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Cloning of Minute Virus of Mice cDNAs and Preliminary Analysis of Individual Viral Proteins Expressed in Murine Cells

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cDNAs corresponding to RNA from the autonomous parvovirus minute virus of mice were cloned into constitutive and inducible expression vectors. These clones generate viral NS2, VP1, and VP2 proteins individually. Initial examination of these clones by transient expression analysis and analysis of stably transformed murine cell lines inducibly expressing these constructs indicated that they will be useful tools for characterizing the function of individual minute virus of mice gene products.

The single-stranded 5-kilobase (kb) genome of the autonomous parvovirus minute virus of mice (MVM) is organized into two overlapping transcription units (Fig. 1A) (1, 2, 20). Transcripts R1 (4.8 kb) and R2 (3.3 kb), which are generated from a promoter at map unit (m.u.) 4 (2, 4), encode the viral nonstructural proteins NS1 (84 kilodaltons) and NS2 (25 kilodaltons), respectively, from open reading frames (ORFs) in the left half of the viral genome (8, 9). The R3 (3.0-kb) transcripts, initiated from a promoter at m.u. 38, encode the overlapping viral capsid proteins VP1 (83 kilodaltons) and VP2 (64 kilodaltons) from the ORF in the right half of the MVM genome (2, 9, 14, 20). All MVM mRNAs are complementary to the genomic DNA strand and are polyadenylated at the far-right-hand end of the genome (6, 20). Three splicing patterns are used to excise a small intron at m.u. 44 to 46 common to all three transcript classes, resulting in nine different spliced RNA species (2, 13, 18). The R2 transcripts are additionally spliced betwen m.u. 10 and 39 (1, 2, 9, 13, 18, 20).

Analysis of MVM RNA, utilizing cDNA clones and junction-specific oligonucleotides, has indicated that, for R1, R2, and R3, the most frequently used splicing pattern of the small 44- to 46-m.u. intron joins nucleotides (nt) 2280 to 2377, a splicing pattern linking nt 2317 to 2399 is used less frequently, and a splicing pattern joining nt 2280 to 2399 has been observed as a rare species (13, 18). A fourth pattern, uniting nt 2317 with 2377, has not been observed and may be too small (59 nt) for efficient splicing (18). The large intron in R2 is excised, using a single donor (nt 514) and acceptor (nt 1990) site (13).

NS1 is encoded in ORF 3, which terminates at nt 2276, upstream from the first small intron donor site at nt 2280 (9). Therefore, synthesis of NS1 is unaffected by differential splicing of the small intron. NS2 shares 84 amino-terminal residues with NS1 in ORF 3 and switches to ORF 2 as the large R2 intron is spliced (9). Alternative splicing of the small intron can theoretically yield three NS2 isoforms differing in the final 6 to 15 amino acids at the carboxyl terminus (1, 2, 13, 18).

VP1 is encoded by the subset of R3 transcripts that excise the small intron at the nt 2317 donor site (1, 9). When this splicing pattern is used, the AUG at nt 2286 is utilized, and a 10-amino-acid ORF is spliced in frame to ORF 1, which encodes the remainder of VP1. VP2 is encoded by the subset of R3 transcripts that excise the small intron at the nt 2280 donor site. This removes the VP1 AUG at 2286, and a downstream AUG at nt 2794 initiates VP2 in ORF 1 (2, 14). The relative ratio in which the capsid proteins appear during infection is proportional to the frequency that the different splice sites are used (7).

Because of the overlapping organization of the parvovirus genome, it has been difficult to assign specific functions exclusively to individual gene products. While some headway has been gained in various parvovirus systems by using standard mutagenesis approaches (12, 22, 26, 27, 29), we have proceeded to isolate MVM cDNAs in order to assay individual MVM proteins in both transient analysis and within stably transformed cell lines. These cell lines might additionally be used to complement viral mutants. In this report, we describe the construction of cDNA copies of the two small intron junctions at m.u. 44 to 46 that are used predominantly, the large intron junction of R2, and their cloning into various constitutive and inducible expression vectors. A preliminary characterization of murine cell lines expressing individual MVM gene products will be presented.

