The EMBO Journal vol.6 no.3 pp.681-688, 1987

Altered transcription of a defective measles virus genome derived from a diseased human brain

Roberto Cattaneo, Gabriela Rebmann, Anita Schmid, Knut Baczko[1], Volker ter Meulen[1] and Martin A.Billeter

Institut für Molekularbiologie I, Universität Zürich, Hönggerberg, 8093 Zürich, Switzerland and [1]Institut für Virologie und Immunbiologie, Universität Würzburg, Versbacherstrasse 7, 8700 Würzburg, FRG

Communicated by M.A.Billeter

Measles virus (MV) is a negative strand RNA virus which usually causes acute disease, but in rare cases its persistence in the human brain induces the lethal disease subacute sclerosing panencephalitis (SSPE). The transcription of MV and of a defective MV derived from autopsy material of a SSPE case was studied in cultured cells. In the lytic infection the levels of the MV mRNAs decreased progressively with the distance of the six cognate genes from the 3' end of the genome, reflecting transcriptional attenuation at every gene junction. Transcripts covering two or three adjacent genes accounted for up to 20% of single gene transcripts; incidentally the MV intergenic transcription signals were found to be less conserved than the analogous signals of other negative strand RNA viruses. Although the analysed SSPE-derived defective MV showed a localized transcription defect at the phosphoprotein-matrix gene junction (substitution of the mRNAs by readthrough transcripts), the corresponding intergenic 'consensus' sequence and the surronding nucleotides were not altered. This implies that factor(s) involved in the transcription of this defective SSPE virus fail to recognize this particular signal sequence, a constellation which in this and other cases might be causally related to the development of MV persistence.

Key words: intergenic sequences/measles virus/persistent infection/SSPE/transcription

Introduction

Measles virus is one of the most medically relevant paramyxoviruses: acute MV infections produce significant morbidity and mortality in third world countries (Mitchell and Balfour, 1985). Furthermore, in rare cases, MV persistence induces a lethal disease of the human central nervous system known as subacute sclerosing panencephalitis, SSPE (Agnarsdottir *et al.*, 1977; ter Meulen *et al.*, 1983). SSPE is among the most thoroughly studied persistent viral infections of the human central nervous system, and analysis of SSPE might provide a general insight into the mechanisms of development of persistent viral infections known or suspected to be the cause of several human syndromes, for example the more common neural disease multiple sclerosis (Haase *et al.*, 1981; Dowling *et al.*, 1986a; Waksman *et al.*, 1985; Koprowski *et al.*, 1985; Hauser *et al.*, 1986; Karpas *et al.*, 1986). MV gene expression in SSPE brains is defective. Infectious virus usually cannot be rescued from brain tissue, but occasionally cell-associated defective viruses can be isolated by co-cultivation of brain cells with fibroblasts (Wechsler and Meissner, 1982; ter Meulen and Carter, 1984). SSPE-defective viruses appear to differ from each other in their gene expression, showing different defects at either the transcriptional or the translational level (Wechsler and Fields, 1978; Hall and Choppin, 1979; Lin and Thormar, 1980; Machamer *et al.*, 1981; Carter *et al.*, 1983; Haase *et al.*, 1985; Norrby *et al.*, 1985; Sheppard *et al.*, 1986; Baczko *et al.*, 1986).

In the present work we have examined the transcription of a defective MV in a cell line originally obtained from a SSPE patient (Kratsch *et al.*, 1977) which produced altered transcripts of the matrix (M) gene (Carter *et al.*, 1984; Baczko *et al.*, 1984b). More detailed qualitative and quantitative analyses of the transcripts produced in this cell line revealed a very specific alteration of either termination/re-initiation or processing at the junction of gene M with the preceding phosphoprotein (P) gene. A similar defect has been observed previously in autopsy brain material from another SSPE patient (Baczko *et al.*, 1984a; Cattaneo *et al.*, 1986). For comparison, we also performed a quantitative analysis of MV transcription in the lytic cycle, which previously had been analysed only qualitatively (Udem and Cook, 1984; Dowling *et al.*, 1986b; Rima *et al.*, 1986). Moreover we completed the inventory of sequences at the MV gene boundaries, thought to act as signals for the generation of MV single gene (monocistronic) transcripts. We found that the MV gene boundaries vary considerably in sequence (see also Bellini *et al.*, 1985). However the analysis of cloned cDNAs from the P-M gene junction surprisingly did not reveal any difference between the sequence of lytic MV and defective SSPE virus. Thus we conclude that in the SSPE-defective virus analysed here, as in another SSPE-defective virus (Cattaneo *et al.*, 1986), the localized transcription alteration is not accounted for by changes in the corresponding genomic sequence. Thus, most likely the transcription machineries of the two defective viruses are no longer able to recognize the particular sequence at the P/M gene boundary as a transcription punctuation signal.

Results

Quantification of the MV RNA species produced during lytic infection

To analyse quantitatively the transcription of MV and of SSPE-defective viruses we needed RNA standards of known concentration. To produce such standards we subcloned inserts corresponding to the six MV genes in vector pGEM-1 (Materials and methods). In this vector the SP6 and T7 bacteriophage promoters flanked the MV inserts. Using these promoters we produced either short RNA segments of MV plus strand polarity, which were used as standards for quantitation, or RNA copies of the minus strands labelled at high specific activity, which were used as probes. Figure 1A shows a Northern blot analysis in which polyadenylated RNA derived from a lytic infection was mixed with six standard RNAs. After electrophoresis the RNA was blotted onto a nitrocellulose filter which was cut into strips; each strip was hybridized with a different probe specific for one of the six MV genes. Since the yield of total RNA was 10 pg/cell and about 8% of this RNA was retained on oligo(dT) cellulose, the 2 μg

