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Avian influenza A virus polymerase gene leads to temperature-resistant genome replication

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ABSTRACT Influenza A viruses (IAV) pose a persistent pandemic threat. Successful cross-species transmission from waterfowl to mammals requires host adaptation for efficient replication and spread. Avian IAVs are adapted to the temperature of the avian gut, approximately 41°C, while human IAVs replicate at 33°C in the upper respiratory tract. PB1, the catalytic core of the viral heterotrimeric RNA-dependent RNA polymerase (RdRp), is essential for viral RNA synthesis. Three pandemic IAV strains (1918, 1957, and 1968) carried an avian-origin PB1 gene, suggesting a selective advantage. Fever, a critical antiviral response, elevates body temperature to 38°C or higher and can restrict viral replication. We hypothesized that avian PB1 may allow IAV replication at febrile temperatures. Using a mini-replicon system, we demonstrate that a polymerase of a seasonal human H1N1 IAV fails to replicate viral RNA efficiently at 40°C, whereas substitution of the PB1 with that from the 1918, 1957, and 1968 pandemic viruses, or a low-pathogenic avian H2N1 virus, restores RNA synthesis. Chimeric seasonal human H1N1 viruses expressing avian or 1918 PB1 replicate at 40°C in MDCK cells, unlike wild-type H1N1. Activity assays with purified chimeric IAV polymerases further suggest that avian-origin PB1 may intrinsically enhance polymerase activity at elevated temperatures. Together, these data show that avian-origin PB1 enables IAV replication at febrile temperatures, highlighting its role in zoonotic emergence and host adaptation. Surveillance of avian-origin PB1 genes in IAVs circulating in mammals may help identify strains with pandemic potential and inform the development of countermeasures.

IMPORTANCE Influenza A viruses of avian origin remain a persistent threat to human health. The 1918, 1957, and 1968 pandemic influenza viruses each acquired an avian-origin PB1 gene, but the adaptive advantage conferred by these genes remains incompletely understood. Here, we show that avian-origin PB1 genes enhance viral RNA replication by a seasonal human influenza virus polymerase at elevated temperature and enable replication of a human influenza virus at 40°C, a temperature relevant to both avian hosts and human febrile illness. PB1-dependent effects were observed in cell-based polymerase assays, with purified polymerase, and during authentic virus infection. These findings identify temperature-resistant genome replication as a potential adaptive property of avian-origin PB1 and suggest that acquisition of an avian PB1 may help influenza viruses overcome temperature barriers encountered during adaptation to mammalian hosts.

KEYWORDS influenza A, viral RNA-dependent RNA polymerase, virus-host interactions, host temperature, replication fitness

Influenza A viruses (IAV) have a broad host range and continue to pose a major threat to public health despite the availability of antivirals and vaccines. Pandemics arise when a zoonotic strain spills over into humans or, more frequently, reassorts with

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circulating human IAVs (1–4). Pandemic IAVs are distinct from seasonal IAVs causing epidemics, with higher infection rates, increased morbidity and mortality, and greater hospital and economic burden (5–7). The 1918 H1N1 pandemic resulted in clinical infections in one-third of the global population and caused an estimated 50 million deaths (5). Subsequent influenza pandemics in 1957, 1968, and 2009, though smaller in scale, also had major impacts and were caused by reassorted viruses with one or more genes from the avian IAV gene pool (1, 8, 9). IAVs are naturally found in wild waterfowl and circulate among migratory birds (10–12). The mammalian host range of select IAV subtypes has diversified in recent years, evidenced by the ongoing global spread of highly pathogenic avian influenza virus (HPAI) H5N1/H5Nx clade 2.3.4.4b and the recent emergence of genotypes B3.13 and D1.1 in dairy cattle across the United States (13–18). These recent events have heightened the threat of avian IAVs to human and animal health, underscoring the critical need to understand the complexities of avian IAV zoonosis and how IAVs overcome the unique environmental, physiological, biological, and immunological barriers to infection and transmission in mammalian hosts.

In contrast to human IAV infections, which predominantly manifest in the upper respiratory tract (URT), low-pathogenic avian influenza viruses (LPAIV) typically cause asymptomatic infections in the gastrointestinal tract of waterfowl (10, 19–21). Both birds and mammals are homeothermic, maintaining a stable body temperature that is metabolically ideal (22). However, the specific homeostatic temperature of an organism is species-specific (Fig. 1). Naturally, avian IAVs have adapted to replicate at the species-specific core temperature of their host reservoir species, approximately 39°C–43°C (23). However, in select species, such as the red-billed quelea (*Quelea quelea*), temperatures can rise to 49°C without host damage. In humans, IAV typically replicates in the URT, where the temperature is approximately 33°C, but can disseminate to the lower respiratory tract (LRT), where temperatures reach 37°C (24, 25). The core temperature of

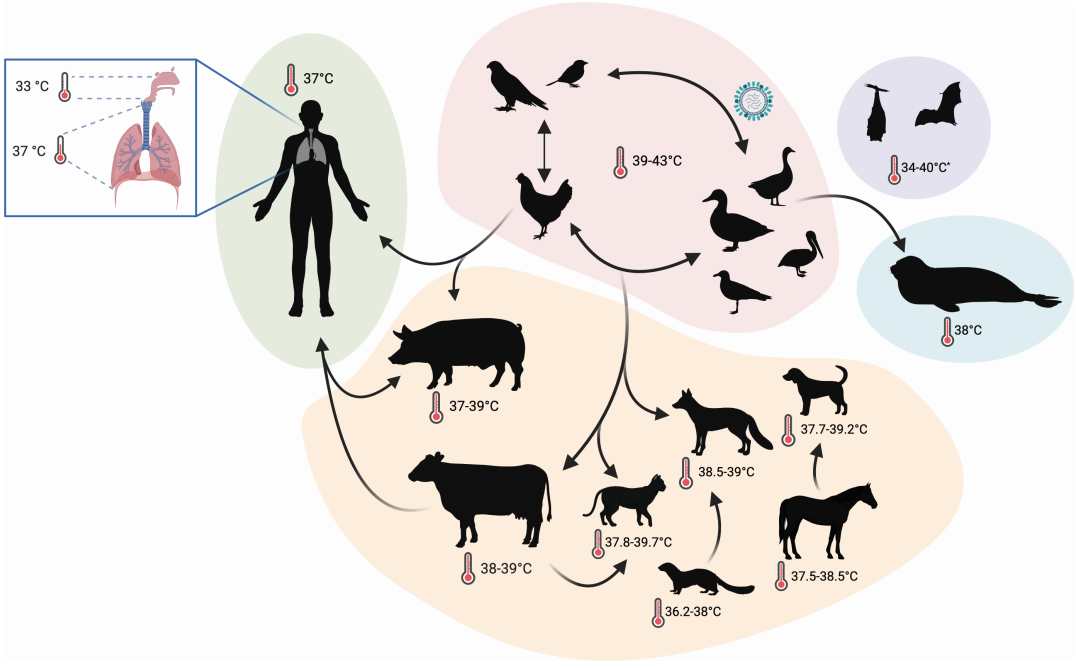


FIG 1 IAV infects a wide range of avian and mammalian hosts with distinct core body temperatures. Wild aquatic birds serve as the primary reservoir and transmit viruses to shorebirds, migratory birds, and poultry. Arrows indicate unidirectional (single) or bidirectional (double) spillover events between host groups. Temperature ranges are shown for key IAV-susceptible hosts: wild aquatic birds, shorebirds, migratory birds, and poultry (red; birds from left to right: raptors, sparrow, chicken, goose, duck, seagull, pelican); terrestrial mammals (yellow; left to right: pig, cow, cat, mink, fox, dog, horse); marine mammals (blue: seal); humans (green); and bats (purple). In humans, body temperature differs between the upper (~33°C) and lower (~37°C) respiratory tracts. For bats (order *Chiroptera*), the temperature range (34°C–40°C) reflects values during torpor and active flight. Created with BioRender.com.

swine, a possible intermediate host for avian, human, and swine IAV reassortment events, is between the temperature range of humans and avian species at 38°C–39°C (2, 26, 27).

LPAI viruses have played a central role in the emergence of pandemic IAVs in humans. A shared feature of the 1918 H1N1, 1957 H2N2, and 1968 H3N2 pandemic strains is that each possessed an avian-origin PB1 gene, encoding the catalytic core of the viral RNA-dependent RNA polymerase (RdRp). The influenza polymerase is a heterotrimeric complex comprising PB1, PB2, and PA and is an essential component of the viral ribonucleoprotein (vRNP) complex, which also includes nucleoprotein (NP) and one segment of negative-sense vRNA. The polymerase functions dually as a replicase and a transcriptase, generating three types of viral RNAs: vRNA, cRNA, and mRNA (28–30). PB1 can markedly influence IAV replication, transmissibility, and polymerase fidelity, with consequences for viral pathogenicity (31–34). Furthermore, polymerase activity and virus pathogenicity can be enhanced by an avian-origin PB1 (31–36). Here, we examine avian-origin PB1 genes from a LPAIV and historical pandemic strains in the context of a contemporary human IAV polymerase to determine if avian-origin PB1 substitutions impact the temperature permissibility of the human IAV polymerase.

