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Characterization of Early Gene Transcripts of Molluscum Contagiosum Virus

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Molluscum contagiosum virus (MCV), a member of the family Poxviridae, replicates well *in vivo* but cannot be propagated in cell culture. The coding capacity of the MCV genome was previously determined by DNA nucleotide sequence analysis. The objective of the present study was to establish experimental systems for the identification and characterization of early MCV gene transcripts. MCV mRNA was obtained in three ways: (1) MCV early mRNA was synthesized *in vitro* using permeabilized virions, (2) MCV mRNA was extracted from MCV-infected skin tissue, and (3) MCV mRNA was extracted from MCV-infected human embryonic fibroblasts. RNA/DNA hybridization experiments showed significant early transcriptional activity in two parts of the MCV genome. Transcripts of 11 early MCV genes located in these parts of the genome, including two subunits of the MCV DNA-dependent RNA polymerase (mc077R and mc079R), the MCV poly(A)$^+$ polymerase gene (mc076R), and the MCV MHC class I homolog (mc080R), were detected in reverse transcription-polymerase chain reaction experiments. Total RNA obtained from MCV-infected skin tissue was used to confirm these results. Three MCV early transcripts, mc002L, mc004.1L, and mc005L, produced distinct bands on rapid amplification of their 3′ ends (3′ RACE). The 5′ mapping of transcription start sites of MCV open reading frames (ORFs) mc002L, mc004.1L, mc005L, and mc148R revealed that the MCV RNA polymerase transcription start sites are consistently located between 11 and 13 nucleotides downstream of the early MCV consensus promoter signal. When cDNA from both 5′ and 3′ mapping experiments was analyzed, MCV ORFs mc004.1L and mc005L were found to be transcribed as a single bicistronic mRNA. The transcript from MCV ORF mc066L, encoding a glutathione peroxidase, was detected in *in vitro* synthesized MCV mRNA as well as in total RNA from MCV-infected human embryonic fibroblasts and MCV-infected skin. This indicates that despite the lack of an early MCV consensus promoter signal immediately proximal to the start codon, this particular gene is transcribed early during MCV infection.

Key Words: molluscum contagiosum virus; DNA nucleotide sequencing; *in vitro* RNA synthesis; transcription; RT-PCR.

INTRODUCTION

Molluscum contagiosum (MC) virus (MCV) is a poxvirus pathogenic for humans. MCV infection is characterized by benign skin tumors that are strictly confined to the human epidermis, causing little inflammation. MC is common in children and immunocompromised individuals (e.g., HIV-infected patients), where it causes widespread disease and is a marker for late stage cellular immunodeficiency. Characterization of the viral properties has been hindered by the fact that MCV cannot be propagated in cell culture. As a first step toward better understanding of the pathogenetic mechanisms of MCV infections, the primary structure and coding capacity of MCV were determined by DNA nucleotide sequencing (Senkevich *et al.,* 1996; 1997). The objective of the present study was to detect and characterize MCV mRNA transcripts, verify the temporal patterns of MCV transcription, and investigate the specific properties of MCV mRNA. Three experimental strategies were used to achieve these goals.

First, *in vitro* synthesis of MCV mRNA was performed using permeabilized virions. Permeabilized poxvirus virions are able to transcribe 5′ capped, polyadenylated, and translatable early mRNAs in an ATP-dependent *in vitro* reaction because they carry a DNA-associated DNA-dependent RNA polymerase, a poly(A)$^+$ polymerase, and a capping enzyme in their virion particles. The mRNAs are presumably of the early temporal class because the MCV virion particles lack full complements of factors that would be required for transcription of intermediate and late genes (Broyles *et al.,* 1988; Kates and McAuslan, 1967; Munyon *et al.,* 1967; Shand *et al.,* 1976). Using this method, we recently characterized the transcript of the molluscum chemokine homolog (MCCH) gene, a β-chemokine antagonist, that was predicted to be an early gene based on its conserved poxviral early promoter signal (Bugert *et al.,* 1998).

Second, total RNA from MCV-infected skin tissue was analyzed. The amount and purity of MCV mRNA that can be obtained from MCV-infected tissue are inferior to the early MCV mRNA that is transcribed *in vitro*. However, the total RNA from MCV-infected tissues contains intermediate and late transcripts, providing the whole range of MCV transcripts. MCV-infected tissue total RNA is

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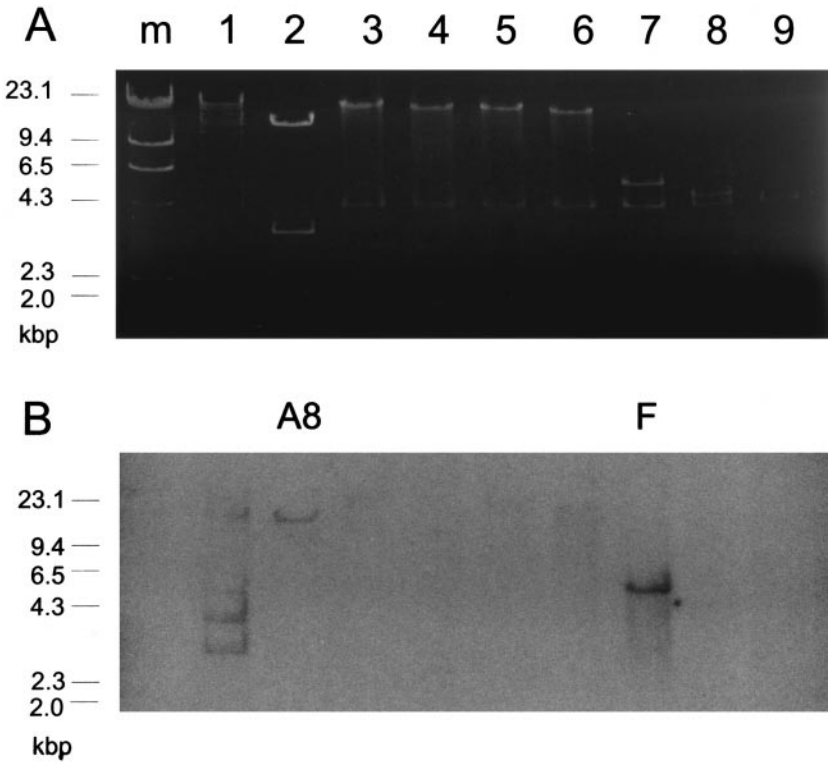

FIG. 1. Southern blot of α-^{32}P-UTP-labeled MCV mRNA against an MCV *Eco*RI DNA fragment library. (A) Slab gel analysis of MCV-1 DNA and MCV-1 recombinant plasmid library: Lambda DNA *Hin*dIII digested; molecular weights are given in kbp (m), MCV type 1 DNA *Hin*dIII digested (lane 1), pMCV-1-BH-A8 (A8), *Bam*HI–*Hin*dIII digested (lane 2), pyMCV-1-E-B, -C, -D, -E, -F (F), -H, -I *Eco*RI digested (lanes 3–9). (B) Southern blot of slab gel shown in A with α-^{32}P-UTP-labeled MCV mRNA as hybridization probe.

therefore suitable for the confirmation of data obtained with *in vitro* early mRNA as well as for control experiments, including late and intermediate MCV mRNA transcripts.