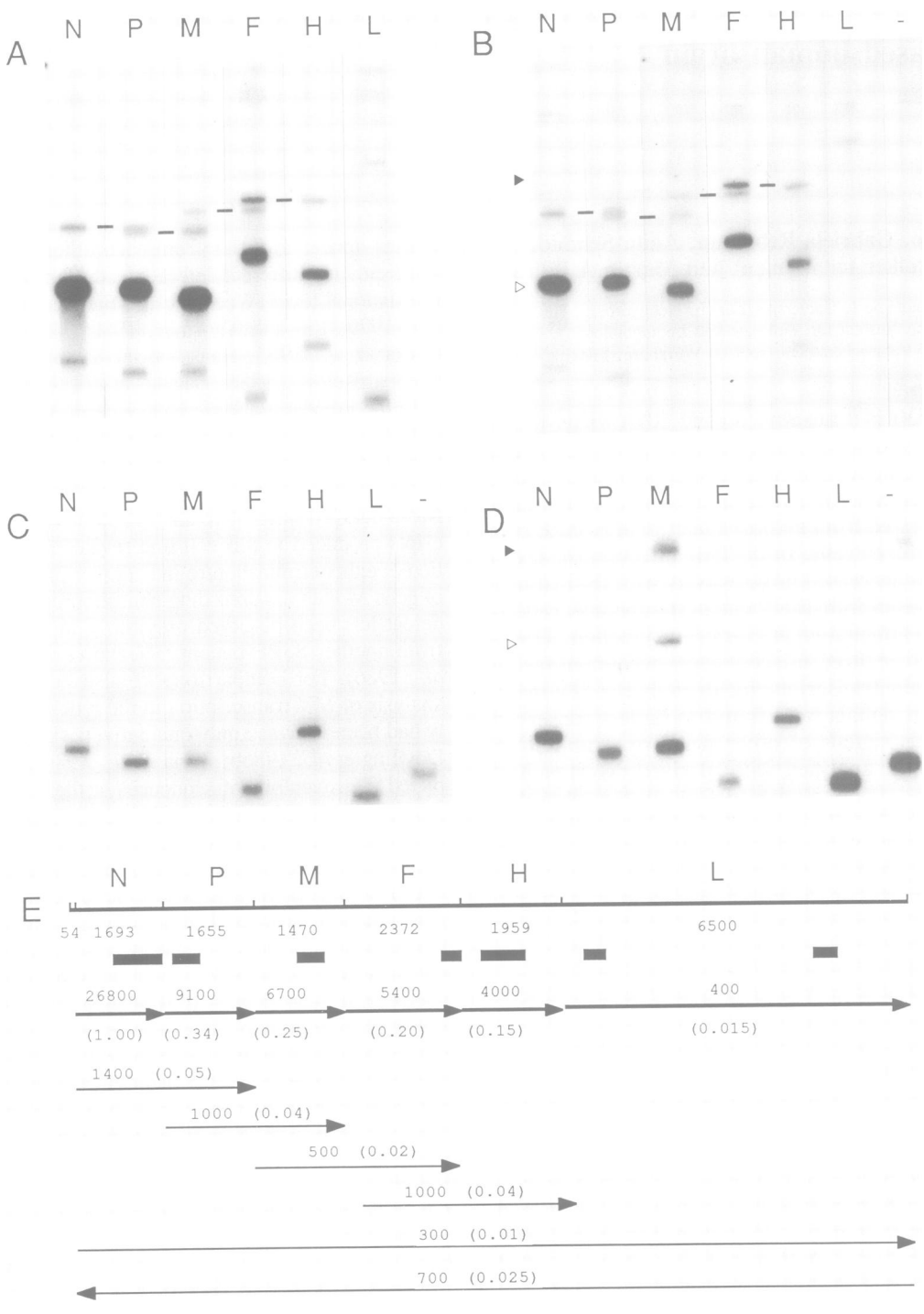

Fig. 1. Northern blot analysis of the RNAs produced in the MV lytic cycle. **(A)** Analysis of polyadenylated RNA from MV infected Hela cells. Two μg of polyadenylated RNA were mixed with 4 fmol of each standard RNA of the six MV genes, applied to a broad slot (60 mm) of a denaturing gel, electrophoresed and blotted onto a nitrocellulose filter. Five mm broad strips were used for hybridization with probes for the six MV genes. The autoradiographs shown are ordered according to the position of the probe with respect to the genome: N (nucleocapsid), P (phosphoprotein), M (matrix), F (fusion), H (hemagglutinin) and L (large or polymerase). The exposure time of single lanes was optimized to give signals of similar strength for the standards, and varied from some to several hours. **(B)** Analysis of 10 μg total RNA from MV infected Hela cells mixed with 1 fmol standard RNAs. The position of 28S rRNA is indicated by a solid triangle, 18S rRNA by an open triangle. The seventh lane corresponds to the genome probe (marked minus, −). **(C)** Analysis of standard RNAs alone. **(D)** Analysis of 10 μg total RNA from uninfected Hela cells mixed with 1 fmol standard RNAs. **(E)** Scheme of the MV genome indicating the length of the genes, the position of probe sequences (boxes), the number of copies per cell of the MV specific RNAs (numbers above the arrows representing the RNAs) and the relative amount of the RNA species in comparison with the N monocistronic transcript (numbers in parentheses). The MV gene order is as in Rima *et al.* (1986) and Dowling *et al.* (1986b). The gene lengths were determined by cloning and sequencing for the first five genes (Rozenblatt *et al.*, 1985; Bellini *et al.*, 1985, 1986; Richardson *et al.*, 1986; Alkhatib and Briedis, 1986) and for the L gene estimated from the mobility of the corresponding mRNA. The 54 nt long potential leader sequence (Billeter *et al.*, 1984) is indicated at the 3′ end of the genome. The copy number per cell of each MV RNA is the average of the determinations for the total and the polyadenylated RNA; these two estimations differed by 2−20% except in the case of the M transcript where the quantity observed in polyadenylated RNA was only 40% of that in total RNA. For the bicistronic RNAs the results obtained with the two probes were also averaged when the bands could be clearly separated; for the antigenome the results obtained with the three probes showing the lowest background around the corresponding band were averaged.

