

Detection of Transcripts of Marek's Disease Virus Serotype 1 ICP4 Homologue (MDV1 ICP4) by *in situ* Hybridization

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ABSTRACT. Homologues of herpes simplex virus ICP4 are important genes for the activation of many herpesviruses. We detected transcripts of the Marek's disease virus serotype 1 homologue of ICP4 (MDV1 ICP4) by *in situ* hybridization (ISH). Using a digoxigenin-labeled-RNA (DIG-RNA) probe, MDV1 ICP4 transcripts were detected in c.a. 90% of MDV1-infected chicken embryo fibroblasts (CEF) cells when cytopathic effect was reached to 90% of the CEF cells and in 0.35% of MDCC-MSB-1 (MSB-1) cells, at a frequency similar to that for MD antigen-positive MSB-1 cells. Using the same *in situ* procedure, we detected abundant MDV1 ICP4 transcripts in the feather follicle epithelium (FFE) and some lymphoid cells in the liver, kidney and peripheral nerve of infected chickens. The subcellular localization of the transcripts appeared to vary: MSB-1 cells had them in the nucleus, infected CEF cells and FFE had them in the nucleus and cytoplasm, and lymphoid cells contained them in the cytoplasm. The MDV1 ICP4 transcripts were also detected in the FFE and lymphoid cells in the liver by reverse-transcriptase polymerase chain reaction (RT-PCR). Detection of MDV1 ICP4 transcripts by RT-PCR indicated the existence of MDV1 ICP4 transcripts-positive cells in these tissues. And these data suggested that DIG-RNA-ISH can detect MDV transcripts on paraffin sections and provide information about their subcellular localization. — **KEY WORDS:** digoxigenin-labeled-RNA probe, ICP4 homologue, *in situ* hybridization, Marek's disease virus serotype 1, paraffin section.

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Marek's disease (MD) is a disease of domestic chickens caused by cell-associated herpesvirus (MD virus serotype 1, MDV1) and characterized principally by T cell lymphoma and demyelinating peripheral neuropathy [5, 12]. Studies on the MDV1-infected chickens have shown that T cells are transformed after a persistent latent infection which is followed by early lytic infection of the primary lymphoid organs [7]. The secondary lytic infection starts mainly in the feather follicle epithelium (FFE) and to a lesser extent in other epithelial cells and lymphoid organs two weeks after infection [6]. Although the host factors such as cell-mediated immune response are involved in the establishment of latency [3, 25], control of viral factors which induce lytic infection required to establish latency.

Immediate-early (IE) proteins are likely to play key roles in the activation of alphaherpesviruses [22]. Among the IE proteins, the homologue to a major regulatory protein of herpes simplex virus-1 (HSV-1)-ICP4 is of primary interest [2]. The MDV1 homologue of the ICP4 gene (MDV1 ICP4) is predicted to have a structure similar to that of the ICP4-like proteins of other herpesviruses [2]. Although its transcription start site was not identified, the full length transcript is reportedly 10 to 15-kbases and its 3' end is in the internal repeat flanking unique short region (IRs region; Fig. 1) [2, 19]. The MDV1 ICP4 transcripts are reportedly abundant in lytically infected cells and can not be detected in a transformed cell line (MSB-1) [8, 21]. Pratt *et al.* have also indicated that MDV1 phosphoproteins pp41, pp38 and pp24 are elevated following the stable transfection of MSB-1 cells with a plasmid harboring an expression construct for

the MDV1 ICP4 gene [21]. These reports suggest that the expression of MDV1 ICP4 enhances the expression of early genes and is prerequisite for the lytic infection cycle.

The gene regulatory mechanisms that reactivate the virus genome *in vivo* can be studied by means of *in situ* hybridization (ISH) [10, 23, 26]. Five to 6 serial sections including the same cells can be prepared from a formalin-fixed paraffin-embedded block and can be applied to 5–6 ISH of different genes [14]. Although viral genomes have been detected by ISH [9, 10, 16, 23], no one has yet detected the MDV1 transcript using this procedure. We used ISH to detect MDV1 genes on formalin-fixed paraffin-embedded sections using a digoxigenin-labeled-RNA (DIG-RNA) probe.

