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Original Article

Predominant Shift of Different P44-Expressing *Anaplasma phagocytophilum* in Infected HL-60, THP-1, NB4, and RF/6A Cell Lines

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SUMMARY: *Anaplasma phagocytophilum*, an agent of human granulocytic anaplasmosis, is an obligatory intracellular bacterium that dominantly produces P44 outer membrane proteins encoded by the *p44*/*msp2* multigene family, which are major antigens for serodiagnosis. However, *A. phagocytophilum* antigens from cultures with different cell lines seem to have varying reactivities with sera. In this study, we performed RNA-seq to investigate the P44 expression of *A. phagocytophilum* propagated in 4 cell lines. In infected HL-60 cells, the P44-2b transcript was predominant in the first RNA-seq analysis (HL-60.1). However, the P44-23 transcript was predominant in the second RNA-seq analysis at 1 month after additional passages (HL-60.2). We further analyzed the P44 expression of *A. phagocytophilum* cultured in THP-1, NB4, and RF/6A cells through consecutive passages in the same cell lines for 1 year after transferring *A. phagocytophilum* from infected HL-60 cells to the respective cell lines. In the long-term cultures, P44-18, P44-78, and P44-51 were predominantly transcribed in infected THP-1, NB4, and RF/6A cells, respectively. Therefore, the predominant shifts of different P44-expressing transcripts of *A. phagocytophilum* might occur during cell culture even in the same cell line at different time points of sample harvest (HL-60.1 and HL-60.2), which may be attributed to host cell adaptation/selection/interaction.

INTRODUCTION

Anaplasma phagocytophilum is an obligatory intracellular bacterium that causes human granulocytic anaplasmosis (HGA), an emerging tick-borne infectious disease (1). This bacterium dominantly produces 44 kDa major outer membrane proteins (P44) encoded by the *p44*/*msp2* multigene family (2–7). Genomic analysis has revealed that there are 113 paralogous gene copies of *p44*/*msp2* including truncated genes in the bacterial genome (8). The *p44*/*msp2* multigene structures consist of a single and central hypervariable region flanked by 5′- and 3′-end conserved regions. *A. phagocytophilum* expresses a variety of P44 proteins in the host during mammalian infection through gene conversion to escape host immune defense responses, resulting in antigenic variation (9–14). In the serodiagnosis of HGA, indirect immunofluorescence assay (IFA) is usually performed using *A. phagocytophilum*-infected HL-60 cells as antigen-presenting cells. The sera of patients with HGA can strongly react with P44 proteins; however, other proteins of *A. phagocytophilum* seem to be less antigenic in human infections (2), suggesting that P44 proteins as antigens are vital for IFA results. Our previous serodiagnostic studies showed that the sera of patients with HGA could react with the IFA antigens of *A. phagocytophilum* HZ (US-human isolate) cultured in THP-1 cells rather than in HL-60 cells (15,16), suggesting that the P44 transcript species predominantly expressed in infected THP-1 cells are likely distinct from those expressed in infected HL-60 cells. Therefore, the P44 species are differentially expressed among different cell lines. In this study, we investigated the P44 expression profile of *A. phagocytophilum* propagated in 4 different cell lines (HL-60, THP-1, NB4, and RF/6A) by RNA-seq.

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MATERIALS AND METHODS

Bacterium, cell lines, and cultures: *A. phagocytophilum* HZ (type strain) was provided by Dr. Rikihisa (The Ohio State University) (17). HL-60 and THP-1 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and NB4 (ACC207) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). These 3 cell lines were human hematopoietic cell lines. RF/6A 135 (rhesus blood vessel endothelial cell line) was obtained from the RIKEN Cell Bank in the RIKEN BioResource Research Center (Tsukuba, Japan). *A. phagocytophilum* HZ was maintained in HL-60 cells using RPMI 1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 (17). For bacterial passage, uninfected and infected cells were combined at a ratio of 3:1 and cultured for 2–3 days. Infectivity was

assessed by Diff-Quick staining. For the infection of other cell lines, *A. phagocytophilum*-infected HL-60 cells (infectivity > 90%) were harvested by centrifugation at 400 × *g* for 5 min, and host cell-free bacteria were released by sonication for 3–5 s with an output setting of 2 using an ultrasonic device (SONIFER 150; Branson Ultrasonics, Danbury, CT, USA). After low-speed centrifugation at 400 × *g* to remove cell debris and nuclei, the supernatant was further centrifuged at 16,000 × *g* for 5 min to obtain a pellet enriched with host cell-free *A. phagocytophilum*. The cell-free bacteria were resuspended, mixed with THP-1, NB4, or RF/6A cells, and maintained for 1–2 h. Then, the cells of each infected cell line were continuously passaged in the same cell lines.