Construction of cDNAs. A 15-μg sample of total RNA taken 24 h postinfection (6) from synchronized (10) MVM(p)-infected human NB324K fibroblasts was hybridized to 7.5 pmol of an oligonucleotide (oligo B, nt 2661 to 2685) complementary to MVM mRNA downstream from the small intron (Fig. 1A) and extended by using 14 U of avian myeloblastosis virus reverse transcriptase (Promega Biotec, Madison, Wis.) for 3 h at 42°C (11). The complementary strand was synthesized by denaturing and annealing the RNA-cDNA hybrid to a second oligonucleotide (oligo A, nt 327 to 346) complementary to the cDNA strand upstream from the large intron donor site (Fig. 1A). The primer was extended by using the thermostable *Taq* (*Thermus aquaticus*) DNA polymerase (Stratagene, La Jolla, Calif.) (25). The double-stranded cDNA was further amplified for 30 consecutive cycles of DNA synthesis in the presence of both oligonucleotide primers (1 μM) and *Taq* polymerase (2 U). Only cDNAs generated from the R1 and R2 transcripts were amplified, since the cDNA-complementary oligonucleotide (oligo A) failed to hybridize to R3-generated cDNAs (Fig. 1A) which terminate at nt 2003 (2, 4, 20).

Amplified cDNAs were digested with *Eco*RV and *Hin*dIII, which cut at MVM nt 385 and 2652, respectively, internal to

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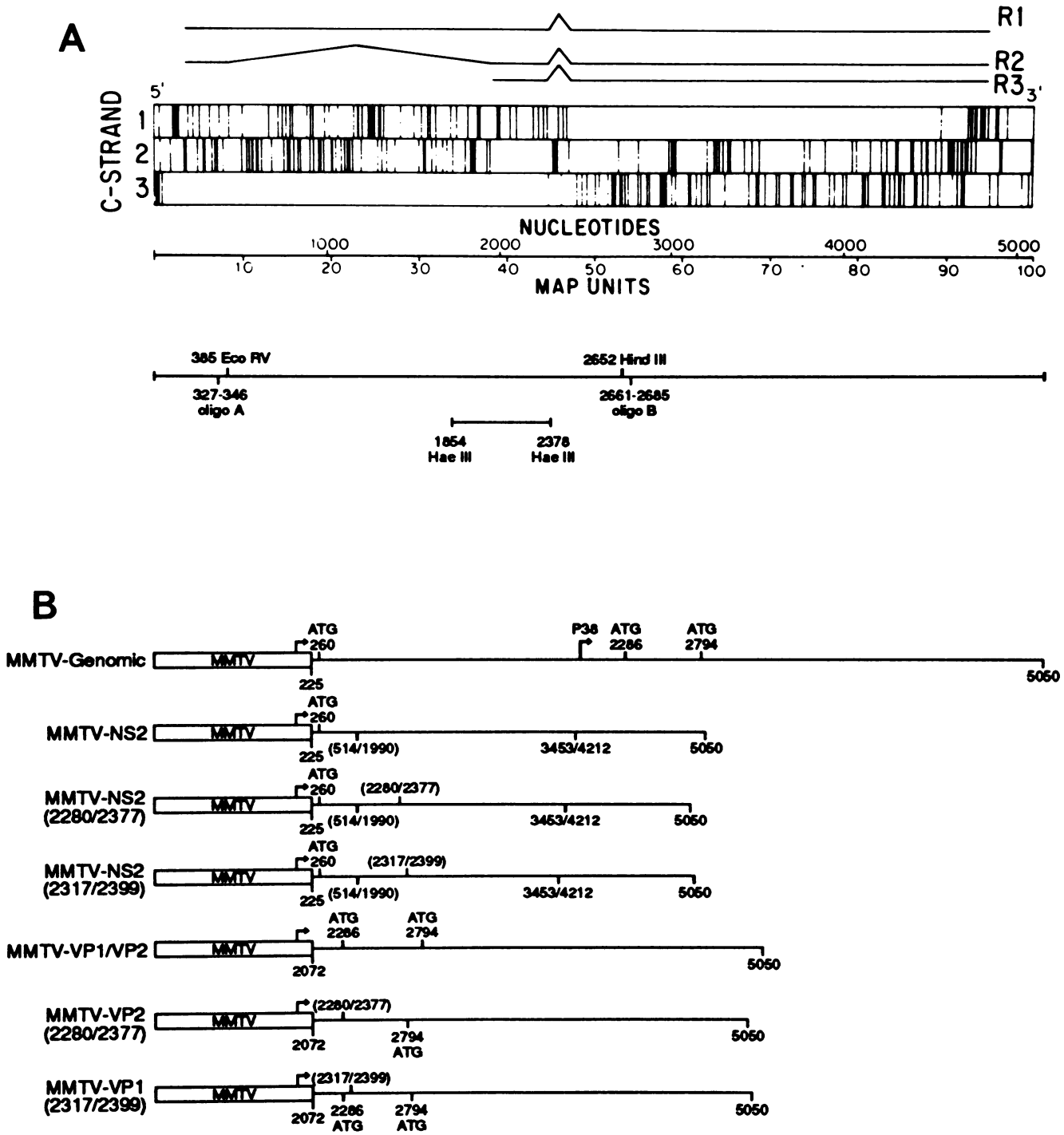

