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Borrelia burgdorferi Population Dynamics and Prototype Gene Expression during Infection of Immunocompetent and Immunodeficient Mice

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The population dynamics of *Borrelia burgdorferi* were quantified by real-time PCR targeting the *flaB* gene in skin (inoculation site, noninoculation site, and ear), heart (heart base and ventricle), quadriceps muscle, and the tibiotarsal joint at 1, 2, 4, 6, and 8 weeks after intradermal inoculation in C3H and C3H-*scid* mice. In addition, RNA transcription was assessed for several prototype genes, including *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42*. Spirochete numbers were equivalent in C3H and C3H-*scid* mice at 1 or 2 weeks and then declined in C3H mice, but they continued to rise and then plateaued in C3H-*scid* mice. Gene transcription was likewise higher in C3H-*scid* mice than in C3H mice, particularly at 4 or more weeks of infection. Gene transcription showed variation among tissues, with the highest levels of transcription in heart and joint tissue, which are sites of inflammation.

Borrelia burgdorferi, the agent of Lyme disease, has a complex life cycle and must rapidly adapt to diverse microenvironments within resting ticks, feeding ticks, and mammalian or avian hosts. Aside from survival, these events must allow maintenance of the bacterium within the dormant tick, proliferation during tick feeding to enhance transmission, migration from the tick midgut to salivary glands during tick feeding, transmission into the host during tick feeding, dissemination within the host, immune evasion within the host, and, finally, successful acquisition by a new vector feeding upon an infected host. It is generally believed that these events are facilitated by the differential expression of selected genes. This has been best exemplified by a widely accepted paradigm of reciprocal expression and down-regulation of outer surface protein A (OspA) and OspC during tick feeding and transmission. OspA is abundantly expressed on spirochetes within unfed ticks (5), but is down-regulated during the course of feeding by the tick and entry into the mammalian host. In contrast, OspC appears to be expressed during feeding and transmission (19, 50).

Among other cues, these events have been shown to be triggered by changes in pH, cell density, temperature, and factors within the mammalian host (1, 13, 14, 20, 25, 37, 39, 40, 44–46, 49, 50, 52, 56). A recent study utilized whole-genome arrays to examine temperature-induced changes in *B. burgdorferi* and found that 215 open reading frames were differentially expressed at two different temperatures: 133 of them were expressed at temperatures greater than 35°C (40). Studies that have artificially mimicked the multiple conditions that take place in the feeding tick (elevated temperature, reduced pH, and increased cell density) have demonstrated the reciprocal down-regulation of OspA, P22, and Lp6.6 and up-regulation of OspC, decorin binding protein (DbpA), OspF, Mlp-8, and RpoS proteins (34, 55). OspE and some, but not all, of the OspE/F-related proteins (Erps) and OspE/F-like proteins (Elps) are also up-regulated under these conditions (29, 30, 51, 52). Furthermore, a number of *B. burgdorferi* genes have been reported to be exclusively expressed in the mammalian host, including PG (54), P21 (53), BBK2.10 (an Erp) (2), and ElpA1 (30). Among the potential factors that influence *B. burgdorferi* gene expression in the host are humoral and cellular immunity (35).

Although *B. burgdorferi* has a relatively small genome, interpreting the complex gene expression events that take place during these adaptive processes remains a daunting task. The present study quantitatively examined the expression of a selected set of prototype genes relative to spirochete population dynamics at intervals of infection and in selected tissues of immunocompetent C3H and immunodeficient C3H-*scid* mice. The use of C3H and C3H-*scid* mice allowed evaluation of the effects of host-acquired immune response on these events during the evolution of acquired immunity and at intervals after development of the immune response.

MATERIALS AND METHODS

Mice. Specific-pathogen-free C3H/HeN (C3H) and C3H/Smn.CIcrHsd-*Prkdc*^{scid} (C3H-*scid*) mice (3 to 5 weeks old) were purchased from Frederick Cancer Research Center (Frederick, Md.) and Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.), respectively. Mice were maintained in an infectious disease containment room, fed commercial mouse diet ad libitum, and humanely killed with carbon dioxide narcosis followed by exsanguination by cardiocentesis.