We detected MDV1 ICP4 transcripts using RNA-ISH in lytically infected chicken embryo fibroblasts (CEF) cells and a few fractions of MSB-1 cells with a low background reaction on uninfected CEF cells. On paraffin-embedded sections from infected chickens, MDV1 ICP4 transcripts were detected in FFE and some lymphoid cells in liver, kidney and peripheral nerve. We showed that RNA-ISH can detect MDV1 transcripts on paraffin sections.

MATERIALS AND METHODS

Experimental chickens and viral inocula: Eggs of the Shaver 288 strain of the White Leghorn were purchased from a breeder (Hokuren Co., Ltd., Sapporo, Japan) and hatched. After infection with MDV, all chickens were held in isolation units. MDV1 strain Md5 was maintained with

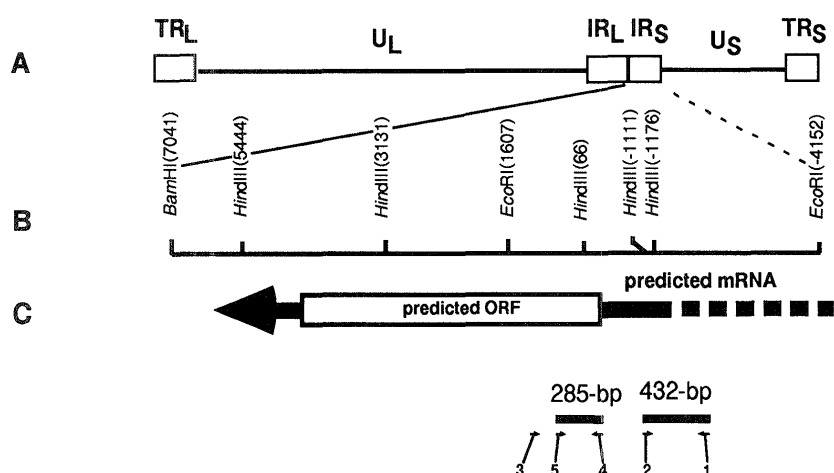


Fig. 1. (A) Diagram of the MDV genome showing the unique long region (U_L) and unique short region (U_S) flanked by repeat regions. TR_L , terminal repeat flanking U_L ; IR_L , internal repeat flanking U_L ; IR_S , internal repeat flanking U_S ; TR_S , terminal repeat flanking U_S . (B) Expanded view of a portion of the *Bam*HI-A genomic fragment. (C) The position and polarity of the predicted coding region for the MDV1 ICP4 gene (arrow). The DNA fragments (432-bp and 285-bp) cloned into pGEM-T (Promega Biotech) to construct pGie1 and pGie2 are indicated with solid bars. Small arrowheads indicate the position of primers used for PCR, 1 and 2, primers for amplification of the 432-bp fragment; 3, primer for RT; 4 and 5, primers for amplification the 285-bp fragment.

the chickens. Inocula for the experiments were from stocks of infected CEF cell cultures. On day 5 post hatching, chickens were injected intra-muscularly with 7,200 plaque-forming units (pfu)/chicken of MDV strain Md5. Tissues for further analysis (skin, liver, kidney and plexus as a peripheral nerve) were obtained from chickens 4 weeks after infection.

Plasmid constructions and selection of the probe for *in situ* hybridization: Cloning was performed by standard procedures [24] using the plasmid p*Bam*H I-A which included *Bam*H I-A fragment of MDV1 genome and was gift from Dr. M. Nonoyama (Tampa Bay Research Institute, St. Petersburg, FL, U.S.A.) [13]. A 432-bp fragment was amplified from p*Bam*H I-A by the PCR amplification and cloned into pGEM-T (Promega Biotech, Madison, WI, U.S.A.). The constructed plasmid was designated pGie1 (Fig. 1). Oligonucleotides for PCR primers were obtained commercially (Science Tanaka, Ebetsu, Japan). The sequence and position of primer 1 (5'-TACTCGAGCTTCTCCCCTCTCCTCTTCT3') was obtained from McKie *et al.* [19], primer 2 (5'-GGGTATCTGGCAGTGTAGG3') was obtained from the MDV1 ICP4 gene sequence reported by Anderson *et al.* [2] (Fig. 1C).