Third, total RNA from human embryonic fibroblasts that are infected with MCV was analyzed. In this unique system, MCV induces a cytopathic effect (CPE) 24 h postinfection that disappears around 72 h postinfection (Brabanti-Brodano *et al.,* 1974). One can assume that MCV gene expression occurs, and consequently, MCV mRNA must be detectable. The MCV infection is aborted because of a failure to uncoat the viral genome before DNA replication (McFadden *et al.,* 1979).

RESULTS

In vitro transcription and detection of early MCV mRNA transcripts

MCV virions were used to synthesize MCV mRNA *in vitro* by the virion- associated early transcription complex. The efficiency of permeabilized MC virions to synthesize mRNA *in vitro* was quantified by α-^{32}P-UTP labeling (data not shown; Bugert *et al.,* 1998). Under the conditions used, α-^{32}P-UTP-labeled MCV mRNA was extruded into the reaction supernatant. For further experiments with *in vitro* synthesized MCV mRNA, the supernatant containing the soluble MCV mRNA fraction was used. The *in vitro* synthesized α-^{32}P-UTP-labeled soluble MCV mRNA was used for screening of an MCV-1 DNA fragment library (Fig. 1A). The labeled MCV mRNA hybridized predominantly to two MCV DNA fragments of the MCV-1 DNA library: MCV-1 *Eco*RI fragment F (Fig. 1B, lane 7) and MCV-1 *Bam*HI–*Hin*dIII DNA fragment A8 (a part of MCV-1 *Bam*HI DNA fragment A, Fig. 1B, lane 2).

Twelve open reading frames (ORFs) with early consensus promoters (Figs. 2A and 2B) encoded by these MCV-1 DNA fragments are shown in Fig. 3 and listed in Table 1. Four MCV genes with predicted late promoters and one with predicted early promoter (mc148R) were used as late negative and early positive controls, respectively (Fig. 2C and Table 1). Oligonucleotide primers specific for these MCV genes were synthesized, and the expected RT-PCR product lengths are listed in Table 2. MCV transcripts were detected by RT-PCR from MCV mRNA synthesized *in vitro* and from total RNA isolated from MCV-infected skin tissue. The results are shown in Fig. 4 and Table 1.

The results of RT-PCR experiments using *in vitro* synthesized MCV mRNA are shown in Fig. 4A. With the exception of ORF mc004L, transcripts from all ORFs within *Eco*RI DNA fragment F were detectable in *in vitro* synthesized RNA. In *Bam*HI–*Hin*dIII DNA fragment A8,

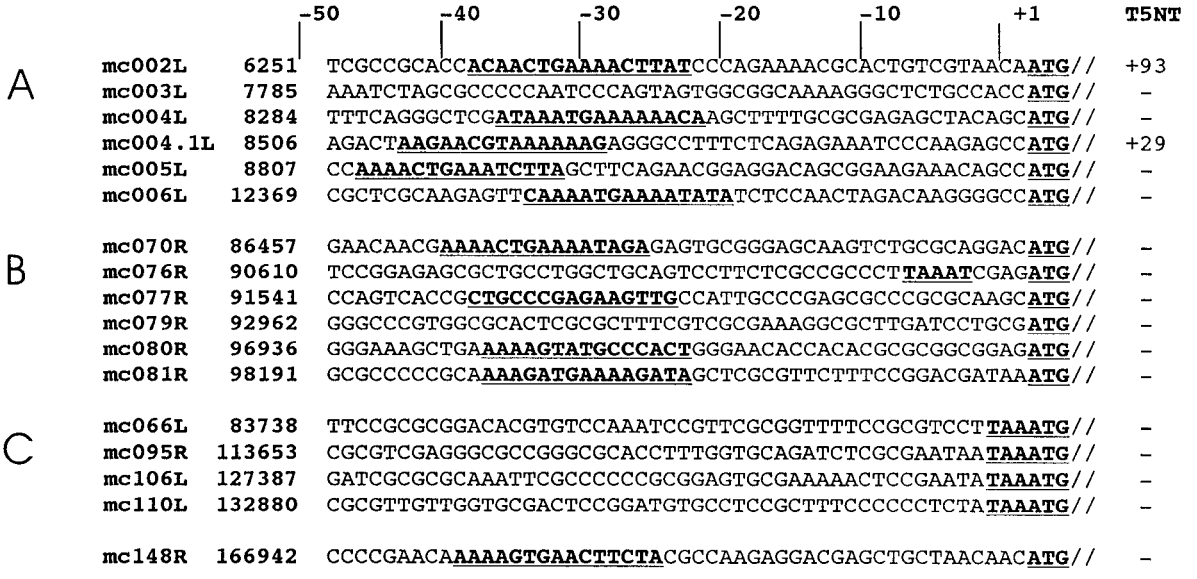

FIG. 2. Alignment of MCV promoter sequences and listing of early transcription termination signal TTTTTNT (T5NT). (A) Promoter sequences of genes encoded by MCV-1 *Eco*RI DNA fragment F. (B) Promoter sequences of genes encoded by MCV-1 *Bam*HI–*Hin*dIII DNA fragment A8. (C) Promoter sequences of four genes with predicted late promoters: MCV ORFs mc066L, mc095R, mc106L, and mc110L. MCV ORFs are identified with their three-digit number and orientation of transcription (L = leftward, R = rightward). The DNA nucleotide sequences 50 bp upstream of the start codon of the individual ORFs are aligned, and the distance to the start codon is shown in negative numbers. The first nucleotide of the start codon has the position +1. The presence of an early transcription termination signal TTTTTNT (T5NT) is indicated by its distance downstream of the stop codon or its absence is noted with a dash.

transcripts from all ORFs with early consensus promoters were detectable in *in vitro* synthesized MCV mRNA.

Results of the same RT-PCR experiments using MCV-infected tissue total RNA are shown in Fig. 4B. The experiments with total RNA from MCV-infected tissue revealed that in this more complex pool of RNA, MCV transcripts from ORFs mc076R, mc080R, and mc081R were below the threshold of detection. However, transcripts from the other ORFs tested, including ORF mc079R, which encodes the largest subunit of the MCV DNA-dependent RNA polymerase, were detected in total RNA from MCV-infected tissue, indicating that these ORFs were naturally transcribed *in vivo*.

The positive control amplification products obtained using MCV genomic DNA as template are shown in Fig. 4C. To exclude DNA contamination of the RNA preparations, *in vitro* synthesized MCV mRNA was tested in PCR experiments, and the results are shown in Fig. 4D. Nucleotide sequence analysis of the RT-PCR products confirmed the identity of the amplified MCV cDNA.