RESULTS

Avian and pandemic PB1 enhance seasonal human IAV polymerase activity and vRNA replication at elevated temperature *in vitro*

PB1 genes of LPAIV-origin have repeatedly contributed to the emergence of pandemic IAVs through reassortment with seasonal human IAV strains. To test whether avian-origin PB1 affects polymerase activity in a human IAV background, we examined PB1 from the A/Brevig Mission/1/1918 (H1N1), A/Japan/305/1957 (H2N2), and A/Hong Kong/1/1968 (H3N2) pandemic strains, as well as from a model LPAIV, A/green-winged teal/Ohio/175/1986 (H2N1) (hereafter referred to as LPAI) (34). The PB2, PA, and NP from the human A/New York/312/2001 (H1N1) strain—hereafter referred to as NY312—were co-expressed with either the cognate NY312 PB1 or a heterologous PB1 derived from the model LPAI or the 1918, 1957, and 1968 pandemic viruses. Hereafter, complexes containing NY312 PB2, PA, and NP together with a heterologous PB1 are referred to as chimeric vRNPs. A luciferase-based mini-genome assay was used to assess polymerase activity in 293T cells that were transfected with plasmids to express vRNP components (Fig. 2A). Polymerase activity was examined at 33°C, 37°C, and 40°C, representing the average temperatures of the human URT, LRT, and the avian gastrointestinal tract, respectively (Fig. 2B). At 33°C, substitution of the cognate PB1 with LPAI- or pandemic virus-derived PB1 had variable effects on polymerase activity. The 1918 PB1 reduced polymerase activity, whereas the other PB1s modestly increased activity, although only the increase conferred by the 1968 PB1 reached statistical significance. At 37°C, polymerase activity was modestly higher for all heterologous PB1-containing polymerase complexes relative to the cognate PB1 complex, but none of these differences reached statistical significance. At 40°C, polymerase activity was also higher for the heterologous PB1-containing complexes than for the cognate PB1 complex, with the largest increase observed for the LPAI-derived PB1, which was the only increase to reach statistical significance. Collectively, these data indicate that the effects of heterologous PB1 segments on NY312 polymerase activity are temperature-dependent, with modest increases observed for most PB1 substitutions and significant enhancement by the 1968 PB1 at 33°C and the LPAI-derived PB1 at 40°C.

The influenza virus polymerase functions both as a transcriptase to generate viral mRNA and as a replicase to amplify vRNA through a complementary RNA (cRNA) intermediate (28–30). Luciferase reporter assays do not differentiate between these distinct polymerase functions. To address this, we used a vRNP reconstitution assay at 33°C, 37°C, and 40°C to determine whether transcription or replication was affected by the avian-origin PB1 by analyzing viral RNA levels by primer extension (Fig. 2C). The polymerase function of the heterologous vRNPs was compared to the NY312 vRNP at 33°C, 37°C, and 40°C. At 33°C, the polymerase with the LPAI PB1 had a statistically

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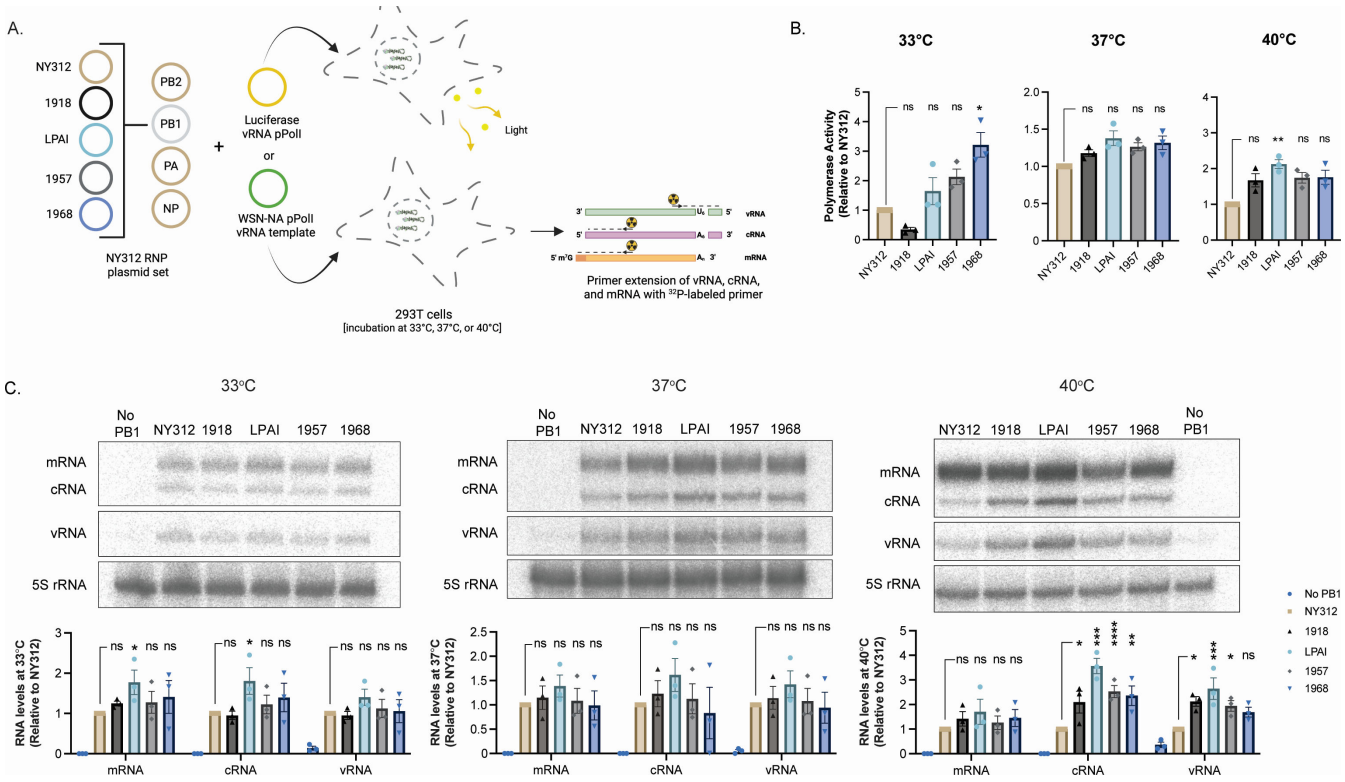


FIG 2 Avian PB1 increases polymerase activity across temperatures. (A) Schematic of minigenome luciferase assay and vRNP reconstitution of chimeric human vRNPs tested at 33°C, 37°C, and 40°C. Created with BioRender.com. (B) Luciferase reporter assay measuring polymerase activity of chimeric NY312 3+1 vRNPs with LPAI, 1918, 1957, or 1968 PB1 at 33°C, 37°C, and 40°C. Chimeric polymerase activities are presented as a percent fold change relative to the NY312 polymerase (mean ± SEM from three biological replicates). For each graph, statistical analysis was performed using a one-sample *t*-test comparing each ratio to 1, with Bonferroni correction applied (α = 0.0125). *P* value: ns, $P \geq 0.0125$; *$P < 0.0125$; **$P < 0.001$. (C) Quantification of vRNA, cRNA, and mRNA levels generated by chimeric vRNPs at 33°C, 37°C, and 40°C using an IAV neuraminidase (NA) vRNA template. Data represent the mean ± SEM of three biological replicates, normalized to 5S rRNA and expressed as a percent fold relative to the NY312 vRNP. Significance was determined by two-way ANOVA with Dunnett's multiple comparison test against the NY312 vRNP at each temperature. *P* values: ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

significant elevation in transcription and cRNA production compared to the cognate NY312 polymerase. At 40°C, all heterologous PB1-containing polymerases produced significantly higher levels of cRNA than the cognate NY312 polymerase. vRNA levels were also significantly increased for the LPAI, 1918, and 1957 PB1-containing polymerases, but not for the 1968 PB1-containing polymerase (Fig. 2C). Western blot analysis confirmed comparable expression levels of PB1 proteins across constructs except for 1918 PB1 at 37°C, showing elevated expression (Fig. S1A). Overall, these findings indicate that the effects of heterologous PB1 at 40°C are primarily associated with increased viral RNA replication rather than transcription.

Avian and pandemic PB1 enable temperature-resistant vRNA replication in a seasonal human IAV polymerase complex at 40°C *in vitro*

The preceding experiments compared the effects of different PB1 genes on polymerase activity at each temperature. We next examined the same experimental conditions by comparing RNA synthesis across temperatures within each PB1 background. To address this, levels of vRNA, cRNA, and mRNA generated by each vRNP were compared across 33°C, 37°C, and 40°C (Fig. 3A).