Northern blot: RNA was isolated from MDV-infected CEF using the TRIZOL reagent according to recommendations of the supplier (GIBCO BRL Life Technology Inc., Gaithersburg, MD, U.S.A.). RNA preparations were digested with 2.5 U of ribonuclease-free RQ1 deoxyribonuclease (Promega Biotech) for 15 min at 37°C and measured at A_{260} . Poly(A)⁺ RNA was prepared by means of two sequential oligo (dT) affinity purifications using the Dynabeads mRNA isolation system according to

the manufacturer's instructions (Dynal A.S., Oslo, Norway). Electrophoresis and Northern blotting proceeded according to basic protocols [24]. RNA size markers were obtained commercially (GIBCO BRL Life Technology Inc.). The RNA probe was synthesized using [³²P]CTP and a commercial *in vitro* transcription system as recommended (Promega Biotech). Hybridization using the RNA probe proceeded at 60°C for 16 hr in 50% formamide, 50 mM potassium phosphate (pH 7.0), 0.05 mg of denatured salmon sperm DNA per ml, 2% blocking reagent (Boehringer-Mannheim GmbH, Mannheim, Germany), 7% sodium dodecyl sulfate, and 5 × SSC. The membrane was washed once for 10 min at room temperature in 2 × SSC and twice at 65°C with 0.1 × SSC-0.1% SDS and autoradiographed using Bio-Imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Reverse-transcriptase polymerase chain reaction (RT-PCR): For RT-PCR, 0.5-μg samples of poly(A)⁺ RNAs were reverse-transcribed with Superscript II (Bethesda Research Lab.) using 0.25 μM primer 3 (5'-TATGGGCCCAAGTGCTTGTCGATGCCAC3'). The resulting cDNAs were amplified with BRL-Taq polymerase under conditions recommended by the manufacturer (GIBCO BRL Life Technology Inc.) with primers 4 (5'-TTTATGCGACTGGCGTGCTG3') and 5 (5'-TCGTGGAGATGAGGTTGTGT3') which amplified a 285-bp fragment located within the predicted coding region of MDV1 ICP4 (Fig. 1). Amplified fragments were resolved by electrophoresis in 1.5% agarose gel according to a basic protocol [24]. To increase the sensitivity and to prevent the false-positive detection, the amplified fragments were detected by Southern blot hybridization. The ³²P-labeled probe was prepared from pGie2 which included the

amplified 285-bp fragment in the pGEM-T (Promega Biotech) (Fig. 1). After construction of pGie2, the sequence of the cloned insert was confirmed by standard protocol [24]. The radioactivity in each band was measured with the Bio-Imaging Analyzer.

In situ hybridization: Tissues were obtained from infected chickens and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. The tissues were embedded in paraffin and cut into 3 μ m sections following a routine procedure. When ISH was performed with cultured cells, they were embedded in 0.5% agarose before fixation. The anti-sense RNA probes were synthesized with T7 RNA polymerase from pGie1 in the presence of DIG-dUTP using a DIG-RNA labeling kit (Boehringer Mannheim GmbH). The dried and deparaffinized sections were incubated with hybridization buffer containing 50% formamide, 10 mM Tris-HCl (pH 7.6), 200 μ g/ml tRNA, 1 \times Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0) and 1% anti-sense RNA probe for 24 hr at 50°C. Rinsing and colorization proceeded as described [17].

RESULTS

Detection of the MDV1 ICP4 transcripts in cultured cells: Before ISH, Northern blots were performed using the 32 P-labelled RNA-probe synthesized from pGie1. An 11 kb RNA was detected in poly(A)⁺RNA from MDV1-infected CEF (Fig. 2). This indicated that the RNA probe synthesized from pGie1 hybridized to the full length transcript of the MDV1 ICP4 gene. Although no signal was detected by Northern blotting (Fig. 2), we found MDV1 ICP4 transcripts in MSB-1 cells using RT-PCR (Fig. 3).