Infectivity assessment: *A. phagocytophilum*-infected cells were stained using the Live/Dead Cell Viability kit (Thermo Fisher Scientific, Waltham, MA, USA). Live *A. phagocytophilum* and host cells (green) as well as dead bacteria/host cells (red) were counted using a LSM700 microscope (Carl Zeiss, Oberkochen, Germany). The infection rate was determined based on a total of 100 or more cells. Three-dimensional imaging of live *A. phagocytophilum*-infected cells was also performed using Imaris 8.3 software (Bitplane AG, Zurich, Switzerland).

Infection for RNA-seq: The experimental design is shown in Fig. 1A. HL-60, THP-1, NB4, and RF/6A cells infected with *A. phagocytophilum* were continuously passaged in the same cell lines for 1 year after the transfer of *A. phagocytophilum* from infected HL-60 cells to the respective cell lines. Cell-free *A. phagocytophilum* from the respective suspension cultures (infectivity of 70–90%) was prepared by sonication as described previously. The cell-free bacteria were mixed with uninfected cell lines and maintained for 1–2 h. The mixed ratio of infected cells (for the preparation of cell-free bacteria) to uninfected cells was approximately 5:1. The inoculated cell lines were further cultured for 20–22 h before host cell lysis. In the case of adherent RF/6A cells, cell-free bacteria in the supernatant, which were spontaneously released from infected monolayer cells, were enriched by centrifugation. After inoculation with uninfected cells for 2 h, the monolayer cells were further cultured for 22 h. Then, the respective infected cells were harvested by centrifugation or cell scraping and kept in RNA later until use for RNA extraction (HL60.1, THP-1, NB4, and RF/6F). In the case of HL-60 cells, the infected cells were further cultured for another month (HL60.2), and total RNA was extracted for replicate experiments.