FIG. 1. (A) Map of MVM indicating the three major transcripts (R1, R2, and R3) in relation to the ORFs deduced from sequence analysis (2). ORFs within the viral complementary strand are depicted as open areas, and termination codons are indicated by vertical lines. Oligo A and oligo B represent oligonucleotides used in MVM cDNA synthesis and amplification as described in text. *Eco*RV (nt 385) and *Hin*dIII (nt 2652) restriction sites were used to generate termini for cloning cDNA sequences into the infectious clone of MVM in pML (16). The designated MVM *Hae*III fragment (nt 1854 to 2378) was cloned into the pGEM vector (Promega Biotec, Madison, Wis.) and used to generate the antisense MVM RNA probe used for the RNase protection assays (see Fig. 4). (B) MMTV-MVM constructs used in the generation of MVM protein-producing cell lines. The open box designates the MMTV long terminal repeat, and the solid line represents MVM sequences. pML vector sequences have been omitted for clarity. MMTV sequences include 976 nt of the long terminal repeat containing the dexamethasone-inducible promoter/enhancer (17). Transcription initates 111 nt upstream of the MMTV-MVM fusion. MVM sequences begin at the MVM nt 225 (2) downstream from the MVM P4 promoter for constructs producing the nonstructural protein(s) and at nt 2072 downstream from the MVM P38 promoter for constructs exclusively producing the structural proteins. Transcription initiation sites are represented as arrows. A 759-nt deletion between two *Bgl*II sites is designated 3453/4212. Nucleotides in parentheses represent intron splice junctions.

the oligonucleotide primers but flanking the large and small intron. This fragment was substituted for the analogous *Eco*RV-*Hin*dIII fragment of infectious MVM cloned into pML (16). cDNA-containing clones generated from RNAs lacking the small intron and those lacking both the large and small intron were differentiated by restriction mapping and were further characterized by sequence analysis of both strands across the entire insert (data not shown). In all clones examined, the large intron splice junction joins nt 514 to 1990, confirming the results of previous studies (13, 18). Two classes of small intron splice junctions were obtained, as expected from previous studies, those joining nt 2280 to 2377 and those joining nt 2317 to 2399. cDNA clones representing the rare splicing pattern which joins nt 2280 to 2399 were not obtained.

Cloning of cDNAs in constitutive and inducible expression vectors and production of expressing cell lines. Cloned cDNAs which lacked the large (514/1990) intron (which removes the P38 TATA box at approximately nt 1978) and retained the small intron or, alternatively, had the small intron excised by either the major or minor splicing pattern, were used to construct clones which produce either all three NS2 isoforms or, uniquely, the major or minor NS2 isoform from either the simian virus 40 early promoter (28) or mouse mammary tumor virus (MMTV) promoter (17) (Fig. 1B). In these constructs, MVM sequences begin at nucleotide 225, downstream from the MVM P4 promoter. Constructs which produce both VP1 and VP2 or VP1 or VP2 individually were obtained by cloning the right-hand end of the MVM genome beginning at nucleotide 2072 downstream from the P38 promoter (Fig. 1B). Constructs that retained the small intron produced both VP1 and VP2. Those containing cDNA representing the major splicing pattern (2280/2377) are predicted to produce VP2, and those containing cDNA representing the minor splicing pattern (2317/2399) are predicted to produce VP1.

When analyzed by immunoprecipitation at 48 h posttransfection as described below, the simian virus 40-driven cDNA clones transfected into COS-1 cells produced the anticipated MVM gene products as identified by comigration with authentic MVM proteins from a wild-type infection (data not shown). Immunofluorescence analysis of simian virus 40-driven NS2 cDNAs transfected into COS-1 cells indicated that without other viral gene products present, NS2 localizes as it does after transfection of wild-type MVM (data not shown).

After verification by transient analysis, cDNA clones were constructed that were driven by the dexamethasone-inducible promoter from MMTV (obtained from J. Majors, Washington University, St. Louis, Mo.) (Fig. 1B) for use in the generation of stable cell lines expressing individual MVM gene products, as had been done previously for the related parvovirus H1 (24). Clones in which the MMTV promoter drives genomic MVM beginning at nucleotide 225 were also constructed. These were expected to produce NS1 and all three isoforms of NS2 under the control of the MMTV promoter and VP1 and VP2 under the control of the MVM P38 promoter. Although the unaltered version of the MMTV promoter has significant activity when uninduced, it was used in an attempt to overcome potential toxic effects of these MVM gene products.