Transcription of predicted MCV late genes

Four MCV genes that were predicted to be transcribed late based on the analysis of their respective promoter signals (Senkevich *et al.,* 1997) were tested by RT-PCR, and the results are shown in Table 1 and Fig. 4 (controls). In these experiments, detection of specific transcripts was expected only from tissue total RNA because in contrast to *in vitro* synthesized MCV mRNA, cellular RNA from MCV-infected skin contains MCV mRNA transcripts of different temporal classes, including late. However, only the transcript of ORF mc066L was detectable by RT-PCR from tissue total RNA (Fig. 4B), confirming that this gene was transcribed *in vivo*. Surprisingly, mRNA transcripts of MCV ORFs mc 066L and mc110L were detectable by RT-PCR using *in vitro* transcribed early MCV mRNA (Fig. 4A)

One possible explanation for the detection of late gene transcripts in *in vitro* synthesized mRNA would be that mRNA classes other than early were transcribed from permeabilized MCV virions. A more orthodox explanation is based on an observation made during *in vitro* mRNA transcription of permeabilized vaccinia virions. Under the conditions of *in vitro* mRNA synthesis, failure of transcription termination occurs when the concentration of ATP is suboptimal in the reaction mix, causing artificially prolonged mRNA molecules, thus increasing the amount of readthrough transcripts (Shuman and Moss, 1989).

To avoid the ATP depletion in *in vitro* transcription reactions, RNA from MCV-infected human cells was tested as an alternative approach for the analysis of MCV early transcripts. Human embryonic fibroblasts were infected with purified MCV. A CPE with cell-rounding was observed after 20 h that disappeared after 72 h, with full recovery of the cell monolayer. Total RNA was extracted from MCV-infected cells after 24 h, and RT-PCR was performed using primers specific for the MCV ORFs

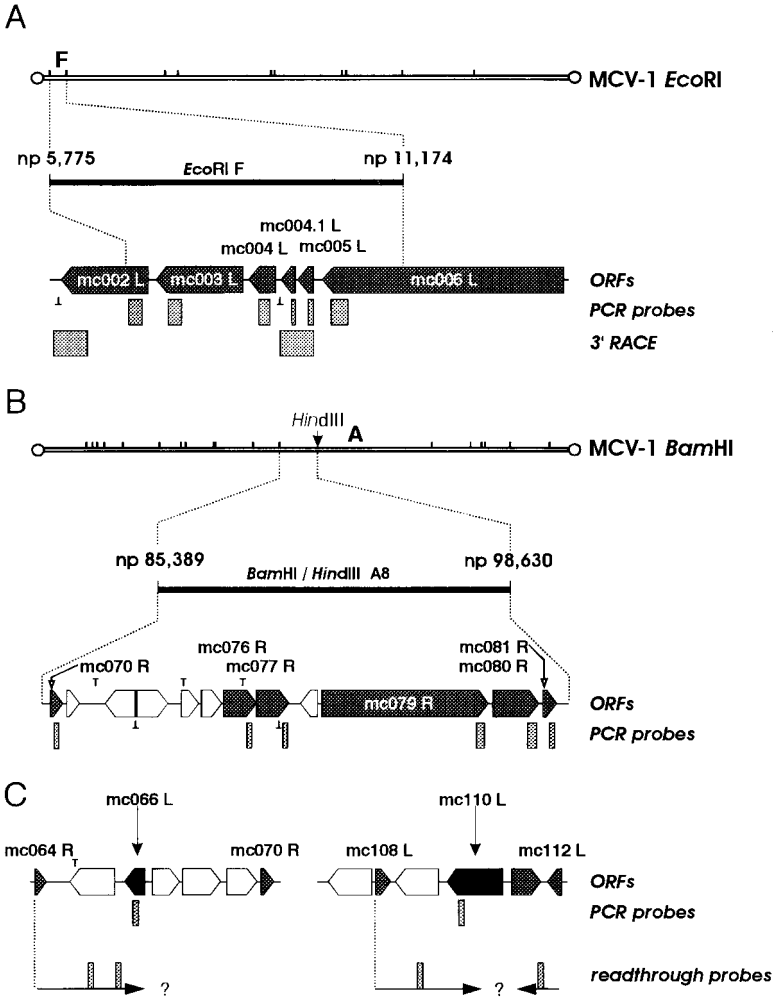

FIG. 3. Diagram showing the genomic localization of the MCV ORFs investigated. ORF orientation is shown as arrows. Black arrows indicate the presence of early consensus promoters, and white arrows indicate the presence of late consensus promoters. Location and length of PCR probes and 3′ RACE amplification products are shown as shaded boxes. T indicates consensus termination sequence (TTTTNT) for early genes transcribed rightward, and inverted T indicates consensus termination sequence for early genes transcribed leftward. (A) *Eco*RI physical map of the MCV-1 genome indicating the genomic position of *Eco*RI DNA fragment F and the position of MCV-1 ORFs mc002L to mc006L. (B) *Bam*HI physical map of the MCV-1 genome indicating the genomic position of *Bam*HI–*Hin*dIII DNA fragment A8 and the position of MCV-1 ORFs mc070R to mc081R. (C) Maps indicating the relative position of MCV-1 ORFs mc064R to mc070R and mc108L to mc112L. Black arrows indicate MCV-1 ORFs mc066L and mc110L that were investigated for readthrough transcription. Shaded arrows indicate ORFs with early represent location and length of PCR probes used to investigate readthrough transcription.

mc004L, mc066L, and mc110L. Primers specific for MCV ORF mc148R were used as a positive control. The results of this experiment are shown in Fig. 5A. Other than transcripts from MCV ORF mc148R (Fig. 5A, lane 1), only transcripts from MCV ORF mc066L were detected in this experiment (Fig. 5A, lane 3). This result indicates that MCV ORF mc066L must be transcribed early, because in MCV-infected human fibroblasts, MCV replication does not take place and late poxviral RNA transcripts are not made.

Furthermore, transcripts from MCV ORF mc110L were not detected, consistent with its late promoter signal (Fig. 5A, lane 4). Transcripts from MCV ORF mc095R, another early transcription factor subunit, also were not detectable (Fig. 5A, lane 5). Finally, mRNA transcripts from MCV ORF mc004L were not detectable in this system (Fig. 5A, lane 2). This confirms previous results from *in vitro* synthesized MCV mRNA (Figs. 4A and 4B, *Eco*RI F).

Because readthrough transcription was suspected to be responsible for the detection of late gene transcripts in *in vitro* synthesized MCV mRNA, oligonucleotide primers upstream and downstream of ORFs mc066L and mc110L were synthesized (Table 2 and Fig. 5B, lanes 1–4).

