YK Cheung 張英傑
SCS Cheng 鄭澤森
Y Ke 柯　岩
Y Xie 謝　雍

Human immunogenic T cell epitopes in nucleoprotein of human influenza A (H5N1) virus

Key Messages

1. Two novel HLA-A2.1 specific H5N1 nucleoprotein epitopes (NP373-381 AMDSNTLEL and NP458-466 FQGRGVFEL) capable of activating cytotoxic T-cells in vitro were identified.
2. When the H5N1 nucleoprotein epitopes (NP373-381 AMDSNTLEL and NP458-466 FQGRGVFEL) were used with the single chain trimer system, they elicited effective cytotoxic T-cell responses against the corresponding nucleoprotein peptide-loaded cells in an HHD transgenic mouse model.

Introduction

An outbreak of a highly pathogenic avian virus, H5N1, occurred in Hong Kong and other Asian countries in 1997, 2003, and 2004 and claimed more than 20 human lives and caused huge economic losses in the poultry industries worldwide.[1] Developing a strategy to combat the H5N1 virus is imperative. One possible solution is to develop vaccines for humans against this virus.

In the influenza A virus, the nucleoprotein (NP) is a comparatively conserved protein, and only a few amino acid differences have been observed from most bird virus strains over the past 90 years. Because of its high conservancy during its evolution, NP is a target for T-cell immunity.[2] One promising approach is to develop direct DNA vaccination to stimulate T-cell immunity against NP.

The single chain trimer (SCT) system has been used to construct DNA vaccines.[3] Two novel HLA-A2 restricted epitopes, H5N1 NP373-381 AMDSNTLEL and NP458-466 FQGRGVFEL, were identified through bioinformatics analysis. The NP peptides NP373 and NP458 showed a high binding affinity towards human MHC class-I in T2-cells, and were capable of activating cytotoxic T-cells in human peripheral blood mononuclear cells. The potential for using the NP373 and NP458 peptide sequences as major DNA vaccine components supplemented with a SCT was investigated in a HHD transgenic mouse model. Results from cytotoxicity and ELISPOT assays indicated that the T-cells obtained from the vaccinated mice secreted a significant amount of IFN-γ in response to NP373 and NP458 and were capable of eliminating the corresponding peptide-loaded T2 cells. The finding of the novel potential immunogenic NP peptides provides valuable information for avian flu vaccine design and construction.

Methods

This study was conducted from 1 August 2006 to 31 October 2008. H5N1 NP peptides from the strain *H5N1-Thailand-human-2004* were predicted by the HLA peptide binding prediction program (SYFPEITHI). Nine 9-mer potential peptides were synthesised by solid-phase strategies. They were NP48 KLSDYEGRL, NP55 RLIQNSITI, NP158 GMDPRMCSL, NP189 MVMELIRMI, NP256 LIFLARSAL, NP275 CLPACVYGL, NP357 QLSTRGVQI, NP373 AMDSNTLEL, and NP458 FQGRGVFEL. LALLLLDRL was used as a positive control.

The selected N-protein peptides were tested by in vitro stimulation of human CD8+ T-cells. Purified CD8+ T-cells were primed with autologous dendritic cells loaded with NP. Autologous dendritic cells were prepared from monocytes cultured in AIM-V medium (Gibco) supplemented with human AB serum and cytokines. Purified NP protein was added on day 5 and maturation of dendritic cells was facilitated by addition of maturation cytokines. NP-loaded mature dendritic cells were co-cultured with the CD8+ T-cells in the presence of cytokines for 7 days. CD8+ T-cells were stimulated three times with protein-loaded dendritic cells. Activation of T-cells was investigated by intracellular cytokine staining and

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Department of Biology, The Hong Kong University of Science and Technology
YK Cheung, SCS Cheng, Y Ke, Y Xie

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Principal applicant and corresponding author:
Prof Yong Xie
Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China
Tel: (852) 2358 7340
Fax: (852) 2358 1559
Email: boyxie@ust.hk

detected by flow cytometrical analysis.

