Virus-specific capping of tobacco mosaic virus RNA: methylation of GTP prior to formation of covalent complex p126-m^{7}GMP

Andres Merits, Reetta Kettunen, Kristiina Mäkinen, Anja Lampio, Petri Auvinen, Leevi Kääriäinen*, Tero Ahola[1]

Institute of Biotechnology, Biocenter Viikki, P.O. Box 56 (Viikinkaari 9), FIN-00014 University of Helsinki, Helsinki, Finland

Received 1 June 1999

Abstract In capping cellular mRNAs, a covalent GMP-enzyme intermediate leads to formation of G(5′)ppp(5′)N at the 5′ end of the RNA, which is modified by methylation catalyzed by guanine-7-methyltransferase. Here we show that isolated membranes from tobacco mosaic virus (TMV)-infected plant or insect cells expressing TMV replicase protein p126, synthesized m^{7}GTP using *S*-adenosylmethionine (AdoMet) as the methyl donor, and catalyzed the formation of a covalent guanylate-p126 complex in the presence of AdoMet. The methyl group from AdoMet was incorporated into p126, suggesting that the complex consisted of m^{7}GMP-p126. Thus, TMV and alphaviruses, despite their evolutionary distance, share the same virus-specific capping mechanism.

Key words: Plant virus; RNA replication; Guanylyltransferase; Methyltransferase; Tobacco mosaic virus

1. Introduction

The large alphavirus-like superfamily of positive-strand RNA viruses consists of animal viruses (alphaviruses, rubella virus and hepatitis E virus), insect viruses (Tetraviridae) and numerous plant virus groups (Bromoviridae, tobamoviruses, tobraviruses, potexviruses and tymoviruses) [1]. The genomes of these viruses consist of one or more single-stranded RNA molecules of positive polarity, which all have a m^{7}GpppN cap structure at their 5′ end [2]. Sequencing of virus genomes derived from divergent taxonomic groups within the superfamily has revealed homologous domains in replicase proteins, such as the polymerase-, helicase- and methyltransferase-like domains [1]. The roles of these domains have been studied mainly in alphaviruses [3]. Recently, the helicase-like domain of Semliki forest virus (SFV) NSP2 has been shown to possess NTPase [4] and RNA helicase activities [5].

The methyltransferase domain, which resides in the amino-terminal part (NSP1) of alphavirus polyprotein, has been shown to be a guanine-7-methyltransferase [6,7]. However, the substrate specificity and subcellular distribution of NSP1 methyltransferase are different from those of the corresponding host cell-encoded enzyme. The alphavirus methyltransferase is membrane-bound [7–9] and it methylates GTP, dGTP and GpppG, but not capped RNAs, whereas the host enzyme is soluble and readily methylates capped RNAs, but not GTP or dGTP [7,10]. NSP1 of SFV and Sindbis virus is also a unique guanylyltransferase, which makes a covalent complex with m^{7}GMP [11,12] unlike the host GTP:mRNA guanylyltransferase (EC 2.7.7.50), which forms a complex only with unmethylated GMP. The virus-specific methyltransferase and guanylyltransferase reactions are potential targets for antiviral substances [13].

Tobacco mosaic virus (TMV) is a prototype member of the tobamovirus group of plant viruses and its genomic structure and molecular biology have been intensively studied [14]. Like alphaviruses, TMV replicates in the cell cytoplasm where the viral RNA polymerase complex is membrane-bound [15]. The replicase of TMV consists of two virus-specific proteins, p126 (1145 aa) and p183 (1665 aa), which have identical N-terminal sequences. The latter represents a read-through product of an amber termination codon and consists of methyltransferase-, helicase- and polymerase-like domains, whereas p126 lacks the polymerase-like domain [16]. The methyltransferase-like domain is in the amino-terminus of p126/p183 [17]. The membrane fraction derived from TMV-infected tobacco cells has been shown to catalyze covalent complex formation between p126 and α-^{32}P-labeled guanylate [18].

Since alphavirus NSP1 and the N-terminal part of TMV p126 have some common features and conserved amino acid residues [17], we wanted to study whether p126 has similar virus-specific methyltransferase and guanylyltransferase activities as SFV NSP1. To this end, we used extracts from TMV-infected plant cells and from insect cells expressing recombinant TMV p126.