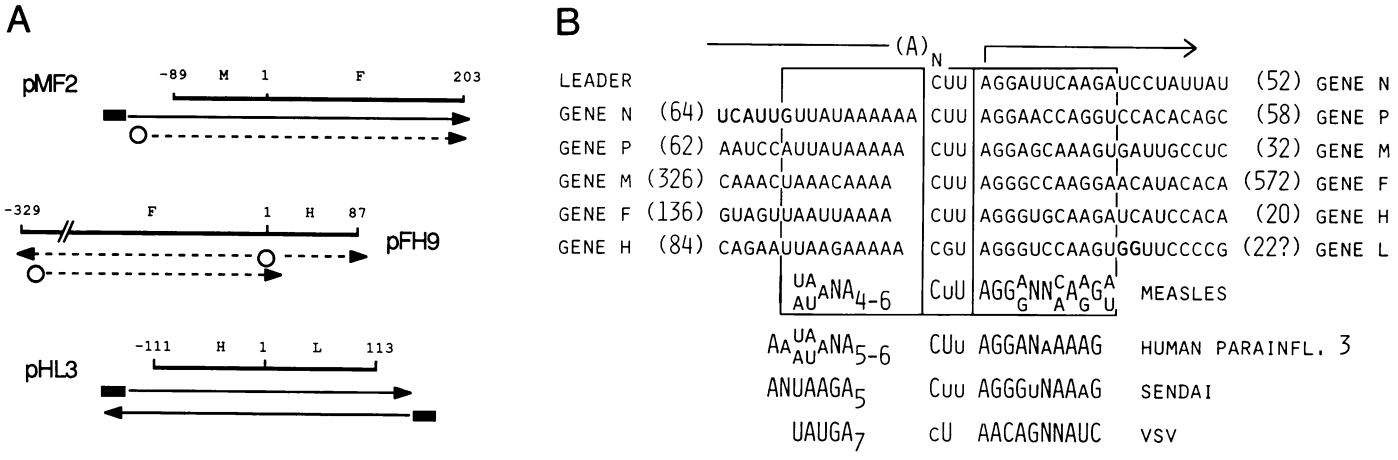

Fig. 2. Sequences of the intercistronic regions of MV and other negative strand RNA viruses. **(A)** Sequencing strategy for the inserts of clones pMF2, pFH9 and pHL3. The insert of these clones are indicated as thick lines and numbered from the first nucleotide of the F, H and L genes respectively. The interrupted lines drawn under the inserts indicate sequences obtained by the chemical degradation method (Maxam and Gilbert, 1980), the continuous lines sequences obtained by the chain termination method (Sanger *et al.*, 1980). The black boxes represent sequencing primers, the open circles restriction sites used for sequencing. Only the relevant parts of the insert sequences are shown in part **(B)**, but the entire sequences can be reconstructed as indicated in Materials and methods. **(B)** Alignment of the intergenic sequences of MV with those of other negative stranded viruses. The MV N−P and P−M intergenic sequences were from Bellini *et al.* (1985), the start of the MV N gene from Billeter *et al.* (1984), the consensus sequences of human parainfluenza 3 virus, Sendai and VSV from Spriggs and Collins (1986), Gupta and Kingsbury (1984) and Rose (1980). The distances of the coding sequences from the intergenic CUU are given in parentheses.

of polyadenylated RNA represented material from about 2.4 $\times$ 10^6 cells. Four fmol (2.4 $\times$ 10^9 molecules) of each of the standard RNAs had been added. Thus an intensity of a band similar to that seen in the internal standard corresponds to 1000 molecules of RNA per cell. As shown in Figure 1A the amount of monocistronic RNAs was much higher, whereas the amount of the more slowly moving bicistronic RNAs (marked with solid lines) was fluctuating around 1000 copies per cell. Faintly visible tricistronic transcripts were also detectable.

To determine precisely the amount of each MV RNA species we cut the corresponding bands from the nitrocellulose filter and measured the radioactivity retained. After subtraction of the background and averaging with the data obtained from total RNA (Figure 1B) we calculated the numbers shown in Figure 1E: the five shorter MV transcripts were present at concentrations ranging from about 27 000 to 4000 molecules per cell and about 400 L transcripts were detected. The amount of transcripts diminished in parallel with the distance of the corresponding gene from the 3′ end of the genome. A similar gradient at levels about 10-fold lower was detected for the bicistronic RNAs. However, the F−H transcript was present at levels higher than the preceding bicistronic RNA.

The analysis of total cellular RNA also allowed the quantitation of the antigenomic RNA to ~300 copies per cell (Figure 1B; the internal standards correspond here to ~600 molecules per cell). To quantify the genomic RNA (minus strand) we used a seventh probe, of plus strand polarity, derived from a region close to the 5′ end of the MV genome (Figure 1B, lane −, and Figure 1E). About 700 molecules of genomic RNA per cell were detected, that is about twice the amount of antigenomic RNA. A similar ratio was determined earlier on MV genomic RNA isolated from nucleocapsids (Udem and Cook, 1984). The estimation of ~1000 molecules of plus and minus genomic RNAs per average cell is also in fairly good agreemment with the yield of ~1200 copies per cell of genomic RNA recovered from nucleocapsid preparations (Materials and methods).

The controls shown in Figure 1C and D established that no background hybridization in the filter area corresponding to MV-specific RNAs is produced from the internal standards (Figure 1C), and that total RNA extracted from uninfected Hela cells does not cross-hybridize with the probes used, except for the G−C rich M probe (Figure 1D). This probe was retained to a small extent by ribosomal RNAs even after very stringent washings (Materials and methods), possibly due to semi-specific hybridization with ribosomal RNA (McClure and Perrault, 1985,1986).

