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Original article

Association of functional influenza viral proteins and RNAs with nuclear chromatin and sub-chromatin structure

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Abstract

Transcription and replication of the influenza virus genome occur in the nucleus. However, the intra-nuclear localization of viral RNP complexes and the function of nuclear domains involved in viral transcription and replication, if any, are not well known. In the present study, we determined the intra-nuclear localization of viral proteins and viral RNAs and the in vitro RNA synthesis activity of viral RNP complexes associated with distinct nuclear fractions prepared from infected nuclei. A majority of viral RNA polymerases and M1 were recovered in DNase-sensitive fractions, whereas some portion of RNA polymerases and approximately 25% of NP were tightly associated with so-called nuclear matrix fractions. The amount of vRNA associated with the nuclear matrix was significantly more than that of cRNA. The in vitro viral RNA synthesis activity was detected in DNase-insensitive fractions, including the nuclear matrix. In contrast, newly synthesized viral RNAs were recovered in the DNase-sensitive fraction. These observations suggest that vRNP complexes are, at least partially, associated with densely packed chromatin, where viral transcription and replication occur, and the newly synthesized vRNP complexes to be transported into the cytoplasm are released into the nucleoplasm.

Keywords: Nuclear domain; Nuclear matrix; Viral RNA synthesis; In vitro system; Host factor

1. Introduction

The influenza A virus genome is composed of eight single-stranded negative-sense RNA segments (vRNA). This genome is complexed with a viral RNA polymerase consisting of three subunits, namely, PB2, PB1, and PA, and nucleoprotein (NP), thus forming RNP complexes (vRNP). Infection by influenza virus starts with the binding of hemagglutinin (HA) on the virions to sialic acids on the surface of the host cell membrane. The virion is then incorporated into the host cell cytoplasm by endocytosis. The vRNP is released into the cytoplasm through HA-mediated membrane fusion between virion envelope and endosome membrane. The vRNP binds to importin α through nuclear localization signals on NP and is transported into the nucleus by the importin α/β-mediated pathway [1].

The replication of the viral genome and the transcription of viral mRNAs occur in the nucleus. The replication of vRNA is a primer-independent and two-step reaction process. First, cRNA, the full-sized copy complementary to vRNA, is synthesized from vRNA. Second, the progeny vRNA is synthesized from cRNA as a template. The viral mRNA transcription is initiated with recognition by PB2 of the capped structure of nuclear pre-mRNAs. The capped RNA bound to PB2 is cleaved by PB1 at 10–15 bases downstream from the 5′ end of the capped RNA, and the RNA fragment serves as a primer for viral mRNA synthesis [2–4]. It has long been known that not only

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viral factors but also host factors are involved in these processes. Previously, we developed an in vitro viral RNA synthesis system [5,6]. By dissecting and reconstituting the system, we identified two host proteins, RAF-1 and RAF-2, which stimulate the viral RNA synthesis [7,8]. RAF-1 is identical to Hsp90. RAF-1/Hsp90 interacts with PB2 and is suggested to act as an acidic chaperone to prevent aggregation and inactivation of the viral polymerase [8]. RAF-2 is composed of two subunits; one of the subunits, namely, RAF-2p48/UAP56/BAT1, interacts with a free form of NP and mediates formation of NP-RNA complexes [7].

Newly synthesized vRNAs form vRNP complexes in the nucleus and are exported from the nucleus to the cytoplasm via a CRM1-dependent pathway [9,10]. It is suggested that viral matrix protein 1 (M1) and nonstructural protein 2 (NS2; also referred to as nuclear export protein (NEP)) are involved in the viral vRNP export mechanism. Since NS2 has a Rev-like nuclear export signal and M1 binds to both vRNP and NS2, it is proposed that NS2 mediates the nuclear export of vRNP by its binding to vRNP via M1 [11–14]. M1 is found to block the re-import of vRNP to the nucleus [14,15].