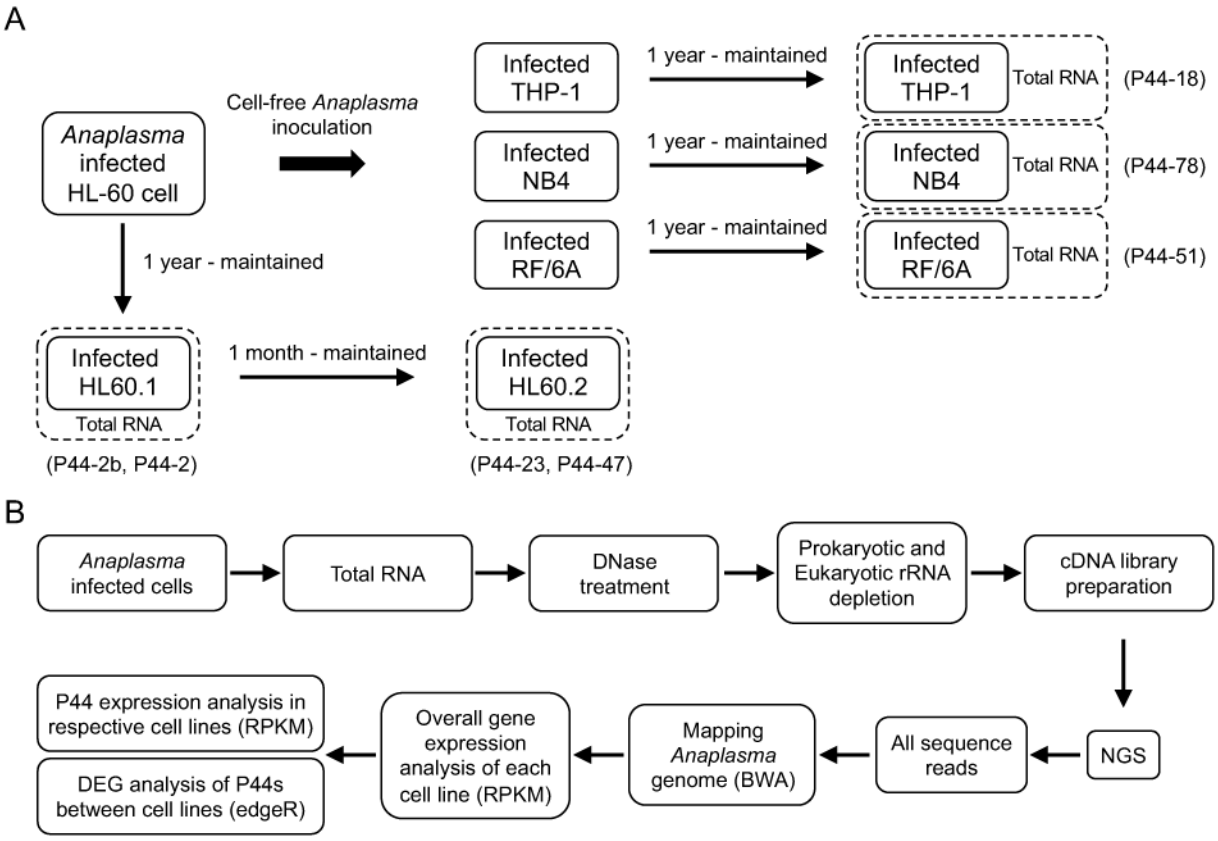

Fig. 1. Experimental design of transcriptional analysis of *p44* multigene family from *A. phagocytophilum* maintained in different host cell lines. (A) Cell cultures for RNA-seq. Frozen *A. phagocytophilum*-infected HL-60 cells under nitrogen liquid were recovered and cultured with medium for several months. Then, cell-free *A. phagocytophilum* prepared from the infected HL-60 cells were inoculated to uninfected HL-60, THP-1, NB4, or RF/6F cells and respective infected cells were continuously-passaged for approx. 1 year with own cell lines for total RNA preparation (HL60.1, THP-1, NB4, and RF/6F). In the case of HL-60 cells, the infected cells were further cultured for additional 1 month for replicate experiments (HL60.2). The predominantly-expressed P44 transcript species in this study are summarized and shown in parentheses. (B) Schematic representation of experimental flow for RNA-seq. The detailed procedures were described in Materials and Methods.

RNA-seq analysis: Total RNA was extracted with TRIzol from approximately 5×10^6 cells per infected cell line, purified using PureLInk RNA Mini Kit, and treated with On-column PureLink DNase according to the manufacturer's protocol (Thermo Fisher Scientific). Ribosomal RNA was depleted from the DNA-free RNA using the RiboMinus Transcriptome Isolation Kit (yeast/bacteria) and the RiboMinus Eukaryote System v2 (Thermo Fisher Scientific). cDNA libraries from the depleted RNA were prepared using Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific), and the quantity/quality of each library was assessed using a Qubit fluorometer with the Qubit DNA assay kit and the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Next-generation sequencing was carried out on 318 v2 chips using the Ion Torrent PGM system with the HiQ Chef kit according to the supplier's instructions (Thermo Fisher Scientific).

Bioinformatics and statistical analyses: By using Metagenomic Pathogen Identification pipeline v. 2.0 (http://mepic.nih.go.jp/) (18), FASTQ reads were mapped on *A. phagocytophilum* HZ reference genome sequences (GenBank accession: NC_007797.1) by Burrows-Wheeler Aligner after quality trimming followed by the subtraction of the reads mapped on human (*Homo sapiens*) or monkey (*Macaca mulatta*) host cell genome. Then, the mapped reads were counted using featureCounts (subread-1. 5. 2-source) from Bioconductor (https://www.bioconductor.org). The reads counted were further normalized using the reads per kilobase per million (RPKM) method (19) to determine the transcriptional levels of P44 species expressed in the respective cell lines. The reads were also mapped and visualized on the reference genome of *A. phagocytophilum* using Geneious software version 9.1.6 (Biomatters, Auckland, New Zealand). Differentially expressed gene (DEG) analysis of *A. phagocytophilum* propagated in different cell lines was conducted using edgeR (version 3.12.1) in R (version 3.2.4) from Bioconductor, and a p value (false positive rate) of < 0.05 and a q value (false discovery rate) of < 0.05 were considered significant for differential expression. To compare the infection rate between different cell lines in the infection experiment, the data were analyzed using SPSS version 20. Differences were considered significant at $p < 0.05$.

