Minute Virus of Mice Non-structural Protein NS-1 Is Necessary and Sufficient for Trans-activation of the Viral P39 Promoter

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SUMMARY

The genome of the autonomous parvovirus minute virus of mice (MVM) is organized in two overlapping transcription units: the genes coding for the two non-structural proteins (NS-1 and NS-2) are transcribed from a promoter (P04) located at map unit 4, whereas the promoter controlling the capsid protein genes (P39) lies at map unit 39. We studied the effect of viral proteins on the activity of the P39 promoter in vivo. By site-directed mutagenesis we constructed clones encoding only one of the two NS proteins. The activity of the P39 promoter was measured in HeLa or EL-4 cells transfected with these clones, either by an RNase protection assay or by following the expression of a reporter gene, CAT (which codes for chloramphenicol acetyltransferase), placed under the control of this promoter. We found that the P39 promoter of strain MVMi is activated in trans by a viral gene product, and evidence to suggest that NS-1 is the only viral gene product responsible for this trans-activation. We also determined that the mechanism of trans-activation is very rapid, since all species of viral mRNAs appear together in non-synchronized infected EL-4 cells within a 2 h interval.

INTRODUCTION

The parvoviruses are small, non-enveloped icosahedral viruses with a genome consisting of a single-stranded DNA molecule 5 kilobases long with hairpin structures at both extremities. The autonomous parvoviruses are able to grow only in actively dividing cells, presumably because they are dependent on a factor specific to the S-phase of the cell cycle (reviewed in Berns, 1984; Ward & Tattersall, 1978).

Two strains of the autonomous parvovirus minute virus of mice (MVM) have been isolated, one of them, MVMp, infecting fibroblasts and the other, MVMi, infecting lymphocytes (Crawford, 1966; Bonnard et al., 1976; Engers et al., 1981; McMaster et al., 1981; Tattersall & Bratton, 1983). The DNA sequences of both strains have been determined (Astell et al., 1983, 1986; Sahli et al., 1985).

It has been shown that the genomic organization of MVM consists of two overlapping transcription units, with promoters at map units 4 and 39 (these promoters will be referred to as P04 and P39 respectively). The leftward part of the genome contains the genes for two non-structural proteins (NS-1 and NS-2) placed under the control of the P04 promoter, whereas the P39 promoter controls the transcription of the capsid genes located in the rightward part of the genome (Pintel et al., 1983; Cotmore & Tattersall, 1986; Jongeneel et al., 1986). The capsid messenger RNAs (two species) all share the same 5' end, and the polyadenylation signal used by all the viral transcripts is located very near the right-hand end of the genome (Clemens & Pintel, 1987).

The trans-activation of viral promoters by viral gene products has been known for a long time in many different systems, e.g. adenoviruses (Nevins, 1981), papovaviruses (Brady et al., 1984;
Keller & Alwine, 1984) and herpesviruses (Post et al., 1981). That this phenomenon also occurs in parvoviruses became evident only in 1985. Rhode (1985) showed that the P38 promoter of the autonomous parvovirus H-1 was activated in trans by a viral gene product encoded on the left part of the genome, and that this activation was lost if the NS-1 protein-coding sequence was altered by a frameshift mutation situated roughly in the middle of the gene. These data provided evidence that in H-1 NS-1 plays a role in the trans-activation of the capsid genes’ promoter. However, it was not possible to exclude the possibility that NS-2 might be the trans-activator, and that its expression or function may be altered if NS-1 is non-functional. Cotmore & Tattersall (1988) have shown that NS-1 of MVMi binds to the 5’ end of the viral DNA which suggests that this protein has at least two functions. In this paper, we show that during infection by the autonomous parvovirus MVMi a trans-activation of the P39 promoter also occurs, and furthermore we present strong evidence that NS-1 is the only viral gene product required for this phenomenon.

**METHODS**

**Molecular cloning.** We followed the protocols given by Maniatis et al. (1982). Restriction endonucleases, T4 DNA ligase and T4 DNA polymerase were purchased from Boehringer Mannheim. The plasmids were amplified in the HB101 strain of *Escherichia coli*.