RNA synthesis by the cognate NY312 polymerase increased from 33°C to 37°C, with significant increases in mRNA, cRNA, and vRNA levels. In contrast, the effects of temperature between 33°C and 37°C varied among the heterologous PB1-containing polymerases. Most importantly, when the temperature was increased from 37°C to

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40°C, vRNA replication by the cognate NY312 polymerase was significantly reduced by approximately 50%. In contrast, no significant reduction in vRNA or cRNA levels between 37°C and 40°C was detected for the polymerases containing LPAI or pandemic PB1. mRNA levels also did not differ significantly between 37°C and 40°C for any of the polymerases tested. Western blot analysis of NP and PB1 revealed increased protein expression at 40°C across all PB1 variants (Fig. S1B).

Taken together, these results demonstrate that genome replication by the cognate NY312 polymerase is temperature sensitive, whereas substitution with LPAI or pandemic PB1 prevents the significant reduction in RNA replication observed for the cognate polymerase at 40°C.

LPAI and wild-type 1918 pandemic vRNPs transcribe and replicate at 40°C *in vitro*

The LPAI and 1918, 1957, and 1968 pandemic-derived PB1 genes confer temperature resistance to a seasonal human IAV polymerase at 40°C. However, the temperature permissibility of the corresponding wild-type polymerases at 40°C is unknown. To determine if the temperature resistance attributed to the avian-origin PB1 persists in the context of wild-type pandemic vRNPs, plasmids encoding PB2, PB1, PA, and NP from the 1918 H1N1, 1957 H2N2, and 1968 H3N2 pandemic strains were used to reconstitute vRNP complexes in 293T cells at 33°C, 37°C, and 40°C. Additionally, the LPAI H2N1 vRNP was reconstituted with PB2 containing the mammalian E627K adaptive mutation (LPAI$^{PB2\text{-}E627K}$). Levels of mRNA, cRNA, and vRNA were quantified to assess polymerase activity at temperatures of 33°C, 37°C, and 40°C.

The LPAI$^{PB2\text{-}E627K}$ vRNP showed no significant differences in mRNA, cRNA, or vRNA synthesis between 37°C and 40°C (Fig. 3B). Similarly, mRNA levels generated by the wild-type pandemic vRNPs did not differ significantly between 37°C and 40°C. In contrast, the effects of elevated temperature on genome replication varied among the polymerases. Notably, for the 1918 polymerase, neither transcription nor replication differed significantly between 37°C and 40°C, whereas significant reductions in replication were observed for some of the other pandemic polymerases.

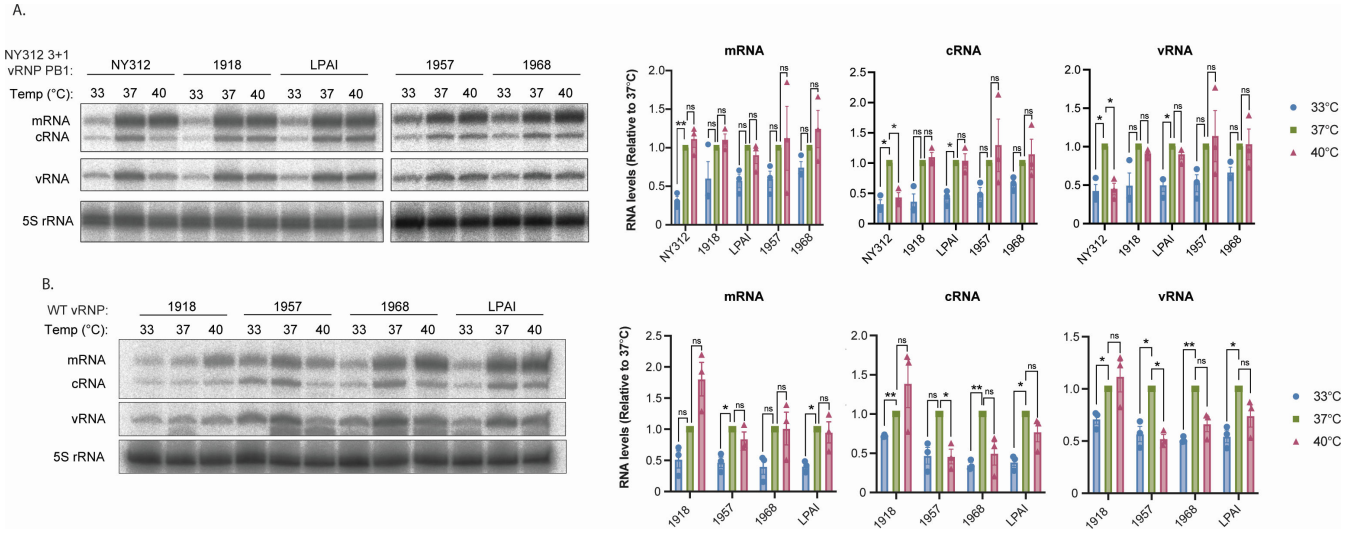


FIG 3 Temperature-dependent RNA synthesis by chimeric and wild-type IAV vRNPs. (A) Quantification of vRNA, cRNA, and mRNA levels generated by chimeric NY312 3+1 vRNPs at 33°C, 37°C, and 40°C. Data represent the mean ± SEM of three biological replicates, normalized to 5S rRNA and expressed as fold change relative to 37°C. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparison test against the 37°C condition. *P* values: ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$. (B) Assessment of temperature permissibility of LPAI$^{PB2\text{-}E627K}$ and wild-type (WT) pandemic IAV vRNPs by measuring mRNA, vRNA, and cRNA levels. LPAI$^{PB2\text{-}E627K}$ data is indicated by the "LPAI" abbreviation. Data represent the mean ± SEM of three biological replicates, normalized to 5S rRNA, and expressed as fold change relative to 37°C. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparison test against the 37°C condition. *P* values: ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

These findings indicate that elevated temperature has differential effects on replication among the pandemic and LPAI polymerases, whereas no significant differences in transcription were detected between 37°C and 40°C. However, despite the ability of the pandemic avian-origin PB1 genes to prevent the significant reduction in replication observed with the NY312 polymerase, this phenotype was not consistently observed in the context of the cognate wild-type pandemic polymerases, aside from the 1918 polymerase.

Role of NP in the temperature dependence of genome replication

IAV NP is a critical factor in IAV genome replication and transcription (37). It has been proposed that NP is recruited to nascent RNA during influenza virus RNA replication through interactions with the unstructured low-complexity acidic region of human ANP32 (huANP32) (38). However, it remains unclear whether there is an interplay between NP and PB1 during genome replication and whether NP contributes to temperature dependence.

To investigate the potential contribution of NP to the temperature-resistant replication phenotype associated with avian-origin PB1, NY312 polymerase with cognate or LPAI PB1 was reconstituted in 293T cells in the absence of NP at 33°C, 37°C, and 40°C (Fig. 4A) (37–39). In this NP-deficient system with a 47-nt long vRNA template, the polymerases generated mRNA and replicated the vRNA. Both the wild-type NY312 polymerase and the LPAI PB1-containing chimera supported transcription at 40°C (Fig. 4B). Replication by the wild-type NY312 polymerase declined at 40°C, but this reduction was not statistically significant compared with 37°C. Thus, in contrast to the temperature-sensitive replication observed for the complete NY312 vRNP, temperature sensitivity was less pronounced when replication was examined using the short NP-independent template. These findings suggest that the temperature dependence of NY312 genome replication is influenced by processes associated with NP-dependent replication, although the underlying mechanism remains unclear.

Purified chimeric human IAV polymerase with an LPAI PB1 retains activity at 40°C

In contrast to the replication differences observed in the vRNP reconstitution assays at 40°C, transcription increased at this temperature independently of the PB1 expressed, compared to 37°C. Cellular genes involved in the heat-shock response, which help stabilize and maintain essential cellular processes, are often upregulated at elevated temperatures (39–41). Such heat-induced transcriptional activation could enhance the pool of host-capped RNAs available for cap-snatching, thereby promoting increased viral transcription.

To determine if IAV transcription increases at 40°C independently of heat-induced cellular responses, we assessed polymerase activity using purified RdRp complexes in a cell-free system. A baculovirus expression system was utilized to produce NY312 wild-type and chimeric NY312 polymerases harboring LPAI PB1 or 1918 PB1. A purified LPAI PB2-E627K polymerase was also produced for comparison (Fig. 5A). Polymerase activity was evaluated using an *in vitro* transcription assay with an ApG primer and short vRNA template, performed at 33°C, 37°C, and 40°C (Fig. 5B) (42). Across all temperatures, the inclusion of LPAI PB1 in the NY312 polymerase variably enhanced transcriptional activity *in vitro*. The LPAI PB1 conferred a statistically significant increase in polymerase activity at 40°C. Similarly, the 1918 PB1 significantly enhanced activity at 37°C, while increases at 33°C and 40°C were modest and not statistically significant (Fig. 5C).