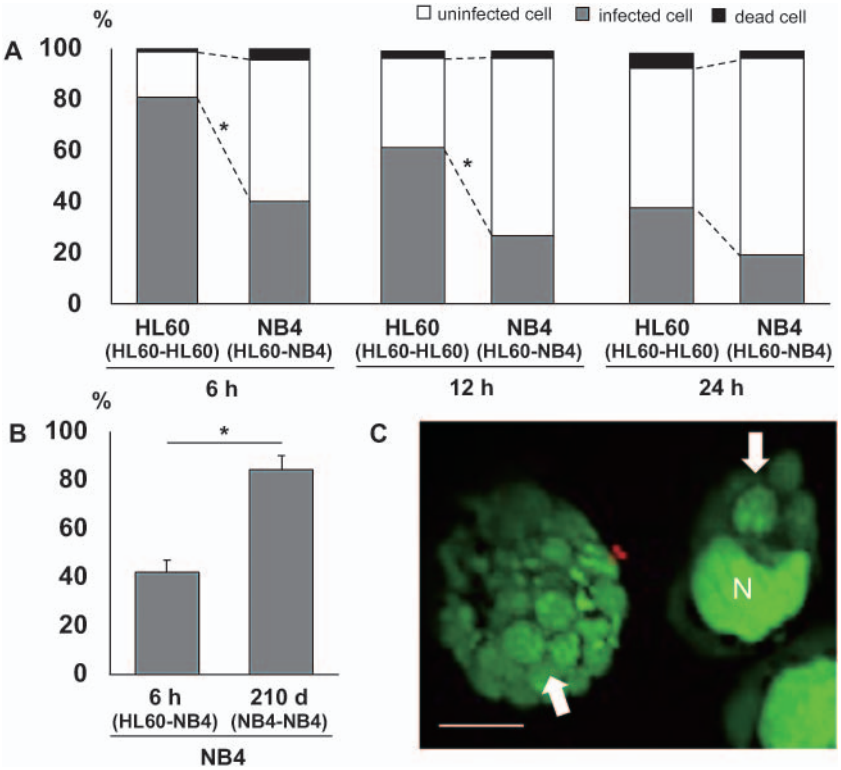

Fig. 2. Comparison of infectivity in early-stage culture and long-term culture after transfer of *A. phagocytophilum* from an infected cell line to own and other cell lines. (A) Infectivities of *A. phagocytophilum* harvested from HL-60 (HL60-HL60) and NB4 (HL60-NB4) at 6 h, 12 h, and 24 h after transfer of *A. phagocytophilum* from infected HL-60 to own cell line and NB4 cell line, respectively. Asterisks show statistical significance (Tukey honestly significant difference test, $p < 0.05$). (B) "Infectivity of *A. phagocytophilum* harvested from NB4 (HL60-NB4) at 6 h after transfer of *A. phagocytophilum* from infected HL-60 to uninfected NB4" and "Infectivity of *A. phagocytophilum* harvested from NB4 (NB4-NB4) at 6 h after inoculation of cell-free *A. phagocytophilum* prepared from infected NB4 to own cell line in long-term culture through repeated-passages for 210 days with own cell line." Asterisks show statistical significance (Student's t-test, $p < 0.05$). (C) (Color online) Three-dimensional imaging of live *A. phagocytophilum*-infected NB4 (NB4-NB4) cell line in long-term culture for 210 days with own cell line using Imaris 8.3 software. Arrows indicate inclusions of intracellular *A. phagocytophilum* (green) and "N" shows host cell nucleus. Small red dots outside of infected cells are probably dead bacteria. Scale bar shows 10 μm.

RESULTS

Increased infectivity of *A. phagocytophilum* in NB4 cells in long-term passage culture with the same cell line: *A. phagocytophilum* is usually maintained through passaging with HL-60 cells. However, when cell-free *A. phagocytophilum* prepared from infected HL-60 cells is inoculated with other cells such as NB4 cells, the infectivity would likely be lower than that in HL-60 cells. Therefore, we first monitored the infectivity during the culture period after the transfer of *A. phagocytophilum* from the infected HL-60 cell line to the NB4 cell line by image scanning. When the infectivity was compared at 6, 12, and 24 h after transferring *A. phagocytophilum* from the infected HL-60 cell line to the same cell line (HL60-HL60) and the NB4 cell line (HL60-NB4), the infectivity at 6 h was higher than that at 12 or 24 h in both cell lines (Fig. 2A). The reduced infectivity in both cell lines over time may be attributed to the proliferation of uninfected cells. When the infectivity of *A. phagocytophilum* harvested from HL-60 cells (HL60-HL60) and NB4 cells (HL60-NB4) was compared at 6, 12, and 24 h post-inoculation, the infectivity in NB4 cells (HL60-NB4) was lower than that in HL-60 cells (HL60-HL60) at 6, 12, and 24 h (Fig. 2A). However, when uninfected NB4 cells were inoculated with cell-free *A. phagocytophilum* prepared from infected NB4 cells (NB4-NB4) for 210 days, the infectivity (84%) was significantly increased compared with that (40%) in NB4 cells inoculated with *A. phagocytophilum* prepared from infected HL-60 cells (HL60-NB4) in the early-stage culture (Fig. 2B). Three-dimensional live imaging revealed that *A. phagocytophilum* was abundant in the long-term passage culture of NB4 cells (NB4-NB4) (Fig. 2C). These results suggest that infectivity was increased in the long-term culture with the same cell line but not in the early-stage culture after bacterial transfer with a different cell line, which may be affected by the host cell adaptation/