The MMTV-MVM constructs were cotransfected at a 30:1 ratio into A9 mouse fibroblasts along with pko-neo (28), in which the neomycin resistance gene is driven by the SV40 early promoter, using the $CaPO_4$ technique (19). Neomycin resistant cell clones were isolated in the presence of 1 mg of Geneticin (G-418 Sulfate; GIBCO, Gaithersburg, Md.) per ml and tested for production of MVM-specific gene products in the presence and absence of dexamethasone by immunoprecipitation as described below.

Characterization of individual MVM gene products expressed in murine cell lines. Approximately 5×10^5 cells from individually isolated cell clones were labeled for 2 h by using 0.1 mCi of [^{35}S]methionine (Tran ^{35}S-label; ICN Radiochemicals, Irvine, Calif.) per ml 15 to 20 h after the addition of dexamethasone (ranging between 0.05 and 0.6 μM in different experiments). A9 cells were infected with MVM at a multiplicity of 10 and labeled similarly at 15 to 20 h postinfection as a control. MVM-specific proteins were immunoprecipitated as previously described (9) by using antisera (kindly provided by S. Cotmore and P. Tattersall) specific for either NS1, NS2, or the amino-terminal ORF common to NS1 and NS2, or antiserum generated in response to purified empty virions. Multiple clonal cell lines obtained by cotransfection with the MVM-expressing constructs diagrammed in Fig. 1B were screened by immunofluorescence as previously described (14). These lines displayed a great variability in the percentage of expressing cells and in the magnitude of their inducibility. Cell lines generating high levels of MVM gene products upon induction were chosen for further study.

Initially, MVM gene products from cell lines transfected with MMTV-Genomic (Fig. 1B) were analyzed. These lines produced NS1, NS2, VP1, and VP2 that comigrated with the corresponding proteins from a wild-type MVM infection and were inducible by dexamethasone (Fig. 2A through C). The enhancement of the capsid gene products in these lines (Fig. 2C) was probably due to elevated levels of NS1, a known transactivator of the P38 promoter (21, 23, 27).

Likewise, cells transfected by the capsid protein-producing constructs MMTV-VP1/VP2, MMTV-VP1 (2317/2399), and MMTV-VP2 (2280/2377) (Fig. 1B) inducibly expressed proteins of the expected size (Fig. 2C). It is interesting that cDNA clones predicted to express VP1 did not express detectable VP2 (despite the retention of the AUG at nt 2794 which is normally used for initiation of VP2) and that already spliced MVM transcripts were apparently transported to the cytoplasm and translated efficiently. In addition, as seen previously (14), cell lines expressing the unspliced R3 transcription unit produced VP1 and VP2 in the same ratio as that seen in viral infection (Fig. 2C), and they generated empty capsids as assayed by hemagglutination of guinea pig erythrocytes (data not shown). Immunofluorescence analysis of these cell lines (data not shown) also indicated that when expressed individually, VP1 and VP2 localize as they do when expressed together.

Cell lines transfected by the NS2-producing cDNA clones were examined next. Proteins immunoprecipitated from cell lines expressing all three NS2 isoforms (MMTV-NS2) and those producing either the major [MMTV-NS2 (2280/2377)] or minor [MMTV-NS2 (2317/2399)] isoform comigrated with the multiple NS2 isoforms normally seen during wild-type infection (Fig. 2B), which were verified by Western blot (immunoblot) analysis (data not shown). In most cases, significantly more NS2 was produced in the presence of dexamethasone. Because the cell lines expressing individual cDNA versions of NS2 generated multiple bands similar to those observed during MVM infection, we chose to investigate the state of NS2 phosphorylation as a possible explanation for the presence of multiple bands.

When NS2 immunocomplexes were treated with 1 U of potato acid phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in MES [0.1 M 2-(*N*-morpholino)

