with 5 μg of total RNA purified from measles virus-infected CV-1 cells. In addition to the 179- and 123-nt RNA species described above, our assay method detected as little as 6 pg of the artificial leader RNA species (Fig. 4A, lanes d to g). Furthermore, when this artificial leader was added to uninfected HeLa cell lysates in an amount equivalent to that of *N* RNA in measles virus-infected cells, it was quantitatively recovered by the GSCN, phenol-SDS, and LiCl-urea methods (Fig. 4B, lanes c to e, respectively).

These reconstruction experiments indicated that the failure to detect a measles virus plus-sense free leader was not due to competition by leader-containing RNA or other technical limitations. Taken together, these lines of evidence strongly suggest that measles virus does not synthesize a detectable leader RNA species. However, the leader sequence is copied as part of the antigenome and some *N*-containing poly(A)$^+$ RNAs.

Antigenomes invariably contain a leader sequence, but monocistronic and bicistronic *N*-containing RNAs are synthesized either with or without a leader. To directly demonstrate the presence of a leader sequence in subgenomic-length RNAs and to determine the distribution of leader-containing RNAs among the monocistronic, bicistronic, and antigenomic RNAs, we purified these species by gel isolation and tested for the presence of a leader sequence in each. Both monocistronic and bicistronic RNAs protected the 179- and 123-nt fragments with the leader-*N* riboprobe (Fig. 5, lanes a and b, respectively). In contrast, antigenomes protected only the 179-nt fragment (Fig. 5, lane c). The gel-purified monocistronic and bicistronic RNA preparations contained no detectable antigenomic RNA (data not shown). Thus, these results unequivocally demonstrate that portions of both monocistronic and bicistronic RNAs contain leader sequences covalently joined to the *N* gene.

Accumulation of measles virus RNAs during infection. (i) Differential accumulation of monocistronic, bicistronic, and antigenomic RNAs. To further examine the relationship among the various measles virus RNA species, accumulation of plus-sense RNAs was examined by Northern blot hybridization with a minus-sense *N*-specific riboprobe (see Materials and Methods). Monocistronic *N* and bicistronic *NP* RNAs were first detected at about 16 and 20 h p.i., respectively (Fig. 6A, lanes e and f, respectively), whereas antigenomes were not detected until about 24 h p.i. (Fig. 6A, lane h). All three RNA species continued to accumulate until about 32 h p.i. Thereafter, monocistronic, bicistronic, and antigenomic *N*-containing RNAs maintained a relatively constant ratio of approximately 18:2.5:1, respectively. A tricistronic *NPM* RNA species was not observed.