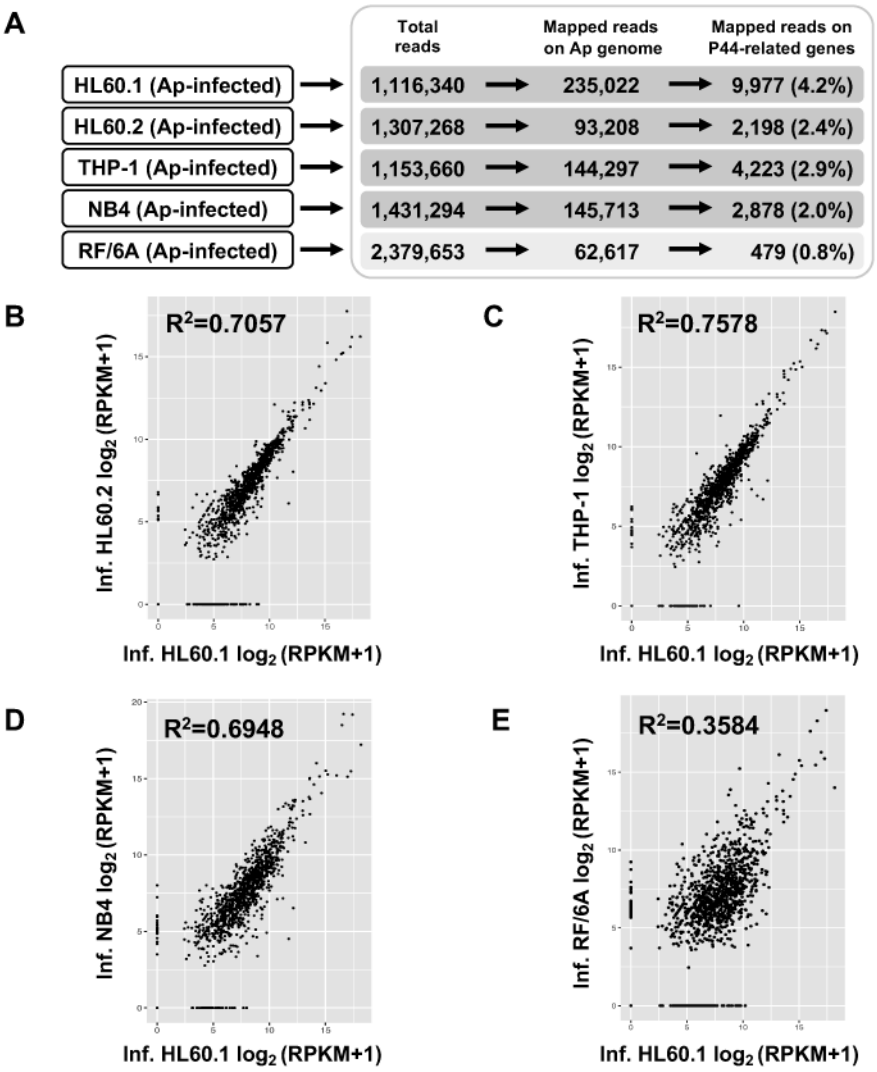

Fig. 3. Overall gene expression of *A. phagocytophilum* propagated in 4 different cell lines. (A) Total reads, mapped reads on *A. phagocytophilum* (Ap) genome, and mapped reads on P44-related gene loci. Percentage in parentheses shows a ratio of P44-related gene/Ap genome of mapped reads. Overall gene expressions of *A. phagocytophilum* are compared between HL60.1 and HL60.2 (B), between HL60.1 and THP-1 (C), between HL60.1 and NB4 (D), and between HL60.1 and RF/6A (E) by linear regression analyses based on RPKM values. The correlation is considered "$R^2 > 0.7$" between the 2 infected cells.

selection/interaction of *A. phagocytophilum*.

Overall gene expression of *A. phagocytophilum* maintained in different cell lines: As shown in Fig. 1, HL-60, THP-1, NB4, and RF/6A cells infected with *A. phagocytophilum* were continuously passaged for approximately 1 year in the same cell lines, and RNA-seq was performed. A total of 1,116,340 to 2,379,653 reads from the respective infected cells were mapped on the *A. phagocytophilum* HZ reference genome sequences (NC_007797.1) (Fig. 3A). The mapped reads (62,617 to 235,022) were further normalized using the RPKM method, and the RPKM values were scattered by ggplot2. The R^2 values (Fig. 3B to 3D) were determined by linear regression analysis using R (version 3.2.4). The overall *A. phagocytophilum* gene expression in HL60.1 cells was better correlated with that in HL60.2 and THP-1 cells ($R^2 > 0.7$) and slightly less correlated with that in NB4 cells ($R^2 = 0.6948$), and the positive correlation between the gene expression in HL60.1 and RF/6A cells was lower ($R^2 = 0.3584$), suggesting that the gene expression profile of *A. phagocytophilum* maintained in the RF/6A cell line might be different from that of *A. phagocytophilum* maintained in the other cell lines.