Sequence of the MV intercistronic regions

The sequences at the gene boundaries of negative strand RNA viruses are thought to act as signals for the generation of transcripts. These sequences are generally highly conserved at every gene junction within one virus species (Rose, 1980; Gupta and Kingsbury, 1984; Spriggs and Collins, 1986). The sequence of the MV N−P and P−M gene boundaries has been determined recently (Bellini *et al.*, 1985). To complete the analysis of the MV intergenic regions, we cloned and sequenced the M−F, F−H and H−L gene junctions of genomic MV RNA as illustrated in Figure 2A. All these MV sequences are aligned in Figure 2B, and a consensus is indicated below them. In this consensus upper case letters represent nucleotides conserved in all the sequences, single lower case letters represent nucleotides conserved in all except one sequence. Two lower case letters signify pairs of nucleotides alternatively conserved. N indicates one non-conserved nucleotide and no gaps are allowed. For comparison the consensus intercistronic sequences of vesicular stomatitis virus (VSV), Sendai virus (SV) and human parainfluenza virus type 3 (PF3) are listed (Rose, 1980; Gupta and Kingsbury, 1984; Spriggs and Collins, 1986). Generally, the area of sequence similarity is extended over a similar range (23−25 nucleotides) in all viruses, but as a whole the MV intergenic sequences are considerably less conserved than the corresponding sequences of the other three viruses. Of the 10 nucleotides transcribed as the 5′ ends of mRNAs, eight are perfectly conserved in VSV and PF3, seven in SV and only five in MV. The degree of conservation of the nucleotides at the 3′ end of mRNAs is even lower: the number of final As varies from four to six, and none of the preceding nucleotides is perfectly conserved. In this respect MV differs drastically from VSV or SV, where both the number of

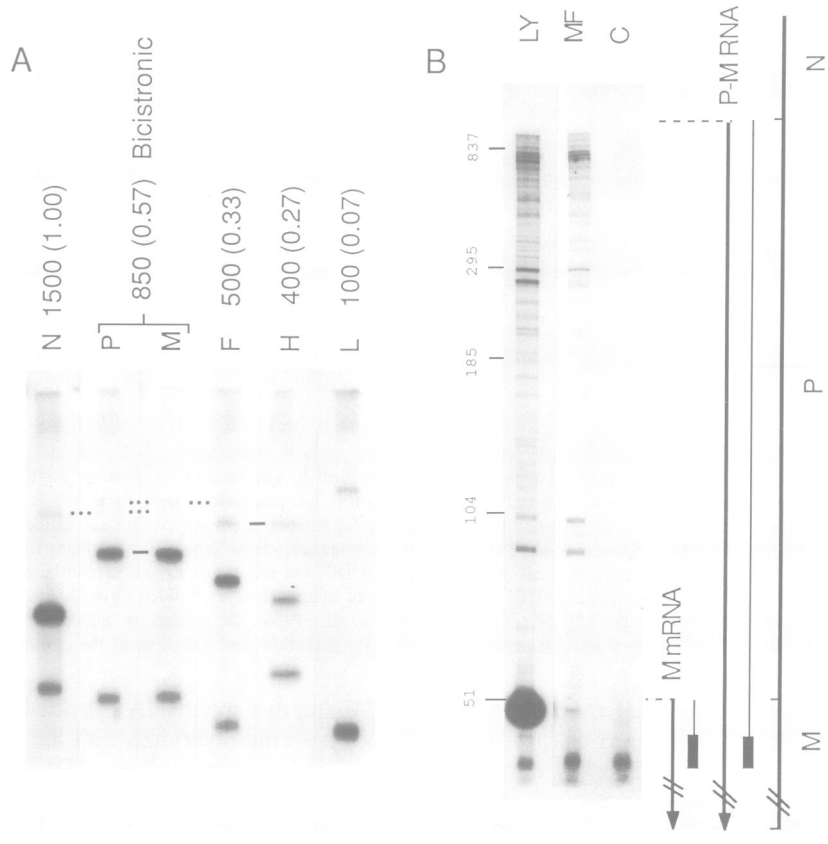

Fig. 3. Analysis of the transcripts produced by cell lines MF and MFL6. **(A)** Northern blot of polyadenylated RNA (1 μg) of cell line MFL6, mixed with 2 fmol standard RNAs. The strength of the signal of the standards corresponds to about 430 molecules of RNA per cell (the yield of polyadenylated RNA was 3.6% of the total). The estimated number of copies per cell of each RNA species is indicated above the corresponding lane. The position of bands due to bicistronic and tricistronic RNAs is indicated by lines and dots, respectively. **(B)** Primer extension analysis of the transcripts from the M gene of cell line MF. A 20-mer primer complementary to nucleotides 50 to 31 of the M mRNA (5′-GTCGTAGATCTCTGTCATTG-3′) was labelled at its 5′ end, hybridized with polyadenylated RNA from lytically infected cells (**lane LY**), from cell line MF (**lane MF**) or from uninfected cells (**lane C**), and extended with reverse transcriptase. The reaction products were analysed by electrophoresis in a 5% acrylamide-7M urea denaturing gel. Mol. wt markers are indicated on the left of the gel. The position of the bands corresponding to the monocistronic M mRNA and to the P−M bicistronic RNA are indicated as interrupted lines; in the drawing RNAs are represented as arrows, the primer as a black box and the extended products as a thin line.

final As and the preceding four nucleotides are always identical. The situation of PF3 is somewhat intermediate: there the degeneration of the consensus sequence at the 3′ end of the mRNAs is almost exclusively due to the M gene, which is markedly different from the other genes. (This correlates with the expression of high amounts of bicistronic M−F RNAs; Spriggs and Collins, 1986). Only the three MV intercistronic nucleotides are conserved at a level comparable to the other three viruses and it is noteworthy that the only non-conventional intercistronic trinucleotide is located at the H−L junction both in MV and SV, and possibly also in PF3.