When evaluating the temperature permissibility of each purified polymerase in cell-free conditions, the LPAI polymerase activity increased significantly from 33°C to 37°C and did not differ significantly between 37°C and 40°C (Fig. 5D). In contrast, the activity of the wild-type NY312 polymerase significantly increased from 33°C to 37°C but significantly decreased at 40°C. This decline contrasts with the consistent transcriptional increases observed in cell-based assays, notably in the NP-independent replication assay,

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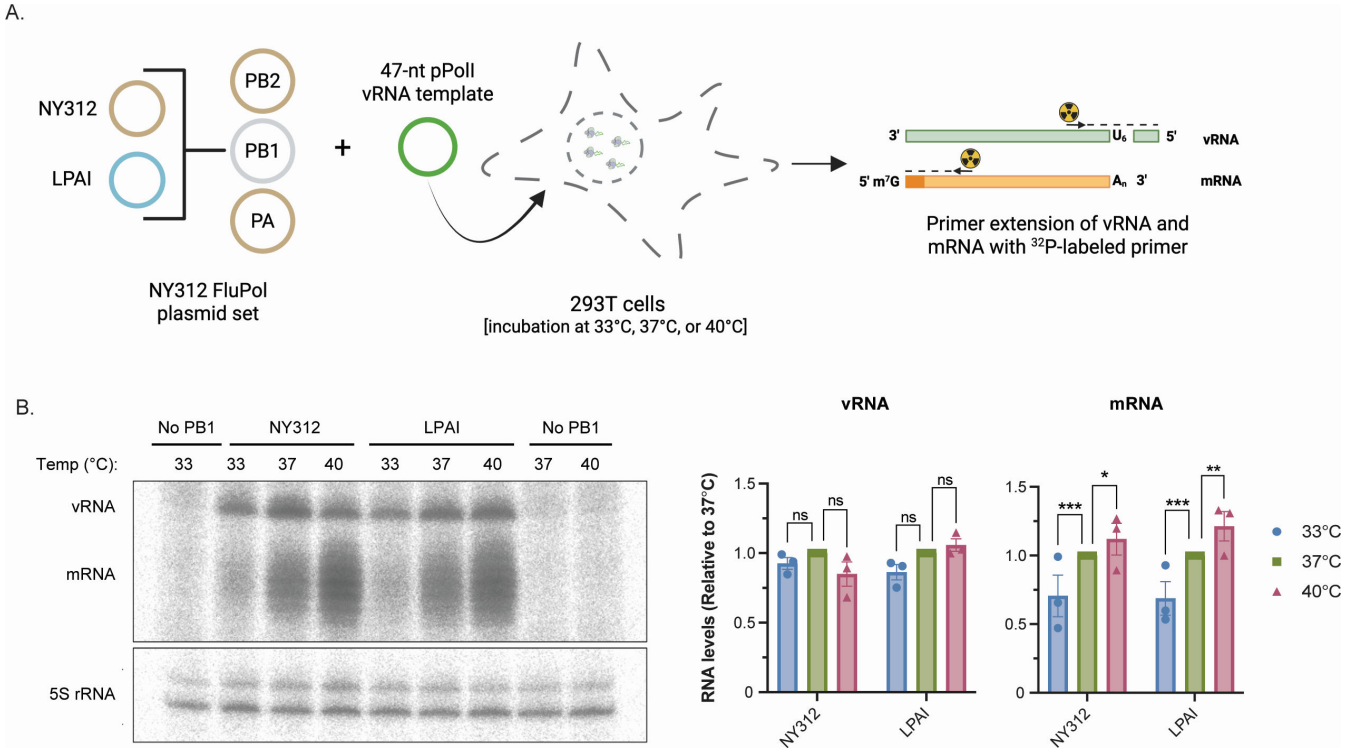


FIG 4 NP-independent RNP reconstitution and RNA synthesis across temperatures. (A) Schematic of the NP-independent RNP reconstitution and primer extension assay. Generated using BioRender.com. (B) Quantification of vRNA and mRNA levels generated from NP-independent replication and transcription of a 47-nt vRNA-like template at 33°C, 37°C, and 40°C by the NY312 IAV polymerase containing either the cognate or LPAI PB1. RNA levels were normalized to the 5S rRNA loading control and expressed as fold change relative to 37°C. Data represent the mean ± SEM from three independent experiments. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparison test against the 37°C condition. *P* values: ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

but aligns with the observed reduction in genome replication. Importantly, chimeric polymerases containing either LPAI or 1918 PB1 showed no significant differences in activity between 37°C and 40°C *in vitro*.

These findings suggest that LPAI PB1 contributes intrinsic properties that influence RdRp activity at elevated temperatures and promote transcriptional activity under elevated thermal conditions, even in the absence of host proteins. Further investigation is warranted to elucidate how physiological changes in cellular metabolism influence viral transcription, particularly in the context of temperature stress.

LPAI and 1918 PB1 genes restore replication of NY312 IAV at 40°C in cell culture and do not enhance virulence in mice

The data above demonstrate that avian-origin PB1 confers temperature resistance in a minigenome assay system. To investigate how PB1 substitution affects viral replication *in vitro*, 7+1 chimeric viruses were generated in which the PB1 gene segment of NY312 was replaced with that of either the LPAI virus or the 1918 pandemic virus, while the remaining seven gene segments were derived from NY312. These viruses are hereafter referred to as NY312$^{\text{LPAI PB1}}$ and NY312$^{\text{1918 PB1}}$, respectively (Fig. 6A). All viruses were handled under enhanced biosafety level 3 (BSL-3) laboratory conditions, in accordance with the biosafety and biosecurity guidelines of the NIH Division of Safety, and with approval from the NIH Institutional Biosafety Committee and the NIH Dual Use Institutional Review Entity (IRE).

To determine whether the LPAI or 1918 PB1 genes enhance viral replication in a human IAV background, multicycle replication kinetics were assessed in MDCK cells at 33°C, 37°C, and 40°C. MDCK cells were infected with the NY312, NY312$^{\text{1918 PB1}}$, or NY312$^{\text{LPAI}}$

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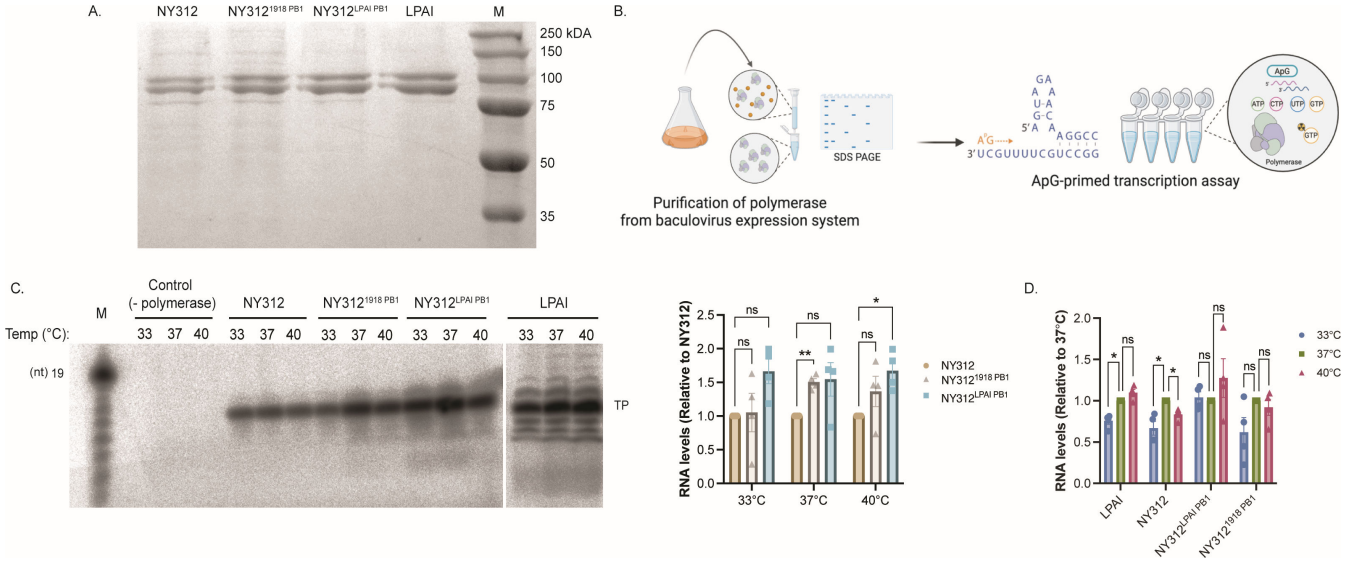


FIG 5 Activity of human IAV NY312 polymerases with cognate LPAI or 1918 PB1 and of a LPAI polymerase. (A) SDS-PAGE analysis of purified IAV NY312 polymerases with cognate, 1918, or LPAI PB1, and of a LPAI polymerase. (B) Schematic of the purification of IAV polymerases expressed in the baculovirus system and subsequent ApG-primed transcription assay. Generated using BioRender.com. (C) *In vitro* ApG-primed short template polymerase activity assay comparing human IAV NY312 polymerases with cognate LPAI or 1918 PB1 and of a LPAI polymerase at 33°C, 37°C, and 40°C. A 19-nucleotide-long ^{32}P-labeled RNA is shown as a size marker, and the position of the transcription product (TP) is indicated. Data are presented as fold change relative to NY312 (set to 1) and represent mean ± SEM from four independent experiments. Statistical significance was determined by two-way ANOVA with Dunnett's correction for multiple comparisons against the NY312 polymerase with cognate PB1. Significant *P* values are noted with an asterisk. *P* values: ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (D) Temperature permissibility of purified polymerase complexes at 33°C, 37°C, and 40°C. Data represent mean ± SEM from four independent experiments. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparison test relative to 37°C. *P* values: ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

^{PB1} viruses at a multiplicity of infection (MOI) of 0.001. Supernatant was collected at 10, 24, 30, 48, and 72 h post-infection, with an additional time point at 90 h for the 40°C condition. Viral titers were determined by plaque assay (Fig. 6B through D).