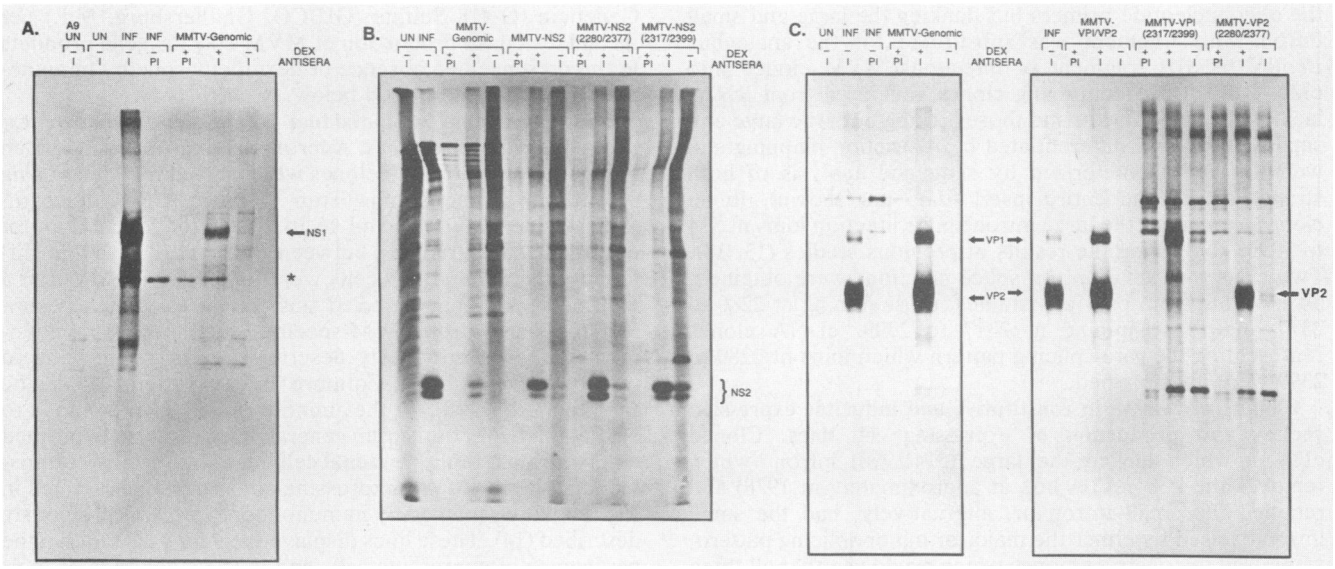

FIG. 2. Characterization of MVM protein-producing cell lines by immunoprecipitation in the absence (−) or presence (+) of 0.6 μM dexamethasone (DEX). Cell lysates from 5×10^5 cells were immunoprecipitated as previously described (9). Control lanes include uninfected (UN) or MVM-infected (INF) pko-neo-transformed or normal A9 mouse fibroblasts (multiplicity of infection, 10) taken 15 to 20 h postinfection. Immunoprecipitated proteins were separated on a 10% (for VP1 and VP2) or 12.5% (for NS2) sodium dodecyl sulfate–polyacrylamide gel. (A) Immunoprecipitations of lysates from cell lines transfected with MMTV-Genomic with either preimmune (PI) or immune (I) serum directed against a region of ORF 3 unique to NS1. The band marked with an asterisk is viral VP2 which has been nonspecifically precipitated in this experiment. (B) Immunoprecipitations of lysates from cell lines transfected with MMTV-Genomic, MMTV-NS2, MMTV-NS2 (2280/2377), or MMTV-NS2 (2317/2399) with either preimmune (PI) or immune (I) serum directed against the carboxyl-terminal ORF common to all three NS2 isoforms (9). (C) Immunoprecipitations of lysates from cell lines transfected with MMTV-Genomic, MMTV-VP1/VP2, MMTV-VP1(2317/2399), or MMTV-VP2(2280/2377) with either preimmune (PI) or immune (I) serum directed against purified empty capsids.

ethanesulfonic acid], pH 6.0, for 15 min at 37°C (3), the amount of the slower-migrating NS2 species was reduced (Fig. 3). When 0.1 M sodium phosphate was included as a phosphatase inhibitor in the reaction mix, the abundance of this species was similar to that found in untreated reactions. In addition, this species was specifically labeled when the cells were incubated for 2 h in the presence of 1 mCi of $H_3{}^{32}PO_4$ (ICN Radiochemicals, Irvine, Calif.) per ml, confirming that NS2 exists in a phosphorylated state (Fig. 3). When cell lines producing either all three NS2 isoforms or exclusively the major or minor isoform were examined, the results were the same, confirming that both the NS2 major and minor isoforms are phosphorylated (Fig. 3). The assignment of individual isoforms of NS2 and their variably phosphorylated states to particular bands on a gel will require further examination of these cDNA clones.