The SSPE-defective virus MF shows a localized transcription alteration

To gain some insight into the mechanisms involved in the formation of MV mRNAs we analysed in detail the transcripts of a cell-associated defective MV originally obtained by cocultivation of brain cells of SSPE patient MF with fibroblasts, and shown to produce abnormally large M transcripts (Kratsch *et al.*, 1977; Carter *et al.*, 1984). We used either RNA from the original MF cell line or from a derivative called MFL6 which was passaged once over a rat brain (Baczko *et al.*, 1984b). Since the transcripts of the two cell lines were qualitatively indistinguishable in Northern blots, but cell line MFL6 expressed a higher level of MV-specific transcripts, RNA from MFL6 was used for most of the following analyses.

To define the precise nature of the larger M transcript we analysed polyadenylated RNA of cell line MFL6 with the set of probes described above (Figure 1E). Figure 3A shows that the relative amounts of a number of MV mono-, bi- and tricistronic transcripts are altered in cell line MFL6: in particular the P and M mRNAs are substituted by a P−M bicistronic transcript. This RNA corresponds to the M transcript of abnormal size detected by Carter *et al.* (1984). In addition the N−P and M−F bicistronic RNAs have been replaced by larger N−P−M and P−M−F transcripts, whereas the M−F−H tricistronic transcript could not be detected. All these differences from the lytic cycle can be reconciled with one single alteration of MV transcription, localized at the P−M junction. This alteration causes a dramatic reduction of all the transcripts initiating with the M gene or terminating with the P gene, and a concomitant increase of the corresponding 'nonprocessed' RNAs. The quantitative data presented at the top of Figure 3A indicate that the steady state concentration of the MV transcripts expressed in cell line MFL6 decreased gradually from 1500 to 100 copies per cell, showing a gradient similar to the one observed in the lytic infection, but at a level about 10-fold lower.

With the Northern analysis presented in Figure 3A no transcript initiating with the M gene could be detected in polyadenylated RNA extracted from cell line MFL6, and the same held true for polyadenylated and total RNA of line MF (data not shown). To test whether some correctly initiated M transcripts could be

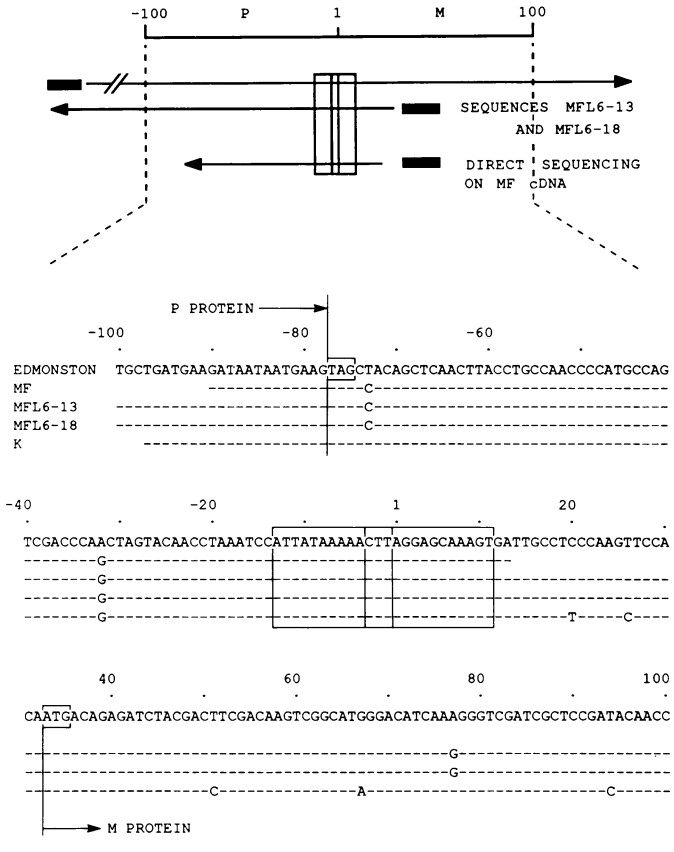

Fig. 4. Sequences flanking the P−M gene junction in MV (Edmonston) and SSPE-defective viruses. In the upper part the strategy for sequencing the inserts of the clones pL6PM-13 and -18, derived from the SSPE-defective virus MF is summarized together with the strategy for directly sequencing the cDNAs obtained after elongation of a specific primer on the polyadenylated RNA of cell line MF. Primers are represented as black boxes, sequenced portions as arrowed lines and the 'consensus' intercistronic sequence is boxed. In the lower part the sequence of the Edmonston strain is shown completely, and only the differences from it are indicated in the other sequences. The first nucleotide of the M gene was chosen as position 1. The sequence indicated as K was derived from RNA extracted from the brain of patient K (Cattaneo *et al.*, 1986).

detected by a more sensitive method we used an end-labeled primer complementary to position 50 to 31 of the M gene. This primer was hybridized to polyadenylated RNA of cell line MF and elongated with reverse transcriptase. As shown in Figure 3B, lane MF, a weak signal was detected at the position corresponding to correctly initiated/processed M transcripts. This signal was more than 50 times weaker than the sum of the signals observed in the upper part of the gel, and corresponding to bicistronic P−M transcripts. As a consequence, cell line MF does not produce detectable amounts of M protein (Carter *et al.*, 1984).

The sequence around the junction of genes P and M of the defective SSPE virus MF is not altered by mutation

The most straightforward explanation of the documented transcription defect would be an alteration of the consensus sequence at the P−M gene boundary. We therefore analysed the RNA of cell line MF by sequencing directly cDNAs obtained from the elongation of a specific end-labeled primer on polyadenylated RNA (top of Figure 4). In parallel we cloned the relevant sequences of cell line MFL6 using polyadenylated RNA and specific primers (Materials and methods). The results of the sequencing of two clones from cell line MFL6 and of the direct sequencing of the cDNA of cell line MF are presented in the lower part of Figure 4. Of 200 nucleotides surrounding the P−M gene junction only three differed between both MFL6 clones (sequences MFL6−13 and MFL6−18) and the Edmonston strain (sequence Edmonston), at a distance of −73, −32 and +77 nucleotides from the first nucleotide of the M gene. The sequence of the cDNA elongated on polyadenylated RNA extracted from cell line MF (line MF) was indistinguishable from the sequence of the two clones obtained from cell line MFL6. Thus the 'consensus' sequence at the P−M gene junction of cell lines MF and MFL6 was not altered by mutation.