Plasmids including NP158HHDpBudCE4.1, NP189HHDpBudCE4.1, NP373HHDpBudCE4.1, and NP458HHDpBudCE4.1 were generated by PCR. The SCT gene—HHD—was constructed by connecting the leader sequence, human β_2- microglobulin, human HLA-A2.1 α-1, α-2 domains and mouse H-2D^b α-3 domain and cloned into pBudCE4.1. Plasmids encoding different peptide sequences were constructed by adding the corresponding DNA fragments after the leader sequence of the HHD by PCR. The N220HHDpBudCE4.1 expressing LALLLLDRL peptide (N220) was used as a control plasmid.[3]

To investigate the cell-mediated immune response triggered by the SCT-DNA vaccine, mice were sacrificed one week after the last vaccination. Splenocytes were cultured with the addition of corresponding target peptides and cytokines for 5 days. Splenocytes were seeded with TDA-labelled, peptide-loaded T2 cells. After incubation for 1 hour at 37ºC, the culture medium was collected and the fluorescent signals from the target cells were detected by Delfia EuTDA cytotoxicity reagents (Perkin Elmer) according to the procedures stated by the manufacturer. An ELISPOT assay was performed by seeding splenocytes in the presence of corresponding target peptides on an anti-mouse IFN-γ antibody coated 96-well nitrocellulose plate that was incubated for 24 hours. Subsequently, the plate was washed and incubated with a biotinylated anti-mouse IFN-γ antibody followed by streptavidin-AP solution. Spots developed after adding BCIP/NBT solution and the visible spots were counted by an ELISPOT reader.

Results and discussion

To find an immunogenic NP peptide sequence, nine potential peptides were predicted using the SYFPEITHI program. The potential for stimulating human CD8+ T-cells was also determined (Fig 1). Results from intracellular cytokine staining showed that the T-cells stimulated with NP189 (MVMELIRMI), NP373 (AMDSNTLEL), and NP458 (FQGRGVFEL) produced the highest number of CD8+ IFN-γ secreting T-cells, whereas the NP158 (GMDPRMCSL) produced the lowest number of CD8+ IFN-γ secreting T-cells. The N160 (LQLPQGTTL) peptide-loaded T2 cells were used as a negative control and did not show any significant changes.[3] As the natural processing and display of peptides on the protein contribute significantly to the level of immune responses,[4] the number of IFN-γ-secreting cells that reacted during intracellular cytokine staining was not high, compared to the number of the whole pool of CD8+ T-cells. During natural processing, the dendritic cells loaded with the whole NP based on its cross-presentation characteristic and presented all epitopes on the protein to the CD8+ T-cells.[5] For this reason, all the activated CD8+ T-cells should be responsive to the immunogenic epitopes on the NP, and the number of CD8+ T-cells responding to a single type of peptide becomes comparably low. In our experiment, our target cells were loaded with a single type of target peptide. This may explain the low percentage of the corresponding peptide-specific CD8+ T-cells.

An animal experiment was performed to test the immunogenicity of the target peptides in vivo after the selection of four target peptides, namely NP158 (GMDPRMCSL), NP189 (MVMELIRMI), NP373 (AMDSNTLEL), and NP458 (FQGRGVFEL) [Fig 2]. Results from the cytotoxicity assay showed that the mice immunised with plasmids encoding NP373 and NP458 produced the highest percentage of cytotoxic response, which was similar to the positive control, N220. During a 1-hour incubation period, NP373 had 14% and 10% killing effect with the effector cell-to-target cell ratios of 50:1 and 10:1, respectively, and the NP458 had 13% and 9% killing effect with the effector cell-to-target cell ratios of 50:1 and 10:1, respectively. However, the killing effect of NP189 was comparatively low (7%) with an effector cell-to-target cell ratio of 50:1, which contradicted the result from the T2 cell binding assay and intracellular cytokine staining of primed CD8+ T cells. The killing effect of NP158 was low as we expected, being 3.7% with a 50:1 ratio. An ELISPOT assay was used to determine the recognition of the splenocytes to their corresponding peptides by secreting IFN-γ after animal immunisation. The largest number of spots was obtained from NP373 and NP458, which were 317 and 286, respectively. In contrast, only 46 and 16 spots were observed with NP189 and NP158, respectively. The results were very close to the negative control (20 spots). The immunogenicity of NP189 in mice was significantly low, which was also not consistent with the results of the intracellular cytokine staining. Nonetheless, the results from NP373 and NP458 were more than 19 times higher than those from NP189 and NP158.