Overall, the introduction of the LPAI PB1 or the 1918 PB1 into NY312 led to modest increases in viral titers at some of the time points post-infection at 33°C and 37°C with varying statistical significance. The NY312$^{LPAI\ PB1}$ virus exhibited significantly elevated titers approximately one log higher than the NY312 H1N1 wild type at 10, 24, and 30 h post-infection. The NY312$^{1918\ PB1}$ virus similarly demonstrated a one-log increase in titer after 24 h at 37°C but continued to have significantly higher titers than the NY312 control through 72 h.

A more striking phenotype emerged at 40°C. The NY312 wild-type virus was severely impaired, exhibiting minimal replication throughout infection. In contrast, substitution of PB1 with either the LPAI or 1918 PB1 in NY312 resulted in a significant increase in replication, with each chimeric virus reaching titers six logs higher than the NY312 wild-type virus. The inability of the NY312 virus to replicate at 40°C persisted through the additional 90 h time point.

These results demonstrate that substitution with LPAI or 1918 PB1 is sufficient to restore replication of the NY312 virus at 40°C. Although this phenotype is consistent with the temperature-dependent differences in genome replication observed in the vRNP reconstitution assays, the substantially greater magnitude of the effect during multicycle infection suggests that relatively modest differences in polymerase function may be amplified over successive rounds of infection and/or that additional temperature-sensitive viral or host processes contribute to the phenotype.

Numerous studies have previously identified PB1 as a virulence factor of IAV. This quality is attributed to the generation of aberrant replication products that are sensed by the innate immune system, as well as the PB1-F2 accessory protein (31, 36, 43, 44).

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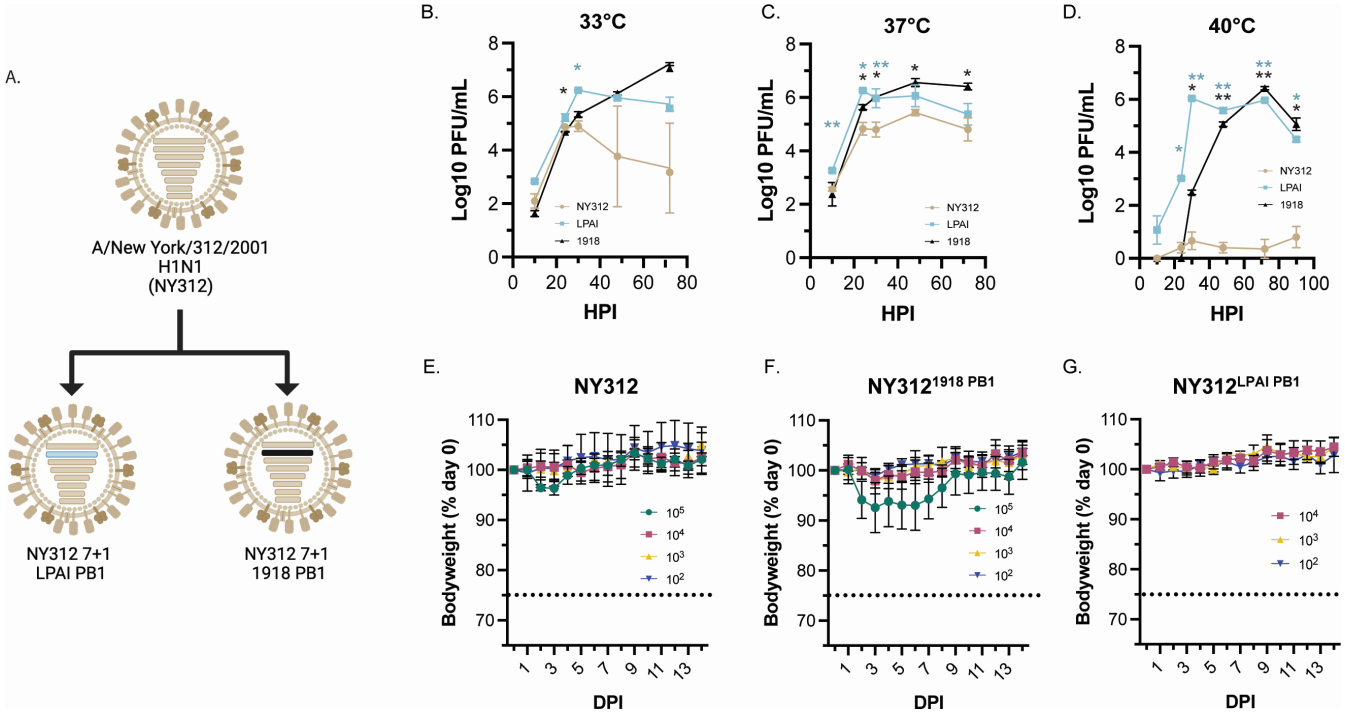


FIG 6 Replication of chimeric human H1N1 viruses in MDCK cells and virulence in mice. (A) Schematic of WT NY312 and chimeric NY312 viruses with the LPAI or 1918 PB1 gene segment. (B–D) Replication kinetics of WT NY312 and chimeric NY312 viruses in MDCK cells at 33°C (B), 37°C (C), and 40°C (D) at a multiplicity of infection (MOI) of 0.001. Viral titers were quantified at the indicated time points post-infection. Error bars represent the standard deviation (SD) of three technical replicates. Titer values were log-transformed and plotted. Titers equaling 0 were set to 1 before log transformation. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparison test relative to WT NY312. Only values comparing titers to the NY312 control are shown. *P* values: ns, $P \geq 0.05$; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Significance to the WT NY312 control is indicated by blue or black asterisks for LPAI or 1918, respectively. For panel D, a mixed-effects analysis with Dunnett's test was used; significance at 10 h could not be calculated due to SD = 0 for the random effect. At 40°C, the NY312 with 1918 PB1 virus was undetectable at 10 and 24 h post-infection, and NY312 WT was undetectable at 10 h post-infection. (E–G) Weight loss in BALB/c mice (*n* = 5 per dose) infected intranasally with WT NY312 (E) or chimeric NY312 viruses expressing 1918 (F) or LPAI PB1 (G) at doses of 10^5, 10^4, 10^3, or 10^2 PFU (LPAI PB1: 10^4, 10^3, or 10^2 PFU only). Mice were monitored for 14 days post-infection (DPI). The dotted line indicates 25% weight loss, the predefined humane endpoint.

To assess whether PB1 substitutions alter virulence in this system, the 50% lethal dose (LD_{50}) for each virus was determined in BALB/c mice. Animal studies were conducted in ABSL-3 laboratory conditions and under the approved NIH Animal Safety Protocol (ASP) LID-6E. Cohorts were intranasally infected with the wild-type NY312, NY312$^{1918\ PB1}$, or NY312$^{LPAI\ PB1}$ viruses and monitored daily for 14 days, with euthanasia criteria set at ≥25% body weight loss.

Independent of the PB1 origin and inoculation dose, all mice survived infection. Moreover, no animals exhibited more than 15% body weight loss prior to recovery (Fig. 6E through G), and LD_{50} values for all three viruses exceeded 10^4 PFU. These findings indicate that replacement of the human PB1 with either the avian LPAI or the 1918 PB1 did not enhance virulence in this model. Consequently, neither potential differences in aberrant RNA production nor variation in PB1-F2 appear to contribute measurably to virulence under these conditions.

Body temperature was not measured during these experiments. However, influenza A virus infection in mice is typically associated with a reduction rather than an increase in core body temperature (45). Thus, this model is unlikely to reproduce the febrile-range temperatures at which the strongest PB1-dependent replication phenotype was observed *in vitro*. This represents a limitation of the mouse model for assessing the physiological consequences of PB1-mediated replication at elevated temperatures and may, in part, explain why the LPAI and 1918 PB1 substitutions did not increase virulence under these conditions.

DISCUSSION

Monitoring and developing countermeasures against future unpredictable pandemic IAVs remain high public health priorities. The rapid spread of panzootic HPAI H5N1/H5Nx clade 2.3.4.4b viruses underscores the expanding host range of avian IAVs, posing a significant threat to global health. Since LPAIs have significantly contributed to past IAV pandemics, understanding how different HPAI or LPAI genes facilitate spillover and adaptation in mammals is critical.

