持って取り組むことが重要と考える。行政の保健部局の努力だけでは95%以上の予防接種率達成は困難であり，教育部局の努力だけでも困難である。双方が連携をとって国が目標に掲げた2012年の国内麻疹排除に向かって，共通の認識で取り組んでいくことが重要と考える。

また，2012年に国内から麻疹を排除する目標で始まった国を挙げての取り組みの成果を継続的に評価しながら，対策方法に適宜改善を加え，世界の一員として，日本も2012年の麻疹排除達成に向けて一層の努力をしたい。

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ウイルス抗体価からみた感染予防

多屋馨子*
Keiko Taya

はじめに

麻疹，風疹，水痘，ムンプスは，小児の疾患という概念が強い。しかし，近年発生している思春期～若年成人の麻疹や風疹の流行について考えてみると[1,2]，決して小児の軽症疾患とはいえないのが現状である。病初期に麻疹や風疹が疑われないと，診断・対応が遅れるため，周りへの感染が拡大してしまう場合がある。水痘やムンプスはその特徴的な症状から，より早期に疑われる可能性が高いが，麻疹・風疹と異なり，定期予防接種対象疾患でないことから，予防接種率が低く，毎年小児を中心として，国内流行をくり返しているのが現状である。

これら4疾患に共通していえることは，予防接種を受けずに成人まで感染・発症を免れていることが少なからずあり，重症となって入院する場合も少なくない。また，どこで感染したかわからない状況で，発症することも十分にありうるのが現在の日本の状況である。

厚生労働省が実施主体となり，都道府県ならびに都道府県衛生研究所と国立感染症研究所が協力して実施している感染症流行予測調査事業では，定期予防接種対象疾患における国民の抗体保有率を，毎年あるいは疾患によっては5年ごとに調査・集計・解析を行っている[3]。感受性者の蓄積状況を把握することで，予防接種が必要な年齢群を見極め，予防接種歴別に解析することで予防接種の効果についても明らかとなる。定期接種に導入されていない水痘，ムンプスについては，本事業は実施されていないため，研究レベルで実施されているのが現状である。

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Ⅰ．抗体保有率

1．麻　疹

感染症流行予測調査では，毎年6,000～7,000人の規模で麻疹の感受性調査（抗体保有率調査）を実施している。直近の結果を紹介すると[3]，2008年度は全国22都道府県で7,013人についてゼラチン粒子凝集（particle agglutination：PA）抗体価が測定された。採血の時期はおおむね当該年度の7～9月である。PA法は健康保険収載がなされている検査方法である。

PA抗体は感度がEIA法と同様に高いため，1：16未満（陰性）の場合は，麻疹に対する免疫をもっていないと考えられる。一方，1：16以上（陽性）であっても，低い抗体価の場合は，発症を予防することができない。1：16，1：32，1：64は発症予防には不十分であり，少なくとも1：128以上は必要である。確実な発症予防には，できれば1：256以上の抗体価を保有しておいて欲しい。すなわち，陽性であっても低い抗体価の場合は，麻疹ウイルスの曝露を受けると，修飾麻疹として発症する可能性があり，感染源になりうることに注意が必要である。これはEIA法でも同様である。すなわち，EIA法で陰性であれば，麻疹に対する免疫をもっていないと考えられるが，低いEIA価の場合は，麻疹ウイルスの曝露を受けると，発症する可能性がある。

世界保健機関（WHO）は，すべての年齢コホートで95％以上の抗体保有率があることを排除（elimination）の定義に含めているが，感染症流行

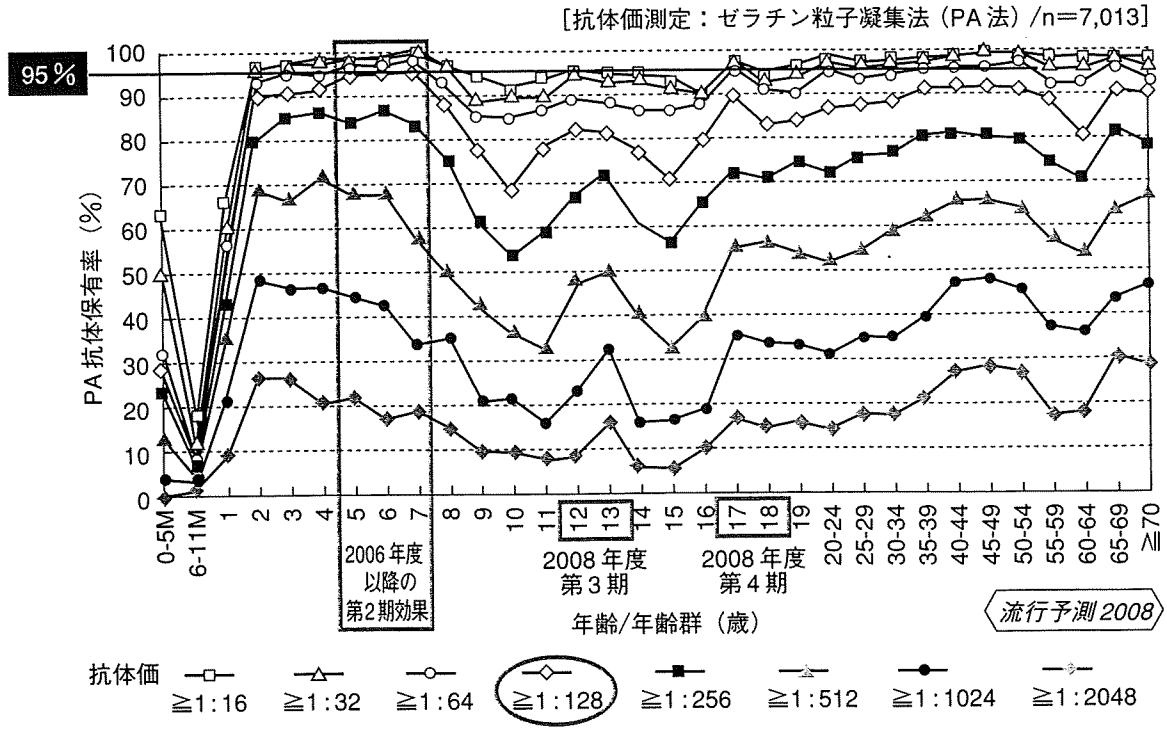

図 1　年齢/年齢群別 麻疹 PA 抗体保有状況　—2008 年度感染症流行予測調査より（暫定値）

予測調査事業より暫定値として得られた 2008 年度の抗体価別・年齢別抗体保有率をみると，**図 1** に示すように，2008 年夏に 9～16 歳であった年齢群では，すべての年齢層に 5～10％の PA 抗体陰性者（1：16 未満）がいることが判明した。これらの年齢群は，多くが学校で集団生活を送っている。ひとたび学校で患者が発生すると，基本再生産数（basic reproductive number〔R_0〕：1 人の患者が周りにいる免疫をもたない人何人にうつしてしまうかを表す数字）が 12～18 と非常に高い麻疹は，迅速な対策が行われないと，麻疹ウイルスの曝露を受けた抗体陰性者（1：16 未満）はほとんどが発症する。とくに，感染可能期間である発症前日から解熱後 3 日を経過するまでの間に登校している麻疹患者がいた場合，さらに，その期間に学校全体で集まる行事が行われていたりすると，低い抗体価である 1：16，1：32，1：64，（1：128）の者を含めた大規模な集団発生につながることが予想される。1：128 以上の抗体保有率でみると，95％以上であったのは 5～7 歳群のみで，2006 年 6 月 2 日から始まった第 2 期の効果と考えられた。8 歳～20 代に存在する多くの抗体陰性者，抗体が不十分な者に対する対策が必要である。

また，0～1 歳児においては，多くの抗体陰性者が存在しており，1 歳になったらすぐの予防接種が求められるところである。0～5 か月児は母親からの移行抗体と考えられるが，約 40％はすでに抗体陰性（1：16 未満）であり，6～11 か月児は，10％台の抗体保有率である。0 歳児が発症しないためには，1 歳以上の者が予防接種により免疫を獲得し，2 回接種を徹底するとともに，国内流行をなくすことで，0 歳児にうつさないよう配慮することが重要である。なお，麻疹流行時の緊急避難的な予防接種は，生後 6 か月以上であれば考慮されている。

次に，一般の人より感染を受ける機会が多く，かつ発症することによる周りへの影響が大きい医療関係者を対象として，日本環境感染学会のワクチン接種プログラム作成委員会では，「院内感染対策としてのワクチンガイドライン」を作成し，現在，ホームページ（2009 年 5 月時点 URL：http://www.kankyokansen.org/iinkai/vacguide.html）に Web 暫定版を公開中である[4]。近日中に，確定版が学会誌に発表される予定であるので，詳細はそちら

表　検査方法と判断基準の目安（案）
—日本環境感染学会ワクチン接種プログラム作成委員会作成—

疾患名	基準を満たさない（陰性）	基準を満たさない（陰性ではない）	基準を満たす
麻疹	中和法で1：4未満 あるいはPA法で1：16未満 あるいはEIA法（IgG）で陰性	中和法で1：4 あるいはPA法で1：16，1：32，1：64，1：128 あるいはEIA法（IgG）で±および16.0未満の陽性	中和法で1：8以上 あるいはPA法で1：256以上 あるいはEIA法（IgG）で16.0以上
風疹	HI法で1：8未満 あるいはEIA法（IgG）で陰性	HI法で1：8，1：16 あるいはEIA法（IgG）で±および8.0未満の陽性	HI法で1：32以上 あるいはEIA法（IgG）で8.0以上
水痘	IAHA法で1：2未満 あるいはEIA法（IgG）で陰性 あるいは水痘抗原皮内テストで陰性	IAHA法で1：2，1：4 あるいはEIA法（IgG）で±	IAHA法で1：8以上 あるいはEIA法（IgG）で陽性 あるいは水痘抗原皮内テストで陽性
ムンプス	EIA法（IgG）で陰性	EIA法（IgG）で±	EIA法（IgG）で陽性

注：このチャートは医療関係者を対象としたものであり，普遍的なものではなく，「基準を満たす」の欄については，値を高く設定している。検査結果はあくまでも検査時点での免疫状態を判断するものであって，長期の免疫状態を証明するものではない。EIA法については，陰性あるいは±の場合は接種が必要であるが，陽性であっても低いEIA価の場合は，発症を予防できない可能性が高く，医療関係者を対象としたチャートであることから，麻疹，風疹については，予防接種によりブースター効果が得られるとする値より高い値に設定した。

を参照して欲しいが，抗体価の考え方については，上記Web版より抜粋して，表に案として紹介するので，参照して欲しい。EIA法の基準は，医療関係者ということから高く設定しているが，加藤ら[5]の研究班の結果から，予防接種によりブースター効果が得られる抗体という観点から決定した。

2．風　疹

麻疹と同様に，風疹に関しても，感染症流行予測調査事業で，毎年4,000～5,000人の規模で感受性調査（抗体保有率調査）が実施されている。直近の結果を紹介すると[3]，2008年度は全国12都県で4,786人について赤血球凝集抑制（hemagglutination inhibition：HI）抗体価が測定された。採血の時期は麻疹と同様，おおむね当該年度の7～9月である。HI法は健康保険収載がなされている検査方法である。