RT-PCR results using *in vitro* synthesized MCV mRNA (Fig. 5B, lanes 5–8) and total RNA isolated from MCV-infected human fibroblasts (Fig. 5C, lanes 1–4) were compared. Fig. 5B, lane 5, shows readthrough transcription, presumably from MCV early gene mc064R, located upstream of MCV ORF mc066L using

TABLE 1

Early Genes Encoded by MCV *Eco*RI DNA Fragment F, *Bam*HI/*Hin*dIII DNA Fragment A8, and Early and Late Controls

Viral DNA Fragment (enzyme) NP[a] Size (bp)	RT-PCR Results Detection +/−	MCV Genes Encoding Hypothetical Viral Proteins			
		ORF[b]	NP[a]	Promoter[c]	Homologies
F	+	mc002L (451)	4849–6201	E	Human signaling lymphocyte activating molecule gi 984969 (335; 20.4%)
(*Eco*RI)	+	mc003L (445)	6401–7735	E	Human carcinoembryonic antigen homolog -CGM2 (265; 16.3%)
5775–11174	−	mc004L (147)	7794–8234	E	mc005L
(5399)	+	mc004.1L (42)	8331–8456	E	
	+	mc005L (85)	8503–8757	E	mc105L (870); VAR-A3L
	+	mc006L (1175)	8801–12,319	E	Large nonglobular protein, Gln-Ala repeats (Hadasch *et al.,* 1993)
A8	+	mc070R (93)	86,507–86,785	E	VV-L2R (87, 23.7%)
(*Bam*HI/*Hin*dIII)[d]	+	mc076R (343)	90,660–91,688	E/L	VV-J3R Poly(A)$^+$ polymerase regulatory subunit, ribose-2′-*O*-methyltransferase
85,389–98,630					(333, 53.3%)
(13,241)	+	mc077R (187)	91,591–92,151	E/L	VV-J4R RNA polymerase subunit
	+	mc079R (1289)	93,012–96,878	E	VV-J6R DNA dependent RNA polymerase largest subunit
	+	mc080R (395)	96,986–98,170	E	Human MHC heavy-chain homolog
	+	mc081R (111)	98,241–98,573	E/L	
Late gene controls	+	mc066L (220)	83,029–83,688	L	Human glutathione peroxidase (201, 73.8%)
	+	mc110L (707)	130,710–132,830	L	VV-A7L early transcription factor subunit (710, 59.9%)
	−	mc095R (635)	113,703–115,607	L	VV-D6R early transcription factor, putative helicase (637, 74.4%)
	−	mc106L (675)	125,313–127,337	L	VV-A3L major core protein (644, 57.8%)
Early control	+	mc148R (104)	166,992–167,303	E	MCV chemokine antagonist (Bugert *et al.,* 1998; Damon *et al.,* 1998)

[a] Nucleotide position referring to MCV-1 genome complete coding sequence (GenBank accession no. U60315).
[b] ORF; open reading frame (protein length in amino acids).
[c] MCV consensus promoter.
[d] A part of MCV-1 *Bam*HI DNA fragment A (nt 85,389–138,491)

in vitro synthesized MCV mRNA. However, no RNA transcripts were detected from DNA sequences immediately 5′ of ORF mc066L (Fig. 5B, lane 6). This excludes readthrough as the cause for the detection of transcripts from mc066L in *in vitro* synthesized MCV mRNA. Readthrough was detected from upstream and downstream early genes in the case of MCV ORF mc110L (Fig. 5B, lanes 7 and 8). This explains the RT-PCR products observed using *in vitro* synthesized MCV mRNA (Fig. 4A, controls). However, when total RNA isolated from MCV-infected human fibroblasts was used, no readthrough transcription was detectable at all (Fig. 5C, lanes 1–4), supporting the previous results that MCV ORF mc066L is transcribed early.

Taken together, these experiments indicate that the molluscum glutathione peroxidase gene is transcribed early in MCV-infected fibroblasts and that it is transcribed in MCV-infected tissue *in vivo*. MCV ORF mc110L is not transcribed early, and readthrough due to failure of transcription termination is rare or absent under biological transcription conditions.

3′ and 5′ RACE characterization of early MCV transcripts

Only three of the MCV early genes investigated have a poxviral early polyadenylation signal 3′ of their coding sequences (Fig. 3A). RT-PCR using a gene-specific 5′ primer and a 3′ poly(A)$^+$ detection primer system (Bugert *et al.,* 1998) produced 3′ transcript-specific amplification products of ~650, ~330, ~550, and ~ 450 bp for MCV ORFs mc002L, mc004.1L, mc005L, and mc148R, respectively. Their identity was confirmed by DNA nucleotide sequence analysis. As shown in Fig. 6, transcripts were terminated between 38 and 118 nucleotides downstream of their respective transcription termination signal TTTTTNT. MCV ORF mc005L has no early poxviral transcription termination signal downstream of its stop codon and preceding the start codon of MCV ORF mc004.1L. In both 5′ and 3′ RACE experiments, only transcripts including both complete MCV ORFs mc004.1L and mc005L were amplified. The bicistronic transcript is terminated between 87 and 118 nucleotides downstream of the transcription termination signal after MCV ORF mc004.1L.

TABLE 2

Primers Used to Analyse MCV Genes Encoded by the MCV-1 *Eco*RI DNA Fragment F, the *Bam*HI/*Hin*dIII DNA Fragment A8, and Early and Late Control Genes