The cytotoxicity assay and ELISPOT assay showed that the NP373-381 AMDSNTLEL and NP458-466 FQGRGVFEL are the most immunogenic peptides (Fig 3). The reactivity of the two peptides was consistent with all the tests during the process of epitope identification. Although NP189 showed good responses after in vitro stimulation of human CD8+ T-cells, it induced weak animal immunisation responses. This indicates that the in vitro system may not truly reflect the situation in vivo. Nonetheless, in vitro experiments are essential for acquiring the basic information on newly found immunogenic peptides since the testing of the peptides generally cannot be performed in clinical trials without extensive investigation. Therefore, we should explore other possible approaches to verify the characteristics of potential peptides. The antigen processing, presentation and CD8+ T-cell responses in an artificial environment can be investigated via in vitro stimulation of human CD8+ T-cells. The HHD transgenic mouse model is an appropriate animal model for investigating the effects of the peptide-HLA complex and corresponding T cell responses in an in vivo system.

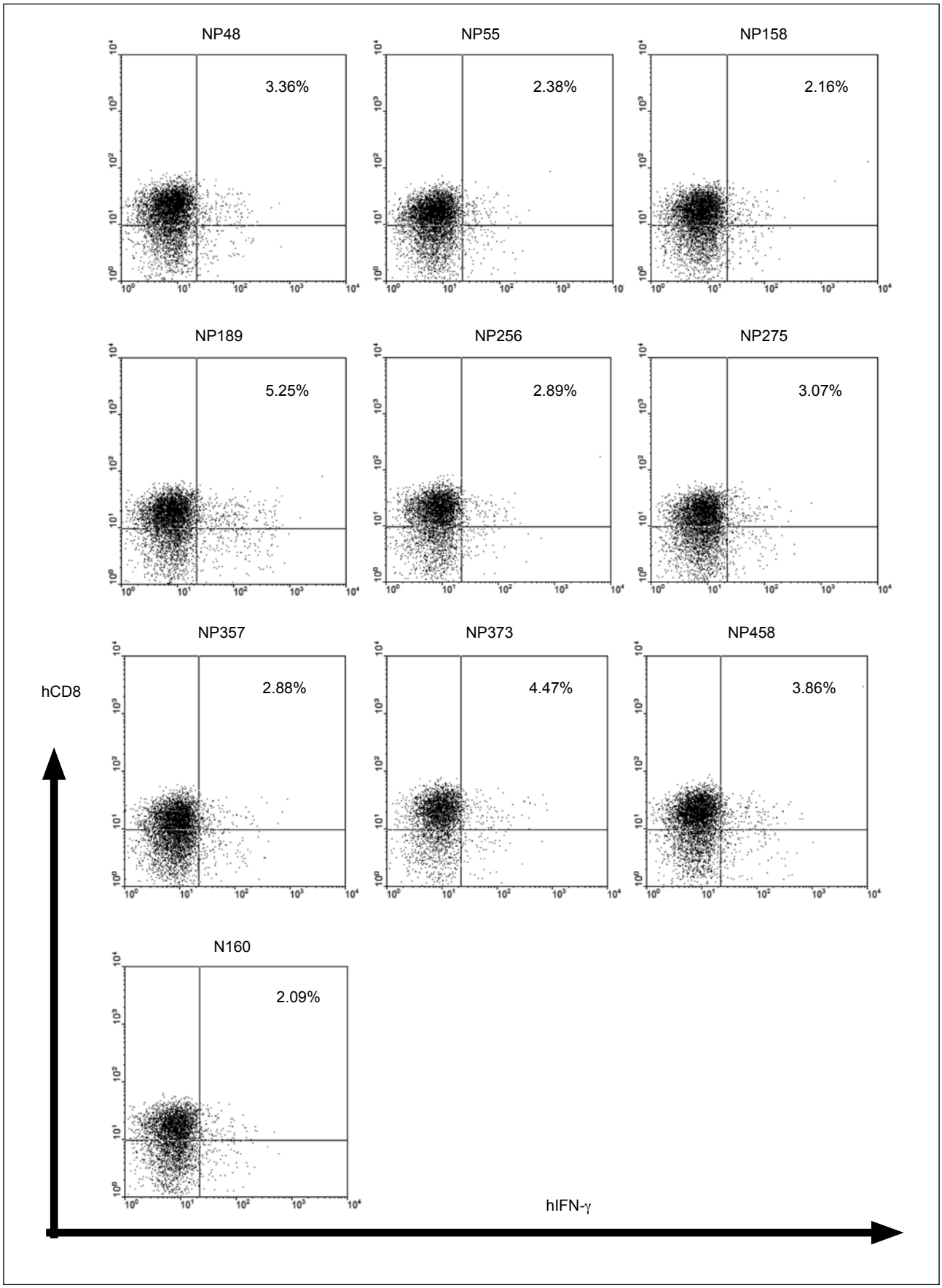

Fig 1. Intracellular cytokine staining owing to the secretion of IFN-γ from human CD8+ T-cells
Primed CD8+ T-cells are cultured with different target peptide-loaded T2 cells for 1 day and the number of IFN-γ secreting CE8+ T-cells is measured by PE-conjugated anti-CD8 and FITC-conjugated anti-IFN-γ antibodies. N160-loaded T2 cells serve as negative controls

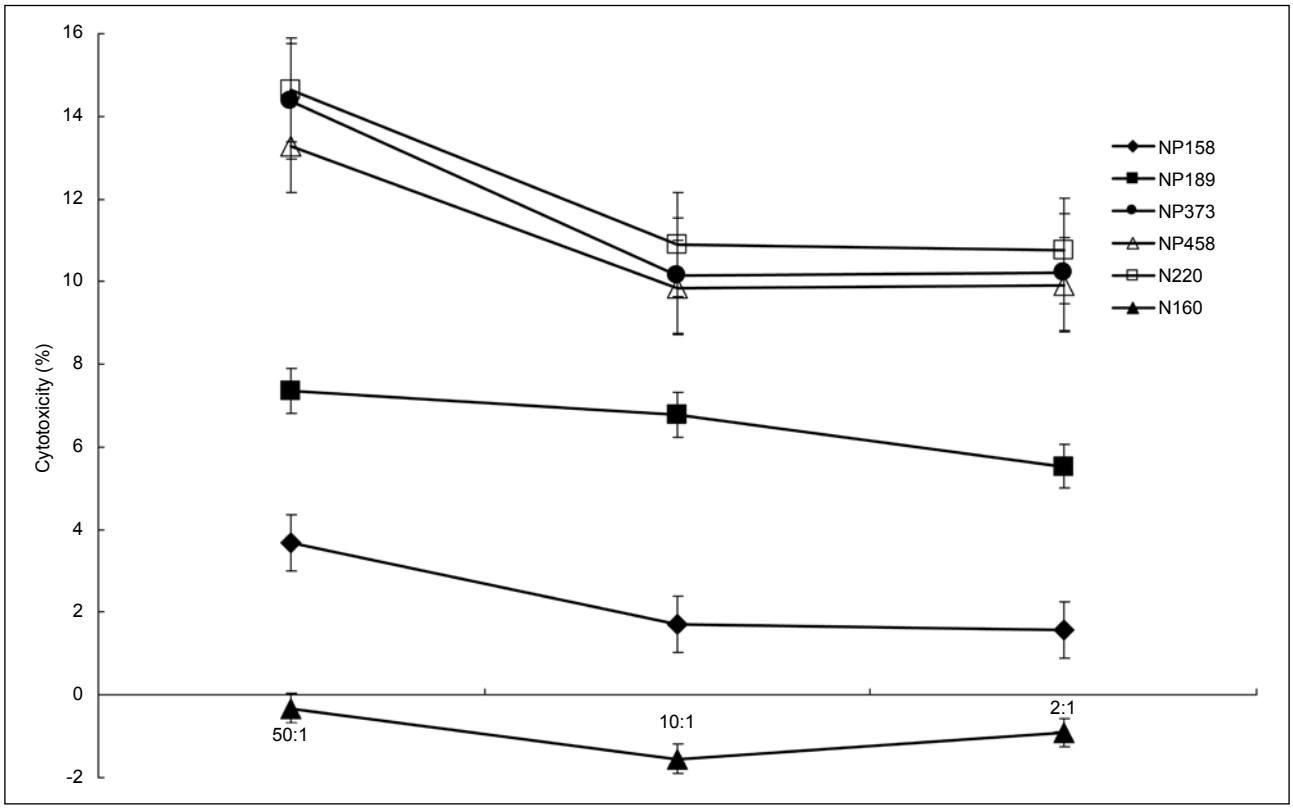

Fig 2. Percentage of specific killing of the target peptide-loaded T2 cells after immunisation