麻疹のHI法はEIA法に比べるとその感度は高くないが，風疹に関しては，EIA法と同等の感度と考えられている。すなわち，1：8未満（陰性）の場合は，風疹に対する免疫をもっていないと考えられる。一方，1：8以上（陽性）であっても，1：8，1：16は不十分と考えられており[2]，とくに女性においては，妊娠初期に風疹を発症すると，先天性風疹症候群の児が生まれる可能性があるため，少なくとも1：32以上の抗体価を保有しておいてほしい[2]。これはEIA法でも同じことがいえる。すなわち，EIA法で陰性であれば，風疹に対する免疫をもっていないと考えられるが，低いEIA価の場合は，風疹ウイルスの曝露を受けると，発症する可能性があるということになる。

2008年度感染症流行予測調査事業より暫定値として得られた抗体価別・年齢別抗体保有率を図2に示す[3]。風疹は麻疹と異なり，1977～1995年までは女子中学生のみが定期予防接種の対象であったために，この年代に中学生であった20～50代では男女差が存在する。上段の女性のHI抗体保有率をみると，0～1歳でHI抗体保有率が低いのは，麻疹と同様であるが，10代には10%台の抗体陰性者が存在し，1：32以上の抗体価を保有しているのは，70%程度にすぎない。一方，男性については，20代までは女性と同等，あるいはわずかに低い結果であるが，30～40代で男女差が大きく，この年代の男性は15～20%が抗体陰性である。

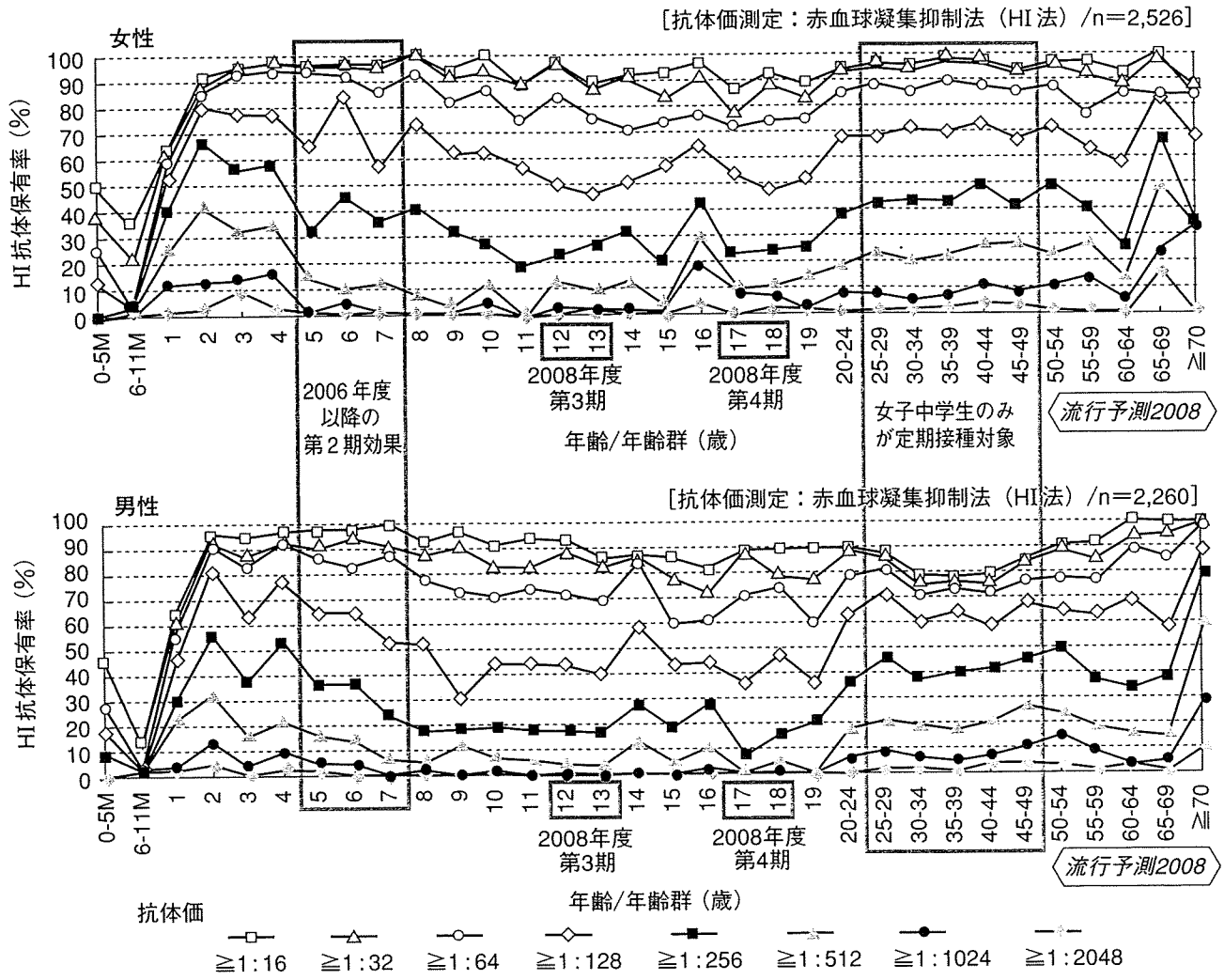

図 2　年齢/年齢群別 風疹 HI 抗体保有状況　—2008 年度感染症流行予測調査より（暫定値）

50 代の男性は約 10%が抗体陰性であった。

2004 年以降，風疹の流行は抑制されているが，これだけの抗体陰性者が蓄積している現状では，ひとたび流行が発生すると，10 代以上の思春期～成人層で流行が拡大することが危惧される。

次に，一般の人より感染を受ける機会が多く，かつ発症することによる周りへの影響が大きい医療関係者については，日本環境感染学会のワクチン接種プログラム作成委員会で，「院内感染対策としてのワクチンガイドライン」として，ホームページ(2009 年 5 月時点 URL：http://www.kankyokansen.org/iinkai/vacguide.html）に Web 暫定版を公開中である。抗体価の考え方については，麻疹同様，表に案として示したので，参照してほしい。EIA 法の基準についても，加藤ら[5]の研究班の結果から，予防接種によりブースター効果が得られる抗体という観点から決定した。

II．予防接種状況

麻疹ワクチンと風疹ワクチンは，それぞれ 1978 年，1977 年に予防接種法に基づく定期接種に導入され，それぞれ 1 回接種が導入されてきたが，より一層の対策強化を目的として，2006 年 6 月 2 日から 1 歳児（第 1 期）と小学校入学前 1 年間の幼児（第 2 期）の 2 回接種制度となり，2007 年の思春期から若年成人を中心とする麻疹流行を受けて，2008 年 4 月 1 日からは，5 年間の時限措置として，中学校 1 年生相当年齢の者（第 3 期）と高校 3 年生相当年齢の者（第 4 期）にも 2 回目の

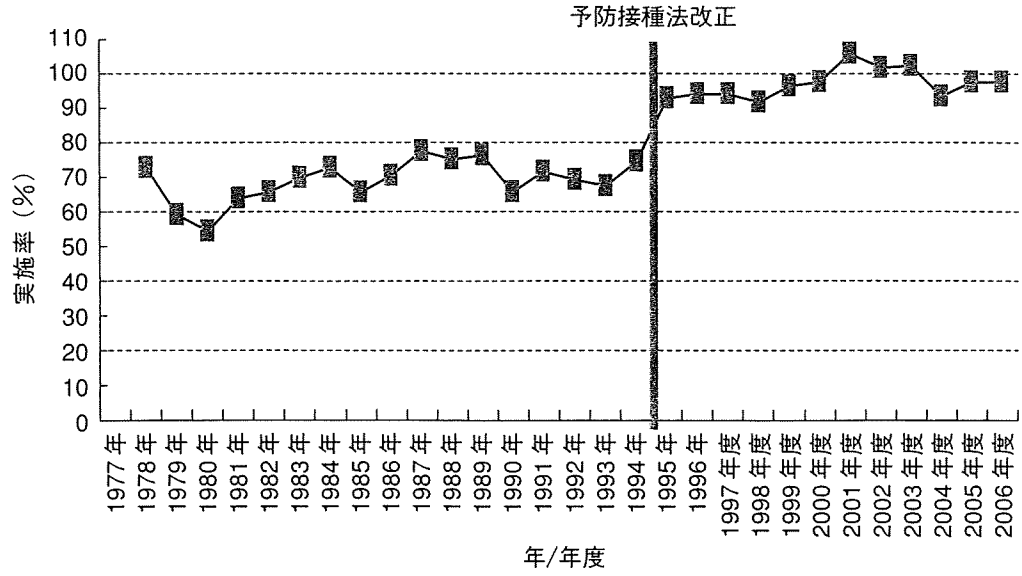

図 3　麻疹定期予防接種実施率
（厚生労働省ホームページ：定期の予防接種実施者数 http://www.mhlw.go.jp/topics/bcg/other/5.html より作図）

麻疹と風疹の予防接種が定期接種に導入されたところである[1]。

水痘とムンプスに関しては，任意接種であるため，接種率は低く，小児でも 25～30％程度の接種率と推定されている[6,7]。

1．麻　疹

厚生労働省によると[8]，定期の麻疹予防接種実施率は**図 3** に示すとおりであり，1994 年の予防接種法改正により，個別接種・努力義務接種になってから実施率は上昇した。なお，1997 年以降は年度結果である。

実施率の計算方法は，標準的な接種年齢期間の総人口を総務省統計局推計人口（各年 10 月 1 日現在）から求め，これを 12 か月相当人口に推計した人数（直近の数値は速報値）を対象人口（分母）とし，1996 年までは保健所運営報告，1997 年以降は地域保健事業報告の「定期の予防接種被接種者数」による「実施人数」を分子として計算している。対象人口は各年度に新規に予防接種対象者に該当した人口であることに対し，実施人口は各年度における定期接種対象者全体の中の予防接種を受けた人員であるため，実施率は 100％をこえる場合がある。

2006 年度から 2 回接種が始まっているため，図 3 の 2006 年度の結果は第 1 期（1 歳児）を示す。図には示していないが，2006 年度の第 2 期（小学校入学前 1 年間）の実施率は 77.9％であった。第 1 期の接種率は高くなっているが，第 2 期は目標の 95％以上に達していない。2008 年度から始まった，中学 1 年生相当年齢の者（第 3 期）と高校 3 年生相当年齢の者（第 4 期）と第 2 期の接種率の中間結果（2008 年 12 月 31 日時点）を**図 4** に示す。目標の 95％に達している都道府県はなく，近年接種率が上昇している第 1 期のみならず，第 2，3，4 期の対象者は忘れずに 2 回目の接種を受けてほしい。

2．風　疹

厚生労働省によると[8]，定期の風疹予防接種実施率は**図 5** に示すとおりであり，1994 年の予防接種法改正により，生後 12～90 か月未満の男女に対する接種（実線）が始まったが，個別接種・努力義務接種になってから中学生の実施率（点線）は激減した。