***B. burgdorferi*.** A clonal strain of *B. burgdorferi* sensu stricto (cN40) was cultured in modified BSK II medium (4). Thirty C3H mice and 25 C3H-*scid* mice were infected by intradermal inoculation at the dorsal thoracic midline with 10^3 mid-log-phase spirochetes each. This dose of spirochetes has been determined to efficiently infect all inoculated mice, yet the introduced antigen is insufficient to induce an OspA antibody response (6, 8). Inoculated mice were randomly divided into groups of five mice each, and at 1, 2, 4, 6, and 8 weeks after inoculation, five C3H and five C3H-*scid* mice were necropsied at each time point. To confirm infection, cells from the urinary bladder, blood, and the inoculation site were cultured from each mouse, as described previously (7).

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TABLE 1. Oligonucleotide PCR primers and TaqMan internal probes of *B. burgdorferi* genes targeted in this study

Gene	Primer	Sequence (5′→3′)	Size (bp)	Probe	Internal probe sequence
flaB	FL-571F FL-677R	GCAGCTAATGTTGCAAATCTTTTC GCAGGTGCTGGCTGTTGA	107	FL-611P	AAACTGCTCAGGCTGCACCGGTTC
ospA	OSPA-288F OSPA-369R	TGAAGGCGTAAAAGCTGACAAA TTCTGTTGATGACTTGTCTTTGGAA	142	OSPA-266P	CAATTTTGAACGATCTAGGTCAAACCACACTTGA
ospC	OSPC-108F OSPC-235R	TGTTAAAGGGCCTAATCTTACAGAAATAA TACCAATAGCTTTGGTAGCAAGTTCAT	128	OSPC-147P	TACAGAATCTAACGCAGTTGTTCTGGCCGT
dbpA	P22-172F P22-266R	GATAACAATGTAAATTTTGCTGCCTTT GTAGCTCGCACTTTTGCTTCAAG	95	P22-203P	ATAGTGAAACAGGTAGCAAGGTATCAGAAAATTCATTCA
arp	P37-663F P37-767R	TACACACCCCATATTTGATCACATTACT TTGCTATCACCACCAATTTCAAGT	105	P37-705P	TCCCGGACAAGATTCTATATCCAATACATGGG
vlsE	VLSE-N40F VLSE-N40R	TGATATGAAGAAGAAGGATAAGGTTGCT TTTAGTACATCCTCCTTCTTACCAACAG	147	VLSE-N40P	ATTAGTAACCGAAAACTTCCCATCTTTAGCCAATCC
fbp	BBK32-101 BBK32-230	AACAACAAGATGAATATAAAGGGATGACT TTTGGCCTTAAATCAGAATCTATAGTAAGA	130	BBK32-167P	TCACCACTTTCACCGCTAAGGGAATTTAAACT
oppA-2	OPPA-1207 OPPA-1304	AACGAAGAATGGACAACATACTTAAACA AATGTCAAAGGATCAGCATAATCG	98	OPPA-1277	ATCCATCCTGCTCTTGCTATTTCATAATTTCCATTT
p37-42	P374-759F P374-894R	TGAGGCTAAGGATAAACTAGCAGAATCT AGCAAAGTCTATAGCTCTGTATGCTAAATT	136	P374-855P	TTGCATATCACGTCCGTTAAAACTGCCA

Analysis of spirochete population dynamics and RNA transcription. For quantitative analysis of spirochetes, samples of the inoculation site, noninoculation site (1 to 2 cm from the site of inoculation), ear, heart base, ventricular myocardium (the heart was bisected through the atria and ventricles, with one-half used for sampling the heart base and ventricles for nucleic acid extraction and the other half processed for histology), quadriceps muscle, and the left tibiotarsal joint were collected from each mouse. The samples were immediately weighed, snap-frozen in liquid nitrogen, pulverized, and homogenized and then were split equally for DNA or RNA extraction. DNA was extracted with DNeasy tissue kits according to the manufacturer's instructions (Qiagen, Valencia, Calif.). The copy number of each *B. burgdorferi* target gene was expressed per milligram of tissue weight. Total RNA was purified with RNeasy mini kits according to the manufacturer's instructions (Qiagen). Samples were homogenized with a QIAshredder and then treated with RNase-free DNase I prior to elution. The concentration and purity of extracted RNA were determined by measuring the A_{260} and A_{280}. The extracted total RNA was stored at −80°C until use. The extracted total RNA was subjected to two separate reactions—one to synthesize cDNA and the other to test for DNA contamination—as described previously (31).