MCV ORF	Genome position (aa)[a]	5′Primer (nucleotide position)	3′Primer (nucleotide position)	Product length (bp)
mc002L	4849–6201 (451)	5′-GCAGCCTGTGCCGGGCACTGCATTC-3′ (np 5111–5135)	5′-TAGCGTAGCATGCTCGTCCCCGTGC-3′ (np 4861–4885)	289
mc003L	6401–7735 (445)	5′-ACCCCCCACGCCTGCGC-3′ (np 6661–6677)	5′-CGTCATCGACAAGTGCCTCCGCTCC-3′ (np 6421–6445)	280
mc004L	7794–8234 (147)	5′-CGCGACCTCGAGCGTGCACG-3′ (np 8041–8060)	5′-GGCGCGCCTACGGGCGGTGCCGCGT-3′ (np 7801–7825)	270
mc004.1L	8331–8456 (42)	5′-ATGTTTCCTGCGGCGCTGCTACAAG-3′ (np 8432–8456)	5′-TTGCCCGCTAGCATGTGTGCCGCAA-3′ (np 8341–8365)	116
mc005L	8503–8757 (85)	5′-ATGTGTCTTGTGGCGCCGATGCAGT-3′ (np 8733–8757)	5′-GCCGGCCACTTCTCCGAGAGAGGCT-3′ (np 8521–8545)	234
mc006L	8801–12,319 (1175)	5′-TGGTCGCTAGCGAGCGCGCA-3′ (np 9078–9097)	5′-CGTACGGCATGGTCTCCAGGCTGGT-3′ (np 8821–8845)	300
mc070R	86,507–86,785 (93)	5′-ATGATCCGCGACGTCCTAGA-3′ (np 86,506–86,526)	5′-TCACGGGCGCCGCCAGTA-3′ (np 86,771–86,788)	279
mc076R	90,660–91,688 (343)	5′-CGATTGCTGAGCGTGCACGCGG-3′ (np 91281–91302)	5′-CGCGGGCGCTCGGGCAATGG-3′ (np 91566–91585)	290
mc077R	91,591–92,151 (187)	5′-CTGCCCGCGCACAACAAGTTC-3′ (np 91,855–91,875)	5′-CTACTCGAGCTCGGCCGCCA-3′ (np 92,135–92,154)	300
mc079R	93,012–96,878 (1289)	5′-GGCCAGCCTGCTCTGCATG-3′ (np 96,572–96,590)	5′-CTAAAAGTCCTCCACGTGCGTAGC-3′ (np 96,858–96,881)	310
mc080R	96,986–98,170 (395)	5′-CCCCGACTACGACTTTGTGG-3′ (np 97,831–97,850)	5′-GCGCCTGCGGAAGCAAGTTC-3′ (np 98,313–98,150)	319
mc081R	98,241–98,573 (111)	5′-AGACGCTGGTGATCACGCAC-3′ (np 98,251–98,270)	5′-CAACGACTAGCGTGCGCAGC-3′ (np 98,502–98,521)	270
mc066L	83,029–83,688 (220)[b]	5′-ATGAACGAGCTGCAGCGG-3′ (np 83,452–83,469)	5′-GGGGAGCAGGGCCTCGATGTC-3′ (np 83,053–83,073)	417
mc095R	113,703–115,607 (635)	5′-ACCTGCTCGCCGCCATCTACGCGGACT-3′ (np 115,115–115,137)	5′-GAGTACAACGTGGGCGCGCCGCTATGA-3′ (np 115,584–115,610)	495
mc106L	125,313–127,337 (675)	5′-CTAGCAGAAGATCTCGGTGATGTTCT-3′ (np 125,310–125,335)	5′-TCACGATGACGGGCGCGTTCATG-3′ (np 125,888–125,910)	600
mc110L	130,710–132,830 (707)	5′-GTACATTCTCATCCAGGAGATCGTG-3′ (np 131,106–131,130)	5′-CTAGCTCAGCCGGTAGTAGCGCTC-3′ (np 130,707–130,730)	423
mc065L	Readthrough probe	5′-ATGACGGAGCCGCGGCAGTCCGAGCT-3′ (np 82,709–82,734)	5′-CAAACTGTCGTGCAGCCGCCA-3′ (np 82,441–82,461)	293
mc065L-066L	Readthrough probe	5′-ACACATAATTCCGGGCACTAC-3′ (np 82,741–82,761)	5′-GATCTTGCGTGCCTTCGCCA-3′ (np 82,991–83,009)	300
mc109L	Readthrough probe	5′-ATGGACCGGTTCCGTGCGCTCT-3′ (np 130,542–130,563)	5′-AGGAGCGCGCGGAAGAGCTC-3′ (np 130,241–130,260)	322
mc110L-111L	Readthrough probe	5′-GCGAGGGCCACCTGCTTGGT-3′ (np 132,901–132,919)	5′-TCACTTCTACGGACTCCGGGT-3′ (np 133,140–133,159)	258
mc148R	166992–167303 (104)	5′-AGGGGCGGAGACGTCTTCGCGAGCGTTGTC-3′ (np 166,992–167,024)	5′-CAGAGACTCGCACCCGGACCATATCTGAGGGC-3′ (np 167,272–167,306)	
Oligo dT detection primer		5′-ATCG-GAATTC-*CTAAAACGACGGCCAG-TTTTTTTTTTTTTTTTTTTTTTTT-3*′		
Smart oligonucleotide		5′-GTAGGG-GAATTC-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3′		
M13 detection primer		5′-ATCG-GAATTC-*CTAAAACGACGGCCAGT*-3′		
mc002L	5′ RACE 3′ gene specific primer	5′-GTAGGG-GAATTC-GAGTCGGTTCCCGAGGCAGACACAGAAATG-3′		
mc004.1L	5′ RACE 3′ gene specific primer	5′-GTAGGG-GAATTC-TTGCCCGCTAGCATGTGTGCCGCAA-3′		
mc005L	5′ RACE 3′ gene specific primer	5′-GTAGGG-GAATTC-TTAGTCTGCCGCTGGCCCCTTGCC-3′		

[a] aa, amino acids.

[b] Genome position and ORF length corrected: Senkevich *et al.,* 1997: mc066L 83,029–83,469 (211 amino acids) Corrected data: mc066L. 83,029–83,688 (220 amino acids)

The three genes mc002L, mc005/004.1L, and mc148R, with early consensus promoter sequences upstream of their start codons (Fig. 3), were used for 5′ RACE transcription start site mapping. Using a 5′ anchor oligonucleotide and 3′ gene specific primers (Table 2), RT-PCR was performed, and the amplification products were molecularly cloned into a bacterial plasmid vector. Recombinant clones were analyzed by DNA nucleotide se-

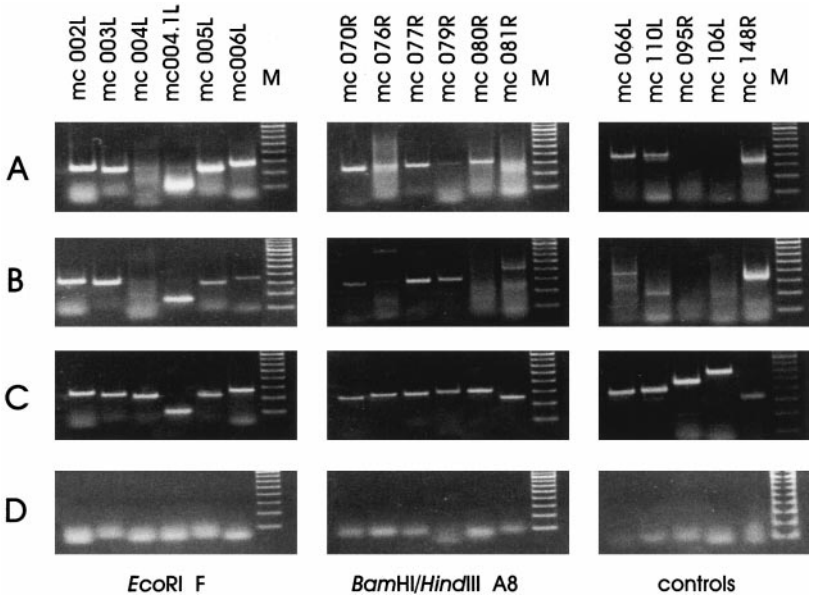

FIG. 4. Detection of MCV-1 transcripts by RT-PCR. Analysis of PCR products by agarose slab gel electrophoresis. MCV ORFs are indicated by their three-digit number and orientation of transcription. Lanes designated with (m) show the 100-bp ladder molecular weight marker. (A) PCR products obtained from cDNA using MCV ORF-specific primers and *in vitro* transcribed viral RNA. (B) PCR products obtained from cDNA using MCV ORF-specific primers and total RNA derived from MCV-1-infected tissue. (C) PCR products obtained from viral DNA serving as positive internal controls. (D) Negative internal controls in which RNA preparations were tested by PCR.

quence analysis. Three to four clones per analyzed MCV ORF were obtained that contained the anchor primer and the MCV gene transcript-specific cDNA transition sequence. The results of the alignments with the 5′ untranslated DNA sequences of these genes are shown in Fig. 7. All transcripts started between 11 and 14 nucleotides downstream of the early poxviral consensus promoter sequence. For the MCV ORFs mc004.1L and mc005L, only bicistronic transcripts consisting of both ORFs were observed.