**Plasmid construction.** For a representation of the plasmids used see Fig. 1.

*pMP39-CAT.* To obtain this plasmid, we isolated the *Bst*II restriction fragment containing nucleotides (nt) 1820 to 2080 from MVMi DNA, rendered it blunt-ended using T4 DNA polymerase and ligated it to *Hind*III linkers. We then inserted it into the *Hind*III cloning site of pSV6-CAT (Gorman et al., 1982a). This vector did not contain any element from the regulatory region of simian virus 40 (SV40). Restriction analysis of the transformants with *Ava*I allowed determination of the orientation of the insert relative to the chloramphenicol acetyltransferase (CAT) gene. pMP39-CAT refers to the plasmid containing the fragment in the orientation needed for transcription of the CAT gene from the P39 promoter.

*pEMBL/i0099.* The construction of this plasmid has already been described (Antonietti et al., 1987); it is an almost full-length genomic clone of MVMi, lacking only some base pairs of the terminal hairpins, inserted through *Sal*I linkers into the pEMBL9 vector (Dente et al., 1983).

*pEMBL/i0052.* This plasmid was made by deleting the *Hind*III fragment (nt 2651 to 5104) from pEMBL/i0099. Thus it contained the two promoters and entire genes for the NS proteins, but lacked the major part of the capsid genes as well as the polyadenylation signals used by all viral mRNAs.

*pMNS-1m.* This plasmid was unable to express NS-1. We obtained it by cutting pEMBL/i0099 with *Bst*EII, filling the 5’ overhangs using T4 DNA polymerase and religating the plasmid. Since the only *Bst*EII site on pEMBL/i0099 was located within the large intron of the NS-2 gene at position 1885 (Jongeneel et al., 1986), we thus created a frameshift mutation affecting only the sequence of NS-1 gene.

*pMNS-2m.* This plasmid was unable to express NS-2, but still expressed NS-1. We produced it by oligonucleotide-directed site-specific mutagenesis. Site-specific mutation was carried out by standard techniques of oligonucleotide-directed mutagenesis using a single-stranded bacteriophage WB239 DNA template (Barnes & Bevan, 1983; Sahli et al., 1985) and a partially mismatched oligonucleotide as a mutagenic primer (Zoller & Smith, 1983). The *Eco*RI-*Eco*RI fragment (positions 1085 to 3523) isolated from pEMBL/i0099 was inserted into the *Eco*RI site of bacteriophage WB239. This clone was used for isolation of single-stranded template DNA, to which the 21-mer mutagenic oligonucleotide (DNA Synthesizer model 380 B, Applied Biosystems) was hybridized. The primer-extended products were used to transform *E. coli* HB373 cells. Since the mutation generated by the oligonucleotide results in the loss of the *Xho*I site at position 2071 (the recognition sequence of *Xho*I is 5’-CTCGAG-3’), we screened the transformants for the lack of this *Xho*I site (Fig. 5a). The mutants were then plaque-purified and sequenced according to Sanger et al. (1977). To reconstitute a clone containing the whole length of MVMi and carrying this mutation, we substituted the mutated *Eco*RI-*Eco*RI fragment to the corresponding *Eco*RI-*Eco*RI (nt 1085 to 3523) fragment of pEMBL/i0099.

*pMNS-2m-cap*.

The only viral protein this clone could express was NS-1. We obtained it by linearizing pMNS-2m with *Bst*EII (position 3001), rendering the extremities blunt-ended using T4 DNA polymerase and inserting into this phosphorylated site a non-phosphorylated decamer *Hind*III linker. This resulted in a frameshift in the 5’ part of the capsid genes. We checked for the presence of a single decamer linker by comparing on a sequencing gel the sizes of the *Bgl*I-*Dra*I fragments (nt 2877 to 3012) obtained by restriction of either pMNS-2m or pMNS-2m-cap, using dyeoxy sequencing reaction products as size markers (data not shown).