実施率の計算方法は麻疹と同じである。また，2006 年度から 2 回接種が始まっているため，図 5 の 2006 年度の結果は第 1 期（1 歳児）を示す。

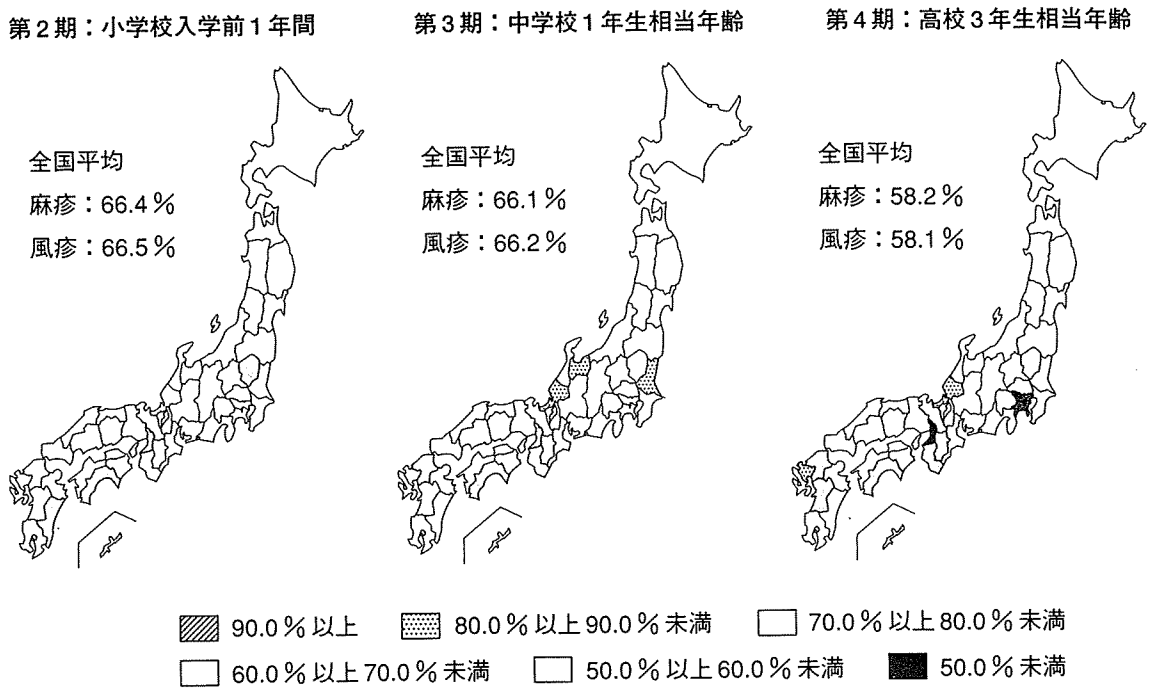

図 4　2008 年度 第 2・3・4 期麻疹風疹ワクチン接種率（中間結果：4 月 1 日～12 月 31 日接種状況評価）　（厚生労働省結核感染症課，国立感染症研究所感染症情報センター）

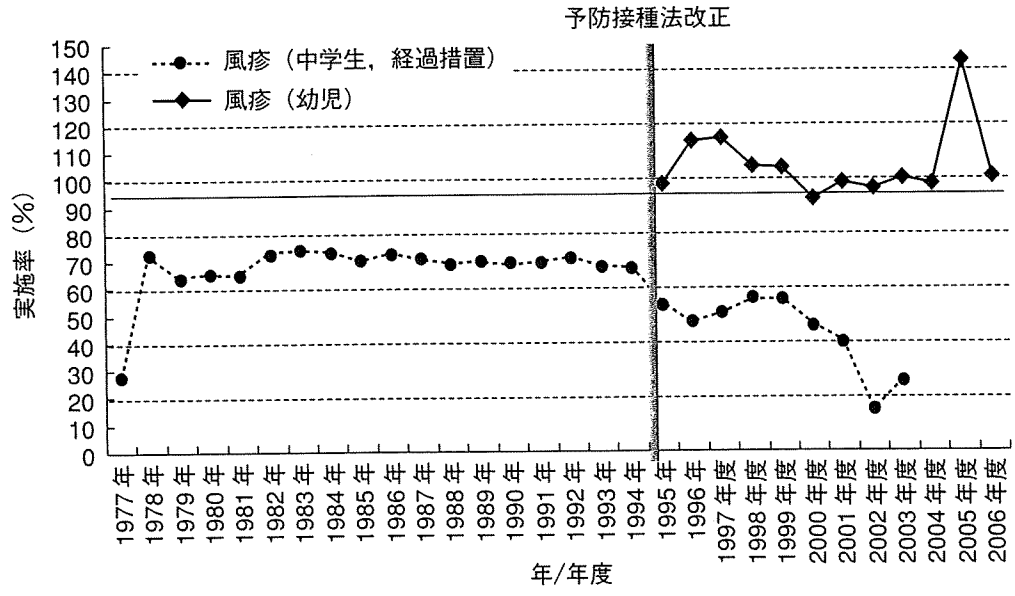

図 5　風疹定期予防接種実施率（中学生・経過措置分および幼児）
（厚生労働省ホームページ：定期の予防接種実施者数 http://www.mhlw.go.jp/topics/bcg/other/5.html より作図）

図には示していないが，2006 年度の第 2 期（小学校入学前 1 年間）の実施率は 79.1％であった。2008 年度から始まった，中学 1 年生相当年齢の者（第 3 期）と高校 3 年生相当年齢の者（第 4 期）と第 2 期の接種率の中間結果（2008 年 12 月 31 日時点）を図 4 に示した。目標の 95％に達している都道府県はなく，対象者は忘れずに接種を受けてほしい。

III. 院内感染対策としての予防接種

Ⅰ. の項にも述べたように，医療関係者を対象としたガイドラインであるが，日本環境感染学会ワクチン接種プログラム作成委員会で作成した抗体価の判断基準を，麻疹・風疹のみならず，水痘・ムンプスについても一緒に表に紹介した。

今後，新たな情報が加わるにしたがって，改正される可能性があるが，抗体価とワクチン接種に関して，医療関係者の一助となることを期待している。とくに，水痘とムンプスの EIA 法による抗体価の見方については，今後さらなる検討が必要であり，新たな情報が加われば，その都度，臨機応変に改訂がなされる予定である。

おわりに

抗体価はあくまでも採血時点の免疫状況を示すものであって，その後も永久にその値が維持されるものではない。国内での流行が抑制され，自然感染のブースター効果を受ける機会が少なくなれば，抗体は減衰していく可能性が高い。

また，抗体測定法にはさまざまな方法があり，それぞれの方法の特徴をよく理解したうえで，結果を解釈していくことがきわめて重要である。

抗体を保有していることを知らずに予防接種を受けた場合，アレルギー反応や血管迷走神経反射といった副反応が起こる可能性は抗体の有無にかかわらずあるものの，医学的には問題ない。

今後は，水痘やムンプスも定期接種対象疾患となり，麻疹，風疹，水痘，ムンプスのすべてが 2 回接種となることを望んでいる。

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The Matrix Protein of Measles Virus Regulates Viral RNA Synthesis and Assembly by Interacting with the Nucleocapsid Protein▽

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The genome of measles virus (MV) is encapsidated by the nucleocapsid (N) protein and associates with RNA-dependent RNA polymerase to form the ribonucleoprotein complex. The matrix (M) protein is believed to play an important role in MV assembly by linking the ribonucleoprotein complex with envelope glycoproteins. Analyses using a yeast two-hybrid system and coimmunoprecipitation in mammalian cells revealed that the M protein interacts with the N protein and that two leucine residues at the carboxyl terminus of the N protein (L523 and L524) are critical for the interaction. In MV minigenome reporter gene assays, the M protein inhibited viral RNA synthesis only when it was able to interact with the N protein. The N protein colocalized with the M protein at the plasma membrane when the proteins were coexpressed in plasmid-transfected or MV-infected cells. In contrast, the N protein formed small dots in the perinuclear area when it was expressed without the M protein, or it was incapable of interacting with the M protein. Furthermore, a recombinant MV possessing a mutant N protein incapable of interacting with the M protein grew much less efficiently than the parental virus. Since the M protein has an intrinsic ability to associate with the plasma membrane, it may retain the ribonucleoprotein complex at the plasma membrane by binding to the N protein, thereby stopping viral RNA synthesis and promoting viral particle production. Consequently, our results indicate that the M protein regulates MV RNA synthesis and assembly via its interaction with the N protein.

Measles is an acute contagious disease characterized by high fever and a maculopapular rash (15). Measles virus (MV), the causative agent, is an enveloped virus classified as a member of the genus *Morbillivirus* in the family *Paramyxoviridae*. The virus has a nonsegmented negative-sense RNA genome, which contains six genes encoding single structural proteins, designated the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The P gene encodes additional gene products, termed the V and C proteins, via an RNA editing process and an alternative translational initiation in a different reading frame, respectively (4, 9). The genome is encapsidated by the N protein and forms a nucleocapsid that exhibits helical symmetry. The amino-terminal region of the N protein (N_{CORE}; amino acids [aa] 1 to 400) constitutes the core region of the helical nucleocapsid while the remaining carboxyl-terminal region (N_{TAIL}; aa 401 to 525) is intrinsically disordered and located outside of the helical nucleocapsid core (36). A viral RNA-dependent RNA polymerase composed of the L and P proteins associates with the nucleocapsid, thereby forming the ribonucleoprotein (RNP) complex. The L protein possesses enzymatic activities that are required for nucleotide polymerization and the capping and polyadenylation of viral mRNAs while the P protein acts as an essential cofactor for the RNA-dependent RNA polymerase functions (15). The P protein directly interacts with N_{TAIL} (2, 16) while the L protein indirectly associates with the nucleocapsid via its interaction with the P protein (10, 22). The RNP complex, but not the naked RNA genome, acts as a template for both transcription and replication. N_{TAIL} has also been shown to interact with multiple host proteins, including the heat shock protein Hsp72, translation initiation factor eIF3-p40, interferon regulatory factor 3 and FcγRII (33, 48, 58, 62). The structural flexibility of the disordered N_{TAIL} is probably responsible for its ability to interact with multiple partners.

The M protein plays a key role in virus assembly. Several lines of evidence indicate that the M protein associates with the inner surface of the plasma membrane (21, 46) as well as the cytoplasmic tails of the H and F glycoproteins (7, 8, 50, 52). The M protein has also been shown to interact with the RNP complex (20, 51) although its binding partner remains unknown. The M protein modulates viral RNA synthesis (51), and a small interfering RNA against M mRNA was reported to increase MV transcription levels in the small interfering RNA-treated cells (43).

In the present study, we analyzed the interactions among MV proteins using a yeast two-hybrid system and coimmunoprecipitation in mammalian cells. In addition to the known interactions, we detected a hitherto unreported interaction between the N and M proteins. Two leucine residues located at the carboxyl terminus of the N protein were found to be critical for this interaction. Further analyses using minigenome assays, plasmid transfection, and recombinant viruses indicated that the M protein regulates viral RNA synthesis and assembly via its interaction with the N protein.