For quantitative analysis of DNA or cDNA extracted from tissue samples, real-time PCR was used and optimized, as previously described (31). Briefly, the sensitivity and reproducibility of real-time PCR for each target gene were determined by spiking normal tissue samples with known numbers of spirochetes or target DNA for each gene to establish standard curves. Each standard assay was performed in triplicate, with nearly identical results derived from replicate samples. The analytical sensitivity for each target gene DNA was in the range of 1 to 10^9 spirochetes and that for RNA was 10^1 to 10^9 spirochetes, with a yield of detection close to 90% of the calculated amount of known target in each sample. All assays were performed with positive and negative control samples, and control results verified the validity of positive and negative findings. False-positive amplification was not detected (the threshold cycle [C_T] was always 40).

Three oligonucleotides, two primers, and an internal probe for each *B. burgdorferi* target gene were selected by using Primer Express software (PE Biosystems, Foster City, Calif.), as shown in Table 1. Internal probes contained 6-carboxy fluorescein as a reporter dye at the 5′ end and 6-carboxy-tetramethyl rhodamine as a quencher dye at the 3′ end. Probes included sequences of genes encoding regions of flagellin, OspA, OspC, DbpA, arthritis-related protein (Arp), variable membrane protein-like expression site protein (VlsE), fibronectin binding protein (Fbp), oligopeptide permease protein A-2 (OppA-2), and a 37-kDa putative lipoprotein of unknown function (P37-42). The gene designation, based upon the published B31 sequence (26), and the GenBank accession number for each gene (prefaced by the *B. burgdorferi* isolate B31 or N40 sequence) are as follows: *flaB*, *bbo294*, B31-AE001126; *ospA*, *bba15*, N40-M57248; *ospC*, *bbb19*, N40-U04240; *dbpA*, *bba24*, N40-U63932; *arp*, *bbf01*, N40-AF050212; *fbp*, *bbk32*, N40-AF050213; *oppA-2*, *bbo329*, B31-AAC66707; and *p37-42*, *bbk47*, N40-AF035553. When B31 accession sequences were used to design primers, it was determined that N40 primer sequences were identical to B31. The *vlsE* primers were designed to target an invariable region (IR4 to IR6) of the N40 *vlsE* cassette (57). The N40-specific sequences for each of these targets were previously determined from clones derived from a N40 genomic expression library, as described previously (21). To quantify the copy number of each DNA gene target, plasmid standards were prepared to create absolute standard curves, as described previously (31).

Pathology. The right knee, right tibiotarsus, left knee, (the left tibiotarsus was used for nucleic acid extraction), and remainder of the heart (including the tissue at the base with thymus) were fixed in formalin, paraffin embedded, and processed by routine histologic techniques. Sections stained with hematoxylin and eosin were coded and blindly examined for lesions (arthritis and carditis). Pathology was utilized to confirm the disease status of C3H and C3H-*scid* mice, as well as the immune phenotype (presence or absence of thymus in tissue at the base of the heart).

Statistics. Statistical comparisons between infected C3H and C3H-*scid* mice were made with StatView (PowerPC version; SAS Institute, Inc., Cary, N.C.). Multiple-comparison analysis was made with one-way analysis of variance followed by a least-squares difference post hoc test. Calculated P values of <0.05 were considered significant; significant differences are noted in the Results section.

RESULTS

All mice that were inoculated with *B. burgdorferi* were subsequently culture positive in one or more tissues at all intervals (Table 2). Thus, all experimental mice were deemed suitable for DNA analysis. Notably, only 1 of 30 C3H mice had a positive blood culture, whereas all C3H-*scid* mouse blood samples were culture positive. Carditis appeared in both groups at the 2-week interval; was regressing at the 4-, 6-, and 8-week

TABLE 2. Culture results from tissues of C3H and C3H-*scid* mice at intervals after intradermal inoculation with 10^3 *B. burgdorferi* cN40 spirochetes

Genotype and tissue	No. of culture-positive samples/no. cultured at wk postinoculation:				
	1	2	4	6	8
C3H					
Inoculation site[a]	5/5	5/5	5/5	5/5	5/5
Blood	0/5	1/5	0/5	0/5	0/5
Urinary bladder	5/5	5/5	5/5	5/5	5/5
C3H-*scid*					
Inoculation site	5/5	6/6	5/5	5/5	5/5
Blood	5/5	6/6	5/5	5/5	5/5
Urinary bladder	5/5	6/6	5/5	5/5	5/5

[a] Subcutaneous tissue beneath the site of inoculation.

intervals in C3H mice; and remained active in the C3H-*scid* mice at 2, 4, 6, and 8 weeks. Arthritis, involving nearly all knees and tibiotarsi examined, appeared in the C3H-*scid* mice at 2 weeks and progressed in severity through subsequent intervals. Arthritis was mild and oligoarticular in the C3H mice; it did not appear until 4 weeks and then regressed. These observations were commensurate with previous events in C3H and C3H-*scid* mice inoculated intradermally with low doses of this clonal strain of *B. burgdorferi* (3, 7, 10, 11).