DISCUSSION

Although the disease of MC has been known since 1814 (Bateman, 1814), it took almost 150 years to identify the causing agent as a poxvirus (Dourmaskin and Bernhard, 1959). Since then, numerous attempts to cultivate the virus *in vitro* have failed, and our knowledge about the virus–host interactions of this important human disease is still very limited. The molecular cloning and the determination of the primary structure and coding capacity of the MCV genome (Bugert and Darai, 1991; Darai *et al.,* 1986; Senkevich *et al.,* 1996; 1997) were major steps in MCV research. The data obtained provide the platform for further analysis of molecular events during MCV infection.

The first information concerning molecular analysis of MCV transcriptional activity came through the detection and characterization of the early mRNA of the molluscum viral chemokine antagonist (ORF mc148R). This was achieved by *in vitro* synthesis of MCV early mRNA and analysis of total RNA from MCV-infected tissue (Bugert *et al.,* 1998). The functional activity of its gene product was determined independently through leukocyte migration and chemokine receptor binding studies (Damon *et al.,* 1998; Krathwohl *et al.,* 1997). In the present study, we demonstrate the transcriptional activity of MCV genes using *in vitro* synthesis of mRNA, extraction of MCV mRNA from MCV-infected tissue, and a cell culture system for the analysis of early MCV transcription products.

Our data indicate that early transcriptional activity occurs over the entire MCV genome. This is in good agreement with previous observations made using *in vitro* transcribed RNA from vaccinia virus and the distribution of early MCV genes as found during the DNA nucleotide sequence analysis of the MCV genome (Gershowitz *et al.,* 1978; Senkevich *et al.,* 1996). However, in the case of MCV, early transcription seems to be particularly strong in two distinct MCV genomic regions: MCV-1 *Eco*RI DNA fragment F and *Bam*HI–*Hin*dIII DNA fragment A8. This raises the question of whether genes encoded by these MCV-1 DNA fragments represent a new immediate early class of MCV transcripts that might be of special importance for the early MCV infection. The predicted functions of the MCV early genes concerned include (1) potential immunodulatory factors (e.g., an MCV homolog to human lymphocyte signaling antigen and an MCV MHC class I homolog) that may be involved in the downregulation of the host NK cell immune answer, and (2) MCV genes associated with transcription [e.g., a poly(A)$^+$ polymerase and two DNA-dependent RNA poly-

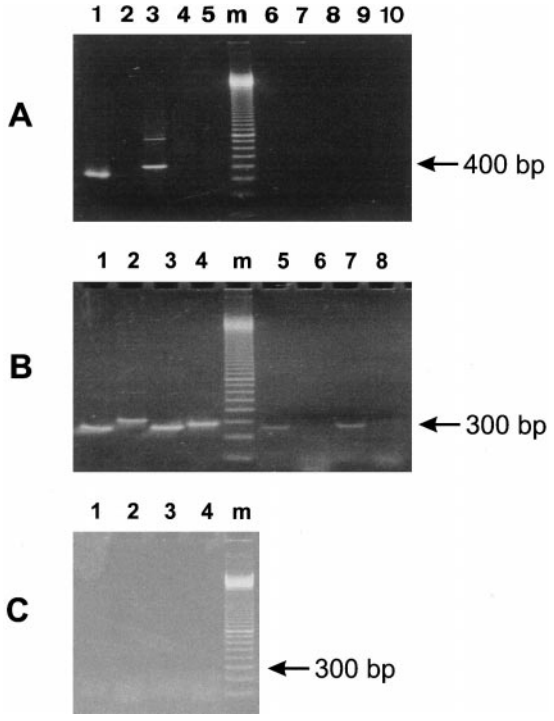

FIG. 5. Readthrough analysis of MCV ORFs. Analysis of PCR products by agarose slab gel electrophoresis. Lanes designated with (m) show the 100-bp ladder molecular weight marker. (A) PCR products obtained from cDNA using MCV ORF-specific primers and total RNA isolated from MCV-infected human embryonic fibroblasts (MRC5), MCV ORFs mc148R (lane 1), mc004L (lane 2), mc066L (lane 3), mc095R (lane 4), mc110L (lane 5), and negative internal controls in which RNA preparations were tested by PCR (lanes 6–10). (B) DNA positive controls for readthrough probes: Primer-pair mc065L (lane 1), mc066/67 (lane 2), mc109L (lane 3), mc110/111 (lane 4), and PCR products obtained from cDNA using readthrough probes and *in vitro* transcribed viral RNA (lanes 5–8). (C) PCR products obtained from cDNA using readthrough probes and total RNA isolated from MCV-infected human embryonic fibroblasts (lanes 1–4).

merase subunits]. Clearly, high RNA transcription levels do not allow predictions regarding the abundance of the translation products. An MCV *in vitro* infection model, must be established in the future that allows the investigation of actual MCV protein expression patterns.

Early poxviral promoter sequences (Davison and Moss, 1989) were found in the majority of the MCV genes investigated (Senkevich *et al.,* 1997). Consequently, we were able to detect transcripts by RT-PCR in *in vitro* synthesized MCV mRNA and in RNA from MCV-infected tissue. The latter finding is good evidence that these genes are transcribed in MCV-infected skin. Transcripts from MCV ORF mc004L could not be detected, raising the possibility that this ORF is only very weakly transcribed or not transcribed at all. The detection of early transcripts from MCV ORF mc066L, an MCV glutathione peroxidase (Shisler *et al.,* 1998), and initially investigated as MCV late gene control was not expected based on analysis of its promoter sequence. Looking for possible explanations, we demonstrated that in the *in vitro* transcription system, readthrough transcription can be observed leading to the synthesis of polycistronic mRNAs. This was shown for the bicistronic transcript of MCV ORFs mc005L and mc004.1L. To exclude readthrough as the cause for the detection of mc066L early transcripts *in vitro*, MCV transcription products had to be analyzed in another transcription system, preferably one that is based on MCV infection of human cells. From early studies by Brabanti-Brodano *et al.* (1974) and McFadden *et al.* (1979), it seemed likely that only MCV early mRNA would be made in MCV-infected human embryonic fibroblasts. This assumption was based on the observation that a CPE was consistently obtained after MCV infection of human cells in culture that was attributed to expression of early MCV gene functions. Replication of MCV viral DNA was never reported (McFadden *et al.,* 1979), precluding synthesis of intermediate and late viral mRNAs. Using MCV-infected human fibroblasts, we succeeded in detecting mRNA transcripts from MCV ORF mc066L, leaving early transcription as the only possibility in the absence of MCV DNA replication. Furthermore, readthrough transcription involving MCV ORFs mc066L and mc110L was not observed in this system. In the light of these experiments, the use of MCV-infected human embryonic fibroblasts holds great promise for the further analysis of early MCV mRNA transcription.