2. Materials and methods

2.1. DNA constructions and preparation of immune serum against TMV p126

A fragment of the TMV genome, corresponding to the N-terminal part of 126 kDa protein (amino acids 1–461, hereafter p51) was created by PCR using oligonucleotides 5′-CCATGGGCATACACACAGACAGCTACC-3′ and 5′-GTCGACTTACAAGCTTAGTATGCAGGTAAAAC-3′ as primers (cloning adapters *Nco*I and *Sal*I underlined). The infectious cDNA (icDNA) clone TMV304 of TMV U1, common strain (a kind gift by Dr. Kirsi Lehto, University of Turku), was used as a template. The synthesized fragment was cloned into pGEM-T vector (Promega), verified by sequencing, and transferred to *Nco*I/*Sal*I-digested expression vector pBAT4 [19]. Recombinant baculovirus expressing p126 was constructed by amplification of a fragment of the TMV genome encoding p126 using *Pfu* DNA polymerase and clone TMV304 as template and oligonucleotides: 5′-GCGGTCGACATGGCATACACACAGACAGCTACC-3′ and 5′-GCGCTGCAGCTATTGTGTTCCTGCATCGACCTTA-3′ as primers (cloning adapters *Sal*I and *Pst*I are underlined). This fragment was cloned into *Sal*I/*Pst*I-linearized vector pFastBac1 (Gibco-BRL). To mutate His-81 to alanine, a *Xma*I-*Afl*II fragment of TMV icDNA

*Corresponding author. Fax: (358) (9) 708 59560.
E-mail: leevi.kaariainen@helsinki.fi

[1] Present address: Institute for Molecular Virology, University of Wisconsin, 1525 Linden Drive, Madison, WI 53706, USA.

PII: S0014-5793(99)00856-X

was cloned to plasmid pSL1190 (Pharmacia). The unique site elimination method (U.S.E. Mutagenesis kit, Pharmacia Biotech) was used and the result was verified by sequencing. The mutated DNA fragment was cloned back to the TMV icDNA to create mutant TMV-A. Capped RNA transcripts from TMV icDNA and TMV-A were transcribed in vitro using SP6 RNA polymerase (Promega) in the presence of m^7GpppG (Pharmacia). The transcripts of TMV U1 and TMV-A were translated in rabbit reticulocyte system and the translational products were analyzed by SDS-PAGE in 10% gels followed by autoradiography.

Protein expression was carried out in *Escherichia coli* strain BL21(DE3) (Novagen). Cells were lysed by two cycles on a French press [7], and inclusion bodies, containing the recombinant protein, were purified using extensive washes and sucrose density gradient centrifugation. For immunization of rabbits, the inclusion bodies containing p51 were solubilized in 8 M urea, 50 mM Na-phosphate (pH 8.0) and mixed 1:1 with Freund's complete adjuvant. The specificity of the antiserum was verified by Western blotting (Amersham ECL-kit) using proteins from TMV-infected and uninfected plant extracts separated by SDS-PAGE.

To create recombinant baculovirus TMV-Bac expressing p126, the Bac-to-Bac system (Gibco-BRL) was used. Production of recombinant p126 was verified by anti-p51 antibody (Fig. 1A). The virus stock was amplified and titrated in *Spodoptera frugiperda* Sf9 cells.

2.2. Isolation of TMV replicase complex from plants and recombinant TMV p126 from insect cells

Young plants of *Nicotiana tabacum* cv. Samsun were inoculated with capped transcripts from icDNA of the TMV U1 (common) strain, and the infection was allowed to become systemic. The uppermost leaves showing visible symptoms were collected and used for isolation of P30 membrane fraction. TMV replicase-enriched membranes were obtained by sucrose gradient (20–60% w/w) centrifugation [15]. About 40×10^6 insect cells (High Five, BTI-TN-FB1-4, Tn5) (Invitrogen) were infected with TMV-Bac by 5 PFU/cell, and incubated at 27°C in serum-free High Five (Invitrogen) medium. At 44 h post infection cells were collected and postmitochondrial pellet (P15) and supernatant (S15) fractions were prepared as described [7].