Avian IAV PB1 has multifactorial impacts on viral fitness, pathogenicity, polymerase fidelity, and transmissibility (31, 33–36, 44, 46). Physiological host temperature can hinder IAV replication and influence viral evolution (24, 47). Avian body temperatures are species-dependent, ranging from 39°C to 43°C (22, 23, 48). Comparatively, humans exhibit lower temperatures, especially in the URT, where the temperature is approximately 33°C, rising to 37°C in the LRT (25). The human core temperature can exceed the homeostatic 37°C through a tightly regulated febrile response to infection. Fever, or pyrexia, is characterized by an elevation in core temperature above 38°C and is one of the symptoms of IAV infection (25, 49). In this study, we have demonstrated that the introduction of an LPAI-origin PB1 gene into a contemporary human H1N1 RdRp enhances viral RNA replication at elevated temperatures and, in the context of infectious virus, enables replication at 40°C, a condition under which the wild-type NY312 virus is highly impaired.

The IAV strains from the 1918, 1957, and 1968 pandemics all possessed avian-origin PB1 genes when they first emerged (1, 50). Each pandemic PB1 gene elicited increased genome replication at 40°C, akin to the model LPAI PB1 across multiple assays. However, the WT pandemic vRNPs did not consistently reproduce the phenotype of their respective PB1s in the NY312 background at the elevated temperature. Only the 1918 vRNP showed no significant reduction in either transcription or replication between 37°C and 40°C. One possible explanation is that this characteristic provided an advantage to the 1918 H1N1 virus during its spillover into humans more than a century ago. Further research is needed to determine whether this temperature resistance contributed to the severe disease pathology for which the 1918 H1N1 virus is infamous. The emergence of the 1918 H1N1 virus ushered in a new "pandemic era" of human IAVs, as ancestral 1918 genes have been found in subsequent pandemic and seasonal IAV strains. The 1957 H2N2 pandemic virus possessed the PB2, PA, and NP genes from the circulating seasonal H1N1 virus—a descendant of the 1918 H1N1 virus. While these vRNP genes were avian-adapted before the emergence of the 1918 H1N1 virus, they did not enable efficient replication at 40°C, even when combined with a PB1 from an LPAI virus. This loss of phenotype may be partially explained by evolutionary changes in the virus between 1918 and 1957, driven by increasing population immunity to the 1918 descendant IAVs. A rise in immunity can lead to milder clinical disease and lower rates of febrile illness upon re-infection. Therefore, the high-temperature replication phenotype in these vRNP genes may have been lost during decades of viral evolution following the 1918 pandemic, potentially in favor of replication at lower temperatures in the upper respiratory tract, which would be optimal for transmission. Although the 1957 and 1968 avian-derived PB1 genes retain some resistance to high temperatures, they cannot compensate for the evolutionary changes in PA, PB2, and NP. These findings suggest that the temperature permissibility of the IAV vRNP is a complex, multifactorial trait not solely determined by the PB1 gene. Further research is necessary to elucidate the complex dynamics of temperature resistance and the interplay between the vRNP genes during viral replication.

Despite clear evidence that an LPAI PB1 can enhance the temperature permissibility of a human IAV polymerase, the exact mechanism remains unresolved. There are 31 non-synonymous changes in the PB1 sequence between the model LPAI PB1 and the human NY312 PB1 (Table 1). The 1918 PB1 shares higher sequence identity with the NY312 PB1 and differs by 26 non-synonymous mutations, 25 of which are present in the model LPAI PB1. The PB1 375N/S change is the only mutation in the 1918 PB1

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sequence not found in its LPAI counterpart, an observation that was noted in the original sequencing of the 1918 H1N1 virus (50). Respectively, these 31 and 26 amino acid changes in the LPAI and 1918 PB1 are scattered across the different PB1 domains spanning the fingers, β-ribbon, palm, thumb, β-hairpin, priming loop, and C-terminus. Reductionist approaches are necessary to identify the constellation of residues linked to PB1's temperature permissibility and how they function in concert within the IAV heterotrimer, the vRNP, and with essential host co-factors, like ANP32, to enable this replicative advantage.

A recent publication by Turnbull et al. reported temperature resistance *in vivo* using a mallard PB1 in a PR8 backbone to model reassorted H1N1 (51). Their study demonstrated that substitutions G180E and S394P in the A/Puerto Rico/8/1934 (H1N1) PB1 or R52K and G216S in A/Texas/50/2012 (H3N2) conferred a replicative advantage in hyperthermic conditions in both cell-based assays and a hyperthermic mouse model that emulates pyretic temperatures. This was the first time discrete mutations could be causally linked to replication at febrile temperatures in a model IAV. Our study uses different H1N1 and LPAI virus models and recapitulates their observation that avian PB1 genes reassorted with human IAV polymerase genes confer a fitness advantage at febrile-range temperatures. However, our NY312 H1N1 strain contains E180 and P394 in its PB1 coding sequence, which are shared with the A/green-winged teal/175/1986 (H2N1) PB1 used in this study. The precise mechanism by which LPAI PB1 enables replication at elevated temperatures remains elusive, and these studies suggest that alternative residues are involved in this phenotype.

In addition to potential intrinsic properties of the polymerase that enhance replication at elevated temperatures, other viral and host factors involved in viral transcription and replication may also contribute. NP is a critical vRNP component that is recruited to nascent RNA during vRNA and cRNA synthesis. In the NP-independent system, the temperature-sensitive replication phenotype of the NY312 polymerase was less pronounced than in the complete vRNP, suggesting that processes associated with NP-dependent genome replication contribute to temperature sensitivity. One possibility is that elevated temperature affects interactions required for NP recruitment, nascent RNA encapsidation, or assembly of replication-competent vRNPs, and that these processes are influenced by the PB1 background. However, the present experiments do not distinguish among these possibilities or demonstrate a direct functional interaction between PB1 and NP. Further experiments using alternative NP-independent templates and different PB1 and NP combinations will be required to determine the molecular basis of this phenotype.

The magnitude of the PB1-dependent phenotype during multicycle virus replication was considerably greater than that observed in the vRNP reconstitution assays. This may reflect amplification of a replication defect over successive rounds of infection,

TABLE 1 Sequence identity and amino acid differences between A/green-winged teal/Ohio/175/1986 (H2N1) LPAI PB1 and human A/New York/312/2001 (H1N1) NY312 PB1

IAV strain	Nucleotide differences from NY312, *n* (identity, %)	Non-synonymous differences from NY312, *n*	Amino acid substitutions relative to NY312[a]	PB1 domain
A/green-winged teal/ Ohio/175/1986 (H2N1)	413 (82%)	31	**R52K**, R54K, **K57T**, **K75E**, **I108L**, **I156T**, **R176K**, **D177E**, **D213N**, **K327R**, **I336V**	Fingers
			V179M, **V195M**, **H210Q**, **K211R**	β-ribbon
			I298L, **Y456H**, **A457E**, L473V	Palm
			D383E, **K386E**	β-hairpin
			V517I, I576L, **D581E**, **V667I**	Thumb
			M645V, N654S	Priming loop
			R691K, **I744M**, **T746I**, **D752E**	C-terminus

[a]Bolded amino acid substitutions are also present in the A/Brevig Mission/1/1918 (H1N1) PB1 sequence.

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but additional temperature-sensitive steps in the viral life cycle or virus–host interactions may also contribute. Recent work has also shown that febrile-range temperatures can enhance innate immune responses to influenza through increased production of aberrant viral RNA species (52, 53), providing another potential mechanism that could contribute to the greater magnitude of the phenotype observed during multicycle infection. Innate immune responses and viral RNA synthesis were not examined during the early stages of authentic virus infection at 40°C in this study, and their contribution to the phenotype therefore remains unresolved. Further studies examining viral RNA synthesis and host responses during single-cycle infection will be required to determine how the PB1-dependent polymerase phenotype translates into the marked difference in infectious virus production at elevated temperature. Differences in the PB1 UTRs could also potentially contribute to the temperature-dependent phenotype. However, the UTR sequences associated with the 1918 PB1 are highly conserved with those of NY312, with an identical 5′ UTR and only two nucleotide differences in the 3′ UTR (54). Thus, while a contribution of the PB1 UTRs at 40°C cannot be excluded, the available evidence supports an important contribution of the PB1 coding sequence.

Despite the replicative advantage conferred by the avian-origin PB1 in a human H1N1 virus at elevated temperatures *in vitro*, neither the 1918 nor the LPAI PB1 substitution in NY312 increased pathogenicity in a BALB/c mouse model. The wild-type NY312 virus is highly attenuated in mice, possibly due to its HA or NA, such that replacing an internal gene like PB1 does not significantly alter virulence *in vivo*. An additional limitation is that body temperature was not measured during these experiments. Influenza virus infection in mice is generally associated with a reduction rather than an increase in core body temperature; therefore, this model would not be expected to reproduce the febrile-range temperatures at which the strongest PB1-dependent phenotype was observed *in vitro* (45). Consequently, the absence of increased virulence in this model does not directly address whether avian-origin PB1 provides a fitness advantage during fever. Future studies using an *in vivo* model in which body temperature can be experimentally elevated to febrile-range temperatures will be required to determine the physiological significance of PB1-mediated temperature resistance. Exploring the relationship between replication, virulence, and zoonotic IAV emergence will expand our understanding of how reassorted IAVs with genome segments from viruses of different hosts emerge and establish themselves.