Although extensive knowledge is available on the molecular mechanism of viral replication and transcription, the involvement and function of nuclear architectures in these processes remain to be clarified. The nuclear structure in the mammalian cell nucleus is compartmentalized based on chromosome territories and inter-chromatin compartments [16]. The inter-chromatin compartment is required for cellular genome functions, including replication and repair of the DNA genome, and transcription and splicing of cellular mRNAs. The biochemical fractionation of the nucleus by using DNases generates DNase-sensitive and DNase-insensitive fractions. The DNase-sensitive fraction is recovered as a soluble fraction after DNase treatment and is believed to be obtained from the inter-chromatin domain and loosely packed chromatin. In contrast, the DNase-insensitive fraction is a residual nuclear fraction and is composed of densely packed chromatin, including the so-called heterochromatin, and a variety of insoluble materials. The nuclear matrix can be experimentally separated by salt extraction from the DNase-insensitive nuclear fraction. The nuclear matrix is a highly complex fibrillar protein network made up of lamin polymers, core filaments, and their associated proteins and RNAs. Furthermore, a variety of nuclear regulatory proteins involved in replication, transcription, repair, and splicing have also been found to be associated with the nuclear matrix [17–20]. Thus, the nuclear matrix is considered to play an important role in the cellular events.

Recently, it has been reported that the influenza virus proteins interact with nucleosomes. Both vRNP and NP free of RNA are capable of binding to core histones in vitro; the histone tail portion is important for interaction between vRNP and nucleosomes [21]. M1 was also found to interact with core histones [21] and with nucleosomes even when the histone tail was removed by trypsin [22]. Based on these results, it is postulated that viral transcription and replication occur in nucleosomes and M1 may be involved in the release of vRNP from nucleosomes at the late phases of infection. However, the RNA synthesis activity of vRNP in nucleosomes and the release mechanism of newly synthesized vRNP from nucleosomes remain to be addressed.

In this paper, we reveal that NP and vRNA interact with DNase-insensitive fractions, including the nuclear matrix fraction, and the viral RNA synthesis activity, when examined in an in vitro assay system, is observed in DNase-insensitive fractions. Furthermore, we found that newly synthesized viral RNAs are recovered in DNase-soluble fractions. These results suggest that viral transcription and replication occur in DNase-insensitive fractions, including chromatin and/or the nuclear matrix and newly synthesized vRNP complexes are ready to be released into the nucleoplasm.

2. Materials and methods

2.1. Biological materials

Madin–Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) (Nissui, Tokyo, Japan) containing 10% fetal bovine serum. Influenza A/PR/8/34 (H1N1) virus was grown at 34 °C for 48 h in allantoic sacs of 11-day-old embryonated eggs, and the virus titer was determined by plaque assay. pcDNA-PB2 and pCAGGS-NP were kindly provided by Y. Kawaoka [23]. DNA fragments corresponding to a region between nucleotide positions 28 and 2307 of segment 1 RNA encoding PB2 (where the nucleotide position 1 is the 5′ terminus of cRNA) were generated by PCR amplification from pcDNA-PB2 using specific primers, as reported previously [24]. The amplified DNA was ligated with pCAGGS-P7 vector [25,26] that had been digested by *Eco*RV between T7 and T3 promoters. Segment 1 RNA of positive polarity was synthesized by T7 RNA polymerase (Promega, Madison, WI, USA) with the plasmid digested using *Sac*I as a template, while segment 1 RNA of negative polarity was synthesized by T3 RNA polymerase (Ambion, Austin, TX, USA) with the plasmid digested using *Kpn*I as a template. pCAGGS-NP was used for expression of NP. MDCK cells in a dish (diameter 35 mm) were transfected with pCAGGS-NP using TransIT LT-1 (Mirus, Madison, WI, USA). TransIT LT-1 (4 μl) was incubated with pCAGGS-NP (2 μg) in 100 μl of OPTI-MEM (Sigma) at room temperature for 15 min and then added dropwise to the cells.

Rabbit polyclonal antibodies against NP and M1 were generated by immunization of 2-month-old female rabbits with 250 μg of purified hexahistidine-tagged M1 (His-M1) and NP (His-NP), and hexahistidine-tagged 119-amino acid C-terminal fragment of DEK (His-DEK-C) in Freund's complete adjuvant; the generation of antibodies was boosted three times by immunization with 150 μg of each protein at 2-week intervals. The constructions of pET14b-M1 and pET14b-NP for expression of His-M1 and His-NP were described previously [7,27]. To construct a plasmid for expression of His-DEK-C, the full-length human DEK cDNA was synthesized by reverse transcriptase (Toyobo, Tokyo, Japan) with 5′-TTGAATTCTCAA GAAATTAGCTCTTTTACAGTTG-3′ as primer (RT primer). Double-stranded cDNA was amplified by the PCR method using primers, 5′-GGAATTCATATGTCCGCCTCGGC-3′ and