Characteristics of P44 transcript species expressed by *A. phagocytophilum* maintained in different cell lines: Among 113 paralogous loci of *p44* on the *A. phagocytophilum* genome, 101, 85, 93, 94, and 81 loci were mapped by the reads from HL60.1, HL60.2, THP-1, NB4, and RF/6A cells, respectively. Although the RPKM values of the overall gene expression of *A. phagocytophilum* were better correlated between HL60.1 and HL60.2 cells (Fig. 3B), the P44 transcript species expressed by *A. phagocytophilum* were markedly different between these infected HL-60 cells (Table 1). In HL60.1 cells, the P44-2b species followed by P44-2 species were predominantly transcribed; however, the P44-23 species followed by P44-47 species were transcriptionally dominant in HL60.2 cells (1 month later). In infected THP-1 cells, the P44-18ES species followed by P44-18 species were predominant at the transcriptional level (Table 1). Indeed, P44-18ES at the expression locus and the P44-18 donor cassette locus on the genome were abundantly mapped by the reads from infected THP-1 cells (Fig. 4A, 4B, and 4C). In infected NB4 cells, although P44-18ES followed by P44-78 had the highest RPKM value (Table 1), the mapping results showed that most sequence reads for P44-18ES were mapped on 5′- and 3′-end conserved regions with recombination at the expression locus and not mapped on a central hypervariable region (Fig. 4A and 4D). In contrast, the entire P44-78 donor cassette locus was mapped by the reads (Fig. 4A and 4E), suggesting that P44-78 transcripts rather than P44-18 transcripts were predominant in the infected NB4 cells. We further compared the

Table 1. Five P44-related genes with high expression ordered by RPKM in each of 5 *A. phagocytophilum*-infected cells

Cell line	Ranking[1)]	P44-related gene[3)]	Locus tag	Old locus tag	RPKM	Ranking in the other cell line				
						HL60.1	HL60.2	THP1	NB4	RF6A
HL60.1	1	P44-2b	APH_RS04960	APH_1229	4,498.0	—	17	23	31	4
	2	P44-2	APH_RS04720	APH_1176	3,392.2	—	46	39	80	28
	3	P44-18ES	APH_RS04925	APH_1221	3,315.0	—	3	1	1	3
	4	P44-47	APH_RS04610	APH_1152	2,375.3	—	2	34	17	55
	5	P44-49	APH_RS05020	APH_1242	1,663.6	—	23	26	21	56
HL60.2[2)]	1	P44-23	APH_RS05075	APH_1256	4,395.8	6	—	21	3	80
	2	P44-47	APH_RS04610	APH_1152	3,316.4	4	—	34	17	55
	3	P44-18ES	APH_RS04925	APH_1221	2,336.0	3	—	1	1	3
	4	P44 C-terminal	APH_RS06100	APH_1185	837.0	13	—	101	28	9
	5	P44-30	APH_RS05505	APH_1352	807.9	9	—	4	6	8
THP-1	1	P44-18ES	APH_RS04925	APH_1221	7,445.9	3	3	—	1	3
	2	P44-18	APH_RS04795	APH_1194	3,975.2	37	24	—	11	45
	3	P44-78	APH_RS05385	APH_1325	1,257.5	7	8	—	2	7
	4	P44-30	APH_RS05505	APH_1352	1,028.4	9	5	—	6	8
	5	P44-4	APH_RS04620	APH_1154	764.1	78	72	—	39	48
NB4	1	P44-18ES	APH_RS04925	APH_1221	2,222.4	3	3	1	—	3
	2	P44-78	APH_RS05385	APH_1325	2,046.2	7	8	3	—	7
	3	P44-23	APH_RS05075	APH_1256	1,840.8	6	1	21	—	80
	4	P44-79	APH_RS05515	APH_1355	921.0	20	11	14	—	15
	5	P44[4)]	APH_RS05530	APH_1359	896.8	8	7	8	—	24
RF/6A	1	P44-51	APH_RS05495	APH_1350	890.0	47	40	15	38	—
	2	P44-6	APH_RS04465	APH_1121	464.1	38	35	16	22	—
	3	P44-18ES	APH_RS04925	APH_1221	429.9	3	3	1	1	—
	4	P44-2b	APH_RS04960	APH_1229	387.4	1	17	23	31	—
	5	P44-13	APH_RS04995	APH_1238	356.8	14	30	29	34	—

[1)]: P44-related genes ranked in the top 5 in the expression of *p44/msp2* multigene family based on RPKM in each of infected cells.
[2)]: *A. phagocytophilum*-infected HL60.2 was continuously passaged for 1 additional month after *A. phagocytophilum*-infected HL60.1.
[3)]: P44-related genes were previously assigned as members of the *p44/msp2* multigene family by Dunning Hotopp et al. (8).
[4)]: P44-related gene, but it has not been specifically assigned in members of the *p44/msp2* multigene family.
RPKM, reads per kilobase per million.