**Cell culture.** HeLa and EL-4 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% foetal bovine serum. EL-4 is a murine lymphoma-derived cell line and is the usual host for lytic infection with MVMi (Bonnard et al., 1976).
Trans-activation in parvoviruses

Fig. 1. Clones used during this study. The top bar represents the viral genome. The main open reading frames are drawn beneath. For each clone a representation is given of its coding capacity. (a) pEMBL/i0099; (b) pEMBL/i0052; (c) pNS-1m; (d) pNS-2m; (e) pNS-2mcapm; (f) pMP39-CAT. Frameshift mutations are represented by a vertical bar with an asterisk above. El, pEMBL; [ ], pSV0-CAT; q-q, functional ORF; [ ], shifted frame.

Transfections. The transfections into HeLa cells were performed by using the calcium phosphate procedure described by Graham & van der Eb (1973). We transfected DNA into EL-4 cells following the DEAE–dextran method described previously (Sompayrac & Danna, 1981; Banerji et al., 1983). The DNA to be transfected was quantified with a DNA minifluorimeter (Hoefer Instruments).

CAT assays. The preparation of extracts from transfected cells and the measurement of their CAT activity were done according to a modification of the procedure described by Gorman et al. (1982a). The cells were harvested 45 h after transfection and resuspended in 100 µl of 250 mM-Tris-HCl pH 7.5; the extracts were obtained by three consecutive cycles of freeze–thaw lysis followed by centrifugation at 4 °C to remove cell debris. The assay mixture (45 µl) contained 20 µl of cell extract, 1 µl of [14C]chloroamphenicol (0.2 mCi/ml, 54 mCi/mmole), 5 µl 4-4 (or up to 44) mM-acetyl-coenzyme A and 19 µl 250 mM-Tris-HCl pH 7.5. The reaction was carried out at 37 °C for 14 h and stopped with 0.5 ml of ethyl acetate, which was also used to extract the chloramphenicol and its acetylated derivatives. The organic layer was lyophilized, and the chloramphenicol taken up in 30 µl of ethyl acetate, spotted on silica gel thin-layer plates and run with chloroform–methanol (95:5). The plate was then dried and exposed to film for autoradiography.

Extraction of cytoplasmic RNA and RNase protection assays. Infected EL-4 cells were lysed with Nonidet P40, the nuclei were removed, and the cytoplasmic RNA was isolated as described by Maniatis et al. (1982).

The RNase protection assays were performed as described previously (Melton et al., 1984; Zinn et al., 1983). The RNA probe (Fig. 2b) was synthesized with SP6 RNA polymerase (Green et al., 1983) from the plasmid pSP64-iHX which was constructed by insertion of the HaeIII-Xhol fragment (nt 1854 to 2071) isolated from MVMi double-stranded replicative form into pSP64 between the SalI and Smal sites (Antonietti et al., 1987).

RESULTS

Time course of viral transcription in MVMi-infected EL-4 cells

We infected non-synchronized EL-4 cells with MVMi and extracted the cytoplasmic RNA 6, 8, 10, 12 and 24 h after infection. The viral mRNAs were detected by hybridization of 5 µg of cytoplasmic RNA with the SP6 RNA probe described in Methods, followed by digestion of the single-stranded RNA with RNase A and RNase T1. We denatured the protected fragments at 100 °C for 5 min in formamide, and analysed their lengths by electrophoresis in polyacrylamide–urea sequencing gels. A fragment of 217 nt should be protected by the NS1 mRNA (R1), the
NS2 mRNA (R2) should protect a fragment of 81 nt, and the capsid (VP1 and VP2) mRNAs (R3) should protect a fragment of about 60 nt (Fig. 2). The results are shown in Fig. 2. We did not detect any viral mRNA 6 and 8 h after infection, but after 10 h the three types of viral mRNAs were visible; the amount of viral transcripts increased between 10 and 24 h. The VP1 and VP2 mRNAs seem to have appeared at the same time as the NS1 and NS2 mRNAs; thus we did not observe an early phase during which only the genes for non-structural proteins were transcribed, followed by a late phase in which most of the mRNA was specific for capsid genes. Instead we observed a burst of all the viral mRNAs between 10 and 12 h after infection. By performing the same kind of experiments using synchronized cells, Clemens & Pintel (1988) did detect such early and late phases.
Trans-activation in parvoviruses