When the RNA samples from this experiment were hy-

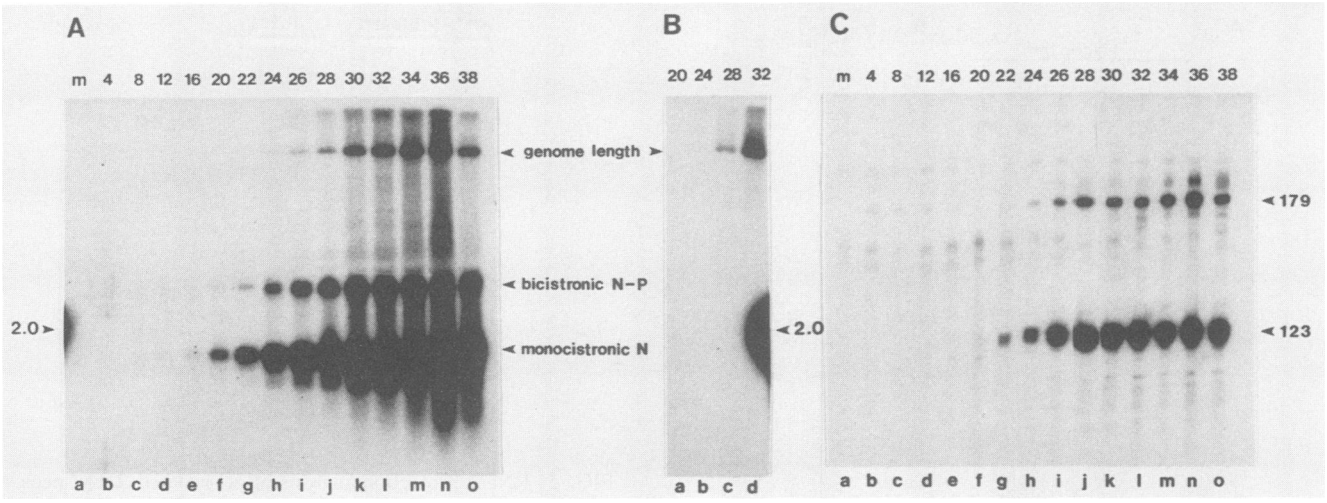

FIG. 6. Time course of *N*-containing RNA accumulation. (A) Equal amounts (2.5 μg) of total RNA isolated at different times p.i. from parallel cultures of measles virus-infected CV-1 cells by the GSCN method were electrophoresed on a denaturing 1% agarose gel and analyzed by Northern blot hybridization with a minus-sense *N*-specific riboprobe. Lanes: a, RNA from mock-infected (m) cells; b to o, RNA isolated from infected cells at the indicated times (hours p.i.). (B) Northern blot hybridization with a plus-sense *N*-specific riboprobe. Lanes a to d, RNA isolated at the indicated times (hours p.i.). The arrowheads indicate monocistronic-, bicistronic-, and antigenomic-length RNAs and a 2.0-kilobase size marker. (C) RNase protection assay with the leader-*N* riboprobe as described in the legend to Fig. 2. Lanes a to o correspond to the RNA samples shown in panel A. The lengths of protected fragments are indicated in nucleotides. Longer exposures of these autoradiograms (data not shown) confirmed the concurrent detection of monocistronic and leaderless *N* RNA species at 16 h p.i. and genome length and leader-containing *N* RNA species at 24 h p.i.

bridized with a plus-sense *N*-specific riboprobe, minus-sense genomes were first detected at about 24 h p.i., corresponding to the time when antigenomes were first observed (Fig. 6B, lane b, and 6A, lane h). Accumulation of genomes and antigenomes proceeded in parallel and simultaneously slowed at about 32 h p.i., when cell lysis began (Fig. 6A, lanes j to l, and 6B, lanes c and d; only representative time points are shown in Fig. 6B).

These results, which were reproduced in three separate experiments, suggest that monocistronic and bicistronic RNAs are detected before antigenomes and genomes, but all three RNA species accumulate concurrently after 24 h p.i.

(ii) Accumulation of leader-containing RNAs. When the RNA preparations used in the time course experiment were examined by RNase protection with the leader-*N* riboprobe, the 123-nt fragment representing leaderless *N* RNA first became detectable at 16 h p.i. (Fig. 6C, lane e), corresponding to the initial detection of monocistronic *N* RNA (Fig. 6A, lane e). Unexpectedly, a low but constant level of the 179-nt fragment, representing leader-containing *N* RNA, was detected in samples collected as early as 4 h p.i. (Fig. 6C, lane b). The origin of this RNA species is currently under investigation. More important, initial detection of antigenomes by Northern blot hybridization at 24 h p.i. was accompanied by an increase in leader-containing RNA (Fig. 6A, lane h, and 6C, lane h). However, the relative ratio of leader-containing RNA to leaderless RNA was significantly higher than the relative ratio of antigenomes to monocistronic and bicistronic RNAs combined (compare the 179- and 123-nt bands with the antigenomic, monocistronic, and bicistronic RNAs in Fig. 6A and C after the 24-h time point). This disparity confirms that a significant portion of leader-containing RNA represents not only antigenomes but also monocistronic and bicistronic RNAs, as demonstrated by the experiments shown above.

Synthesis of leader-containing RNA and antigenomes requires protein synthesis. To investigate whether synthesis of the different viral RNA species requires protein synthesis, a sublethal dose of cycloheximide was added at different time points to measles virus-infected cells, and RNA harvested at 32 h p.i. was analyzed by Northern blot hybridization with an *N*-specific probe.