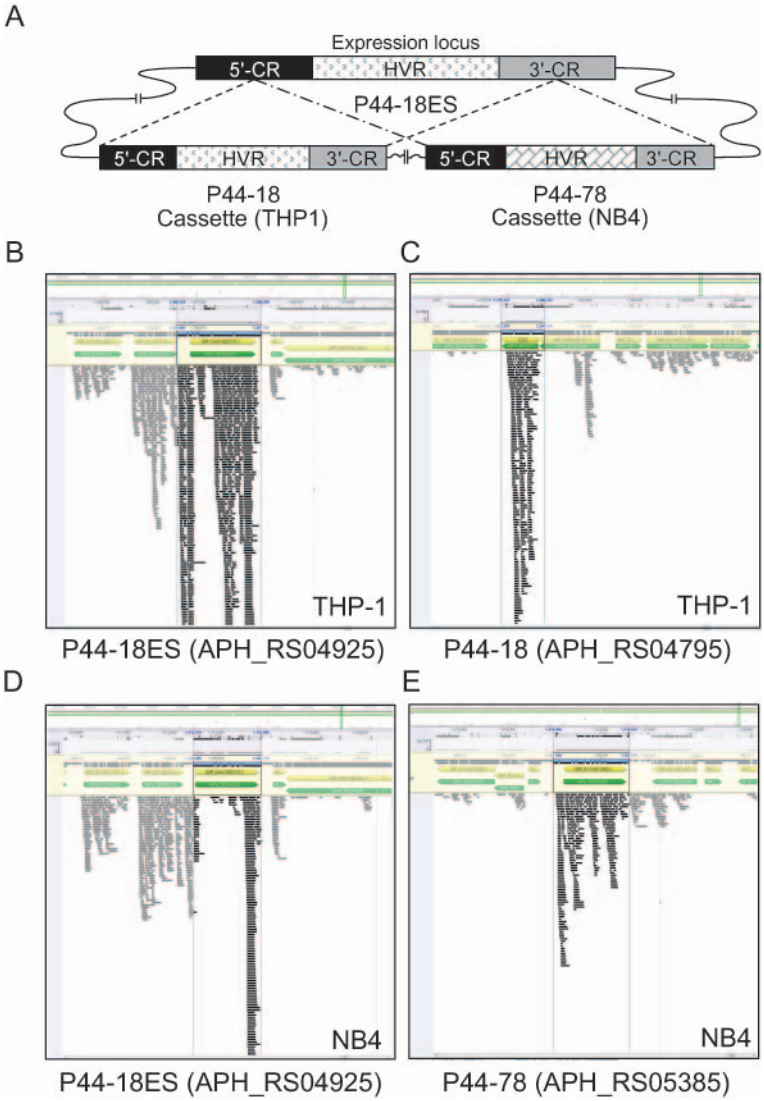

Fig. 4. (Color online) Representative P44-related loci of *A. phagocytophilum* genome mapped by RNA-seq reads. (A) P44-expression locus (P44-18ES) and 2 donor cassette loci (P44-18 and P44-78) on *A. phagocytophilum* HZ genome (NC_007797.1) were illustrated. "HVR": hypervariable region, "5′-CR": 5′-end conserved region, and "3′-CR": 3′ end -conserved region. Mapping images show (B) P44-18ES at the expression locus in infected THP-1, (C) P44-18 donor cassette locus in infected THP-1, (D) P44-18ES at the expression locus in infected NB4, and (E) P44-78 donor cassette locus in infected NB4. Locus tag is shown in parenthesis.

DEGs of P44 transcripts between the HL60.1 cell line and other cell lines using edgeR. The transcriptional level of P44-23 was higher in HL60.2 cells than in HL60.1 cells; however, the levels of P44-2b and P44-2 in HL60.2 cells were downregulated (Table 2). Furthermore, the expression level of P44-18 was highly upregulated in THP-1 cells, and the levels of P44-2b, P44-2, P44-47, and P44-49 with high RPKM values in HL60.1 cells were downregulated. Therefore, the results of DEG analysis were consistent with the results (RPKM values) of expression profile analysis in Table 1. In the case of NB4 cells, all 5 P44-related genes with statistically significant differential expression were highly downregulated at the transcriptional level (Table 2); however, these 5 genes were not part of the P44-related genes of infected NB4 cells with high RPKM values (Table 1). The predominant shifts of P44 transcript species in the respective cell lines are summarized in Fig. 1A.

DISCUSSION

The P44 proteins of *A. phagocytophilum* were first identified as immunodominant proteins recognized by the sera of patients in 1998 (2). Subsequently, P44 proteins were found to be encoded by the *p44/msp2* multigene family (3–5), and *A. phagocytophilum* can express a variety of P44 transcript species (similar but not identical to each other) in patients with HGA as well as in infected mammalian hosts and ticks (6,7,11,12,20–22). The differential expression of the *p44/msp2* multigene family may be attributed to gene conversion through the RecF pathway; thus, *A. phagocytophilum* could use antigenic variation to escape from host immune defense (9,10,14). Genomic and proteomic analyses have revealed that P44-18 is likely to be predominantly expressed by *A. phagocytophilum* isolates cultured in HL-

Table 2. DEG analysis of P44-related genes between *A. phagocytophilum*-infected HL60.1 and each of other infected cells by edgeR