The P39 promoter of MVMi is activated in trans by a viral gene product

In order to test the possibility of trans-activation of the P39 promoter by a viral gene product, we used the bacterial CAT gene as a marker gene to monitor the transcriptional activity of MVMi P39 promoter (Gorman et al., 1982). For this purpose we constructed the plasmid pMP39-CAT, which contained a 260 bp fragment carrying the P39 promoter TATA box (with about 160 bp of the upstream region and 100 bp of the downstream region) in front of the CAT gene, in the orientation allowing transcription of this gene from the viral promoter. We first chose to test the activity of P39 in HeLa cells, since we had already shown that this promoter yields run-off transcripts in HeLa cell extracts (unpublished observations). Upon transfection of pM39-CAT into HeLa cells, the amount of CAT activity in the cells was very low (Fig. 3, lane 1). However, when different amounts of pEMBL/i0099 (a plasmid containing both complete transcription units of MVMi) were included in the transfected DNA, a dramatic increase in the expression of CAT was observed; the extent of this increase depended on the amount of pEMBL/i0099 added in the transfection. This effect was not seen when the 260 bp fragment was inserted in the other orientation relative to the CAT gene (data not shown). From these observations we conclude that a viral gene product was able to activate the P39 promoter in trans. We repeated this experiment using the MVMi host cell line EL-4 (Fig. 4, lanes 3 and 4). By counting the radioactivity in the fastest migrating spots, we determined that the activity of the P39 promoter was enhanced about 60-fold in the presence of viral gene products.

One or both NS proteins play a role in the trans-activation of the P39 promoter

From the experiment described above it was not possible to deduce which of the viral proteins was responsible for the trans-activation. One could imagine that it was due to one of the non-structural proteins controlled by the P04 promoter, or to a positive feedback regulation by the capsid proteins. To discriminate between these possibilities, we transfected EL-4 cells with a
mixture of pMP39-CAT and pEMBL/i0052, a clone containing only the left part of MVMi genome, from nt 0 to the HindIII site at nt 2651. The coding capacity of this clone was restricted to the non-structural proteins, since it contained only about 300 nucleotides of capsid 5' coding sequence. The level of CAT activity in such transfected EL-4 cells is shown in Fig. 4, lane 5. This clearly demonstrates that the viral genes located in the left part of the genome played a role in the trans-activation of the P39 promoter. The trans-activation observed in cells cotransfected with pEMBL/i0052 and pMP39-CAT (lane 5) was somewhat less efficient than that observed when the added DNA was pEMBL/i0099 (lane 4). This is presumably because the polyadenylation signals used for all the viral transcripts (including those coding for NS-1 and NS-2) were located very near the right end of the genome and thus absent from pEMBL/i0052. However, we cannot exclude that the capsid proteins are needed in conjunction with one or both NS proteins to restore a full level of trans-activation.

At this point there remained four possibilities: the viral protein(s) functioning as a trans-activator could be (i) NS-1, (ii) NS-2 or (iii) both of them in conjunction, or (iv) the capsid proteins are needed in addition to one or both NS proteins to generate maximal trans-activation. To determine which was in fact involved, we had to construct plasmids expressing only one of the two non-structural proteins and test their capacity to activate the P39 promoter in transfected cells.