Using the DIG-RNA probe synthesized from pGie1, the MDV1 ICP4 transcripts were detected in c.a. 90% of MDV1-infected CEF cells when cytopathic effect was reached to 90% of the CEF cells by ISH (Fig. 4A). On the other hand, 0.35% of MSB-1 cells also indicated positive signals for transcription of MDV1 ICP4 (Fig. 4C). On the same hybridization slide, there were no signals from uninfected CEF cells (data not shown). In the infected CEF cells, the MDV1 ICP4 transcripts were detected in the cytoplasm and the nucleus (Fig. 4B). On the other hand, the transcripts located mainly in the nucleus of MSB-1 cells (Fig. 4D).

Detection of MDV1 ICP4 transcripts in lymphoid cells in vivo: The organs and tissues including liver, kidney, skin and peripheral nerves, which were subjected to ISH, were sampled from five chickens exhibiting paralysis. Lymphoid cells were found in them. All skin tissues were subjected to ISH had intra-nuclear inclusion bodies in their FFE. The ISH using the DIG-RNA probe revealed MDV1 ICP4 transcripts in FFE, lymphoid cells of the liver, kidney and peripheral nerves (Fig. 5). Signals located in the nucleus and cytoplasm in the FFE (Fig. 5B), but mainly in the cytoplasm of lymphoid cells (Fig. 5H). Using the RT-PCR, the MDV1 ICP4 transcripts were also detected in the organs

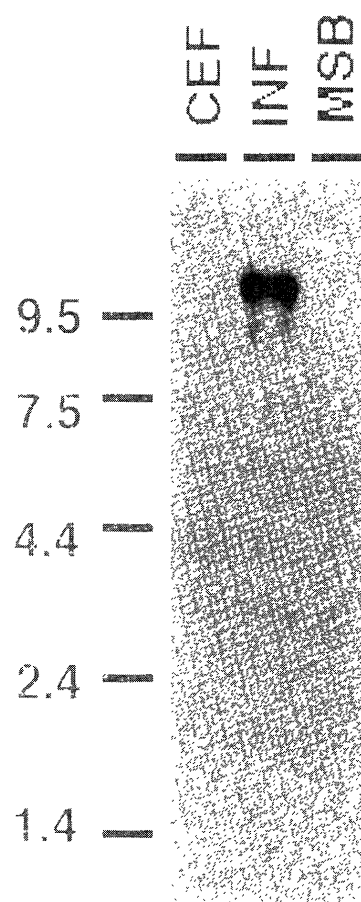


Fig. 2. Northern blot of MDV-infected cells for the ICP4 transcript. Hybridization to a 32 P-labelled RNA probe to detect sense transcripts synthesized from pGie1. Each lane contains poly(A)⁺ RNAs (0.5 μ g/lane) extracted from uninfected CEF cells (CEF), MDV(Md5)-infected CEF cells (INF), or MSB-1 cells (MSB). The positions of molecular weight markers are indicated.

or tissues subjected to ISH as above mentioned (Fig. 3). These data showed that the RNA probe detected MDV1 ICP4 transcripts in the FFE cells, some lymphoid cells infiltrated in the liver, kidney and peripheral nerves.

DISCUSSION

The use of ISH to identify the topographic distribution of the MDV1 ICP4 transcripts within MDV1 infected cells has not been described. In this study, we performed RNA-ISH on viral infected formalin-fixed paraffin-embedded tissues obtained from MDV1 infected chickens. We used a 0.55-kb DIG-RNA probe to detect the MDV1 ICP4 transcripts in MDV1 infected cells *in vitro* and *in vivo*.