MATERIALS AND METHODS

Cells and viruses. Vero, CV-1, and HeLa cells constitutively expressing human signaling lymphocyte activation molecule (hSLAM) (Vero/hSLAM [41], CV-1/hSLAM [55], and HeLa/hSLAM [55] cells, respectively) were maintained in

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Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals, Aurora, OH) supplemented with 7.5% fetal bovine serum (FBS) and 500 μg/ml Geneticin (G418; Nacalai Tesque, Tokyo, Japan). 293T cells were maintained in DMEM supplemented with 7.5% FBS. B95a cells (31) and VV5-4 cells (a derivative of CHO cells) (1) were maintained in RPMI 1640 medium (ICN Biomedicals) supplemented with 7.5% FBS. Recombinant MVs based on the virulent IC-B strain were generated from cloned cDNAs as described previously (53, 57).

Reagents and antibodies. A fusion-blocking peptide, Z-D-Phe-Phe-Gly (45), was purchased from the Peptide Institute (Osaka, Japan). A rabbit polyclonal antibody raised against the MV N protein was purchased from Novus Biologicals (Littleton, CO). Rabbit polyclonal and mouse monoclonal (clone E388) antibodies raised against the MV M protein were kindly provided by T. Kohama and T. A. Sato, respectively. The serum of a patient with subacute sclerosing panencephalitis (SSPE) (61) was kindly provided by M. B. A. Oldstone.

Construction of plasmids. p(+)MV323-EGFP and p(+)MV323-Luci were derived from p(+)MV323, which encodes the full-length antigenomic cDNA of the virulent IC-B strain of MV (57). They encode an additional transcriptional unit of enhanced green fluorescence protein (EGFP) and *Renilla* luciferase, respectively (17, 56). p(+)MV-NΔ3-EGFP and p(+)MV-NΔ3-Luci were generated by deleting nine nucleotides encoding 3 aa at the carboxyl terminus of the N protein from p(+)MV323-EGFP and p(+)MV323-Luci, respectively. Three nucleotides (TAG) were inserted into the deletion site to maintain the genome length in multiples of six nucleotides. The recombinant MVs generated from p(+)MV323-EGFP, p(+)MV323-Luci, p(+)MV-NΔ3-EGFP, and p(+)MV-NΔ3-Luci were designated IC323-EGFP, IC323-Luci, IC-NΔ3-EGFP, and IC-NΔ3-Luci, respectively.

DNA fragments encoding mutant N proteins with carboxyl-terminal truncations of 3 and 15 aa (NΔ3 and NΔ15, respectively) were cloned into the eukaryotic cell expression vector pCA7, a derivative of pCAGGS (40), thereby generating pCA7-IC-NΔ3 and pCA7-IC-NΔ15, respectively. The expression plasmids pCA7-IC-C and pCA7-IC-M encoding the MV C and M proteins, respectively, were reported previously (39, 52). In yeast two-hybrid assays, a bait vector, pDBLeu (Invitrogen Life Technologies, Carlsbad, CA), and a prey vector, pPC86 (Invitrogen Life Technologies), were used. DNA fragments encoding the entire reading frames of the N, P, V, C, and M proteins of the MV IC-B strain were cloned into the pDBLeu bait vector. The cytoplasmic domains of the F protein (aa 518 to 550 at the carboxyl terminus [$F_{518-550}$]) and H protein (aa 1 to 34 at the amino-terminus [H_{1-34}]) of the IC-B strain were also cloned into pDBLeu. The L proteins of morbilliviruses have three conserved domains (D1, D2, and D3), which are linked by two variable hinges (H1 and H2) (13). DNA fragments encoding 1,707 aa of the amino terminus of the L protein (residues 1 to 1707 [L_{1-1707}]; the region containing D1 and D2) and 476 aa at the carboxyl terminus of the L protein (residues 1708 to 2183 [$L_{1708-2183}$]; the region containing D3) were cloned into the pDBLeu bait vector. DNA fragments encoding the N, P, V, C, and M proteins were also cloned into the pPC86 prey vector. DNA fragments encoding mutant N proteins with truncations of 3, 6, 9, 12, and 15 aa (NΔ3, NΔ6, NΔ9, NΔ12, and NΔ15, respectively) at the carboxyl-terminal end were cloned into both the pDBLeu bait and pPC86 prey vectors.

The plasmids used for minigenome assays of MV (32), Sendai virus (SeV) (28), and parainfluenza virus type 5 (PIV5) (18, 34) were kindly provided by K. Komase, A. Kato, and B. He, respectively. Expression plasmids pCA7-SeV-C and pCA7-PIV5-V for the SeV C and PIV5 V proteins, respectively, were generated by inserting DNA fragments encoding these proteins into the pCA7 vector. The cDNAs encoding these proteins were provided by A. Kato and B. He, respectively.

Plaque assay. Monolayers of Vero/hSLAM cells in 12-well cluster plates were infected with serially diluted virus samples. After 1 h of incubation at 37°C, the virus samples were removed, and the cells were overlaid with DMEM containing 7.5% FBS and 1% methylcellulose. At 5 days postinfection (p.i.), the cells were washed with phosphate-buffered saline (PBS). After cells were stained with 0.01% neutral red, the numbers of PFU were counted. Monolayers of CV-1/hSLAM cells in 12-well cluster plates were cultured with 100 μl of virus samples containing 50 PFU of MV for 1 h at 37°C, cultured with DMEM containing 7.5% FBS and 1% methylcellulose, and stained with 0.01% neutral red at 5 days p.i., as described above. After high-resolution digital images of the plaques were obtained, the sizes of the plaques were measured.

Minigenome assay. Reporter gene expression from viral minigenomes encoding the luciferase gene was analyzed in VV5-4 cells (1). Monolayers of VV5-4 cells cultured in 24-well cluster plates were infected with vTF7-3 (a recombinant vaccinia virus expressing the T7 RNA polymerase) (14), at a multiplicity of infection (MOI) of 0.5 and then transfected with minigenome plasmids (p18MGFLuc01, pHvLuciRT4, and pMG-Rluc for MV, SeV and PIV5, respectively) together with appropriate support plasmids, which were described previously (18, 28, 32, 34, 39). At 2 or 3 days posttransfection, the enzymatic activities were measured by a luciferase assay system (Promega, Madison, WI) or *Renilla* luciferase assay system (Promega) and a luminometer (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany).

Yeast two-hybrid assay. MaV203 yeast cells (Invitrogen Life Technologies) transformed with two plasmids comprising a bait plasmid (pDBLeu) and a prey plasmid (pPC86) were grown on synthetic complete (SC) medium plates lacking leucine and tryptophan (SC/−Leu/−Trp). After 2 days, yeast cells forming colonies on the plates were further selected on four different kinds of plates according to the manufacturer's instructions: SC/−Leu/−Trp plates lacking uracil (SC/−Leu/−Trp/−Ura), SC/−Leu/−Trp plates supplemented with 0.2% 5-fluoroorotic acid (SC/−Leu/−Trp/5FOA), SC/−Leu/−Trp plates lacking histidine and containing 10 mM of 3-aminotriazole (SC/−Leu/−Trp/−His/3AT), and YPAD medium (a rich medium for routine growth of yeast) plates supplemented with 5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (YPAD/X-Gal). Retention of both the bait and prey vectors by yeast cells was confirmed by their growth on SC/−Leu/−Trp plates. Interactions between proteins fused to the *GAL4* activation domain (AD) and the *GAL4* DNA binding domain (BD) encoded in the prey and bait vectors, respectively, resulted in activation of transcription of the *HIS3*, *URA3*, and *lacZ* genes. When transcription of these genes was activated strongly, yeast cells produced blue colonies on the YPAD/X-Gal plates and formed colonies on the SC/−Leu/−Trp/−Ura and SC/−Leu/−Trp/−His/3AT plates but not on the SC/−Leu/−Trp/5FOA plates (yeast cells were killed by the toxicity of 5-fluorouracil converted from 5FOA). If the transcription of these genes was only weakly activated, yeast cells formed colonies on the SC/−Leu/−Trp/−His/3AT and SC/−Leu/−Trp/5FOA plates. However, they produced white colonies on YPAD/X-Gal plates and failed to form colonies on SC/−Leu/−Trp/−Ura plates.

Immunoprecipitation and Western blot analyses. Subconfluent monolayers of 293T cells in six-well cluster plates were transfected with pCA7-IC-N (2 μg) or pCA7-IC-NΔ3 (2 μg) together with pCA7-IC-M (2 μg) (pCA7-IC-N has been referred to as pCAG-T7-IC-N in previous papers [38, 39]). At 48 h posttransfection, the cells were washed with PBS and lysed in 1 ml of coimmunoprecipitation buffer (10 mM HEPES, pH 7.4, 50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM sodium vanadate, 1% Triton X-100) (42) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO). The lysates were centrifuged at 20,630 × *g* for 90 min at 4°C. A small amount (30 μl) of each supernatant was mixed with sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. The rest of the supernatant was incubated for 90 min at 4°C with a 1:1 mixture of protein A-Sepharose and protein G-Sepharose (GE Healthcare AB, Uppsala, Sweden), which had been pretreated with an anti-MV M protein monoclonal antibody (E388) for 90 min at 4°C. Complexes with the Sepharose were obtained by centrifugation and sequentially washed with buffer 1 (100 mM Tris, pH 7.6, 500 mM LiCl, 0.1% Triton X-100, 1 mM DTT) and buffer 2 (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT) (42). The polypeptides in the precipitated complexes were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Piscataway, NJ). The membranes were incubated with the SSPE patient serum and an anti-M antibody, followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G (IgG) or anti-rabbit IgG (Invitrogen Life Technologies) for detection of the MV N and M proteins, respectively. The ECL plus reagent (Amersham Biosciences) was used to elicit chemiluminescent signals, and the signals on the membranes were detected and visualized using a VersaDoc 3000 imager (Bio-Rad, Hercules, CA).

Indirect immunofluorescence assay. HeLa/hSLAM cells were seeded on coverslips in six-well cluster plates and infected with IC323-Luci or IC-NΔ3-Luci in the presence of the fusion-blocking peptide. CV-1/hSLAM cells were seeded on coverslips in six-well cluster plates and transfected with pCA7-IC-N or pCA7-IC-NΔ3, together with pCA7-IC-PΔC or pCA7-IC-PΔC plus pCA7-IC-M. VV5-4 cells seeded on coverslips in six-well cluster plates were infected with vTF7-3 (14) and transfected with an MV minigenome plasmid (p18MGFLuc01) and support plasmids (pCA7-IC-N or pCA7-IC-NΔ3, pCA7-IC-PΔC, and pGEMCR-9301B-L) with or without pCA7-IC-M. At 24 h p.i. or 48 h posttransfection, the cells were fixed and permeabilized with PBS containing 2.5% formaldehyde and 0.5% Triton X-100. The cells were then washed with PBS and incubated with a rabbit polyclonal antibody against the MV N protein (Novus Biologicals) and a mouse monoclonal antibody against the MV M protein (E388), followed by incubation with Alexa Fluor 488-conjugated donkey anti-rabbit IgG(H+L) and Alexa Fluor 594-conjugated donkey anti-mouse

TABLE 1. Interactions between MV proteins in yeast

pDBLeu-encoded protein (DNA BD)	pPC86-encoded protein (AD) interaction[a]					
	Empty	N	P	V	C	M
Empty	−	−	−	−	−	−
N	−	+	+++	+	−	+
P	+	ND	ND	ND	ND	ND
V	+	ND	ND	ND	ND	ND
C	−	−	−	−	−	−
M	−	+	−	−	−	+
$F_{518-550}$	−	−	−	−	−	−
H_{1-34}	−	−	−	−	−	−
L_{1-1707}	−	−	+	−	−	−
$L_{1708-2183}$	−	−	+	−	−	−

[a] +, weak interaction; +++, strong interaction; −, no interaction; ND, not done.