Cycloheximide added at or before 8 h p.i. blocked accumulation of all viral RNAs (Fig. 7A, lanes a to j). Adding cycloheximide at 16 h p.i. greatly decreased the levels of monocistronic and bicistronic *N*-containing RNAs and reduced antigenomes to an undetectable level (Fig. 7A, lanes k to o). Interestingly, if cycloheximide was added at 24 h p.i., after significant levels of monocistronic and bicistronic RNAs had accumulated (Fig. 6), these two RNA species continued to accumulate to a level two- to threefold higher than that in infected cells without cycloheximide treatment, while antigenomes were reduced by fourfold in the same cells (Fig. 7A, lanes p to t).

Analysis of the same RNA samples by RNase protection assay revealed that cycloheximide added at up to 16 h p.i. reduced accumulation of both leader-containing and leaderless RNAs (Fig. 7B, lanes a to o). However, adding cycloheximide at 24 h p.i. significantly reduced the 179-nt fragment but caused a slight increase in the 123-nt fragment (Fig. 7B, lanes p to t).

These results imply that synthesis of leaderless monocistronic and bicistronic RNAs is insensitive to cycloheximide once sufficient viral proteins have accumulated. Since measles virus carries an active RNA polymerase into host cells (57), cycloheximide treatment early in infection most likely affects amplification of virus-encoded products rather than inhibiting primary transcription. However, late in infection, preferential inhibition of antigenome accumulation and, to a lesser extent, leader-containing RNA accumulation suggests that synthesis of these species requires a late protein function or continuous protein synthesis or both.

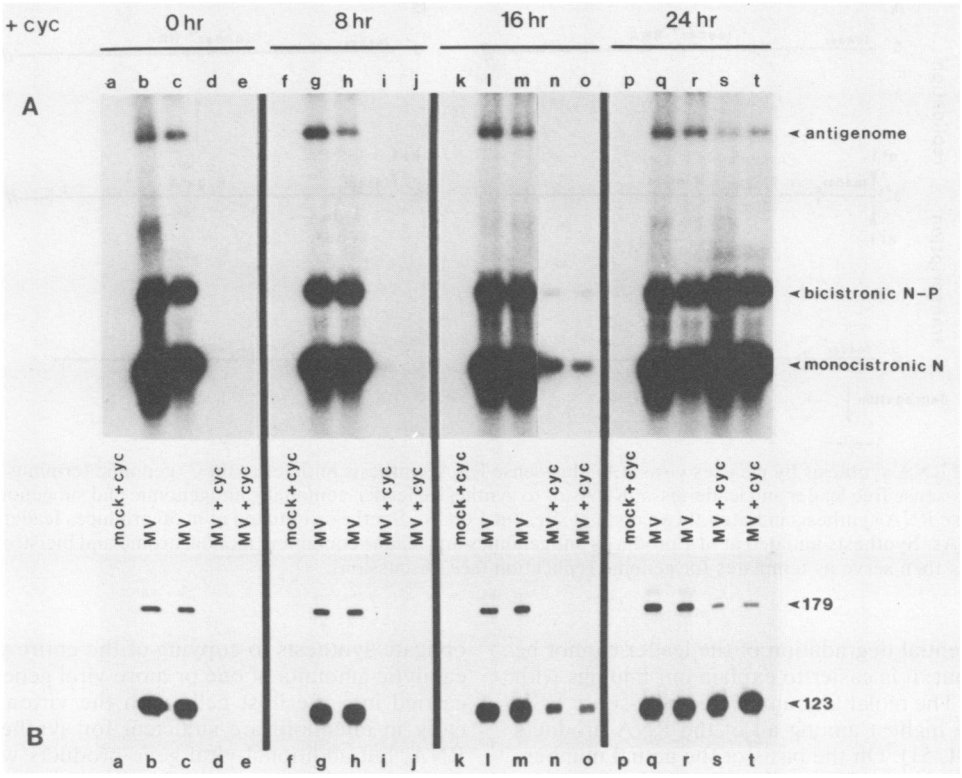

FIG. 7. Effect of cycloheximide (cyc) on accumulation of *N*-containing RNAs. At 8-h intervals, duplicate cultures of measles virus (MV)-infected CV-1 cells were replenished with fresh medium with or without 10 μg of cycloheximide per ml. Total RNA was purified by the GSCN method from all of the cultures at 32 h p.i. (A) A 5-μg portion of RNA from each sample was electrophoresed in a denaturing 1% agarose gel and analyzed by Northern blot hybridization with a minus-sense *N*-specific riboprobe. (B) A 5-μg portion of RNA from each sample was also examined by an RNase protection assay with the leader-*N* riboprobe as described in the legend to Fig. 2. Lanes: a, f, k, and p, mock-infected cells treated with cycloheximide; b, c, g, h, l, m, q, and r, RNAs from duplicate cultures of measles virus-infected cells without cycloheximide treatment; d, e, i, j, n, o, s, and t, RNAs from duplicate cultures of measles virus-infected cells treated with cycloheximide. The sizes of protected fragments are indicated in nucleotides.