In Figure 4 the sequence of the P−M gene junction of another SSPE-defective virus shown previously to substitute the M and P mRNAs with a P−M bicistronic transcript is also shown (sequence K, Cattaneo *et al.*, 1986). Also in case K, all the changes are situated outside the MV 'consensus' sequence (boxed in Figure 4). Although it is formally possible that the only difference common in both SSPE viruses as compared to the Edmonston strain (G instead of A at position −32) would be responsible for the defect in P- and M-mRNA formation we consider this explanation unlikely, among others because this nucleotide would not be involved in the formation of a stable secondary structure which can be drawn theoretically for this RNA region. Furthermore it should be mentioned that in case MF the M reading frame was not interrupted by a stop codon (R.Cattaneo, unpublished data). This finding eliminates the possibility (proposed for case K) that the defect of transcription could be linked to lack of translation of the region downstream of the RNA processing site. It is most likely not a *cis*-acting signal, therefore, but a factor acting in *trans* that is responsible for the transcription defect common to both SSPE viruses.

Discussion

MV transcription and regulation of gene expression

In VSV, the most extensively studied nonsegmented negative strand RNA virus, a gradient of decreasing mRNA abundance was observed (Villareal *et al.*, 1976; Iverson and Rose, 1981) and it was found that the rates of degradation and the translation efficiencies of VSV RNAs are similar (Pennica *et al.*, 1979; Lodish and Froshauer, 1977). Thus it was concluded that, in VSV, attenuation of transcription is the principal regulatory mechanism used for the differential production of the viral proteins. In MV the same seems to hold true: the amount of viral protein detected in the lytic infection roughly reflects the gene order (Udem, 1984), the rates of degradation are similar for all MV mRNAs (Yoshikawa *et al.*, 1986), and we have now established that a gradient of transcription exists in the lytic infection.

In the MF persistent infection the transcription gradient is similar, but all mRNA levels are about 10-fold lower than in the lytic infection. In brain material of two SSPE and one inclusion body encephalitis patients transcription gradients were also monitored (unpublished results). However they started at levels about 10-fold lower than in the lytic infection and particularly showed a more pronounced attenuation at every gene junction. These data indicate that in addition to individual MV gene expression defects in different SSPE cases (Baczko *et al.*, 1986), a general quantitative alteration of MV transcription and thus protein synthesis exists in MV brain infections, leading to an unbalanced low expression of the viral envelope proteins.

The bicistronic transcript overexpressed in the MF defective virus does not contain internal polyadenylate stretches. The same was observed in the minor species of readthrough transcripts expressed *in vivo* by other negative strand RNA viruses including VSV, Sendai and Newcastle disease virus (Masters and Samuel,

1984; Gupta and Kingsbury, 1985; Wilde and Morrison, 1984). Pulse-chase experiments indicate that MV polycistronic transcripts are not precursors of the monocistronic RNAs (Yoshikawa *et al.*, 1986). Taken together, these observations indicate that transcripts of negative strand RNA viruses are most likely synthesized by a polymerase stop−start mechanism (Gupta and Kingsbury, 1985) and not by cleavage of internally polyadenylated precursors (Herman *et al.*, 1978 and 1980).

It remains speculative whether bicistronic transcripts fulfill a template function to produce minority proteins. Viral bicistronic transcripts are associated with ribosomes (Wilde and Morrison, 1984), and the analysis of the MV sequence at the junction of genes M and F presented here revealed that an open reading frame starting downstream of the M coding region extends into the F 5′ 'untranslated' region. This open reading frame totals 336 amino acids and could be translated only from a bicistronic RNA.

The molecular basis of MV persistence

The stability of the MV transcription defect characteristic for the SSPE-defective virus MF should be emphasized. The original defect was perpetuated over several dozens of passages in fibroblasts permissive for the MV lytic cycle (Carter *et al.*, 1984), transmitted to a rat brain (Baczko *et al.*, 1984b), recovered from it and propagated again over several passages in cultured cells. Thus the defect appears to be genetic and not imposed by the cellular environment. One might therefore conclude that cellular factors modulating viral gene expression are not necessarily involved in maintenance of MV persistence, even if they possibly influence its establishment (Oldstone *et al.*, 1980; Rammohan *et al.*, 1981; Fujinami *et al.*, 1984; Barret *et al.*, 1985).

The stable phenotype of SSPE-defective virus MF is remarkable considering the infidelity of RNA virus genome replication (Holland *et al.*, 1982; Domingo *et al.*, 1985). Sequencing of two clones obtained from the same MFL6 RNA preparation over more than 1200 overlapping positions revealed one difference (R.Cattaneo, unpublished results). This indicates that despite the stability of the phenotype, genomic variants are present in defective virus MF.

The specific alteration of transcription in defective virus MF is another example of the disparate lesions of MV gene expression characterizing different SSPE cases.Initially, alterations of M protein expression were observed in several SSPE-defective viruses (Wechsler and Fields, 1978; Hall and Choppin, 1979). More recently it became evident that M protein can be expressed in SSPE brains and that generally one or more of the envelope proteins (M, F or H) can be altered in MV persistent infections (Young *et al.*, 1985; Norrby *et al.*, 1985; Sheppard *et al.*, 1986; Baczko *et al.*, 1986; Liebert *et al.*, 1986). The diversity of the defects restricting MV gene expression in SSPE brains and the detection of 1% nucleotide differences in sibling clones derived from the same brain (Cattaneo *et al.*, 1986) suggests that the cause underlying these defects may be a particularly error-prone SSPE-defective virus replication machinery.