Spirochete populations were quantified by determining the copy number of *flaB* DNA per mg of tissue, and quantitative *flaB* DNA means and standard deviations are shown in the top panel of Fig. 1 through 7 for each target tissue. These tissues were selected to represent sites with inflammation (heart base and tibiotarsus) and minimal or no inflammation (skin, ventricular myocardium, and quadriceps).

For quantitative analysis of RNA transcription, several prototype genes were selected for study (*flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42*). Results of quantitative mRNA analysis are provided in the lower 9 panels of Fig. 1 through 7 for each target tissue. Because DNA and RNA samples from each mouse tissue represented different target aliquots (despite their common collection, pulverization, and homogenization), we likewise plotted individual RNA copy numbers to depict variation among samples and for comparison with quantitative DNA values from each tissue.

Quantitative values for spirochete numbers (*flaB* copy numbers) in C3H and C3H-*scid* mice appeared to overlap in the first 2 weeks of infection (prior to full effervescence of acquired immunity), and spirochete numbers subsequently rose and then plateaued in C3H-*scid* mice (in the absence of acquired immunity). In tissues that were prone to inflammation (heart base and tibiotarsus), there was no evidence of tissue-specific decline in spirochete populations that coincided with disease resolution in C3H mice, and in fact, spirochetes in these tissues seemed to follow the same dynamics as spirochetes in noninflamed tissues in both C3H and C3H-*scid* mice. The accuracy of quantitative DNA results that were obtained with *flaB* primers was confirmed by processing the same target DNA with *ospA* primers. Similar patterns and levels of spirochete numbers were found with both primer sets (data not shown).

Gene expression patterns were also not absolute as to tissue, stage of infection, or immune status of the host, but RNA levels generally followed the same trends as spirochete population (*flaB* DNA) dynamics, with lower values in C3H mice than in C3H-*scid* mice at 2 weeks and beyond. The only gene that was not expressed in the context of the immunocompetent C3H mouse was *ospA*. Unexpectedly, *ospA* expression was found in many of the tissue samples from C3H-*scid* mice.

Spirochete dynamics and gene expression in skin. Three different skin samples were evaluated: inoculation site (Fig. 1), nearby noninoculation site (Fig. 2), and ear (Fig. 3). Following inoculation, spirochetal *flaB* DNA was detected in the inoculation site at the 1 week, declined at 2 and 4 weeks, and was no longer detectable at 6 or 8 weeks in either C3H or C3H-*scid* mice. This was apparently in contrast to inoculation site culture results, which were positive in all mice. However, PCR results were based on the actual skin (dermis and epidermis) from the site of inoculation, whereas cultures were obtained from the underlying subcutis. Spirochetal *flaB* DNA was first detected in the noninoculation skin site at 1 week in a single C3H-*scid* mouse, but was present at all subsequent intervals. Spirochetes appeared to reach the ear at a more rapid rate, because many samples were *flaB* DNA positive at 1 week. Gene expression profiles of the ear and the noninoculation site of C3H mice suggested that *flaB* and *oppA-2* were expressed at low levels at all intervals, whereas other genes were expressed at early, but not later, intervals. All were expressed at all intervals in C3H-*scid* mice. Skin appeared to harbor somewhat fewer spirochetes compared to the other tissues.