IgG(H+L) antibodies (Molecular Probes, Eugene, OR). The stained cells were observed using a confocal microscope (Radiance 2100; Bio-Rad).

Reverse transcription-quantitative PCR. Subconfluent monolayers of CV-1/hSLAM and Vero/hSLAM cells were infected with IC323-EGFP or IC-NΔ3-EGFP at an MOI of 0.001 or left uninfected in the presence of the fusion-blocking peptide. At 24 h p.i., total RNA was extracted from the cells using the TRIzol reagent (Invitrogen Life Technologies). The total RNA extracts were treated with RQ1 DNase (Promega) and then reverse transcribed into cDNAs using SuperScript III reverse transcriptase (Invitrogen Life Technologies) with an oligo(dT) primer. The amounts of cDNAs for the MV mRNAs were quantified using SYBR Premix Ex *Taq* II (TaKaRa Bio, Shiga, Japan) and a LightCycler instrument (Roche Diagnostics, Indianapolis, IN) as described previously (54). The levels of β-actin mRNA were quantified as an internal control, as reported previously (39).

Growth kinetics. Monolayers of Vero/hSLAM or CV-1/hSLAM cells on six-well cluster plates were infected with IC323-EGFP or IC-NΔ3-EGFP at an MOI of 0.001 and cultured in 2 ml of medium. At various time intervals, the medium was harvested and centrifuged at 400 × *g* for 5 min at 4°C. The viral titer of the supernatant (cell-free titer) was determined by a plaque assay on Vero/hSLAM cells. After the medium was removed from the infected cells, 2 ml of fresh medium was added to each well. The infected cells were scraped into the medium, and the viral titer (cell-associated titer) was determined by a plaque assay.

RESULTS

The MV M protein interacts with the N protein. We attempted to analyze the interactions among MV proteins comprehensively using a yeast two-hybrid system (Table 1). DNA fragments encoding the full-length N, P, V, C, and M proteins, cytoplasmic domains of the F and H proteins ($F_{518-550}$ and H_{1-34}, respectively), and the amino- and carboxyl-terminal regions of the L protein (L_{1-1707} and $L_{1708-2183}$, respectively) were individually inserted in-frame downstream of the *GAL4* DNA BD in the bait vector (pDBLeu) or the *GAL4* AD in the prey vector (pPC86). The empty pDBLeu and pPC86 vectors were used as controls. MaV203 yeast cells grew on SC/−Leu/−Trp/−His/3AT plates when they were transfected with a bait vector encoding either the P or V protein (pDBLeu-P or -V) together with the empty pPC86 prey vector. A similar finding has been reported previously (11). Therefore, these bait vectors could not be used for further analyses. Experiments using pDBLeu-N as the bait indicated that, in addition to the known N-V (59) and N-N (2, 25) protein interactions, there was a weak, but significant, interaction between the N and M proteins (Table 1). Furthermore, when pDBLeu-N and pPC86-P were used, transcription of all three reporter genes, *HIS3*, *lacZ*, and *URA3*, was strongly activated (Table 1). This was consistent with the previously reported strong interaction between the N and P proteins (2, 11, 16). Similarly, data using pDBLeu-M and pPC86-N or -M confirmed the weak, but significant, N-M protein interaction and indicated the self-association of the M protein (47), which is similar to the case for the SeV M protein (19). Analysis using pPC86-P together with pDBLeu-L_{1-1707} or -$L_{1708-2183}$ indicated that the P protein interacts with both the amino-terminal and carboxyl-terminal regions of the L protein. The interaction with the amino-terminal region was previously demonstrated by analyzing L protein mutants whose amino acid residues were progressively deleted from the carboxyl-terminal end (10, 22). On the other hand, the present study is the first to demonstrate that the P protein interacts with the carboxyl-terminal region (aa 1708 to 2183) of the L protein.

The carboxyl-terminal residues of the N protein are essential for the N-M protein interaction. The N protein can be divided into two regions, N_{CORE} and N_{TAIL} (36). We predicted that the M protein may interact with N_{TAIL}, like the P protein and multiple host factors. pDBLeu bait vectors expressing the mutant N proteins NΔ3, NΔ6, NΔ9, NΔ12, and NΔ15 with stepwise deletions from the carboxyl-terminal end were generated. The interactions of these mutant N proteins with the M protein were analyzed in the yeast two-hybrid system. All five mutants were unable to interact with the M protein (Fig. 1A). On the other hand, all of the mutants retained the abilities to interact with the P protein and to self-associate (Fig. 1B and C). Since even the NΔ3 protein was unable to interact with the M protein, the 3 aa residues at the carboxyl-terminal end of the N protein (two leucines and an aspartic acid, in the sequence order of LLD) were further examined. A single-amino acid deletion (NΔ1 [LL-]) did not affect the N-M protein interaction, whereas a 2-aa deletion (NΔ2 [L–]) abolished the interaction (Table 2). Substitution of an alanine for the individual leucine residues at positions 523 and 524 (N-ALD and N-LAD, respectively; substitutions are underlined) abolished the N-M protein interaction, while substitution of a serine or glutamic acid for the aspartic acid at position 525 (N-LLS and N-LLE, respectively) had little effect on the interaction (Table 2). These data indicate that the leucine residues at positions 523 and 524 in N_{TAIL} are essential for the N-M protein interaction.

The N-M protein interaction was also assessed in mammalian cells. 293T cells were individually transfected with pCA7 vectors encoding the wild-type (wt) N, NΔ3, and NΔ15 proteins with or without a pCA7 plasmid encoding the M protein. When the whole-cell lysates were subjected to SDS-PAGE and Western blotting, all the proteins were detected at high levels (Fig. 1D). In another experiment, the cell lysates were incubated with protein A/G conjugated to Sepharose beads, which had been pretreated with a monoclonal antibody against the M protein, and immune complexes were retrieved by centrifugation. When the immune complexes were subjected to SDS-PAGE and Western blotting, the wt N protein, but not the NΔ3 and NΔ15 proteins, was detected in complexes that contained the M protein (Fig. 1D). These data indicate that the N protein also interacts with the M protein in mammalian cells and that its carboxyl-terminal residues are essential for the interaction.

The MV M protein inhibits RNA synthesis in the MV minigenome system via its interaction with the N protein. Next, we

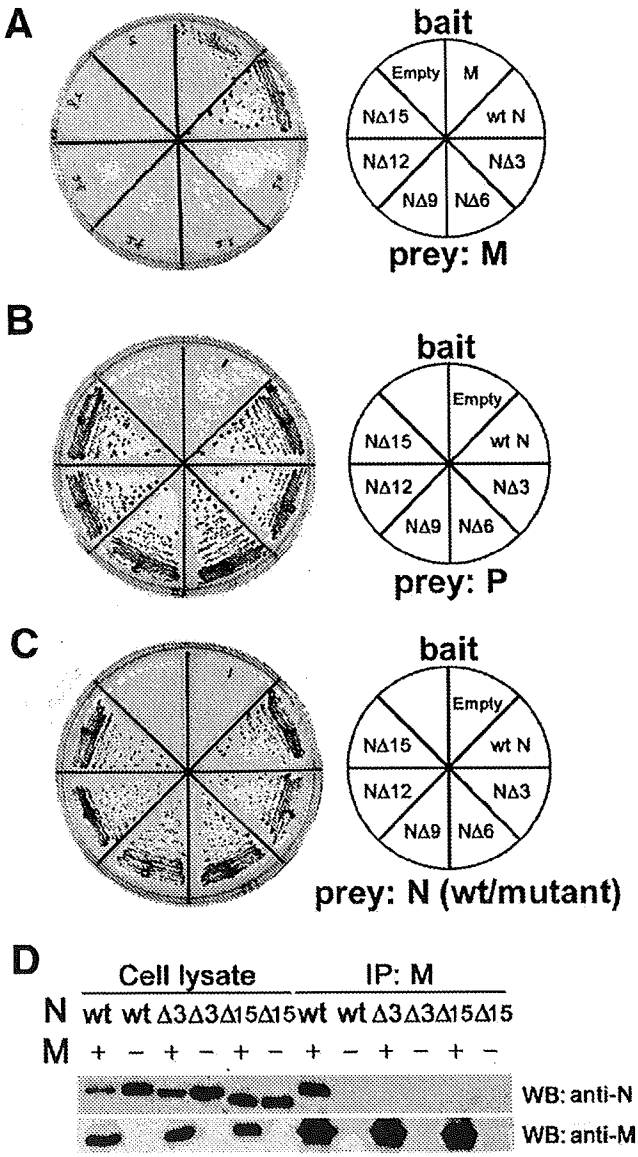

FIG. 1. The carboxyl-terminal end of the N protein is essential for the N-M protein interaction. (A to C) Yeast two-hybrid assay. MaV203 yeast cells were transfected with a bait vector expressing the *GAL4* DNA BD fused with the M, wt N, NΔ3, NΔ6, NΔ9, NΔ12, or NΔ15 protein or the *GAL4* DNA BD alone (Empty) (the bait used is shown in the explanatory figure on the right) together with a prey vector expressing the *GAL4* AD fused with the M protein (A), P protein (B), or wt N protein or corresponding N protein deletion mutant (C). Self-association of the wt N protein and its deletion mutants was examined. Reporter gene (*HIS3*) expression, which results in the growth of MaV203 cells on SC/−Leu/−Trp/−His/3AT plates, was analyzed. (D) Coimmunoprecipitation assay. 293T cells were transfected with a plasmid expressing the wt N, NΔ3, or NΔ15 protein with (+) or without (−) a plasmid expressing the M protein. At 48 h posttransfection, small amounts of cell lysates obtained from the transfected cells (Cell lysate) were subjected to SDS-PAGE and Western blotting (WB) for detection of the N and M proteins using a human polyclonal antibody against the N protein and a rabbit polyclonal antibody against the M protein. The remaining cell lysates were incubated with protein A/G-conjugated Sepharose beads, which had been pretreated with a mouse monoclonal antibody against the M protein (E388). The immune complexes were obtained by centrifugation. The precipitated immune complexes were subjected to SDS-PAGE and Western blotting for detection of the N and M proteins. IP, immunoprecipitation.