Temperature is a critical determinant of influenza virus replication and transmission. This phenomenon is multifactorial, as it is shaped by complex interactions at the viral-host interface (55–59). Understanding viral temperature sensitivity has been central to the development of live-attenuated influenza vaccines (56, 60, 61). Temperature also influences tropism and host adaptation of IAV. This study shows that an LPAI PB1 or pandemic avian-origin PB1 enables a human IAV to replicate efficiently at 40°C (Fig. 7). Although these experiments demonstrate a PB1-dependent replication advantage at elevated temperatures *in vitro*, its physiological significance during fever remains to be established in an appropriate *in vivo* model. Fever is a highly conserved, tightly regulated innate immune response in mammals (49). In humans, pyrexia is defined as a rise in body temperature above the body's regulated set point. Fevers can be categorized as low-grade, moderate-grade, and high-grade based on a patient's baseline temperature and the site where the temperature is measured. Typically, the temperature benchmark for mild febrile illness is 38°C (Fig. 7A). This complex inflammatory response elevates cellular metabolism, boosts immune activity, and creates a hostile environment for pathogens, ideally reducing viral replication as temperature increases (Fig. 7B). However, avian IAVs may inherently tolerate higher temperatures, as their reservoir hosts maintain core body temperatures of 39°C–43°C (Fig. 7C). Consequently, when an avian IAV spills over or reassorts with a circulating human IAV strain, acquisition of an avian PB1 could enable the new virus strain to sustain replication despite fever. This may confer a substantial advantage for an emerging zoonotic virus, allowing it to establish in an

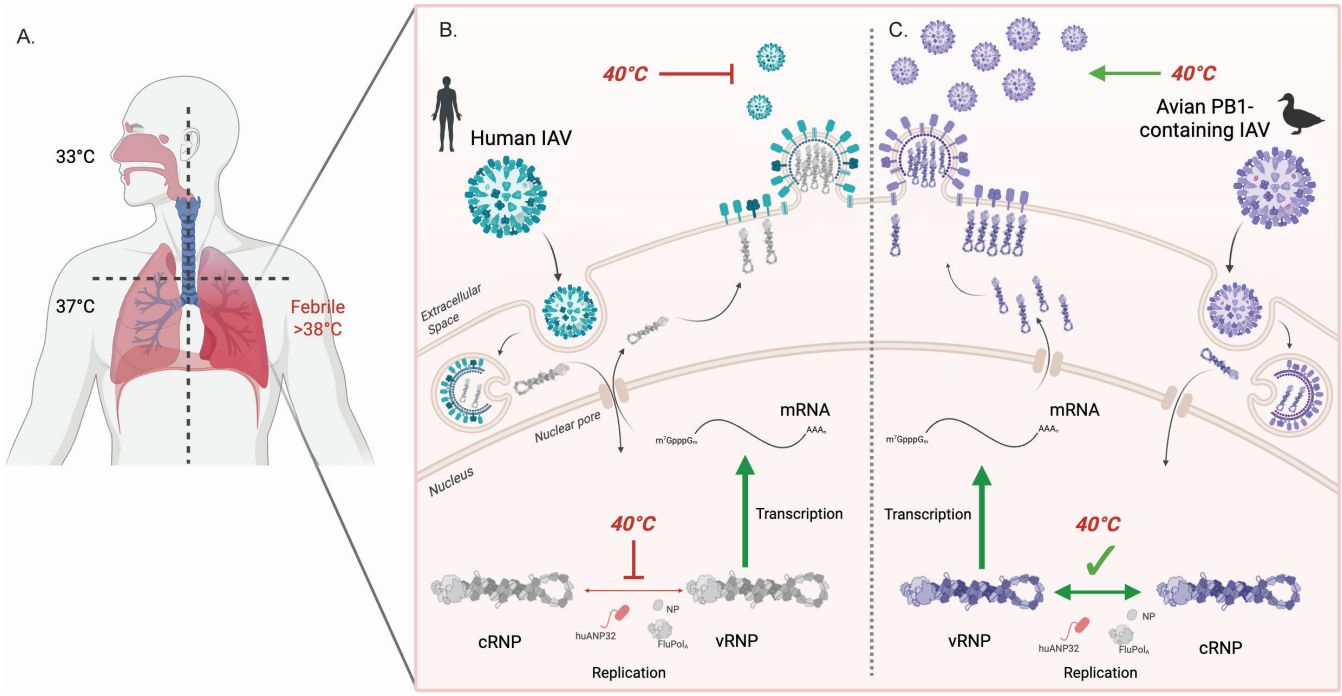


FIG 7 Effect of febrile temperature on IAV transcription and genome replication. (A) Temperature distribution within the human respiratory tract. The URT maintains approximately 33°C, while the LRT approximates the core body temperature of around 37°C. During febrile illness, core temperature rises above 38°C, enhancing immune responses and creating a physiologically restrictive environment for pathogens. (B) Model depicting the impact of 40°C on transcription and replication of a human IAV. Elevated temperature increases transcription but markedly reduces replication of vRNA through cRNA, which may reduce vRNP accumulation and contribute to reduced production of infectious progeny virions. (C) Model illustrating the response of an avian IAV following spillover into humans. At 40°C, both transcription and genome replication are sustained or enhanced, which may support continued vRNP accumulation and infectious virus production at elevated temperature. This temperature resilience may permit ongoing replication and dissemination in the febrile host.

immunologically naïve population, potentially influencing transmissibility, pathogenesis, and pandemic potential.

Overall, this study expands our understanding of PB1's role in modulating the temperature permissibility of the influenza virus polymerase complex and highlights how avian-origin PB1 may overcome febrile barriers in mammalian hosts. Given the historical re-emergence of avian PB1 in the 1918 H1N1, 1957 H2N2, and 1968 H3N2 pandemics, avian PB1 may influence the pandemic potential and transmissibility of an emerging IAV in humans. Understanding how avian PB1 and other avian IAV genes help overcome such barriers will be valuable for ongoing surveillance of avian IAVs circulating in mammals. Insights from this study and further research could inform IAV surveillance efforts and preparedness, particularly as LPAI and HPAI continue to emerge in birds and spill over into humans and other mammals.

MATERIALS AND METHODS

Analysis of polymerase activity using reporter gene expression

293T cells were seeded the day before transfection in 96-well plates to achieve 70%–80% confluency. The pCAGGS plasmid set for the expression of A/New York/312/2001 (H1N1) (NY312) RNP genes and plasmids to express PB1 of A/green-winged teal/Ohio/175/1986 (H2N1), A/Brevig Mission/1/1918 (H1N1), A/Japan/305/1957 (H2N2), and A/Hong Kong/1/1968 (H3N2) have been described (36, 62–64). Chimeric 3+1 vRNPs comprised the NY312 PB2, PA, and NP and a heterologous PB1. A pPolI Luc plasmid expressing a firefly luciferase gene flanked by IAV noncoding UTRs was used as a

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reporter for polymerase activity (65). Cells were transfected with plasmids expressing RNP components PB1, PB2, PA, and NP, and the luciferase reporter plasmid at 40 ng each using Lipofectamine 2000 (Invitrogen). An empty pCAGGS vector was transfected instead of the PB1 plasmid for a negative control. Cells were maintained at 37°C before transfection. Immediately following transfection, plates were transferred to 33°C, 37°C, or 40°C and incubated at the indicated temperature for 24 h. The Promega ONE-Glo Luciferase assay kit (#E6120) was used to measure luciferase activity 24 h post-transfection following the manufacturer's protocols. The data shown are from three independent experiments. The background signal of the negative control was subtracted from the experimental data. The data were presented as percent fold changes relative to the NY312 RNP value. A one-sample *t*-test with Bonferroni's correction for multiple comparisons was used ($\alpha = 0.0125$).

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Analysis of polymerase activity by primer extension

Approximately 3.0×10^5 to 4.5×10^5 293T cells/well were seeded in 24-well plates 24 h before transfection to achieve 70%–80% confluency. The pCAGGS plasmid set for the expression of the NY312 RNP components and plasmids to express LPAI or pandemic PB1s were transfected along with a pPolI plasmid encoding the NA vRNA segment of A/WSN/33 (H1N1) influenza virus at 200 ng of each plasmid (42, 62–64). Cells were maintained at 37°C before transfection. Immediately following transfection, plates were transferred to 33°C, 37°C, or 40°C and incubated at the indicated temperature for 24 h. TRI Reagent (Sigma) was used to isolate total RNA 24 h post-transfection following the manufacturer's protocols. First-strand RNA synthesis using SuperScript III (Thermo Fisher Scientific Inc.) was conducted with NA-specific primers radiolabeled with [γ-^{32}P] ATP (Perkin-Elmer) as described (42). A primer specific to 5S rRNA was used as an internal control. Products were resolved on a 6% or 12% denaturing PAGE containing 7 M urea. The resulting gels were imaged using a FLA-5000 scanner (Fuji) phosphorimager, and targets were quantified using Fiji ImageJ2. Because the number of experimental conditions exceeded the number of samples that could be resolved on a single PAGE gel, comparisons examining the effect of PB1 within each temperature (Fig. 2C) and the effect of temperature within each PB1 background (Fig. 3A) were resolved on separate gels and quantified independently. The data shown are from three independent experiments.