Spirochete dynamics and gene expression in heart. Two different heart sites were evaluated: heart base (Fig. 4) and ventricular muscle (Fig. 5). Heart base was selected because this is the site where carditis occurs, and ventricular myocardium was selected because this portion of the heart has minimal or no inflammation (3, 11). Based upon *flaB* DNA copy numbers, there were consistently fewer spirochetes per milligram of heart base tissue weight in C3H mice than in to C3H-*scid* mice at weeks 2, 4, 6, and 8 ($P = 0.017$, 0.021, 0.0001, and P 0.001, respectively). There was also a significant decrease in spirochetes in C3H mice at week 8 compared to the numbers at weeks 2 ($P = 0.023$) and 6 ($P = 0.017$), suggesting that acquisition of immunity and carditis regression in C3H mice correlated with declining spirochete numbers. Evaluation of gene expression in C3H and C3H-*scid* mice revealed RNA transcription of *flaB*, *ospC*, *dbpA*, *arp*, *fbp*, *oppA-2*, and *p37-42* at all intervals, with generally higher levels of expression in C3H-*scid* mice at 4 weeks and beyond compared with C3H mice. In contrast, *ospA* mRNA was not detected in the heart tissue of C3H mice at any time point, but was detected in C3H-*scid* mice. In comparison to other tissues, *ospC* was expressed at higher levels in both C3H and C3H-*scid* mice, but expression declined in C3H mice at 4 weeks and beyond. At week 1 after inoculation, *vlsE* mRNA was at an undetectable level, and at later time points, it was detected inconsistently and at low levels in C3H mice.

Comparison of spirochete numbers and gene expression in the ventricular myocardium between C3H and C3H-*scid* mice revealed patterns similar to those in the heart base, with significantly lower numbers of spirochetes in C3H mice than in C3H-*scid* mice at weeks 4, 6, and 8 ($P = 0.049$, 0.0053, and 0042, respectively).