Cell lines that express either all MVM gene products (transfected with MMTV-Genomic), NS2, or capsid proteins were stable uninduced for at least 3 weeks in culture, retaining full inducibility (data not shown). Upon continued induction however, cell lines that express either all MVM gene products (transfected by MMTV-Genomic), NS2 alone [transfected by either MMTV-NS2 or MMTV-NS2 (2280/2377)], or VP1 [transfected by MMTV-VP1 (2317/2399)] demonstrated decreased viability (cell lines expressing the minor NS2 isoform or VP2 alone have not yet been tested). All lines have reduced plating efficiencies and lose MVM expression during prolonged culture (K. E. Clemens and D. J. Pintel, unpublished data). The toxicity of parvovirus nonstructural proteins has been noted previously (5, 15, 24). A quantitative characterization of the toxic effect of these MVM proteins is in progress.

MVM-specific mRNAs produced by the murine cell lines. MVM-specific mRNAs produced by the MMTV-MVM cDNA-generated cell lines were investigated by using RNase protection analysis of hybrids formed between total RNA isolated from dexamethasone-induced cells and an SP6-generated antisense MVM RNA probe spanning nt 1854 to 2378 (Fig. 1A) as previously described (7). The 5′ terminus of the probe resides within the R2 large intron, and the 3′ terminus is within the small intron common to all three transcripts. This allows discrimination between the R1, R2, and R3 transcript classes as well as those subspecies using alternate small splice donor sites. Briefly, 10 μg of total RNA was hybridized to a 40-fold molar excess of probe at 50°C for 16 to 20 h, followed by digestion with 4.5 μg of RNase A and 0.2 μg of RNase T1 per ml at 37°C for 45 min. The nuclease-protected hybrids were separated on an 8% polyacrylamide–urea gel (Fig. 4).

Cell lines expressing the MMTV-Genomic construct produced transcripts identical in size and similar in abundance to those found in a wild-type MVM infection (Fig. 4, lanes INF and 1). Cell lines producing all three NS2 isoforms (transfected with MMTV-NS2) produced spliced R2 transcripts with both the major and minor small intron splicing pattern (Fig. 4, lane 2). As predicted, cell lines producing either the major [transfected by MMTV-NS2 (2280/2377)] or the minor [transfected by MMTV-NS2 (2317/2399)] NS2 isoform produced R2 transcripts bearing the major and the minor small intron-splicing pattern, respectively (Fig. 4, lanes 3 and 4).

In constructs used to make the MVM capsid-producing cell lines, MVM sequences begin at nt 2072, downstream from the native MVM R3 transcript start site at nt 2003. As