2.3. Enzyme assays

Formation of TMV 126 kDa-[α-^{32}P]guanylate complex was assayed as follows. TMV replicase or mock preparations (6 μl) were incubated with 10 μCi of [α-^{32}P]GTP in 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 5 mM DTT in the presence or absence of 100 μM *S*-adenosylmethionine (AdoMet) in a total volume of 30 μl. To reveal incorporation of the methyl group from ^{3}H-labeled AdoMet into p126, the reaction was modified as follows: 100 μl of TMV replicase preparations were incubated with 45 μCi of [^{3}H]AdoMet in the presence of 30 μM GTP in a total volume of 700 μl. In both assays the reaction was stopped by boiling in the presence of 1% SDS. The reaction mixtures were subjected to immunoprecipitation with anti-p51 antiserum after dilution with NET buffer [19] followed by analysis by SDS-PAGE in 10% gel. For detection of tritium-labeled p126, the gel slice representing the position of p126 was dissolved in OptiSolv (Wallac), and the radioactivity was determined by liquid scintillation counting. Methyltransferase activity was assayed as described using GTP as a substrate [7].

2.4. Infection experiments in plants

To analyze the infectivity of RNA transcripts, originating from the mutated and non-mutated cDNA clones, systemic (nn genotype) and hypersensitive (NN genotype) *N. tabacum* test plants were used. Leaves were infected with 5 μg of RNA transcripts by the carborund-dust method. Formation of necrosis in the hypersensitive host was monitored 4 days after infection. Systemic infection was revealed by symptom development in systemic leaves of susceptible plants 12 days after infection and confirmed by a Western blot analysis, using an anti-TMV coat protein antibody (Biorega).

3. Results and discussion

3.1. Identification of p126 in plant and insect cells

Membranes from TMV-infected tobacco leaves were isolated from the $30\,000\times g$ cytoplasmic pellet fraction (P30) by sucrose gradient centrifugation according to Osman and Buck [15]. Anti-p51 antiserum recognized a protein of about 130 kDa and some smaller proteins (Fig. 1A, lane 1), which were not detected in the P30 fraction of control cells (Fig. 1A, lane 2). A protein band with the same mobility was also detected in sucrose gradient fractions 3 and 4 (Fig. 1A, lanes 5 and 6). To verify the the identity of the 130 kDa protein as TMV-specific non-structural protein p126, we constructed a recombinant baculovirus, which encodes p126 protein of TMV under the polyhedrin promoter. The recombinant p126 was expressed in Tn5 insect cells. Western blotting with anti-p51 antiserum showed the presence of a 130 kDa protein and a smaller, 40 kDa protein enriched in the P15 fraction (Fig. 1A, lane 10). These proteins were not seen in insect cells infected with the wild type baculovirus (not shown). Thus, the larger 130 kDa protein present in both plant and insect cells was indeed the TMV-specific replicase protein p126. The smaller proteins were presumably degradation products of p126, since their sizes and amounts varied in different cells and in different experiments.