The probe detected the full length transcript of MDV1 ICP4 (Fig. 2). A comparison of the results of the Northern

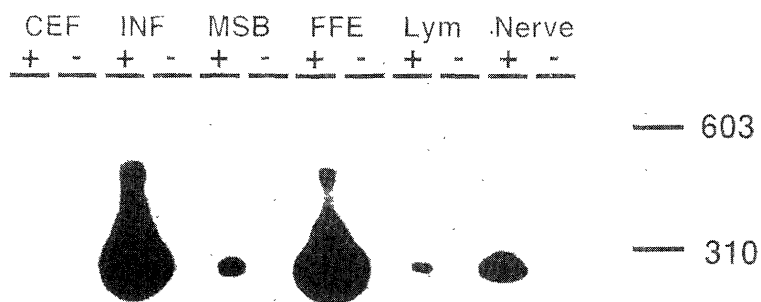


Fig. 3. RT-PCR of MDV1 ICP4 transcripts in mock infected CEF (CEF), infected CEF (INF), MSB-1 cells (MSB), skin including FFE (FFE), cumulated lymphocyte foci in infected chickens (Lym) and peripheral nerves including nerve fibers and lymphoid cells (Nerve). An autoradiogram of a probed blot of ICP4 RT-PCR products separated on a 1.5 % agarose gels is shown. Poly(A)⁺RNAs (0.5 μ g) were reacted with (+) or without (-) RT primed with primer 3 as described in Materials and Methods. Primers 4 and 5 were used to amplify 285-bp fragment from cDNAs. The molecular weight of the PCR product was verified upon the molecular weight marker (pBR322 digested with *Msp*I, migrated positions of 603-bp and 310-bp fragments are indicated on the left).