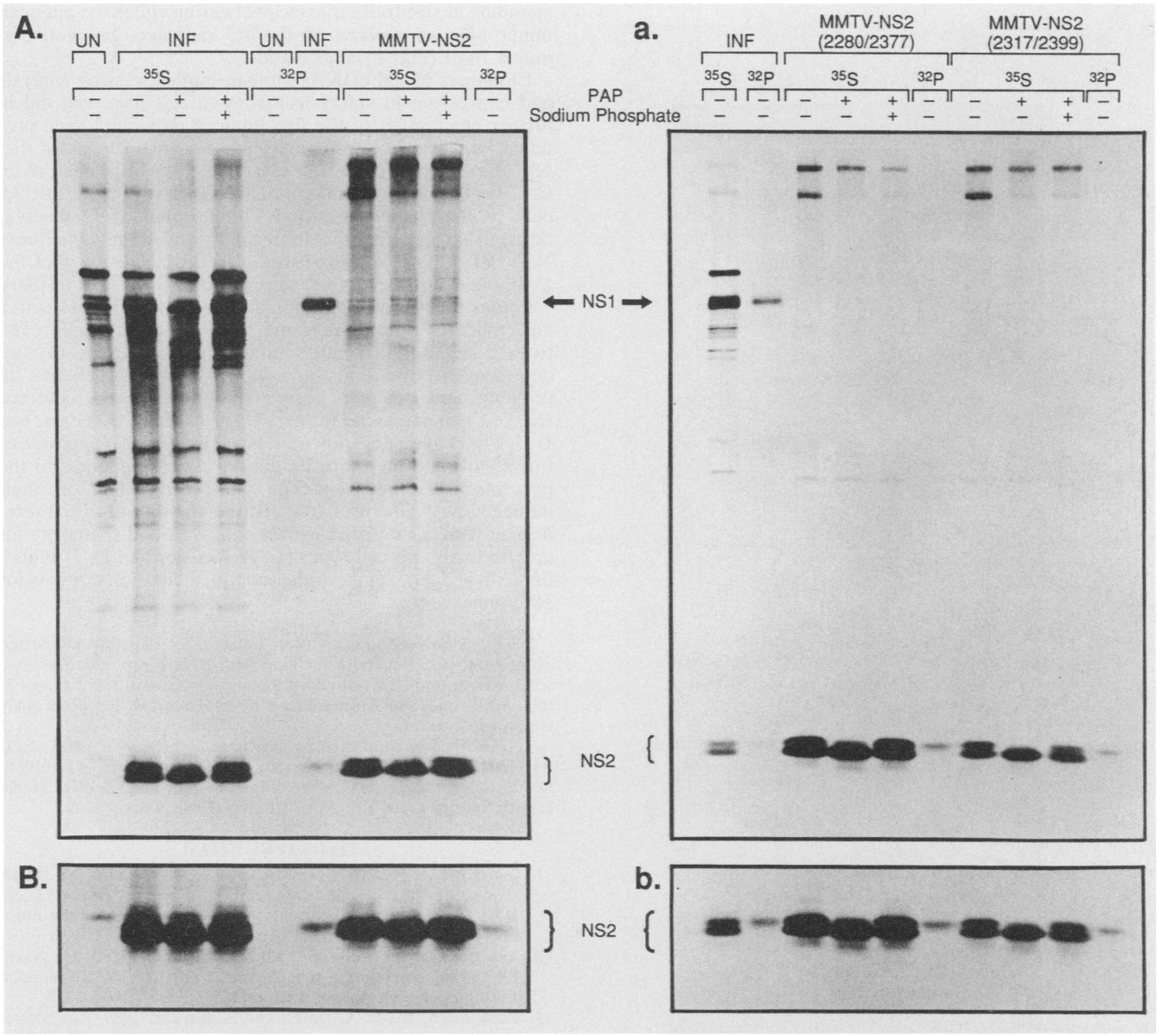

FIG. 3. Examination of the phosphorylated state of NS2 in cell lines expressing all three NS2 isoforms (transfected with MMTV-NS2) or exclusively the major [transfected with MMTV-NS2 (2280/2377)] or minor [transfected with MMTV-NS2 (2317/2399)] NS2 isoform. Cell lysates from 5×10^5 cells labeled with 0.1 mCi of [^{35}S]methionine per ml or 1×10^6 cells labeled with 1 mCi of $H_3{}^{32}PO_4$ per ml were immunoprecipitated by using antisera directed against the ORF common to the amino termini of NS1 and NS2 (9). Controls include uninfected (UN) or MVM-infected (INF) pko-neo-transformed A9 mouse fibroblasts taken 15 to 20 h postinfection. Immunocomplexes bound to *Staphylococcus* A cells were washed in 500 μl of 0.1 M MES, pH 6.0, buffer containing 1 mM phenylmethylsulfonyl fluoride. One unit of potato acid phosphatase (PAP), an ammonium sulfate suspension (Boehringer Mannheim), was pelleted by centrifugation, suspended in 100 μl of 0.1 M MES or 0.1 M MES–0.1 M sodium phosphate, pH 6.0 (an inhibitor of PAP), and added to the indicated experimental sample. Control samples were incubated in MES buffer without PAP. Control and experimental samples were incubated for 15 min at 37°C, pelleted by centrifugation, suspended in loading dye, and electrophoresed in a 12.5% sodium dodecyl sulfate–polyacrylamide gel. The exposure required to visualize additional NS2 in all lanes of infected samples revealed a number of bands which were nonspecifically precipitated in these samples. (A) Immunoprecipitations using uninfected (UN) and MVM-infected (INF) pko-neo-transformed A9 mouse fibroblasts and a cell line expressing all three NS2 isoforms (transfected with MMTV-NS2). (B) Longer exposure of immunoprecipitations shown in panel A. (a) Immunoprecipitations using cell lines expressing either the major [transfected with MMTV-NS2 (2280/2377)] or minor [transfected with MMTV-NS2 (2317/2399)] NS2 isoform. (b) Longer exposure of immunoprecipitations shown in panel a.

a result, nuclease-protected hybrids using RNA from the capsid-producing cell lines should be approximately 70 nt smaller than MVM-specific RNA from a wild-type infection. As predicted, RNA from cell lines producing both VP1 and VP2 (MMTV-VP1/VP2) protected fragments the correct size for the unspliced and the major and minor spliced R3 messages; each was reduced by approximately 70 nt (Fig. 4, lane 5). RNA from cell lines producing exclusively VP2 [transfected by MMTV-VP2(2280/2377)] or VP1 [transfected by MMTV-VP1(2317/2399)] protected fragments corre-