Examination of NP-independent polymerase activity

293T cells were seeded into 24-well plates 24 h before transfection to achieve 70%–80% confluency. The pCAGGs plasmids expressing NY312 PB2 and PA and plasmids expressing LPAI or pandemic PB1s were transfected at 100 ng each along with a pPolI plasmid expressing a 47-nt vRNA-like template derived from the NP segment of A/WSN/33 (H1N1) transfected at 50 ng. Lipofectamine 2000 (Thermo Fisher Scientific Inc.) was used for transfection according to the manufacturer's protocols. Cells were maintained at 37°C before transfection. Immediately following transfection, plates were transferred to 33°C, 37°C, or 40°C and incubated at the indicated temperature for 24 h. After 24 h, TRI Reagent (Sigma) was added, and total RNA was extracted following the manufacturer's protocols. Primer extension analysis of viral RNAs was performed as previously described (42). The data shown are the result of three independent experiments.

Immunoblotting

Cells were lysed using Promega lysis buffer (Promega), and protein samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Primary antibodies for PB1 (GTX125923) and NP (GTX125989) were purchased from GeneTex. The primary β-actin antibody was supplied by Santa Cruz Biotechnology (sc-47778). Secondary goat anti-rabbit IgG (Li-COR 926-32211) and goat anti-mouse IgG (Li-COR 926-32220)

antibodies were used for detection with the Li-COR Odyssey DLx platform. Images were processed with the affiliated software (LI-COR, Inc., 2022).

Purification of chimeric recombinant IAV polymerases

The MultiBac system was used to express the polymerase complex of the A/New York/312/2001 (H1N1) (NY312) and A/green-winged teal/Ohio/175/1986 (H2N1) (LPAI) influenza viruses (66). To express chimeric polymerases, the NY312 PB1 gene was replaced with that of the A/Green-winged teal/Ohio/175/1986 (H2N1) PB1 and the A/Brevig Mission/1/1918 (H1N1) PB1 in the recombinant baculovirus. Recombinant IAV polymerases were expressed in Sf9 insect cells and subsequently purified via IgG affinity chromatography using a protein-A tag on the NY312 PB2 protein. Following cleavage with TEV protease and release from IgG Sepharose, the polymerase complexes were further purified by size-exclusion chromatography in a low-glycerol buffer (25 mM Hepes-NaOH [pH 7.5], 500 mM NaCl, and 5% [vol/vol] glycerol) as previously described (67). Fractions containing purified influenza polymerase were concentrated to 0.5–5.0 mg/mL for downstream applications.

Polymerase *in vitro* transcription assays

Polymerase activity of purified recombinant IAV polymerase was assessed using an α-^{32}P GTP incorporation assay as previously described (67). A reaction mixture containing α-^{32}P GTP (3,000 Ci/mmol), 1.5 µL of purified recombinant polymerase (284 nM), and 2.5 µM of 5′ and 3′ vRNA promoter was prepared and incubated at 33°C, 37°C, and 40°C for 1 h. The promoter sequences have been described (67). RNA products were resolved on an 18% denaturing PAGE with 7 M urea after the addition of formamide to halt the reaction. The gels were imaged by phosphorimaging through the FLA-5000 scanner (Fuji), and the products were quantified using Fiji ImageJ2. The data shown are from four independent experiments.

Generation of chimeric IAVs by reverse genetics

All work with the chimeric IAVs was performed in enhanced biosafety level 3 (BSL-3) laboratory conditions at the National Institutes of Health (NIH). Biosafety practices were aligned with NIH and the Centers for Disease Control and Prevention Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th ed., the Federal Select Agent program, and the NIH Guidelines for Research Involving Recombinant and Synthetic Nucleic Acids, as well as NIH policies and requirements set forth by the NIH Division of Safety and the NIH Institutional Biosafety Committee. This project was periodically reviewed by the NIH Dual Use Research of Concern Institutional Review Entity (IRE), and the manuscript was reviewed and approved by the IRE before submission. The IRE determined that this research did not meet the definition of dual-use research of concern or dangerous gain of function and that the work did not require elevation as part of the P3CO framework.

A reverse genetics system based on the pHH21 plasmid for the A/New York/312/2001 (H1N1) (NY312) has been established previously (68). This strain was selected as a model contemporary, non-lab-adapted human H1N1 virus. The pHH21 plasmid encoding the PB1 gene segment of A/green-winged teal/Ohio/175/1986 (H2N1), hereafter referred to as LPAI, was provided by R. Slemons (Ohio State University, Columbus, Ohio) and has been described previously (11, 63, 69). The pHH21 plasmids encoding the PB1 gene segments of A/Brevig Mission/1/1918 (H1N1) have also been described previously (36). The NY312 and LPAI PB1 rescue plasmids contain the cognate 5′ and 3′ UTRs of their respective PB1 gene segments, whereas the 1918 PB1 rescue plasmid contains the A/WSN/1933 (H1N1) PB1 5′ and 3′ UTRs. The NY312 7+1 chimeric viruses containing the PB1 gene segments of the A/green-winged teal/Ohio/175/1986 (H2N1) and A/Brevig Mission/1/1918 (H1N1) were generated in the NY312 backbone as previously described (36, 70). The recombinant viruses were cultured and quantified by plaque assay. The LPAI

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and 1918 PB1-containing viruses were passaged 1–2 times before use. All viruses were verified by sequencing.

Viral growth kinetics in MDCK cells

MDCK cells were seeded in 6-well plates in DMEM containing 10% FBS and grown to 90% confluency at 37°C over a period of 24 h. Virus stocks were diluted in DMEM, and MDCK cells were infected at an MOI of 0.001 in three technical replicates. Virus adsorption was performed for 1 h at 37°C, after which the infected cells were transferred to 33°C, 37°C, and 40°C and maintained for the duration of the experiment. Virus titers were measured at 10, 24, 30, 48, and 72 h post-infection using plaque assays. An additional 90 h time point was taken for the samples incubated at 40°C.

Mouse studies

Groups of mice (n = 5) were intranasally infected with the NY312 WT or NY312 7+1 1918 PB1 chimeric virus at 10^5, 10^4, 10^3, and 10^2 PFU doses and the NY312 7+1 LPAI PB1 chimeric virus at 10^4, 10^3, and 10^2 PFU doses. Natural history of disease was monitored via daily weights taken for 14 days post-infection. Mice were ethically euthanized if they lost more than 25% of their initial body weight.

Quantification and statistics

All statistical analyses were performed using Prism 10. Statistical tests were selected according to the experimental design and the comparisons of interest. For experiments comparing multiple groups with a defined control, ANOVA followed by Dunnett's multiple-comparison test was used. For the luciferase reporter assay in Fig. 2B, activities of individual heterologous PB1-containing polymerases were expressed relative to the cognate NY312 polymerase and compared with a theoretical value of 1 using one-sample t-tests. Because four PB1 variants were compared with NY312 at each temperature, a Bonferroni correction was applied, resulting in an adjusted significance threshold of $\alpha = 0.0125$. Statistical significance was defined using the thresholds specified in the corresponding figure legends. Unless otherwise indicated, nonsignificant differences were not interpreted as evidence of equivalence. Statistical analyses were performed in consultation with a statistician at the National Institutes of Health.

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AUTHOR CONTRIBUTIONS

Stephanie L. Williams, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Sebastian M. Gygli, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Jacob C. Bjorgen, Data curation, Formal analysis, Investigation, Validation, Writing – review and editing | Haitian

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Fan, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – review and editing | Munashe Mudiwa, Data curation, Investigation, Methodology, Writing – review and editing | Lex Matthews, Data curation, Investigation, Methodology, Writing – review and editing | Yongli Xiao, Data curation, Formal analysis, Methodology, Software, Writing – review and editing | Ashley Freeman, Data curation, Investigation, Methodology, Writing – review and editing | Sharon F. Legaspi, Data curation, Investigation, Methodology, Writing – review and editing | Li Qi, Conceptualization, Methodology, Resources, Supervision, Writing – review and editing | Jeffery K. Taubenberger, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing | Ervin Fodor, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

The National Institutes of Health can provide all experimental materials pending scientific review and a completed material transfer agreement. Requests for any materials should be submitted to E.F. or J.K.T. All sequences used in phylogenetic analysis exist in the public domain and are available on the Influenza Research Database (IRD). All data are available in the main text or the supplemental material.

ETHICS APPROVAL

Animal studies complied with the approval of the NIAID Animal Care and Use Committee (ACUC) under the NIH Animal Safety Protocol (ASP) LID-6E.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1 (JVI00978-26-s0001.pdf). Western blot analysis of PB1 and NP in vRNP reconstitution assays.

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