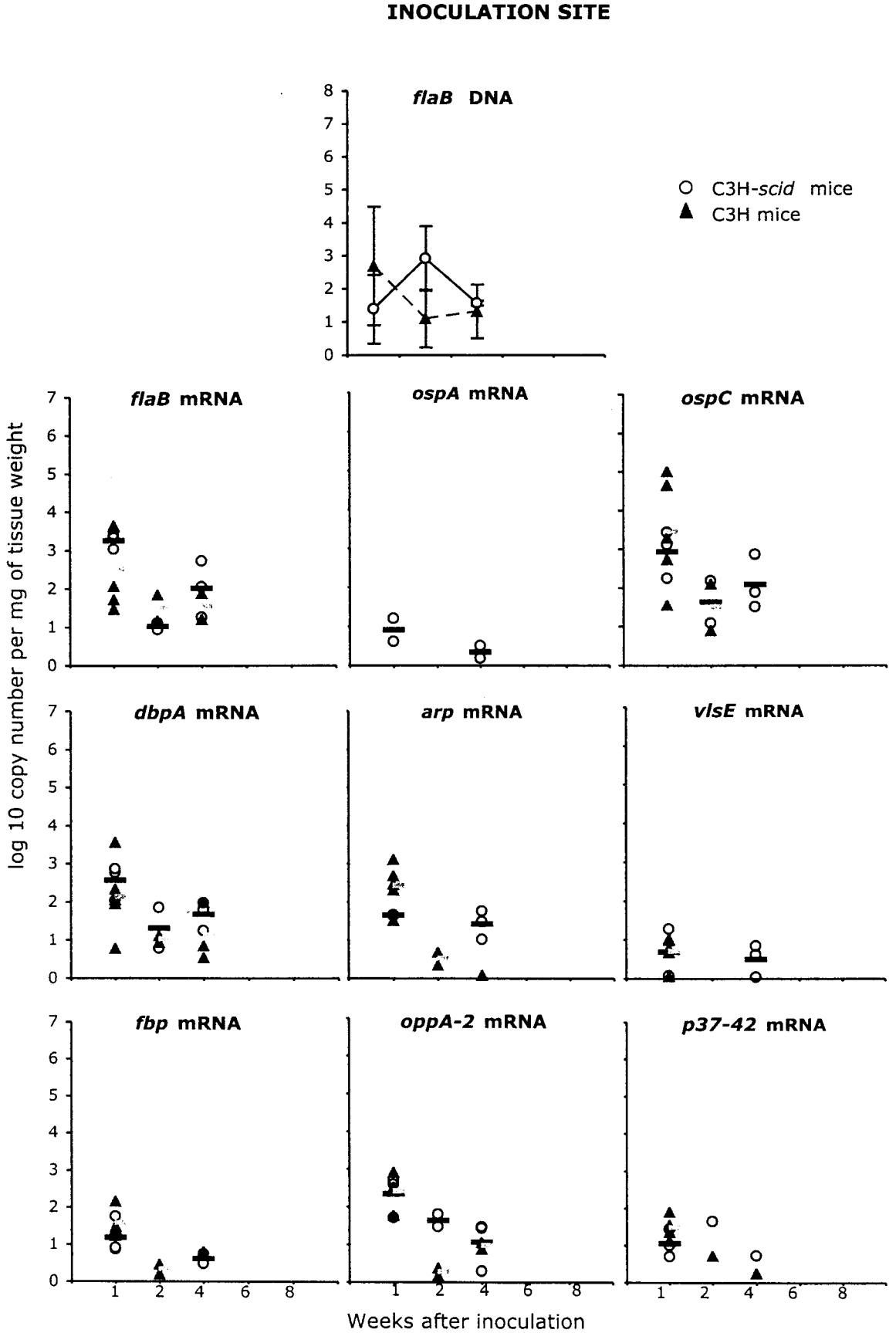

FIG. 1. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2* and *p37-42* RNA in skin at the site of intradermal inoculation at 1, 2, 4, 6, and 8 weeks in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

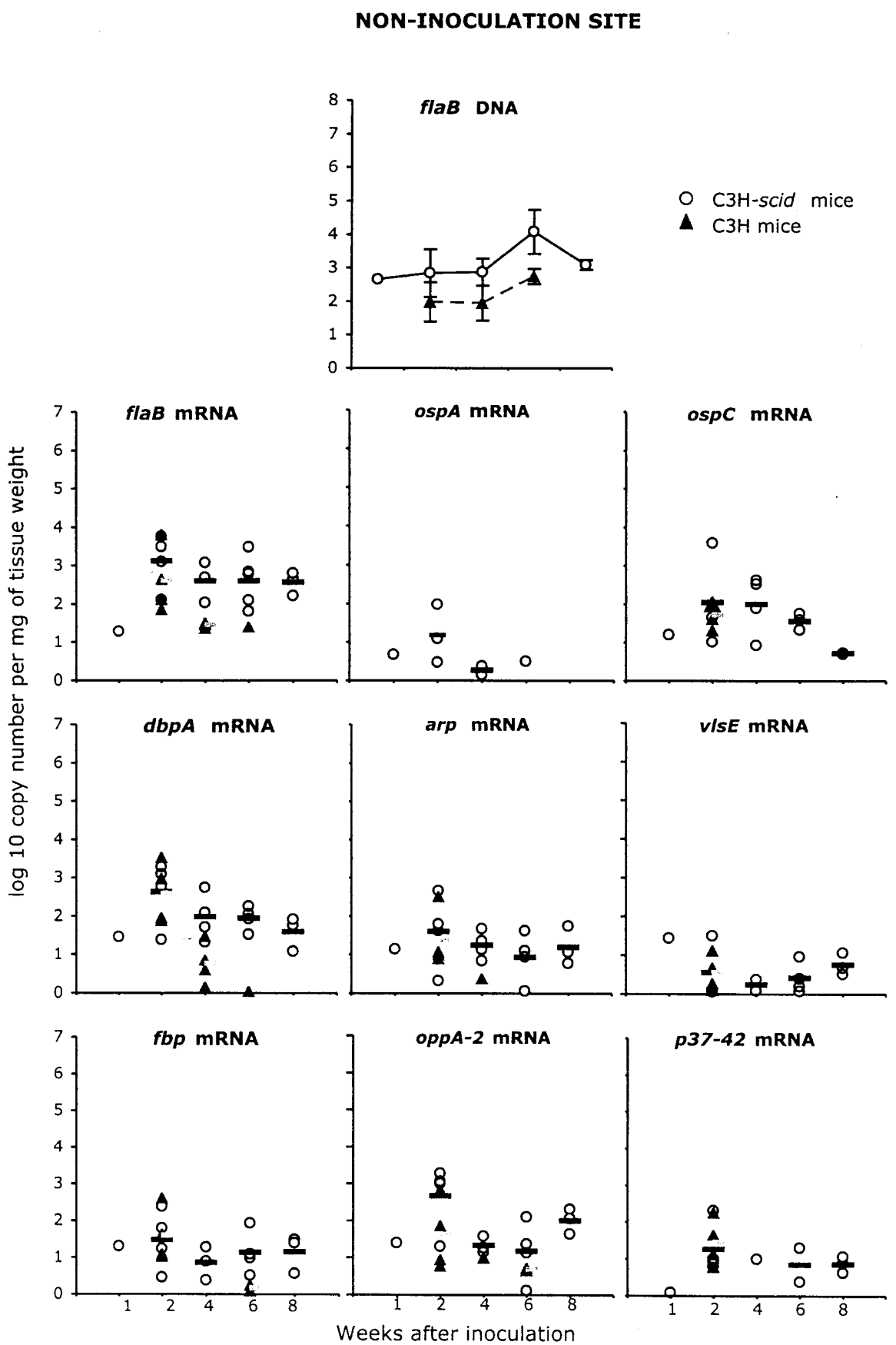

FIG. 2. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42* RNA in skin distant from the site of intradermal inoculation at 1, 2, 4, 6, and 8 weeks in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