Sample1 vs Sample2[1)]	P44-related gene	Locus tag	Old locus tag	$\log_2$ (fold change)[2)]	p[3)]	q[3)]
HL60.1 vs HL60.2	P44-23	APH_RS05075	APH_1256	1.6	9.06E−10	3.56E−07
HL60.1 vs HL60.2	P44-49	APH_RS05020	APH_1242	−3.0	4.37E−06	1.03E−03
HL60.1 vs HL60.2	P44-32	APH_RS05210	APH_1287	−3.4	2.44E−05	3.59E−03
HL60.1 vs HL60.2	P44-15	APH_RS06105	APH_1226	−3.7	1.99E−04	2.35E−02
HL60.1 vs HL60.2	P44-2b	APH_RS04960	APH_1229	−4.1	2.06E−13	1.21E−10
HL60.1 vs HL60.2	P44-2	APH_RS04720	APH_1176	−5.6	2.74E−17	3.23E−14
HL60.1 vs THP1	P44-18	APH_RS04795	APH_1194	4.0	4.73E−13	2.79E−10
HL60.1 vs THP1	P44-4	APH_RS04620	APH_1154	3.8	1.33E−07	2.60E−05
HL60.1 vs THP1	P44-23	APH_RS05075	APH_1256	−2.5	6.39E−05	9.41E−03
HL60.1 vs THP1	P44-57	APH_RS03560	APH_0900	−3.1	2.09E−04	2.73E−02
HL60.1 vs THP1	P44-49	APH_RS05020	APH_1242	−3.4	2.75E−07	4.63E−05
HL60.1 vs THP1	P44-2b	APH_RS04960	APH_1229	−4.3	1.13E−11	4.42E−09
HL60.1 vs THP1	P44-47	APH_RS04610	APH_1152	−4.3	1.80E−10	5.29E−08
HL60.1 vs THP1	P44-12	APH_RS05055	APH_1249	−4.4	5.26E−09	1.24E−06
HL60.1 vs THP1	P44-2	APH_RS04720	APH_1176	−5.0	1.76E−13	2.07E−10
HL60.1 vs NB4	P44-47	APH_RS04610	APH_1152	−4.1	1.26E−04	3.42E−02
HL60.1 vs NB4	P44-12	APH_RS05055	APH_1249	−4.9	2.87E−05	1.13E−02
HL60.1 vs NB4	P44-2b	APH_RS04960	APH_1229	−5.6	5.12E−07	3.02E−04
HL60.1 vs NB4	P44-57	APH_RS03560	APH_0900	−5.7	1.45E−04	3.42E−02
HL60.1 vs NB4	P44-2	APH_RS04720	APH_1176	−7.3	1.02E−08	1.20E−05

[1)]: Differential expressed gene analysis (DEG) for RF/6A is not available, because of low mapped read counts.
[2)]: Log_2 (fold change) = $\log_2$ (sample2_RPKM)/$\log_2$ (sample1_RPKM).
[3)]: Statistical significances of each P44-related gene ($p < 0.05$ and $q < 0.05$ in edgeR).

60 cells (8,23). On the other hand, tiling array analysis of *A. phagocytophilum* isolates has demonstrated the strong fluorescent signals of P44-47 and P44-35 in infected HL-60 cells and those of P44-39 and P44-51 in infected HMEC-1 cells, associated with the respective hypervariable regions (24). Furthermore, global proteomic analysis has revealed that 110 out of 113 *p44*/*msp2* paralogs including *p44* donor cassettes are differentially expressed in infected HL-60 cells at the protein level (25). As there is no host immune pressure in the cell culture, a shift in the population of different P44-expressing *A. phagocytophilum* may occur and involve host cell fitness (adaptation/selection/interaction) rather than gene conversion. In this study, the improved infectivity of *A. phagocytophilum* in the long-term passage culture with the same cell lines may be attributed to cell culture bias due to host cell adaptation/selection/interaction. We also demonstrated the predominant shift of different P44-expressing *A. phagocytophilum* among 4 cell lines and even in the same cell line (HL60.1 and HL60.2). Previously, P44-18 was found to be predominant in HL-60 cells (23), probably due to host cell adaptation/selection/interaction after isolation; however, it was not predominant in the culture of HL-60 cells in the current study. Interestingly, in our previous studies, the P44-47 and P44-60 transcripts were found to be predominantly expressed in infected THP-1 cells, and the expressed antigens could react with the sera of patients (15,16); however, the P44-18 transcript was predominant in the current study. In addition to P44 expression, the overall gene expression of *A. phagocytophilum* among HL-60, THP-1, and NB4 cells (human hematopoietic cell lines) appeared to be correlated. However, the correlation between HL-60 and RF/6A cells (rhesus blood vessel endothelial cell line) was lower ($R^2 = 0.3584$) even though the mapped reads on the *p44* loci were not abundant, suggesting that the gene expression of *A. phagocytophilum* may be affected by host cell origin such as animal species and/or cell type.

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Conflict of interest None to declare.

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