The exact nature of the mutation(s) causing the localized alteration of transcription in cases MF and K remains a matter of speculation. Most likely defect(s) of viral protein(s) implicated in transcription (the polymerase itself or the proteins N or P) underly these phenotypes. It should be possible to identify these defects by setting up an *in vitro* or a complementation system. The establishment of an *in vitro* system based on synthetic MV genomes is complicated by the fact that MV genomes are synthesized and transcribed *in vivo* in the form of nucleocapsids. It might be simpler to rescue infectious viruses from SSPE cell lines by transfecting them with functional MV genes; the use of genes variously recombined from different cDNAs should then allow the identification of mutations accounting for defective phenotypes. The central question will then still remain whether such mutations caused SSPE or if they were introduced in the course of the disease, contributing marginally if at all to the pathogenicity of the virus. To answer this question reconstitution experiments employing whole animals are needed.

Materials and methods

Cells and virus

The MV Edmonston strain was propagated in Hela S3 cells basically as described (Udem, 1984; Cattaneo *et al.*, 1986). The titer of infectious virus liberated by freeze-thawing at collection ranged between 10^6 and 10^7 p.f.u./ml medium (corresponding to 2−20 p.f.u./average cell).

The SSPE-defective virus MF was isolated from a SSPE patient by co-cultivation of brain cells with Vero cells (Kratzsch *et al.*, 1977), and RNA was isolated from passage 52. The MFL6 variant was generated by injecting infected Vero cells intracerebrally into a rat. The diseased animal was sacrificed and the brain removed. Brain material was minced, co-cultivated with Vero cells and a persistently infected cell culture established. RNA was extracted after several passages.

Preparation of total cellular RNA and of genomic MV RNA from nucleocapsids

Total RNA was extracted from Hela cells using a modified lithium chloride−urea protocol (Auffray and Rougeon, 1980; Cattaneo *et al.*, 1984). Yields were of 5 mg total RNA from 5×10^8 cells. Polyadenylated RNA was selected by passage through a column of oligo(dT) cellulose basically as described (Maniatis *et al.*, 1982), except that Hepes pH 7.0 was used to substitute Tris−HCl pH 7.5 and a cushion of Chelex 100 chelating resin (Bio-Rad, Richmond, CA, USA) was on the bottom of the oligo(dT) column to adsorb residual heavy metal ions.

Total RNA was extracted from MF and MFL6 cells basically as described (Chirgwin *et al.*, 1979; Baczko *et al.*, 1986) with additional proteinase K and DNase digestions. After pelleting through CsCl total cellular RNA was dissolved in HE (10 mM Hepes, pH 7.0, 0.5 M EDTA) containing 0.5% SDS and digested for 10 min at 37°C with 50 μg/ml proteinase K to eliminate residual protein contamination. This preparation was phenol extracted twice and ethanol precipitated. To eliminate residual DNA the pellet was dissolved in HE supplemented with 2.5 mM $CaCl_2$ and digested with RNase-free DNase purified as described (Picard and Schaffner, 1983).

Intracellular nucleocapsid RNA was prepared basically as described (Udem and Cook, 1984), with yields of about 5 μg RNA from 5×10^8 cells, corresponding to about 1200 copies of genomic RNA per average cell.

Cloning from MV genomic RNA and from polyadenylated cellular RNA

Clones pMF2 and pHL3 were obtained after annealing of a 20-mer primer 5′-CCGAACCGCACAAGCGACCG-3′, complementary to nucleotides 86−67 from the 3′ end of the M gene, to MV genomic RNA and cDNA synthesis. First strand synthesis was done with reverse transcriptase, and second strand synthesis with *E. coli* polymerase I, *E. coli* ligase and RNase H using standard procedures (Maniatis *et al.*, 1982; Gubler and Hoffman, 1983). The cDNAs were inserted in the *Sma*I site of vector pEMBL 18^- (Dente *et al.*, 1983; Yanisch-Perron *et al.*, 1985). Clone pFH9 has been described (Billeter *et al.*, 1984).

Clones pL6-PM13 and pL6-PM18 were obtained from polyadenylated RNA from cell line MFL6 by a cloning procedure involving MV-specific primers with additional C and G nucleotides at their 5′ ends to fit into *Acc*I and *Xcy*I sites. This procedure will be described in detail elsewhere and involves use of specific primers for both first and second strand synthesis and subsequent tailoring of the cDNA ends with T4 polymerase in presence of dATP and dTTP and absence of dCTP and dGTP to yield appropriate 5′ overhanging ends. The cDNAs were then cloned in plasmid bluescript M13 (Stratagene, San Diego, California) digested with *Acc*I and *Xcy*I.

Sequences of the MV intercistronic regions

The sequences of clones pMF2 and pFH9, shown only in part in Figure 2, were very similar to the corresponding M, F and H sequences published previously (Bellini *et al.*, 1986; Richardson *et al.*, 1986; Alkhatib and Briedis, 1986). Over 695 positions compared, three base substitutions were noticed, two in non-translated regions and one resulting in a silent mutation. Moreover a GC pair at position 1426−1427 in the M sequence of Bellini *et al.* (1986) was sequenced as a single A residue in our clone pMF2 as well as in clone CL-M, originally used by Bellini *et al.*