DISCUSSION

Although details of the transcription mechanism for any negative-strand RNA virus have not been completely understood, studies with VSV have led to several models. An early processing model hypothesized that mRNAs were derived from cleavage and modification of polycistronic precursors (23, 24), but several findings have rendered this model unlikely (reviewed in reference 8). An alternative stop-and-start model suggests that transcription is invariably initiated at nt 1 and mRNAs are generated by sequential terminations and reinitiations at intergenic regions (9, 28). Although some in vitro evidence supports such a mechanism (28, 37, 52), other studies have suggested a multiple initiation site model in which transcription is initiated at intergenic regions as well as at the 3′ genomic terminus (19, 48, 61). Although differing in the number of polymerase entry sites, both models postulate that transcription of each gene requires prior transcription of the preceding sequence, and thus synthesis of a free leader RNA is a requisite for transcription of all subsequent genes (9, 61).

Intensive searching has failed to detect a free leader RNA species in measles virus-infected cells (12, 25; Fig. 3 and 4). Instead, measles virus copies the leader sequence as the 5′ termini of antigenomes and a subpopulation of monocistronic and bicistronic *N*-containing RNAs (Fig. 2 and 5). The absence of a free leader RNA thus distinguishes measles virus from all other nonsegmented negative-strand RNA viruses analyzed and raises questions about the applicability of the VSV transcription models to measles virus.

Our current data are consistent with at least two alternative mechanisms depicted in Fig. 8. In the first model, measles virus initiates plus-sense RNA synthesis only at the 3′ genomic terminus and transcribes mRNA through a stop-and-start mechanism. However, the plus-sense leader RNA is rapidly degraded unless specifically protected by some viral gene product, such as nucleoprotein. In the latter case, termination at the leader-*N* junction is suppressed, resulting in synthesis of leader-containing antigenomic and subgenomic RNAs (Fig. 8A). Except for synthesis of an unstable free leader RNA, this model is similar to the stop-and-start model for VSV (9, 28). In the second model, measles virus synthesizes plus-sense RNAs from at least two independent initiation sites (Fig. 8B). Initiation at the extreme 3′ terminus of the genome (nt 1) generates leader-containing antigenomic and subgenomic RNAs, whereas synthesis initiated at the beginning of the *N* gene (nt 56) produces leaderless mRNAs. This second model differs significantly from the models proposed for VSV, since transcription of the leader sequence is not necessary for transcription of the *N* gene. Neither of these models addresses how internal genes might be transcribed, and in both cases, leader-containing monocistronic *N* and bicistronic *NP* RNAs could represent aborted attempts at antigenome replication.