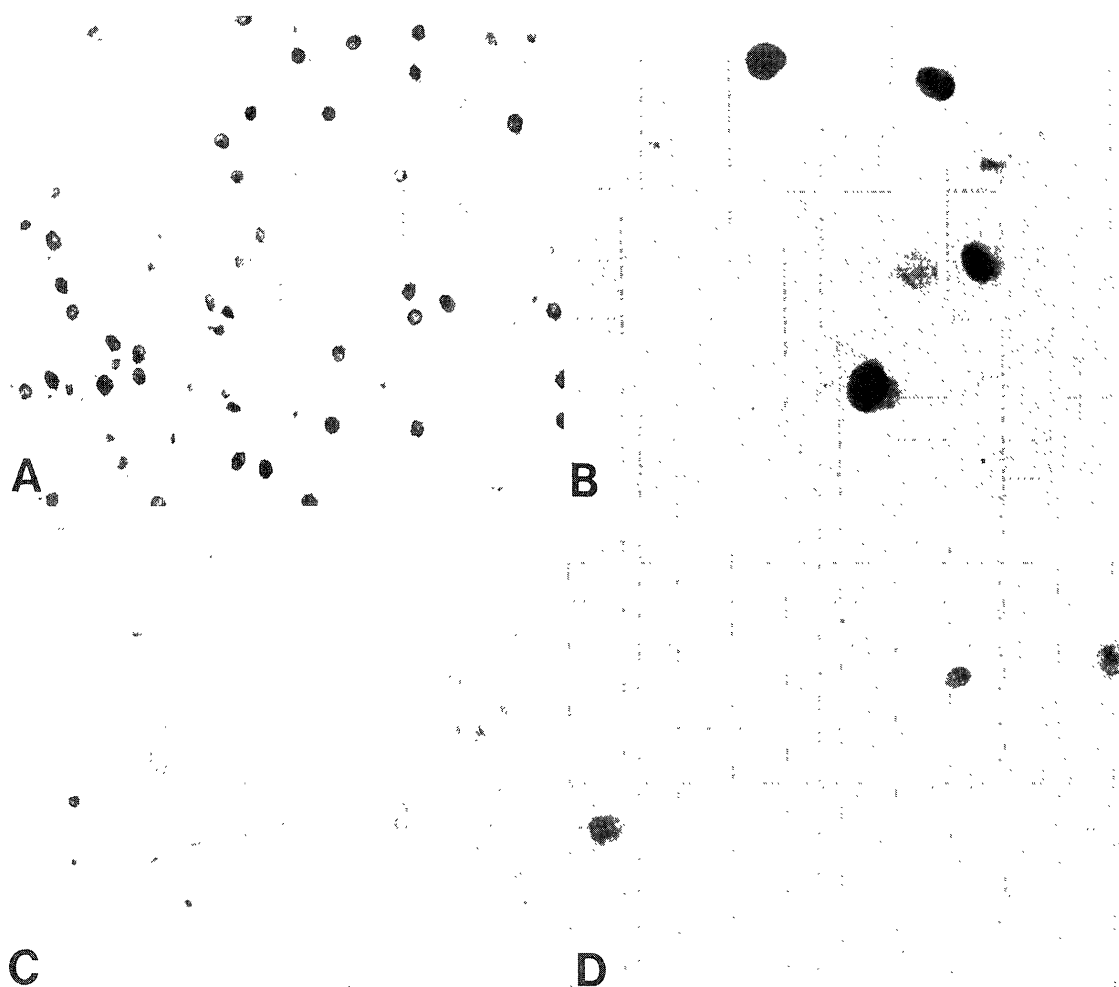


Fig. 4. ISH on agarose-embedded, fixed and paraffin-embedded sections showing the transcript of the MDV1 ICP4 gene in infected CEF (A and B) and MSB-1 cells (C and D). Uninfected CEF were hybridized with DIG-RNA probe on the same slide. The hybridized probe was detected with alkali-phosphatase labeled anti-DIG-antibody after a rinse. Magnification is $\times 123$ (A and C) or $\times 756$ (B and D).