5′ RACE experiments on 3 MCV genes with highly conserved early poxviral consensus promoter sequences revealed that the MCV RNA polymerase complex initiated transcription between 11 to 13 nucleotides downstream of the respective early poxviral promoter signal. These results are in agreement with data obtained from 5′ transcript mapping experiments of vaccinia virus early genes (Coupar *et al.,* 1987; Lee-Chen *et al.,* 1988). The MCV 3′ RACE results are consistent with previous studies with MCV and vaccinia virus (Bugert *et al.,* 1998; Yuen and Moss, 1987).

In conclusion, the majority of MCV genes analyzed in this study is transcribed as predicted by DNA sequence analysis of their promoter signals. The MCV glutathione peroxidase gene (mc066L), which was predicted to be a late gene, seems to be transcribed early in the infectious cycle. This transcription was found to be independent of readthrough in MCV-infected human fibroblasts.

MATERIALS AND METHODS

Cells, virus, and recombinant plasmids

The MCV-1 isolate HD 94-4 was obtained from an MCV-infected individual and used for the *in vitro* synthesis of MCV mRNA and preparation of infectious virions. The recombinant plasmids harboring the specific DNA sequences of the MCV-1 prototype used in this study were described previously (Bugert and Darai, 1991; Darai *et al.,* 1986). Human MRC-5 embryonic fibroblasts

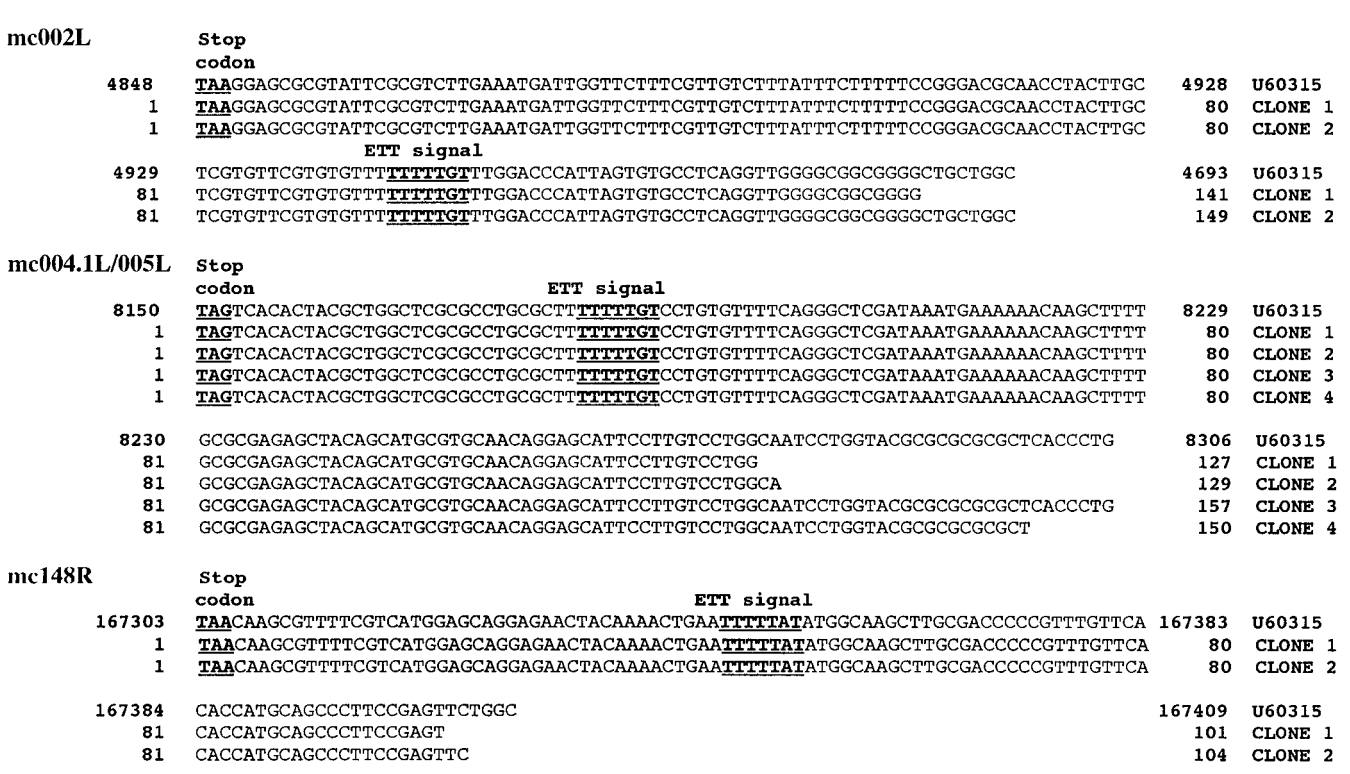

FIG. 6. Alignment of the DNA nucleotide sequence of the cDNA of the 3′ end of MCV-1 early transcripts from ORFs mc002L, mc004.1/005L, and mc148R with the corresponding DNA sequence of the MCV-1 prototype (GenBank accession no. U60315). cDNA sequences of MCV chemokine transcripts were obtained by 3′ RACE from total RNA derived from MCV-1-infected human skin tissue and inserted into the TA site of plasmid vector pCR2.1, and individual clones were analyzed by DNA nucleotide sequencing. Stop codons and poxviral early transcription termination (ETT) signals are shown underlined and in bold letters.

(CCL171; American Type Culture Collection) were used for transient infection with MCV. Cells were grown in DMEM with 10% FCS. Infections were carried out in 6-well dishes. Each well was infected with an infectious dose of 0.025 *A* purified MCV and then incubated until a CPE developed.

In vitro RNA synthesis by permeabilized virions

MCV virions were isolated from MCV lesion material by centrifugation through a 36% sucrose cushion. The virions were resuspended in 1 mM Tris–HCl, pH 8.0, adjusted to the absorbance of 1 A_{260}, and stored at −80°C. The *in vitro* transcription assay is based on a protocol described by Shand *et al.* (1976) and has been modified for the production of mRNA suitable for RT-PCR. Virus (50 μl) suspension (A_{260} = 1) was incubated in a volume of 200 μl with *in vitro* transcription buffer containing 1.0 M Tris–HCl, pH 8.5, 50 mM $MgCl_2$, 0.25% 2-mercaptoethanol, 0.05% Nonidet P-40, 20 μl of 75 mM phosphoenolpyruvate, 4.0 μg of pyruvate kinase, 1.0 μl of RNasin (40 U/μl; Boehringer-Mannheim, Mannheim, Germany), 4.0 mM ATP, and 2.0 mM concentration of CTP, UTP, and GTP at 35°C for 120 min. For synthesis of radioactively labeled MCV mRNA, the reaction was incubated for 180 min, and UTP was substituted by 0.2 mM [α-^{32}P]UTP (specific activity, 1–3 × 10^3 Ci/mmol; New England Nuclear, Dreieich, Germany). Four aliquots (each 20 μl) were taken at different times (0, 60, 120, and 180 min); soluble and core associated RNAs were separated by centrifugation, filtrated on nitrocellulose, and washed three times with 10% trichloroacetic acid, and the incorporated radioactivity was measured in a Beckman scintillation counter. The remaining supernatant was phenol/chloroform extracted and ethanol precipitated in the presence of 3.0 M sodium acetate, pH 5.3. Unincorporated nucleotides were removed over Sephadex G50 columns. The α-^{32}P-UTP-labeled *in vitro* transcribed MCV mRNA was hybridized to MCV and vaccinia viral DNA and recombinant plasmids harboring MCV viral DNA fragments immobilized on nitrocelluose membranes. Unlabeled *in vitro* transcription reactions were treated under the same conditions as described above and used for RT-PCR. Twenty units of RNase inhibitor were added to the RNA sample, and the reaction was treated with 1.0 μl (40 U/μl) DNase/RNase free.