TABLE 2. Effect of the C-terminal mutations of the N protein on interactions in yeast

pDBLeu-encoded protein (DNA BD)[a]	pPC86-encoded protein (AD) interaction[b]		
	Empty	M	P
Empty	−	−	−
wt N [LLD]	−	+	+++
NΔ1 [LL-]	−	+	+++
NΔ2 [L-]	−	−	+++
NΔ3 [—]	−	−	+++
N-ALD	−	−	+++
N-LAD	−	−	+++
N-LLS	−	+	+++
N-LLE	−	+	+++

[a] Substituted nucleotides are underlined. The carboxyl-terminal deletions of the N mutant proteins are indicated by dashes.
[b] +, weak interaction; +++, strong interaction; −, no interaction.

examined the activity of the M protein in viral RNA synthesis. Consistent with previous reports that the M protein inhibits MV RNA synthesis (51), our results confirmed that coexpression of the M protein reduced the level of the reporter (luciferase) gene expression from an MV minigenome by ~95% (Fig. 2A). The C protein also inhibited minigenome RNA synthesis, which is consistent with previous reports (3, 39, 44). In contrast, the MV M protein hardly affected reporter gene expression levels in minigenome systems of other paramyxoviruses (SeV and PIV5) while the SeV C protein and PIV5 V protein inhibited reporter gene expression from their own minigenomes (Fig. 2A), as reported previously (26, 27, 34). These data indicate that the ability of the MV M protein to inhibit minigenome expression is specific for MV RNA.

We hypothesized that the N-M protein interaction may be involved in this specific inhibitory activity of the M protein. The NΔ3 and NΔ15 proteins induced reporter gene expression from the MV minigenome as efficiently as the wt N protein when they were expressed together with the P and L proteins (Fig. 2B). However, coexpression of the M protein no longer inhibited reporter gene expression from the RNP complexes formed with the NΔ3 and NΔ15 proteins (Fig. 2B). These results indicate that the M protein regulates MV RNA synthesis by interacting with the carboxyl-terminal residues of the N protein. In contrast, the MV C protein still efficiently inhibited reporter gene expression from the RNP complexes formed with the NΔ3 and NΔ15 proteins (Fig. 2C), indicating that the M and C proteins regulate viral RNA synthesis by different means.

The N-M protein interaction causes the redistribution of the N protein within the cell. Many lines of evidence have indicated that paramyxovirus M proteins play major roles in virus assembly (49). The MV M protein, but not the N protein, has an intrinsic ability to associate with the lipid membrane or lipid raft (37, 46, 60). It has been proposed that the M protein recruits the nucleocapsid to the plasma membrane (47) or lipid raft for assembly (60). To assess their intracellular distributions, the wt N, NΔ3, and NΔ15 proteins were expressed in CV1/hSLAM cells, together with the P protein. The P protein is required for cytoplasmic retention of the N protein (23). The wt N, NΔ3, and NΔ15 proteins were all mainly localized in the cytoplasm, forming small dots in the perinuclear area (Fig.

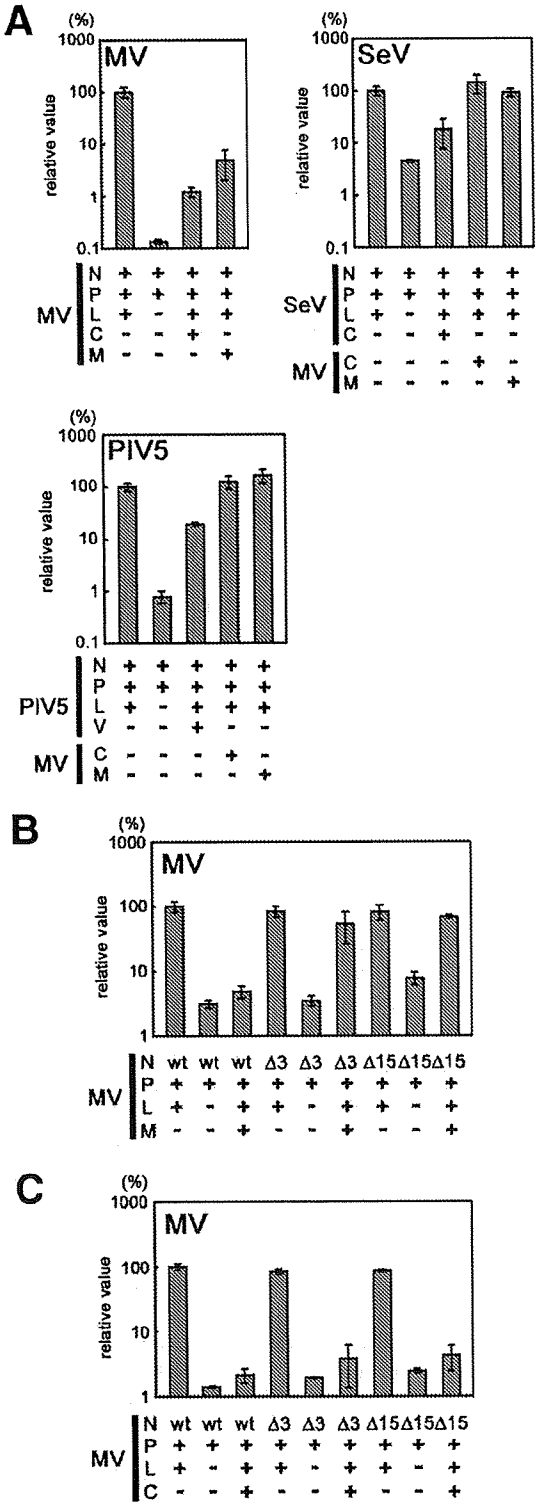

FIG. 2. The interaction with the carboxyl-terminal end of the N protein is required for the M protein to inhibit MV minigenome gene expression. (A) The MV M and C proteins specifically inhibit MV minigenome gene expression. VV5-4 cells were infected with vTF7-3 at an MOI of 0.5 and then transfected with a minigenome plasmid (p18MGFLuc01 [32], pHvLuciRT4 [28], and pMG-Rluc [34] for MV, SeV, and PIV5, respectively) and three support plasmids expressing the N, P, and L proteins of each virus. +, expression of the protein; −, no expression of the protein. The L protein expression plasmid was omitted from the transfection mixtures for negative control cells (L−). The MV M and C protein expression plasmids (pCA7-IC-M and pCA7-IC-C, respectively) were included in the transfection mixtures for some cells (MV M+ and MV C+, respectively). At 72 h posttransfection, luciferase activity was measured. The luciferase activity in control cells transfected only with a minigenome plasmid and three support plasmids (N, P, and L) for each virus was set to 100%. Data represent the means ± standard deviation of triplicate samples. (B) Effects of carboxyl-terminal deletion of the N protein on the activity of the M protein to inhibit MV minigenome gene expression. VV5-4 cells infected with vTF7-3 were transfected with the MV minigenome plasmid (p18MGFLuc01) and three support plasmids (pCA7-IC-N, pCA7-IC-PΔC, and pGEMCR-9301B-L). As replacements for pCA7-IC-N (wt), pCA7-IC-NΔ3 (Δ3) and pCA7-IC-NΔ15 (Δ15) were also used as support plasmids. pGEMCR-9301B-L was omitted from the transfection mixture for negative control cells (L−). pCA7-IC-M was added to the transfection mixture for some cells (M+). At 48 h posttransfection, luciferase activity was measured. The luciferase activity in cells transfected with p18MGFLuc01, pCA7-IC-N, pCA7-IC-PΔC, and pGEMCR-9301B-L (N wt, P+, L+, and M−) was set to 100%. Data represent the means ± standard deviations of triplicate samples. (C) Effects of carboxyl-terminal deletion of the N protein on the activity of the C protein to inhibit MV minigenome gene expression. Experiments were performed as described for panel B, except that pCA7-IC-C was used instead of pCA7-IC-M.

3A). When the M protein was coexpressed, the wt N protein was colocalized with the M protein and became redistributed. In contrast, the intracellular distributions of the NΔ3 and NΔ15 proteins were hardly affected by coexpression of the M protein (Fig. 3A).

Similar experiments were performed in VV5-4 cells in which the MV minigenome was replicated and transcribed by the N, P, and L proteins, as shown in Fig. 2B. Both the wt N and NΔ3 proteins were mostly localized in the perinuclear area, forming small dots (Fig. 3B). When the M protein was coexpressed, the wt N protein became redistributed (Fig. 3B). Notably, in cells expressing the M protein at low levels, the wt N protein remained as small dots in the perinuclear area (Fig. 3B). In contrast, the intracellular distribution of the NΔ3 protein remained unaltered even in cells highly expressing the M protein (Fig. 3B). Therefore, the M protein neither inhibits viral RNA synthesis nor causes redistribution of the N protein when it is unable to interact with the N protein (e.g., NΔ3).

The N-M protein interaction is required for efficient production of infectious MV particles. The relevance of the N-M protein interaction was analyzed in the context of viral particles using reverse genetics. A recombinant MV possessing the NΔ3 protein (IC-NΔ3-EGFP) replicated much less efficiently than the parental virus possessing the wt N protein (IC323-EGFP) in Vero/hSLAM and CV1/hSLAM cells (Fig. 4A), indicating that the N-M protein interaction is indeed important for efficient virus production. The MV transcript levels examined in the presence of the fusion-blocking peptide were comparable between IC323-EGFP- and IC-NΔ3-EGFP-infected Vero/hSLAM cells, whereas the transcript levels in IC-NΔ3-EGFP-infected CV-1/hSLAM cells tended to be severalfold lower than those in IC323-EGFP-infected cells (Fig. 4B). The difference in the transcript levels between the two viruses did not appear to account for the large difference in viral growth. Since only very low titers of IC-NΔ3-EGFP were obtained, we