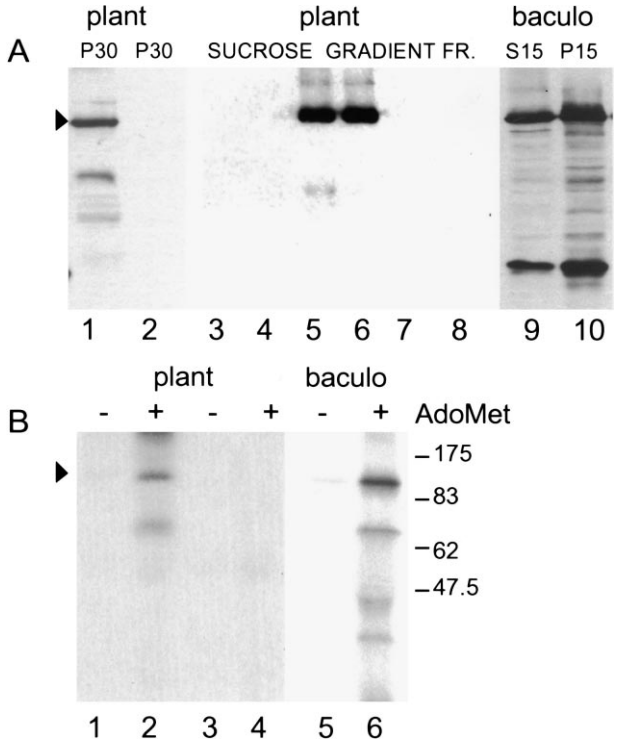

Fig. 1. Expression and guanylate complex formation of TMV-specific p126 protein in subcellular fractions of TMV-infected tobacco and in TMV-Bac-infected insect cells. A: Western blotting after SDS-PAGE analysis of proteins from the following: P30 from TMV-U1-infected plant (lane 1), control plant (lane 2), sucrose gradient (20–60%, w/w) fractions 1–6 from bottom (lanes 3–8) and from TMV-Bac-infected insect cell fractions (lanes 9 and 10). B: Autoradiogram of covalent complex of [^{32}P]guanylate-p126 in the absence and presence of AdoMet as indicated. Pooled membrane fractions (A, lanes 5 and 6) from TMV-infected tobacco plants (lanes 1 and 2), from control tobacco plants (lanes 3 and 4) and P15 from TMV-Bac-infected insect cells (lanes 5 and 6).

3.2. TMV p126 as guanylyltransferase and methyltransferase

The membrane fractions from TMV-infected plant cells and TMV p126-expressing insect cells were exposed to α-^{32}P-la-

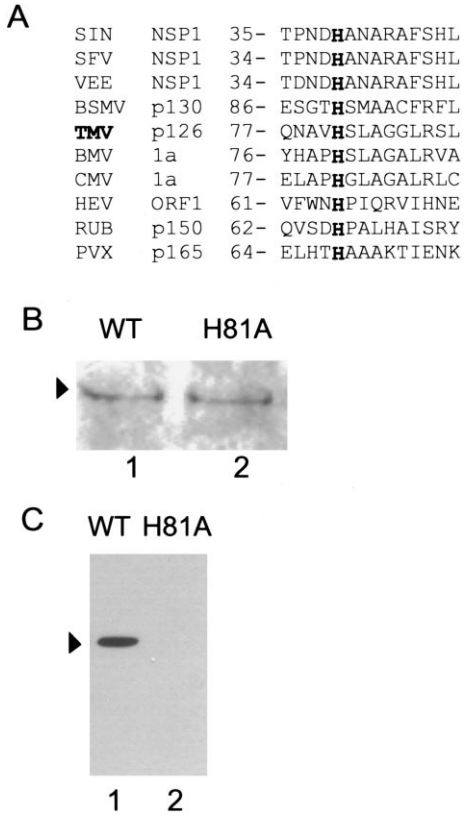

Fig. 2. Effects of TMV His-81 to alanine point mutation. A: Amino acid sequence comparison according to Rozanov et al. [17] highlighting a conserved His residue within the alphavirus-like superfamily. The abbreviations are as follows: SIN, Sindbis virus; SFV, Semliki forest virus; VEE, Venezuelan equine encephalitis virus; BSMV, barley stripe mosaic virus; TMV, tobacco mosaic virus; BMV, brome mosaic virus; CMV, cucumber mosaic virus; HEV, hepatitis E virus; RUB, rubella virus; PVX, potato virus X. B: SDS-PAGE analysis of in vitro translation products of iRNAs from TMV-U1 (WT, lane 1) and TMV-A (mutant H81A, lane 2). Arrowhead indicates p126. C: Western blotting by anti-coat protein antiserum of proteins isolated from systemic leaves of *N. tabacum* (nn) 12 days after inoculation with iRNAs of TMV-U1 (WT, lane 1) and TMV-A (lane 2). Arrowhead indicates the TMV coat protein.

beled GTP in the presence and absence of 100 μM AdoMet to see whether p126 can form a covalent guanylate complex under similar conditions as alphavirus NSP1 [11,12]. The pooled fractions 3 and 4 shown in Fig. 1A (lanes 5 and 6), generated a labeled band migrating at the position of p126 (Fig. 1B, lane 2) when the reaction mixture contained AdoMet, whereas in its absence no label was detectable (Fig. 1B, lane 1). No label was detected in mock-infected plants either (Fig. 1B, lanes 3 and 4). Similar results were obtained with TMV-Bac-infected insect cell membranes (Fig. 1B, lanes 5 and 6). These results suggest that a covalent complex had been formed between p126 and m^7GMP, as has been reported for SFV NSP1 [11].