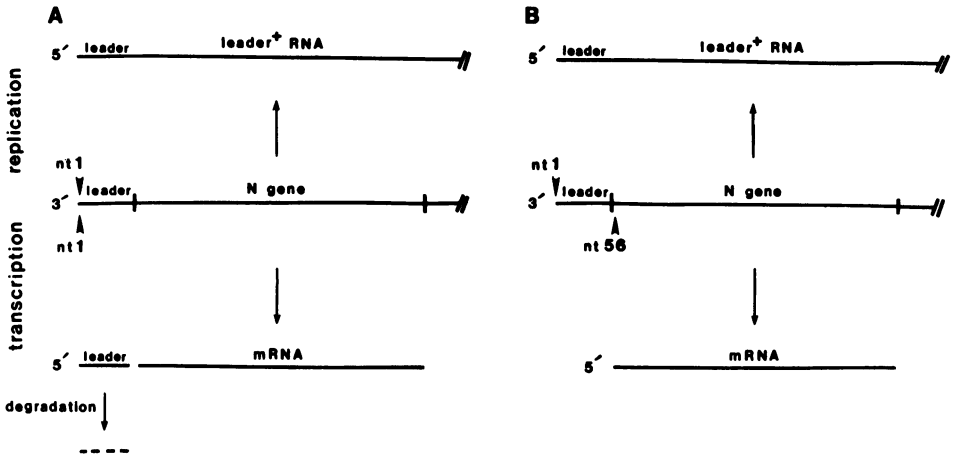

FIG. 8. Models of RNA synthesis by measles virus. (A) Plus-sense RNA synthesis initiates at the 3′ genomic terminus (nt 1) to transcribe a highly unstable plus-sense free leader and leaderless mRNAs or to synthesize leader-containing antigenomic and subgenomic RNAs (leader$^+$ RNAs). (B) Plus-sense RNA synthesis initiates at two distinct sites (nt 1 or 56). Synthesis initiated at nt 56 produces leaderless monocistronic and bicistronic mRNAs. Synthesis initiated at nt 1 produces antigenomes and leader-containing monocistronic and bicistronic RNAs (leader$^+$ RNAs). Antigenomes then serve as templates for genome replication (see Discussion).

Although preferential degradation of the leader cannot be completely ruled out, it is easier to explain our findings with the second model. The molar amount of free plus-sense VSV leader RNA is the highest among all of the RNA products (Fig. 3A; 16, 24, 34, 51). On the basis of the actual numbers of *N*-specific cDNA clones in our library and Northern blot comparison with a known amount of *N*-specific RNA transcribed in vitro (see Materials and Methods), we estimated that 10 μg of total RNA from measles virus-infected HeLa cells contains about 80 ng of the monocistronic 1,690-nt *N* mRNA. An equimolar amount of a putative 55-nt free leader RNA would thus be equivalent to (55/1,690) × 80 ng = 2.6 ng. Since our RNase protection assay detected 6 pg of the artificial leader RNA (Fig. 4A), we would have detected a putative free leader RNA species even if it were present at less than 1/400 of the molar level of monocistronic *N* RNA. Furthermore, reconstruction experiments efficiently recovered and detected an artificial measles virus leader RNA species (Fig. 4B). It is difficult to explain why VSV leader RNA is stable while a putative measles virus leader RNA is not, since both the free VSV leader and leader-containing measles virus RNAs complex with nucleoproteins in a manner which protects them from RNase digestion (14, 15; manuscript in preparation). If mRNA transcription requires prior transcription of the leader sequence, then a free leader species should be present at times when both leaderless and leader-containing RNAs are detected, since the mechanism which protects leader-containing RNAs should similarly protect the putative free leader. However, both leaderless and leader-containing RNAs are synthesized concurrently in the apparent absence of a free leader RNA during measles virus infection (Fig. 3B).

Transcription and replication of measles virus RNA might have different protein requirements, since late in infection cycloheximide reduced leader-containing and antigenomic RNAs but not leaderless or monocistronic and bicistronic RNAs (Fig. 7). Interestingly, synthesis of leader-containing RNA was more sensitive to cycloheximide than was that of leaderless RNA, and synthesis of antigenomes was still more sensitive to cycloheximide than was that of leader-containing RNA (Fig. 7). Thus, copying of the leader sequence is required for antigenome synthesis but does not necessarily obligate synthesis to copying of the entire genome. Perhaps catalytic amounts of one or more viral gene products (either carried into the host cells with the virions or synthesized early in infection) are sufficient for synthesis of leaderless RNA, but additional viral gene products which accumulate during infection are required for initiation at nt 1. Elongation of the resulting leader-containing RNA into full-length antigenomes might further require stoichiometric amounts of additional viral gene products, perhaps in a structural role. Several studies with VSV and other paramyxoviruses indicate that nucleoprotein might serve this latter function (3, 6, 26, 36, 50). Consistent with this hypothesis is the fact that the leader sequence appears to be involved in encapsidation of measles virus RNAs by nucleoprotein (manuscript in preparation).

In conclusion, measles virus exhibits a unique pattern of RNA synthesis previously undescribed for a nonsegmented negative-strand RNA virus. In light of these results, the transcription and replication mechanisms of other negative-strand RNA viruses might warrant further investigation.

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