The X-axis indicates the ratios of the effector cells (splenocytes) to the target cells (peptide-loaded T2 cells). The Y-axis indicates the percentage of cytotoxicity. The cytotoxicity for the six groups of mice vaccinated with the various plasmids: NP158HHDpBudCE4.1, NP189 HHDpBudCE4.1, NP373HHDpBudCE4.1, and NP458HHDpBudCE4.1. N220HHDpBudCE4.1 is a positive control, whereas the mice vaccinated with the N220HHDpBud CE4.1 plasmid but tested with N160-loaded T2 cells are used as negative controls. Splenocytes from vaccinated animals are cultured with the corresponding peptides for 5 days. They are incubated with the corresponding peptide-loaded T2 cells for 1 hour. Means and SDs are shown (n=2, $P<0.05$)

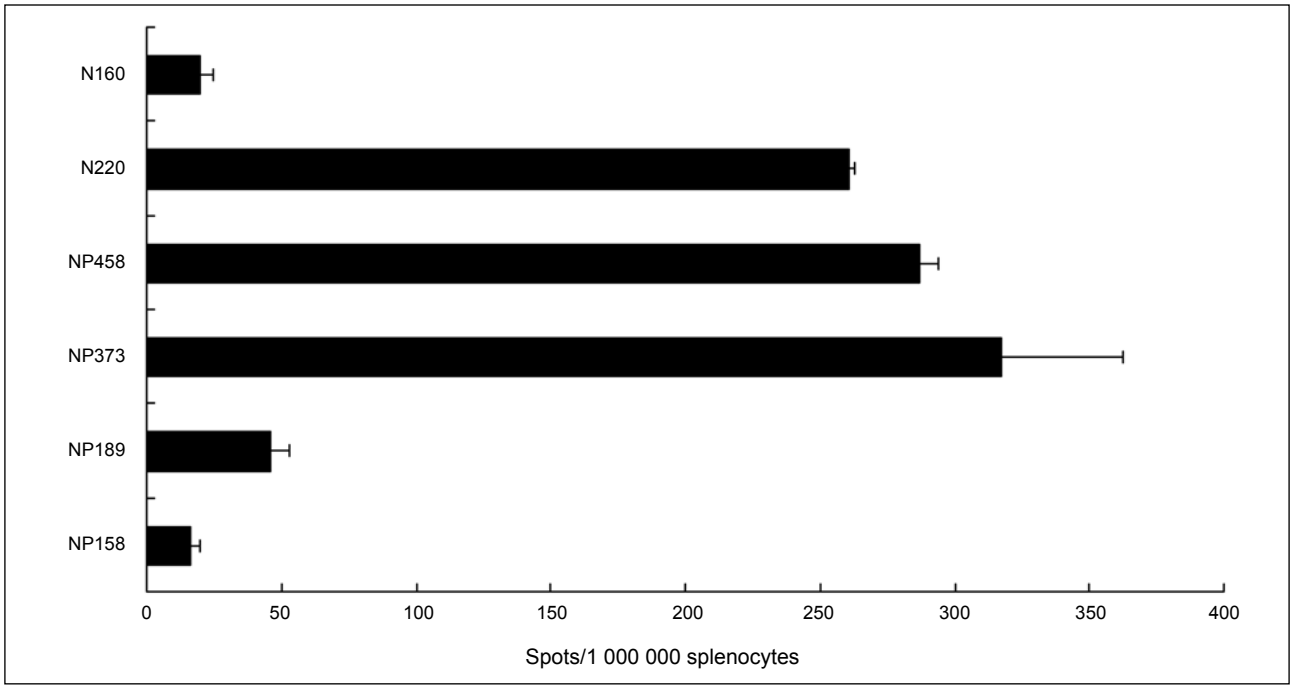

Fig 3. Results of the ELISPOT assay

Splenocytes obtained from mice vaccinated with the plasmids are cultured with corresponding peptides for 1 day and the secretion of IFN-γ is measured by the ELISPOT assay. The N160 peptide (LQLPQGTTL) is used as a negative control for the mice vaccinated with N220HHDpBudCE4.1. Means and SDs are shown (n=2) [three mice per group]

All our assays provided some information about the various peptide vaccine candidates and the results indicated that the NP373 and NP458 were capable of inducing strong immune responses both in vitro and in vivo. The NP373-381 AMDSNTLEL and NP458-466 FQGRGVFEL were two novel HLA-A2 T-cell epitopes found in (H5N1) viral NP.

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