To further verify that the p126-guanylate complex was methylated, we used AdoMet, which was labeled with tritium at the methyl group. An isolated membrane fraction, corresponding to that shown in Fig. 1A, lanes 5 and 6, was used as enzyme source. The reaction product was immunoprecipitated and separated by SDS-PAGE. Gel fractions were solubilized and their radioactivity determined by liquid scintillation. Similarly treated membranes from uninfected tobacco plants served as controls. A fraction from TMV-infected plant cells migrating at the position of p126 contained 11 000 cpm of ^{3}H, whereas the corresponding fraction from control plant had background activity (500 cpm). Thus, we conclude that the p126-guanylate complex had received a methyl group from AdoMet during the covalent complex formation, and consisted of p126-m^7GMP. Previously, Dunigan and Zaitlin [18] reported that p126 can form a covalent guanylate complex in the absence of added AdoMet. However, it is likely that the concentrated cell lysates used by these authors provided the necessary AdoMet for the reaction.

The formation of the methylated guanylate complex in the presence of AdoMet would imply that p126 has also guanine-7-methyltransferase activity, which is able to methylate GTP to yield m^7GTP. Therefore, membrane preparations and the respective supernatants from TMV-infected tobacco plant (P30) and TMV-Bac-infected Tn5 cells (P15) were tested for methyltransferase activity [7]. Respective fractions from uninfected tobacco and wild type baculovirus-infected insect cells served as controls. In both cases methyltransferase activity was associated with the membrane fraction and was approximately 1.5- and 2-fold higher than in mock-infected plants or in wild-type baculovirus-infected insect cell extracts, respectively. These results were obtained reproducibly. Taken together, these results suggest that capping reactions catalyzed by TMV p126 take place in the following order: p126 catalyzes first the methylation of GTP followed by formation of p126-m^7GMP complex, exactly as with alphaviruses [11].

Mutation of a conserved His-38 to alanine in SFV NSP1 resulted in specific inhibition of the guanylyltransferase reaction, while the methyltransferase activity persisted [12]. This mutation is also lethal for virus replication [20]. Comparative analysis of replicase proteins within the alphavirus-like superfamily has suggested that His-38 of SFV has a conserved counterpart in His-81 of TMV p126 [1,17] (Fig. 2A). To see if His-81 is vital for TMV, a mutant (TMV-A) was constructed, in which this amino acid was changed into alanine. The icDNAs from wild type (TMV-U1) and TMV-A mutant were transcribed and capped to yield infectious RNAs. Both RNAs were translated in a reticulocyte cell-free system to verify that their major translational product is p126 [14]. As expected, both RNAs directed the synthesis of a protein, which migrated like p126 (Fig. 2B). However, when these RNAs were inoculated into tobacco plants, only the wild type was able to elicit TMV replication. This was verified by two biological assays: (i) iRNAs were inoculated to necrotic

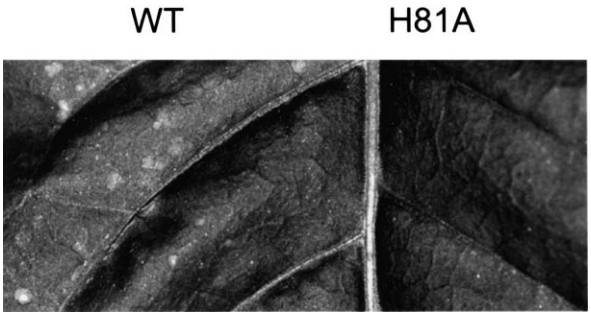

Fig. 3. Photographs of leaves from *N. tabacum* cv. Samsun (NN) 4 days after inoculation with iRNAs of TMV-U1 (WT) and TMV-A (H81A).

tobacco plants carrying the N resistance gene. These plants show hypersensitive response (HR) when challenged with TMV-U1. In the case of TMV-A, no HR was observed, whereas infection with TMV-U1 iRNA resulted in the formation of clear HR lesions (Fig. 3). (ii) To eliminate the possibility that the ability to elicit HR response rather than the ability to replicate was altered in TMV-A, we also infected susceptible plants (nn genotype) with TMV-U1 and TMV-A RNAs. Only TMV-U1 was able to infect plants systemically, as shown by symptoms appearing in the systemic leaves (data not shown), and by detection of virus coat proteins in these leaves (Fig. 2C). These in planta experiments give circumstantial evidence that capping reactions catalyzed by TMV p126 are essential for viral replication, and support the putative role of the conserved His-81 in the capping of viral RNAs. In fact, the phylogenetic relationship within the alphavirus supergroup has been questioned by Paolo et al. [21]. However, our present experiments with TMV indicate that the advanced computational methods used by Koonin and collaborators [1,17] have been able to reveal subtle but important relationships between viruses infecting plants and animals.