The insert of pHL3 (total length 270 nt) contains 45 nucleotides of the M gene (including the 20-mer), but these are followed by 225 nucleotides not belonging to the M gene. The origin of these additional nucleotides was found by a computer search of published MV sequences: 107 of the following nucleotides showed exact homology, except for one position, to the last 108 nucleotides of the H

gene (Alkhatib and Briedis, 1986; the single difference concerned nucleotide 1894 in the untranslated region, which in our sequence is an A and not a T). To ascertain whether the following nucleotides of pHL9 are co-linear with the MV genome we synthesized a 20-mer oligonucleotide 5'-TCTCATGATGTCACCCAGAC-3' corresponding to nucleotides 1892–1911 of the H gene. This primer was elongated on genomic RNA and the cDNA products were sequenced by the chemical degradation method (Maxam and Gilbert, 1980). The sequence obtained directly from the cDNA was in complete agreement with the sequence of the pHL3 insert, thus we conclude that the 225 nt of the pHL3 insert not derived from the M gene are co-linear to the MV genome, and that 116 of these nucleotides are due to the L cistron. The sequence of clone pHL3 after the end of the H gene was the following: CGTAGGGTCC'AAGTGGTTCC'CCGTTATGGA'CTCGC-TATCT'GTCAACCAGA'TCTTATACCC'TGAAGTTCAC'CTAGATAGC-C'CGATAGTTAC'CAATAAGATA'GTAGCCATCC'TGGAGTA. The intercistronic 'consensus' sequence is underlined, as well as a potential initiation codon, which is followed by an open reading frame of at least 30 amino acids.

Subcloning in SP6-T7 vectors

Fragments from each MV gene were subcloned in a plasmid bearing the promoters for the SP6 and T7 bacteriophages RNA polymerases on both sides of a polylinker (pGEM-1, Schenborn and Mierendorf, 1985). The position of the fragments on the MV genome is indicated in Figure 1E. A 851 bp *Eco*RV–*Xba*I fragment of the N gene derived from plasmid CL-15 (Rozenblatt *et al.*, 1985) was used for subcloning; a 531 bp *Bal*HI–*Hin*dIII fragment from plasmid pP1 (A.Schmid and M.A.Billeter, unpublished; see Bellini *et al.*, 1985 for the sequence of the P gene) was used for the P gene; a 520 bp *Pst*I–*Sma*I fragment derived from plasmid pM1 (A.Schmid and M.A.Billeter, unpublished; see Bellini *et al.*, 1986 for the sequence of the M gene) was used for the M gene; a 320 bp *Taq*I–*Dde*I fragment from plasmid pFH9 (this work; see Richardson *et al.*, 1986, for the F sequence) was used for the F gene; a 787 bp *Bgl*II–*Ava*I fragment derived from pH8 (Billeter *et al.*, 1984; see Alkhatib and Briedis, 1986 for the sequence of the H gene) was used for the H gene and a 342 bp *Eco*RI–*Hin*fI fragment was used for the L gene. This fragment was derived from pL6, which hybridized with the MV L mRNA (Billeter *et al.*, 1984; Rima *et al.*, 1986) and shows 70% homology with positions 712–787 of the Sendai virus L gene (Morgan and Rakestraw, 1986). Thus it most likely maps near the 5' end of the MV L gene. Finally a *Eco*RV–*Pst*I fragment derived from plasmid pL11 (Billeter *et al.*, 1984) was used as probe for the negative-stranded MV genome. This insert was shown to hybridize with one strand to the L-mRNA and with both strands to small, possibly defective-interfering-like RNAs (K.Baczko, M.A.Billeter and Ursula Ernst, unpublished). For this reason this fragment was drawn in Figure 1E near the 5' end of the genome, but its precise location remains unknown.

Synthesis of RNA standards and of specific probes

To synthesize RNA standards and probes of high specific activity the SP6 or the T7 promoters were used, depending on the orientation of the insert in the linker of pGEM-1. The RNAs were synthesized using standard procedures (Melton *et al.*, 1984; Schenborn and Mierendorf, 1985). The exact molar amount of transcripts produced as standards was determined by adding trace amounts of [α-^{32}P]-rUTP to a mixture containing the four ribonucleotides at a concentration of 0.5 mM. The percentage of labeled nucleotides incorporated into the transcripts was determined, and the number of transcripts produced calculated considering the number of Us present in each of the different gene fragments. Usually in such a reaction a 15- to 80-fold molar excess of RNA products over DNA template was obtained. To produce RNA probes a final concentration of 12 μM [α-^{32}P]rUTP with a specific activity of 400 Ci/mmol and 0.5 mM of the unlabeled ribonucleotides were used in 20 μl reactions. Typically a molar ratio of approximately 1:1 of RNA product to DNA template was obtained while specific activities ranged from 3 to 7 $\times$ 10^8 c.p.m./μg RNA. The exact lengths of the standard RNAs used were of 670, 560, 543, 351, 822, 362 and 413 bases for the N, P, M, F, H, L and the minus strand standards. Maps of the plasmids and lists of the enzymes used for linearization are available on request.

Northern blotting

Northern blotting was performed using 1.2% agarose, 2.2 M formaldehyde gels and nitrocellulose paper (Schleicher and Schüll, Dassel, FRG) basically as described (Maniatis *et al.*, 1982). Hybridization and washings were as modified for the use of RNA probes (Melton *et al.*, 1984). To lower the problems of background hybridization with ribosomal RNA observed with the G–C rich M probe, washings in 0.1 $\times$ SSC, 0.1% SDS were performed three times for 20 min at 75°C. The signals were quantified by liquid scintillation counting of the corresponding pieces of the filters. The background was estimated from similar sized pieces of the filters from regions immediately above and below the bands.

Acknowledgements

We thank Horst Hornig for synthesis of oligonucleotides, Fritz Ochsenbein for the photographs, Jeannette Roller for excellent technical assistence, Stephen Udem for MV Edmonston virus adapted to growth in Hela S3 cells, William Bellini, Christopher Richardson and Exeen Morgan for sharing unpublished sequence data, Walter Schaffner and Charles Samuel for critical reading of the manuscript and Charles Weissmann for discussions. This work was supported by grant 3.141-085 of the Schweizerische Nationalfonds to M.A.B. and by a grant of the Deutsche Forschungsgemeinschaft.

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Received on November 28, 1986; revised on January 9, 1987