Construction of defective mutants

To construct a clone of MVMi unable to express NS-2 but still expressing NS-1, we introduced a mutation in a region where NS-1 and NS-2 used different reading frames. We substituted a thymidine for a cytosine at position 2073, so that the amino acid sequence of NS-1 was not altered (because both CTC and CTT code for a leucine residue), but the codon CGA coding for an arginine in NS-2 was changed into a TGA stop codon (see Fig. 5a). The mutant and the wild-type DNA were compared by dideoxy sequencing, and the results are shown in Fig. 5(b). They
(a) 2068 2081
DNA sequence 5'-GATC TCGAGGACCT-3'
NS-1 protein Asp Leu Glu Asp
NS-2 protein Ser Arg Gly Pro

(b) G--
T C G A T C G A
**~--A
-C

Fig. 5. Sequence of the pMNS-2m cloned DNA. (a) Nucleotide sequence and predicted amino acid sequence of the DNA regions of MVMi (upper sequence) and the mutant clone pMNS-2m (lower sequence) unable to direct the synthesis of a non-truncated NS-2 polypeptide. The arrow at position 2073 indicates the site of the mutation. (b) Autoradiograms of the portions of DNA sequencing gels showing some of the MVMi nucleotide sequence (left-hand side) and the corresponding sequence of the mutant pMNS-2m (right-hand side). The nucleotide sequence is shown for nt 2087 to 2056, reading on the strand complementary to those shown in (a). The base altered in the mutant relative to the wild-type virus is indicated in bold. The letters T, C, G and A above each lane indicate which chain-terminating dideoxynucleoside triphosphate was present in the sequencing reaction mixture loaded.
clearly show that the mutant had the expected DNA sequence. In this figure the synthesized strands have a polarity opposite to that of the viral mRNA, so we read 5'-TCCTCGAGATC-3' for the wild-type and 5'-TCCTCAAGATC-3' for the mutant. This mutant will be referred to as pMNS-2m. We also constructed a mutant (pMNS-1m) which was able to express NS-2 and capsid proteins, but not NS-1 (see Methods). pMNS-1m no longer contained the BstEII site, and clearly had a mutant phenotype (see below).

**NS-1 is the only NS protein needed for the trans-activation of the P39 promoter**

When we tested the ability of pMNS-1m (which was able to express NS-2 but not NS-1) to trans-activate the P39 promoter, by cotransferring it into EL-4 cells with pMP39-CAT and subsequently assaying CAT activity in cell extracts (Fig. 4, lane 7), we observed a signal similar in strength to that provided by extracts of cells transfected by pMP39 alone (lane 3). In a similar experiment we replaced pMNS-1m by pMNS-2m (which was able to express NS-1 but not NS-2); this restored the trans-activation of the P39 promoter, and caused a signal (lane 6) similar to that observed when the DNA cotransfected with pMP39-CAT was the whole length clone pEMBL/i0099 (lane 4). Thus we conclude that NS-1 is implicated in the trans-activation, and that NS-2 plays no role in this phenomenon.

**There is no positive feedback regulation of the P39 promoter by capsid proteins**

The last point to clarify was that the CAT activity was lower in cells transfected with a 'trans-activator' clone lacking the right-hand part of the genome (pEMBL/i0052; Fig. 4, lane 5) than in cells transfected with a clone containing the whole length of the viral genome (pEMBL/i0099 or pMNS-2m; Fig. 4, lanes 4 and 6). To determine whether or not this was due to a role of the capsid proteins in the trans-activation, we modified the plasmid pMNS-2m in such a way that the altered clone (pMNS-2mcapm) could no longer express capsid proteins. This was done by insertion of a linker in the 5' region of the capsid protein-coding sequence (position 3001), which resulted in a frameshift in this gene (see Methods). Upon cotransfection of pMP39-CAT and pMNS-2mcapm into EL-4 cells, we observed a trans-activation of the P39 promoter that was equivalent to that generated by pEMBL/i0099 or pMNS-2m (Fig. 4, lane 7). We obtained this result with two independent pMNS-2mcapm clones. The experiments presented in Fig. 4 were repeated several times and the results were highly reproducible. From these data we conclude that NS-1 is the only viral protein required for the trans-activation of the P39 promoter.