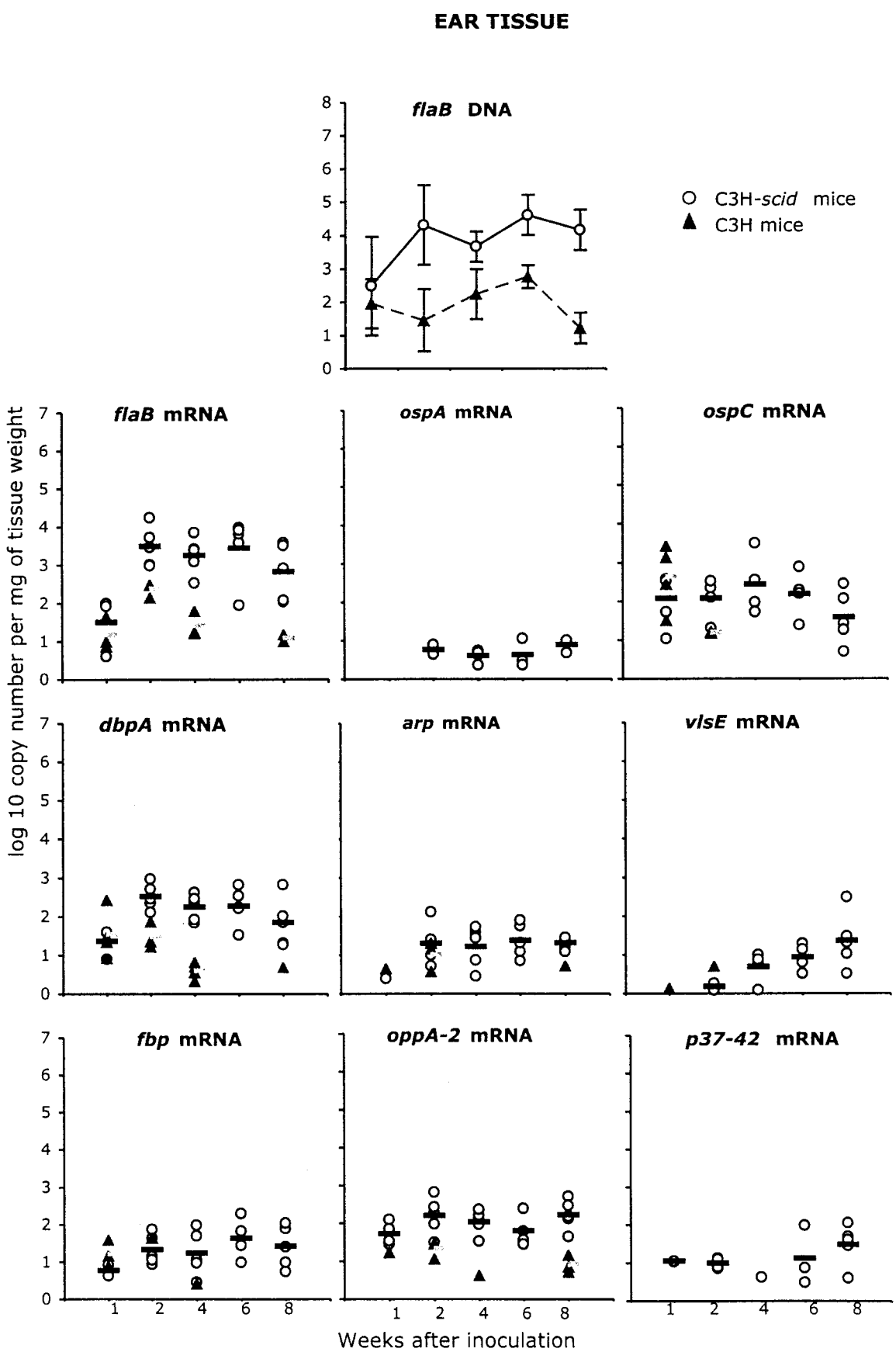

FIG. 3. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42* RNA in ear tissue at 1, 2, 4, 6, and 8 weeks after inoculation in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