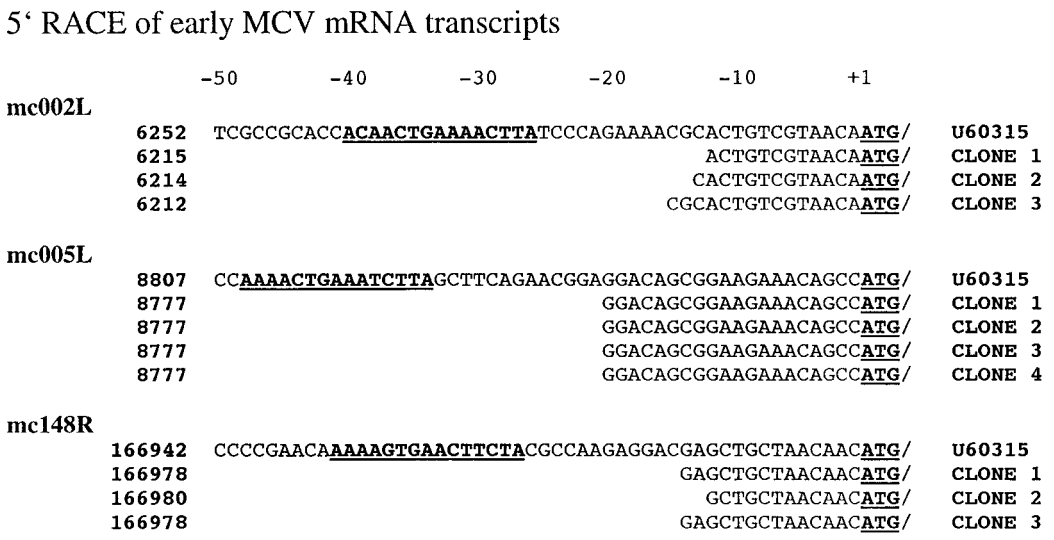

FIG. 7. Alignment of the DNA nucleotide sequence of the cDNA of the 5′ end of MCV-1 early transcripts from ORFs mc002L, 005L, and mc148R with the corresponding DNA sequence of the MCV-1 prototype (GenBank accession no. U60315). cDNA sequences of MCV chemokine transcripts were obtained by 5′ RACE from total RNA derived from MCV-1-infected human skin tissue using a 5′-anchor oligonucleotide, inserted into the TA site of plasmid vector pCR2.1, and individual clones were analyzed by DNA nucleotide sequencing. MCV consensus promoter sequences and start codons are shown underlined and in bold letters.

RNA extraction from MCV-infected human tissue and MCV-infected human fibroblasts

MCV-1-infected human skin samples were immediately frozen at −20°C. MCV-infected MRC5 cells were lysed and kept in guanidium isothiocyanate. Total cellular RNA was extracted from individual MCV lesions and infected human embryonic fibroblasts by the guanidinium/cesium chloride method (Glisin *et al.,* 1974) as described by Sambrook *et al.* (1989).

RT-PCR

Oligonucleotide primers used for PCR were synthesized according to the coding sequences of the respective MCV-1 ORFs. The properties of the primers used in this study are summarized in Table 2. PCR was performed using 0.01 fmol of MCV DNA in 50-μl volumes containing 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1–2 mM $MgCl_2$, 1.5% formamide, 200 μmol concentration of each dNTP, 200 pmol of each primer, and 0.5 U of *Taq* Polymerase (Perkin–Elmer). Twenty-five cycles were run in an automated temperature cycling reactor (Perkin–Elmer Geneamp 2400), which provided 30 s of incubation at 96°C and 4 min at 60°C per cycle. A final incubation step at 72°C for 7 min was used to extend the unfinished products. The amplified product was phenol/chloroform extracted and precipitated in ethanol. For RT-PCR experiments, the AMV reverse transcriptase and the LA *Taq* Polymerase (TaKaRa-Shuzo Co., Ltd., distributed by Boehringer Ingelheim, Germany) were used. For cDNA synthesis, an oligo(dT) primer was designed with an universal M13 primer binding site (Table 2).

Rapid amplification of MCV transcript 3′ ends (3′ RACE)

For 3′ RACE experiments, an M13 detection primer recognized the universal M13 primer binding sequence incorporated into the oligo(dT) adapter primer (Table 2). PCR products were analyzed by slab gel electrophoresis, and cDNA from RT-PCRs with a specific product was cloned into the pCR2.1 TA-cloning vector (InVitrogen). The DNA nucleotide sequence analysis of the cDNAs corresponding to the 3′ ends of MCV mRNA transcripts was performed using the gene-specific 5′ primers (Table 2).

Mapping of MCV transcript 5′ ends (5′ RACE)

For 5′ RACE experiments, an anchor oligonucleotide (Table 2) was attached to MCV transcript 5′ ends in the cDNA first-strand reaction using the SMART technology (Switching Mechanism at 5′ End of RNA Transcript; Clontech Laboratories, Inc). The MCV transcript 5′ ends were then PCR amplified using the anchor oligonucleotide as 5′ primer and an MCV gene-specific 3′ primer. cDNA from RT-PCRs with a specific product was cloned into the pCR2.1 TA-cloning vector (InVitrogen). The DNA nucleotide sequence analysis of the cDNAs corresponding to the 5′ ends of MCV mRNA transcripts was performed using the gene-specific 3′ primers (Table 2).

DNA sequence analysis

The DNA sequence of the PCR products in this study was determined with an automated 373 "Extended" DNA sequencer using the standard terminator cycle sequenc-

ing technique (Applied Biosystems GmbH, Perkin–Elmer Corporation, Germany).

Computer-assisted sequence analysis

Nucleotide sequences were compiled using the ABI sequence navigator software version 1.2. Nucleotide and amino acid sequences were analyzed using the PCGENE program release 6.85 (Intelligenetics Inc., Mountain View, CA), Omiga release 1.1, and the NIH BLAST server (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/).

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