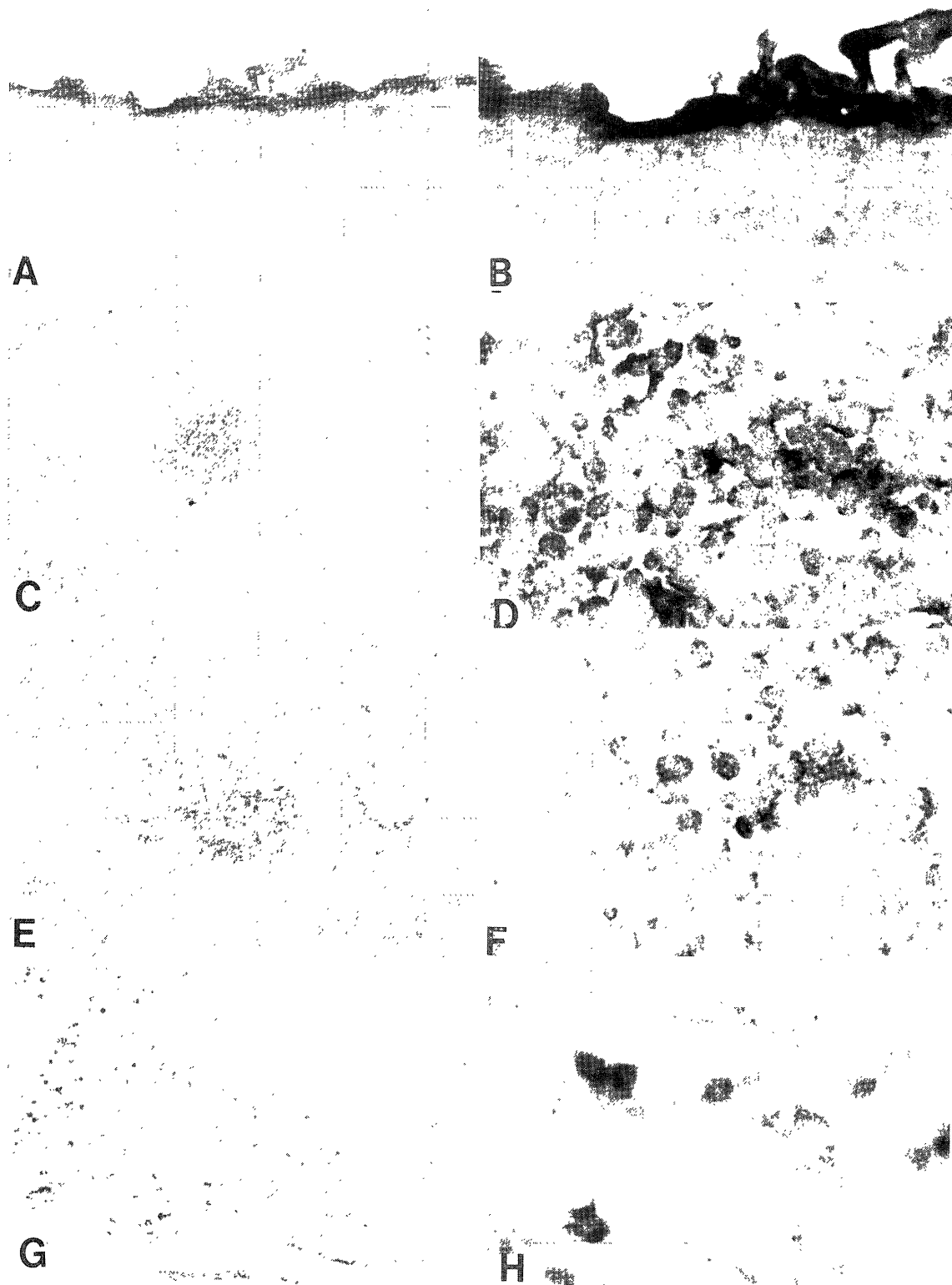


Fig. 5. ISH showing the transcript of MDV1 ICP4 gene. Sections of skin including FFE (A and B), liver (C and D), kidney (E and F) and peripheral nerve including nerve fibers and lymphoid cells (G and H) were hybridized with an RNA probe to detect the MDV1 ICP4 transcript synthesized from pGiel. Magnification is $\times 123$ (A, C, E and G) or $\times 756$ (B, D, F and H).

blots and ISH showed that the former was unable to detect the MDV1 ICP4 transcripts in MSB-1 cells (Figs. 2, 4 C and D). This would be due to the low frequency (0.35%) of MDV1 ICP4 transcript-positive MSB-1 cells indicated by the ISH (Fig. 4E). This signal was confirmed by the results from the RT-PCR (Fig. 3) which was more sensitive than Northern blotting. These data indicated that ISH method is more sensitive than Northern blotting on the samples with low frequency of positive cells. On the other hand, although MDV1 reportedly exist in every MSB-1 cells [15, 20], the ratio of MDV1 antigen-positive cells in MSB-1 cells is reportedly 0.5% [11] or 1–2% [1]. The similarity of the frequency between MDV1 ICP4 transcripts-positive cells and MDV1 antigen-positive cells might indicate the close relationship between these cells.

We examined the applicability of our probe to formalin-fixed paraffin-embedded tissue specimens (Fig. 5). The results revealed topographic information about the distribution of MDV1-infected cells in tissue sections (Figs. 5 B, D, F and H). The strength of the signal is most likely a reflection of the abundance of MDV1 ICP4 transcripts, which accumulate during lytic infection such as in FFE cells. ISH of FFE cells showed basically the same topographic distribution of the MDV1 ICP4 transcripts as that seen in infected CEF cells. In these FFEs hybridization was detected in the cytoplasm and nucleus. The intense signal within the cytoplasmic region of some lymphoid cells and FFE cells suggested the efficient transport of MDV1 ICP4 transcripts to the cytoplasm. Although effective translation in FFE cells is plausible, the production of MDV1 ICP4 protein should be studied using the anti-MDV1 ICP4 antibody.

In some lymphoid cells hybridization was confined to the nucleus of the cells as seen in MSB-1 cells. Kramer and Coen have described the partly activated state of HSV-1 infection in latently infected nerve cells [18]. In these cells ICP4 was transcribed and it activated the early genes, whereas in the same cells, the late genes were not activated or fully progressed the lytic infection cycle. As MDV1 latently infects lymphocytes [4, 11], it may assume a partially activated state in infected lymphocytes. Activation of early and late genes of MDV1 should be studied by ISH.

In conclusion, this study demonstrated that ISH using a strand-specific DIG-RNA probe derived from MDV1 ICP4 transcripts is a useful means of detecting lytically and latently-infected cells, especially when the frequency of infected cells is low. With low frequency of expression cells, RNA detection method for whole cells such as Northern blotting occasionally fail. Using DIG-RNA-ISH, we can detect MDV1 ICP4 transcripts on formalin-fixed paraffin embedded tissues with the information about subcellular localization of the transcripts.

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