In this communication, we have shown that despite the large evolutionary distance of TMV and alphaviruses, members in the large alphavirus-like superfamily, two essential reactions, guanine-7-methyltransferase and guanylyltransferase, in the capping of viral mRNAs, are strikingly similar. Our findings may also help explain why replication of a number of alphavirus-like plant viruses is suppressed in AdoMet-deficient transgenic plants, which express *S*-adenosylhomocysteine hydrolase in antisense orientation [22].

Acknowledgements: We thank Airi Sinkko and Tarja Välimäki for technical assistance, and Dr. Kirsi Lehto for infectious cDNA TMV304 clone. This work was supported by the Technology Development Centre (TEKES) and the Academy of Finland (Grant 8397). L.K. is a Biocentrum Helsinki fellow.

References

[1] Koonin, E.V. and Dolja, V.V. (1993) Crit. Rev. Biochem. Mol. Biol. 28, 375–430.
[2] Buck, K.W. (1996) Adv. Virus Res. 47, 159–251.
[3] Strauss, J.H. and Strauss, E.G. (1994) Microbiol. Rev. 58, 491–562.
[4] Rikkonen, M., Peränen, J. and Kääriäinen, L. (1994) J. Virol. 68, 5804–5810.
[5] Gomez de Cedrón, M., Ehsani, N., Mikkola, M.L., García, J.A. and Kääriäinen, L. (1999) FEBS Lett. 448, 19–22.
[6] Mi, S. and Stollar, V. (1991) Virology 184, 423–427.
[7] Laakkonen, P., Hyvönen, M., Peränen, J. and Kääriäinen, L. (1994) J. Virol. 68, 7418–7425.
[8] Laakkonen, P., Ahola, T. and Kääriäinen, L. (1996) J. Biol. Chem. 271, 28567–28571.
[9] Ahola, T., Lampio, A., Auvinen, P. and Kääriäinen, L. (1999) EMBO J. 18 (in press).
[10] Cross, R.K. and Gomatos, P.J. (1981) Virology 114, 542–554.
[11] Ahola, T. and Kääriäinen, L. (1995) Proc. Natl. Acad. Sci. USA 92, 507–511.
[12] Ahola, T., Laakkonen, P., Vihinen, H. and Kääriäinen, L. (1997) J. Virol. 71, 392–397.
[13] Lampio, A., Ahola, T., Darzynkiewicz, E., Stepinski, J., Jankowska-Anyszka, M. and Kääriäinen, L. (1999) Antiviral Res. 42, 35–46.
[14] Dawson, W.O. (1992) Virology 186, 359–367.
[15] Osman, T.A.M. and Buck, K.W. (1996) J. Virol. 70, 6227–6234.
[16] Goelet, P., Lomonossoff, G.P., Butler, P.J., Akam, M.E., Gait, M.J. and Karn, J. (1982) Proc. Natl. Acad. Sci. USA 79, 5818–5822.
[17] Rozanov, M.N., Koonin, E.V. and Gorbalenya, A.E. (1992) J. Gen. Virol. 73, 2129–2134.
[18] Dunigan, D.D. and Zaitlin, M. (1990) J. Biol. Chem. 265, 7779–7786.
[19] Peränen, J., Rikkonen, M., Hyvönen, M. and Kääriäinen, L. (1996) Anal. Biochem. 236, 371–373.
[20] Wang, H-L., O'Rear, J. and Stollar, V. (1996) Virology 217, 527–531.
[21] Paolo, M., Zanotto, A., Gibbss, M.J., Gould, E.A. and Holmes, E.C. (1996) J. Virol. 70, 6083–6096.
[22] Masuta, C., Tanaka, H., Uehara, K., Kuwata, S., Koiwai, A. and Noma, M. (1995) Proc. Natl. Acad. Sci. USA 92, 6117–6121.