**Evidence that the activation by NS-1 increases mRNA concentration**

It could be imagined that the increase in the CAT signal in the presence of NS-1 in transfected cells resulted from some mechanism other than enhancement of transcription, e.g. increased efficiency of translation of the RNA. To test this possibility, we transfected EL-4 cells with pEMBL/i0099, pMNS-1m or pMNS-2m; after 24 h we extracted the cytoplasmic RNA and analysed viral mRNAs by the RNase protection method described above. The results are shown in Fig. 6. By comparing the abundance of the capsid mRNA in the presence or absence of functional NS-2 (Fig. 6, lanes 1 and 3) we observed that the P39 promoter had the same activity in both cases. On the other hand, the transcription pattern of the transfected clone coding for an abortive NS-1 (pMNS-1m) was drastically modified: in this case we observed neither NS-2 mRNA nor capsid mRNA (lane 2). Thus this kind of approach confirms the data obtained with the CAT assays, and gives evidence that the activator effect of NS-1 on the products controlled by the P39 promoter was at the level of mRNA concentration.

**DISCUSSION**

We have studied the regulation of transcription in the autonomous parvovirus MVMi. In particular we have shown that the non-structural protein NS-1 is necessary and sufficient for the trans-activation of the P39 promoter. This was shown by using a plasmid that coded for NS-1, but not for NS-2 or the capsid proteins. Cotransfection with a plasmid that contains the CAT gene under the control of the P39 promoter led to a strong increase in the production of CAT.
Analysis by RNase protection assays of viral transcripts expressed in transfected cells leads to the same conclusion. Rhode (1985) has shown that the P38 of parvovirus H-1 is trans-activated by a non-structural gene product, and that this trans-activation disappears if the NS-1 gene is specifically altered. Our study with MVMi confirms these data and goes further by providing evidence that NS-1 is directly responsible for the trans-activation, and that neither NS-2 nor capsid proteins play any role in this process. Moreover we have checked (by RNase protection analysis) that the effect of NS-1 on expression of the CAT gene placed under the control of the P39 promoter really does reflect an increase in the concentration of the RNA transcribed from this promoter. This increase in the capsid mRNA level in the presence of NS-1 could have been due either to a faster transcription rate, or to increased stability of the mRNA. Since the stability of mRNA is in general modulated by the 3' end of the molecule (Owen & Kühn, 1987), and since in our CAT experiments the only viral sequence present in the mRNA is a short part of the 5' end, we suppose that the presence of NS-1 results in an increased transcription rate from the P39 promoter. Providing evidence for this will require run-on transcript analysis.
Rhode has defined a short sequence placed upstream of the H-1 P38 promoter which is necessary for the trans-activation and which he named the TAR element (for trans-activation responsive) (Rhode & Richard, 1987). A closely related sequence is present upstream of the P39 promoter on the MVMi 260 bp fragment that we inserted into pSV0-CAT (around nt 1870).

The transcription pattern of pMNS-1m is such that the NS-1 and NS-2 mRNAs are present in lesser amounts than in the case of clones which are able to express NS-1 (Fig. 6). This indicates that NS-1 may play a role in the regulation of the P04 promoter. We are currently testing this hypothesis. It is also noticeable that in the transcription pattern of pMNS-2m the relative amount of NS-2 mRNA is lower than in the transcription pattern of pEMBL/i0099 (Fig. 6). This could mean that NS-2 has a positive feedback action on the production of its own mRNA. More work is needed to clarify this point.

When we analysed the time of appearance of specific viral mRNAs during an infection of EL-4 cells by MVMi, we did not detect two phases of viral transcription, with the non-structural genes being expressed first and the capsid genes being activated later on, as can be observed in other viral systems. Instead, we observed that all species of viral transcripts seem to appear simultaneously. This indicates that under our experimental conditions the time needed for the activation of the P39 promoter by NS-1 was too short to be noticed. By analysing the appearance of viral mRNA in synchronized infected cells, Clemens & Pintel (1988) have recently demonstrated that early and late phases do exist.

Cotmore & Tattersall (1988) have recently shown that NS-1 is bound covalently to the 5' end of the viral genome; the functional aspect of this interaction is not yet understood. Our data show that this association is not required for the trans-activation, since this phenomenon occurs when only a small fragment of viral DNA carrying the promoter is inserted into a plasmid. This suggests that this protein plays at least two roles during the infection. We are currently studying the interaction between NS-1 and the P39 promoter.

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