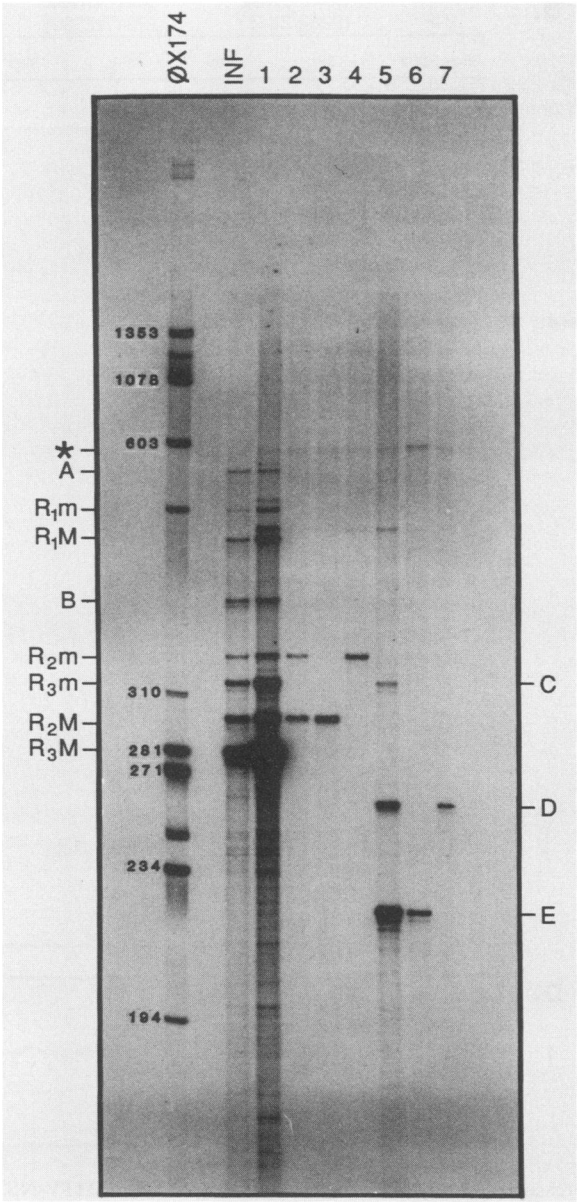

FIG. 4. RNase protection assay using RNA from cell lines expressing all MVM proteins (transfected with MMTV-Genomic, lane 1), all three NS2 isoforms (transfected with MMTV-NS2, lane 2), the major NS2 isoform [transfected with MMTV-NS2 (2280/2377), lane 3], the minor NS2 isoform [transfected with MMTV-NS2 (2317/2399), lane 4], VP1 and VP2 (transfected with MMTV-VP1/VP2, lane 5), VP2 [transfected with MMTV-VP2 (2280/2377), lane 6], or VP1 [transfected with MMTV-NS2 (2317/2399), lane 7]. ϕX174 digested with *Hae*III (leftmost lane) and dideoxy sequencing reactions (not shown) were used to size the protected fragments. RNase-protected hybrids with RNA from MVM-infected A9 mouse fibroblasts (INF) concurred with previous results (7). The unlabeled lane between the lanes labeled ϕX174 and INF shows results obtained with RNA from uninfected cells. The bands are identified by transcript class (R1, R2, and R3) and small intron donor site (M-2280 and m-2317). Bands A and B are the sizes expected to be protected by unspliced R1 and R3, respectively. Bands C, D, and E represent unspliced R3, R3m, and R3M, each reduced by approximately 70 nt because of the altered MVM initiation site. The band designated by an asterisk is undigested probe.

sponding in size to R3 transcripts bearing either the major or minor splicing pattern of the R3 message, respectively, minus 70 nt (Fig. 4, lanes 6 and 7).

Cloning of MVM cDNAs for transient expression analysis and expression in stably transformed cell lines will aid in further elucidation of the functions of individual viral proteins. For instance, preliminary studies have suggested that NS2 cDNAs are unable to transactivate MVM(p) P38-driven CAT vectors in transient cotransfection assays in murine A9 cells (K. E. Clemens and D. J. Pintel, unpublished observations). Also, cell lines that individually and inducibly express NS2, VP1, or VP2 will be useful in further analysis of the toxic effects of these viral gene products. The use of these cell lines for complementation and propagation of defective viral mutants after DNA transfection, however, has proven to be complex. Although limited complementation of both defective NS1 mutants and capsid mutants deficient in progeny single-strand accumulation has been detected (K. E. Clemens, L. R. Burger, D. R. Cerutis, G. Tullis, and D. J. Pintel, data not shown), it is variable. The magnitude of this effect, and therefore its utility, clearly depends on the percentage of expressing cells within the population, their induced levels of expression, and the viability of the culture. Recent results that demonstrate that NS2 is dispensable for growth in certain cell types (L. K. Naeger and D. J. Pintel, unpublished data) makes other complementation systems for NS2 unnecessary.

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