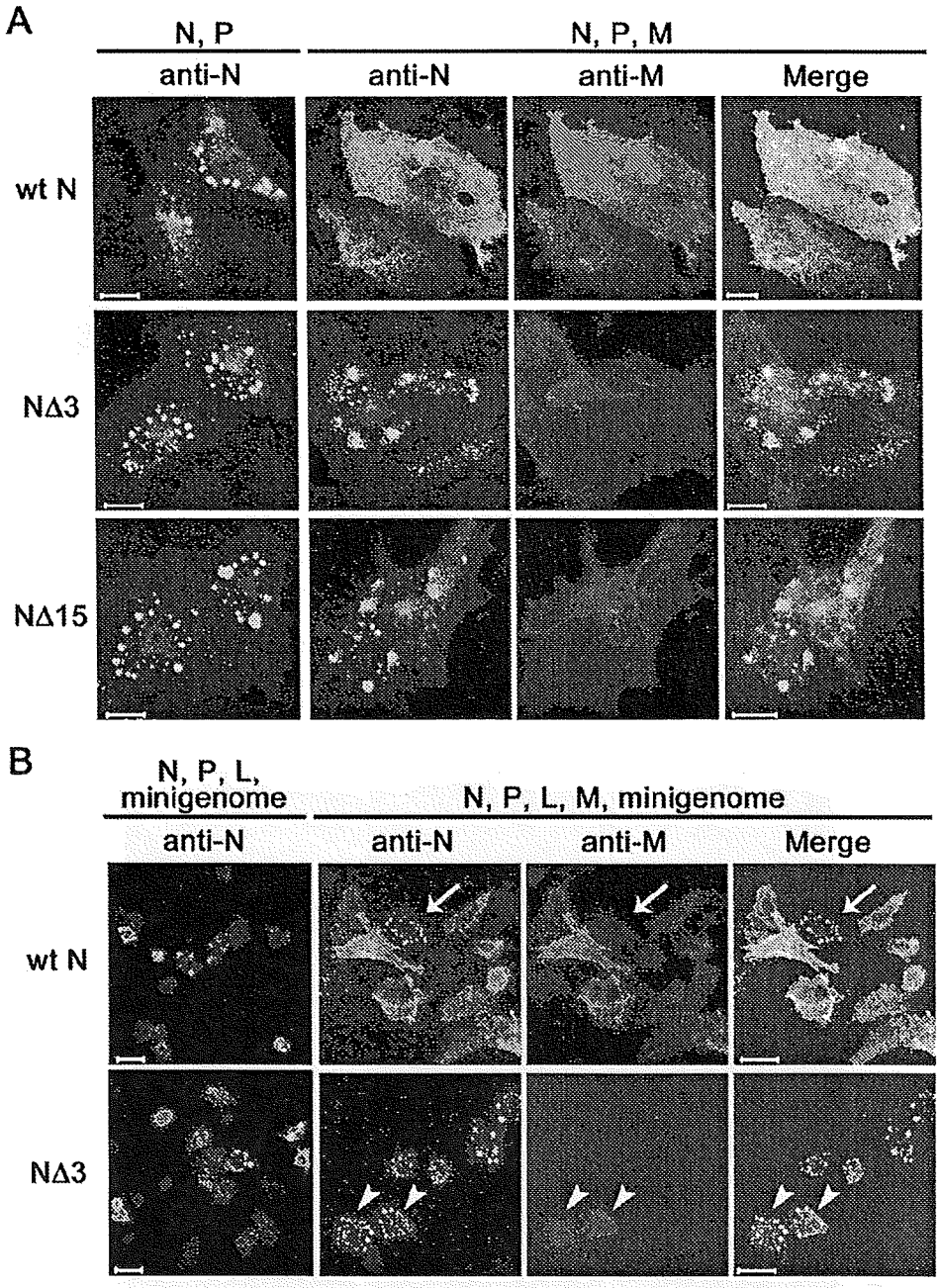

FIG. 3. Intracellular distributions of the N and M proteins analyzed by indirect immunofluorescence and confocal microscopy. (A) CV-1/hSLAM cells were transfected with pCA7-IC-N (wt N), pCA7-IC-NΔ3 (NΔ3), or pCA7-IC-NΔ15 (NΔ15) together with pCA7-IC-PΔC (N, P). Cells were also transfected with pCA7-IC-N (wt N), pCA7-IC-NΔ3 (NΔ3), or pCA7-IC-NΔ15 (NΔ15) together with pCA7-IC-PΔC and pCA7-IC-M (N, P, M). At 48 h posttransfection, the intracellular distributions of the N and M proteins were analyzed by indirect immunofluorescence and confocal microscopy. The primary antibodies used were a rabbit polyclonal antibody against the N protein and a mouse monoclonal antibody against the M protein. The secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-mouse antibodies, respectively. Bar, 20 μm. (B) VV5-4 cells infected with vTF7-3 were transfected with the MV minigenome plasmid (p18MGFLuc01) together with three support plasmids expressing the N, P, and L proteins (N, P, L, minigenome). Either pCA7-IC-N (wt N) or pCA7-IC-NΔ3 (NΔ3) was used as the N protein expression plasmid. Cells were also transfected with pCA7-IC-M together with the minigenome plasmid and three support plasmids (N, P, L, M, minigenome). At 48 h posttransfection, the intracellular distributions of the N and M proteins were analyzed by indirect immunofluorescence and confocal microscopy as described for panel A. Arrows and arrowheads indicate cells expressing M protein at low and high levels, respectively. Bar, 20 μm.

could not reliably compare the viral protein production levels between IC323-EGFP- and IC-NΔ3-EGFP-infected cells using Western blot analysis. Instead, we compared the sizes of the plaques produced by the two viruses. In both CV-1/hSLAM and Vero/hSLAM cells, IC-NΔ3-EGFP produced large plaques similar to those of IC323-EGFP (Fig. 4C and data not shown). These results suggest that the multiplication of IC-NΔ3-EGFP is adversely affected at a step later than viral pro-

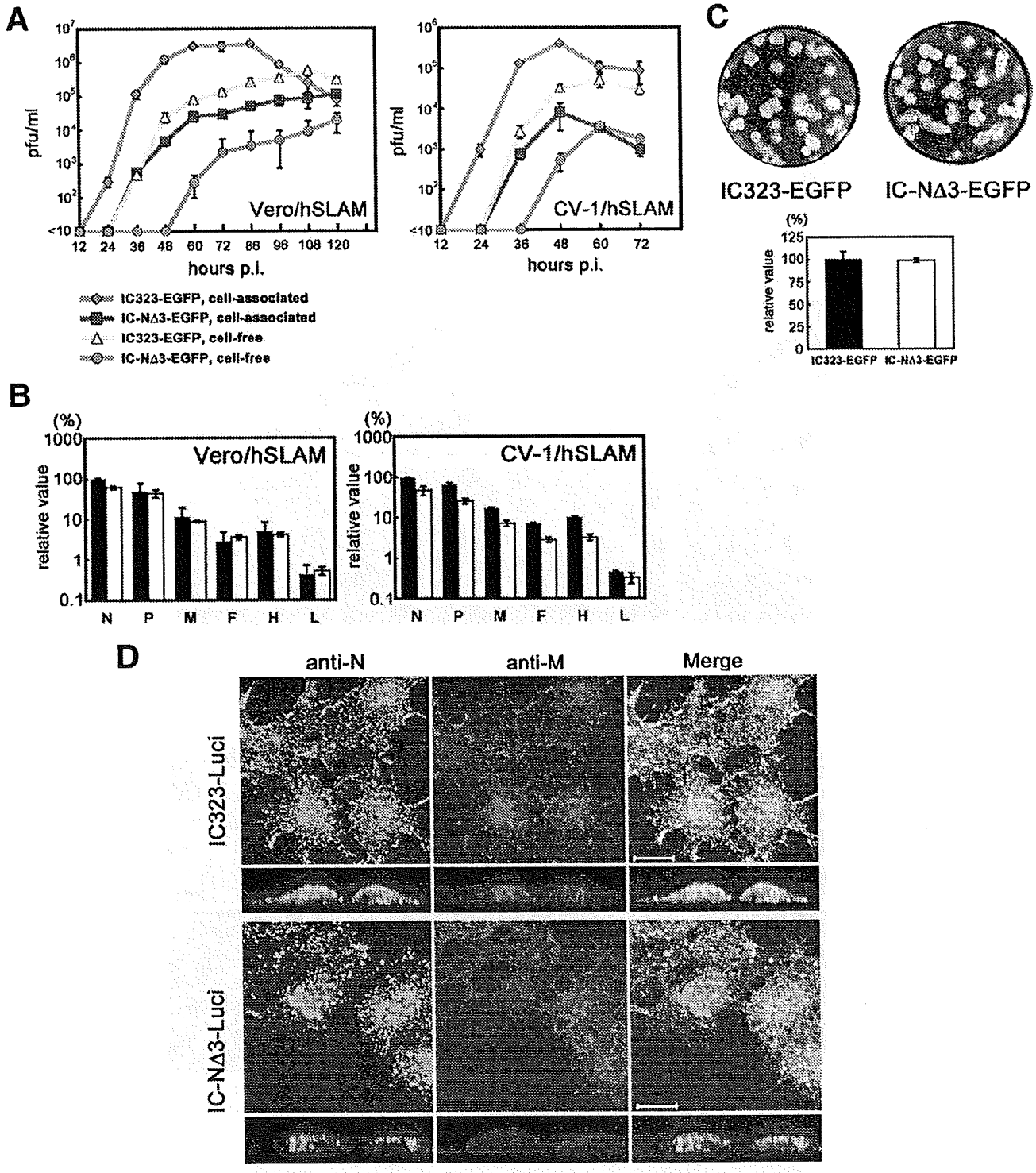

FIG. 4. Analyses of recombinant MVs possessing carboxyl-terminal deletion of the N protein. (A) Growth kinetics. Vero/hSLAM and CV-1/hSLAM cells were infected with recombinant MVs (IC323-EGFP and IC-NΔ3-EGFP) at an MOI of 0.001. The infectious titers in culture medium (cell free) and cells (cell associated) were determined at various time points. (B) Quantification of viral mRNAs. Vero/hSLAM and CV-1/hSLAM cells were infected with IC323-EGFP (black bar) or IC-NΔ3-EGFP (white bar) at an MOI of 0.001 in the presence of a fusion-blocking peptide. At 24 h p.i., the levels of the N, P, M, F, H, and L mRNAs of MV in the infected cells were analyzed by reverse transcription-quantitative PCR. Data were normalized by the levels of β-actin mRNA and represent the means ± standard deviations of triplicate samples. The N mRNA level in IC323-EGFP-infected cells was set to 100%. (C) Plaque assays. Monolayers of CV-1/hSLAM cells on 12-well cluster plates were infected with 50 PFU of IC323-EGFP or IC-NΔ3-EGFP and overlaid with DMEM containing 7.5% FBS and 1% methylcellulose. At 5 days p.i., the cells were stained with 0.01% neutral red, and the sizes of the plaques were measured. The mean diameters ± standard deviations are shown in the bar graph. (D) HeLa/hSLAM cells were infected with IC323-Luci (upper panels) or IC-NΔ3-Luci (lower panels). At 24 h p.i., the intracellular distributions of the N and M proteins were analyzed by indirect immunofluorescence and confocal microscopy as described in the legend of Fig. 3A. Longitudinal sections are also shown at the bottom. Bar, 10 μm.

tein production, presumably virus assembly, by the impaired interaction between the N and M proteins.

The intracellular distributions of the N and M proteins were examined in cells infected with recombinant MVs possessing the wt N or NΔ3 protein (Fig. 4D). Since a green fluorescent Alexa Fluor 488-conjugated secondary antibody was used to detect the N protein, IC323-Luci and IC-NΔ3-Luci, rather than IC323-EGFP and IC-NΔ3-EGFP, respectively, were used for this experiment. In IC323-Luci-infected cells, the wt N protein was mostly colocalized with the M protein at the cell surface or as small dots in the perinuclear area (as observed in transfected cells expressing low levels of the M protein). Although the NΔ3 protein was also localized in small dots in infected cells, its distribution was totally unrelated to that of the M protein.

DISCUSSION

In the present study, we have demonstrated that the M protein regulates MV RNA synthesis and assembly via its direct interaction with the N protein. The N protein is biologically divided into two regions. N_{CORE} has a rigid structure and contains all of the regions necessary for self-assembly and binding to viral genomic and antigenomic RNA (2, 25, 35). On the other hand, the intrinsically disordered N_{TAIL} protrudes from the viral nucleocapsid (25, 29, 36) and interacts with many viral and cellular proteins (2, 16, 33, 62). The present study indicates that N_{TAIL} also binds to the M protein. N_{TAIL} contains an α-helical molecular recognition element (aa 488 to 499), which interacts with three α-helical strands of the carboxyl-terminal module (XD; aa 459 to 507) of the P protein (24, 30). Surface plasmon resonance analyses suggested that the BOX3 region (aa 517 to 525) of the N protein also interacts with the P protein (6). However, another study using nuclear magnetic resonance reported that the BOX3 region does not directly contribute to the interaction with the P protein (5). Our present study supports the findings of the latter study since deletion of up to 15 aa residues from the carboxyl-terminal end of the N protein did not affect its interaction with the P protein, as examined by the yeast two-hybrid system. Then, the question arises as to whether the carboxyl-terminal region of the N protein participates in viral RNA synthesis. We performed minigenome assays and found that the levels of viral RNA synthesis did not change when a mutant N protein lacking the carboxyl-terminal 15 aa residues was used for the assay. This finding is consistent with a previous report that truncation of the carboxyl-terminal 24 aa residues of the MV Edmonston N protein does not diminish transcription and replication of the minigenome (62).

Using the minigenome assay, we further found that the ability of the M protein to regulate viral RNA synthesis was mediated by its interaction with the N protein. The C protein of MV inhibits viral RNA synthesis (3, 39, 44). However, in our yeast two-hybrid system, we found that the C protein did not interact with any of the MV proteins including the N protein. Furthermore, the C protein still exerted its inhibitory activity when the mutant N proteins that were unable to interact with the M protein were used for the minigenome assay. Therefore, the C protein is likely to regulate viral RNA synthesis in a different manner from the M protein. The host protein Hsp72 has been shown to stimulate MV minigenome reporter gene expression by interacting with the carboxyl-terminal BOX3 region of the N protein (62). It is possible that the M protein exerts its inhibitory activity by modulating the interaction between the N protein and Hsp72.

Although MV assembly has been studied for many years, its precise mechanisms, including the viral and host proteins involved, are largely unknown. In the *Paramyxoviridae*, the M protein underlies the viral envelope and forms electron-dense layers in electron micrographs (15). The M protein has been shown to interact with the viral RNP complex (20, 51) as well as the cytoplasmic domains of viral envelope glycoproteins (7, 8, 50, 52). Although the recombinant MV lacking the M protein (MV-ΔM) was successfully rescued, virus titers were reduced ~250-fold compared with the parental virus (7), as observed for IC-NΔ3-EGFP (possessing a mutant N protein that was incapable of interacting with the M protein) in the present study (2-log reduction). Furthermore, the inability of defective M proteins to associate with the viral nucleocapsid is thought to be responsible for the low level of infectious viral particle production in patients with SSPE (20). Consequently, the M protein and its interaction with the RNP complex are believed to play important roles in MV assembly. The present study has clearly demonstrated that the N protein is the RNP complex component that interacts with the M protein. A similar finding has been obtained for another paramyxovirus, human PIV1 (hPIV1). When cells were infected with SeV and transfected with the nucleoprotein (NP) cDNA of hPIV1, nucleocapsids composed of a mixture of NP molecules as well as those composed of NPs solely from SeV or solely from hPIV1 were detected in the cytoplasm (12). However, most of the NPs in the nucleocapsids of the progeny SeV virions were found to be from SeV. Coexpression of the hPIV1 M protein greatly increased the incorporation of the nucleocapsids containing hPIV1 NP into SeV virions. Analysis using SeV-hPIV1 chimera NP cDNAs indicated that the hPIV1 M protein caused specific incorporation of the nucleocapsid containing hPIV1 NP into SeV virions by interacting with the carboxyl-terminal domain of hPIV1 NP (aa position 420 to 466) (12).

Interestingly, previous studies have shown that MV-ΔM and viruses bearing the H and F proteins with shortened cytoplasmic tails are more efficient in inducing cell-cell fusion than the parental MV (7, 8). In contrast, IC-NΔ3-EGFP and IC323-EGFP produced similar sizes of plaques. The observations suggest that the interaction between the M and envelope proteins, but not that between the M and N proteins, critically affects MV-induced membrane fusion.

The present study has also shed new light on the mechanism by which the M protein promotes MV particle production. The N and M proteins were expressed in cultured cells by transfection with expression plasmids or by infection with recombinant viruses, and their intracellular distributions were investigated by indirect immunofluorescence staining and confocal microscopy. Our results revealed that in the presence of the P protein, the N protein formed small dots in the perinuclear area when it was expressed without the M protein or when it was incapable of interacting with the M protein. However, when the N protein was expressed in the presence of the P protein and in a form that was able to interact with the M protein, the N protein colocalized with the M protein in the cytoplasm as

well as at the plasma membrane. We further found that IC-NΔ3-EGFP produced similar or slightly reduced levels of viral RNA and comparable sizes of plaques (and therefore probably comparable levels of viral proteins) compared with the parental IC323-EGFP although its production of infectious viral particles was markedly impaired. Taken together, these observations may suggest that the M protein retains the viral RNP complex at the plasma membrane by interacting with the N protein and promotes the production of infectious viral particles.

Unlike the recombinant MV lacking the C protein (39), IC-NΔ3-EGFP did not produce an increased level of viral RNA in infected cells. This observation may be explained if we presume that, in addition to the interaction between the N and M proteins, retention of the RNP complex by the M protein at the plasma membrane is required to stop viral RNA synthesis. If this hypothesis is correct, the M protein inhibits RNA synthesis only from the RNP complexes that are to become incorporated into progeny virions at the plasma membrane and does not affect viral RNA synthesis from the RNP complexes in the cytoplasm. In contrast, the nonstructural C protein may inhibit overall viral RNA synthesis in the cells. In MV-infected cells, the level of the M protein may be regulated (unlike the case in transfected cells) such that viral RNA synthesis continues at a sufficient level in the perinuclear area of the cytoplasm.

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AUTHOR'S CORRECTION

The Matrix Protein of Measles Virus Regulates Viral RNA Synthesis and Assembly by Interacting with the Nucleocapsid Protein

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Volume 83, no. 20, p. 10374–10383, 2009. Page 10374, column 2, lines 4–6: "Hsp72, translation initiation factor eIF3-p40, interferon regulatory factor 3 and FcγRII" should read "Hsp72 and interferon regulatory factor 3."

IMMUNOLOGY

Mitofusin 2 Inhibits Mitochondrial Antiviral Signaling

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The innate immune response to viral infection involves the activation of multiple signaling steps that culminate in the production of type I interferons (IFNs). Mitochondrial antiviral signaling (MAVS), a mitochondrial outer membrane adaptor protein, plays an important role in this process. Here, we report that mitofusin 2 (Mfn2), a mediator of mitochondrial fusion, interacts with MAVS to modulate antiviral immunity. Overexpression of Mfn2 resulted in the inhibition of retinoic acid–inducible gene I (RIG-I) and melanoma differentiation–associated gene 5 (MDA-5), two cytosolic sensors of viral RNA, as well as of MAVS-mediated activation of the transcription factors interferon regulatory factor 3 (IRF-3) and nuclear factor κB (NF-κB). In contrast, loss of endogenous Mfn2 enhanced virus-induced production of IFN-β and thereby decreased viral replication. Structure-function analysis revealed that Mfn2 interacted with the carboxyl-terminal region of MAVS through a heptad repeat region, providing a structural perspective on the regulation of the mitochondrial antiviral response. Our results suggest that Mfn2 acts as an inhibitor of antiviral signaling, a function that may be distinct from its role in mitochondrial dynamics.

INTRODUCTION

Innate immunity is an essential and ubiquitous system that defends organisms from infectious pathogens. Initiation of the innate immune response is typically triggered by the recognition of broadly conserved elements of the invading pathogen known as pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs by germline-encoded pattern recognition receptors ultimately activates intracellular signaling cascades that result in the clearance and killing of infectious microbes (*1*, *2*). Viral infection of host cells is detected by the cell's recognition of PAMPs such as double-stranded RNA (dsRNA), which initiates two distinct signaling pathways (*3*). The first, mediated by endosomal Toll-like receptor 3, recognizes viral dsRNA that enters the cell by endocytosis, whereas the second pathway detects cytoplasmic, virus-derived dsRNA through the involvement of two RNA helicases, retinoic acid–inducible gene I (RIG-I) and melanoma differentiation–associated gene 5 (MDA-5) (*3*). Although these two pathways differ with respect to their initiating stimuli and downstream effectors, they converge at the point of transcriptional activation, resulting in the rapid production of type I interferons (IFN-α and IFN-β) and other cytokines that promote the subsequent development of adaptive antiviral immunity (*4*, *5*).

Mitochondria, in addition to serving as the powerhouses of eukaryotic cells, are well characterized as crucial players in numerous cellular processes, including apoptosis (*6*), aging (*7*), and calcium homeostasis (*8*). Other studies, however, have revealed that mitochondria also play a fundamental role in antiviral immunity in mammals (*9*–*13*). Mitochondrial antiviral immunity depends on both the upstream activation of the RIG-I or MDA-5 pathway and the participation of mitochondrial antiviral signaling protein [MAVS (*9*), also known as IPS-1 (*14*), VISA (*15*), and Cardif (*16*)], a mitochondrial outer membrane protein that is a member of the caspase activation and recruitment domain (CARD) family. Cellular deficiency in MAVS abrogates the production of type I IFNs and prevents the activation of the transcription factors interferon regulatory factor 3 (IRF-3) and nuclear factor κB (NF-κB) after viral infection (*17*, *18*), thus underscoring the importance of the linkage between antiviral immunity and mitochondria. Although several cytoplasmic proteins have been functionally linked to the MAVS-dependent antiviral signaling pathway (*3*), the importance of other mitochondrial integral membrane proteins that potentially cooperate with MAVS has remained unclear. Here, we describe our findings that mitofusin 2 (Mfn2), a mediator of mitochondrial fusion, negatively regulates antiviral signaling through MAVS.

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RESULTS

MAVS assembles into a high molecular mass complex on mitochondria

We reasoned that additional mitochondrial membrane proteins could functionally and physically interact with MAVS to regulate mitochondrial antiviral immunity. To test this hypothesis, we examined the molecular mass of endogenous MAVS by size exclusion chromatography. Despite having a predicted molecular mass of 56 kD, endogenous MAVS extracted from the mitochondrial fraction of human embryonic kidney (HEK) 293 cells eluted in a high molecular mass fraction that corresponded to ~600 kD at physiological pH (pH 7.2) (Fig. 1A). A similar result was obtained with N-terminal Myc-tagged MAVS that was stably expressed in HEK 293 cells (Fig. 1A). To verify that the observed molecular mass of the MAVS complex was not due to nonspecific aggregation under these experimental conditions, we analyzed Fis1, a C-terminal tail–anchored mitochondrial outer membrane protein that similarly forms higher-order complexes. Fis1 derived from mitochondrial extracts of HEK 293 cells eluted at a position corresponding to less than 230 kD (Fig. 1A), consistent with previous studies (*19*). These findings indicated that MAVS ordinarily forms a stable higher-order complex on the outer mitochondrial membrane, and they raised the possibility that unidentified mitochondrial components of this complex could be relevant to the mitochondrial antiviral response.