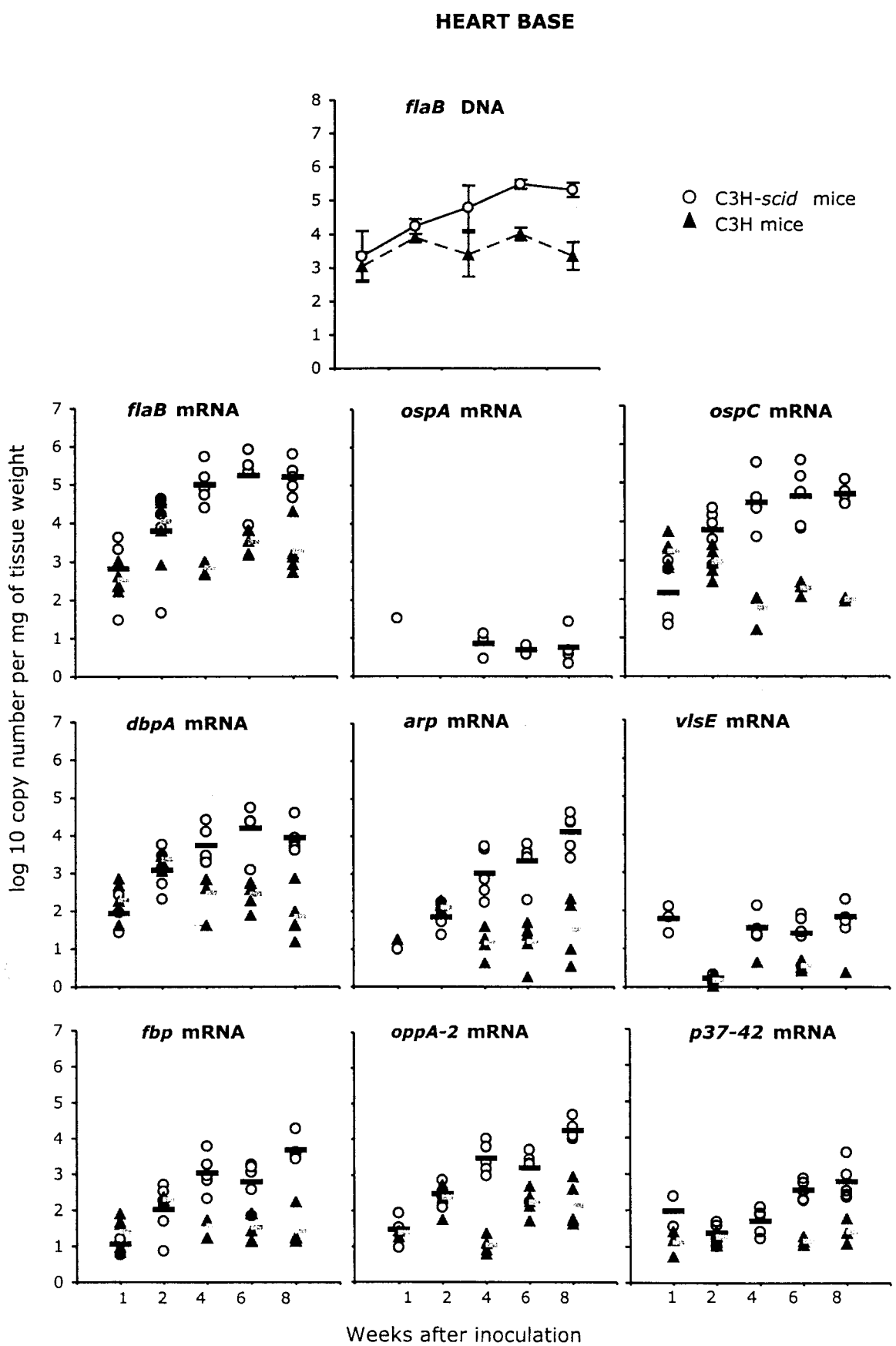

FIG. 4. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42* RNA in heart base tissue at 1, 2, 4, 6, and 8 weeks after inoculation in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

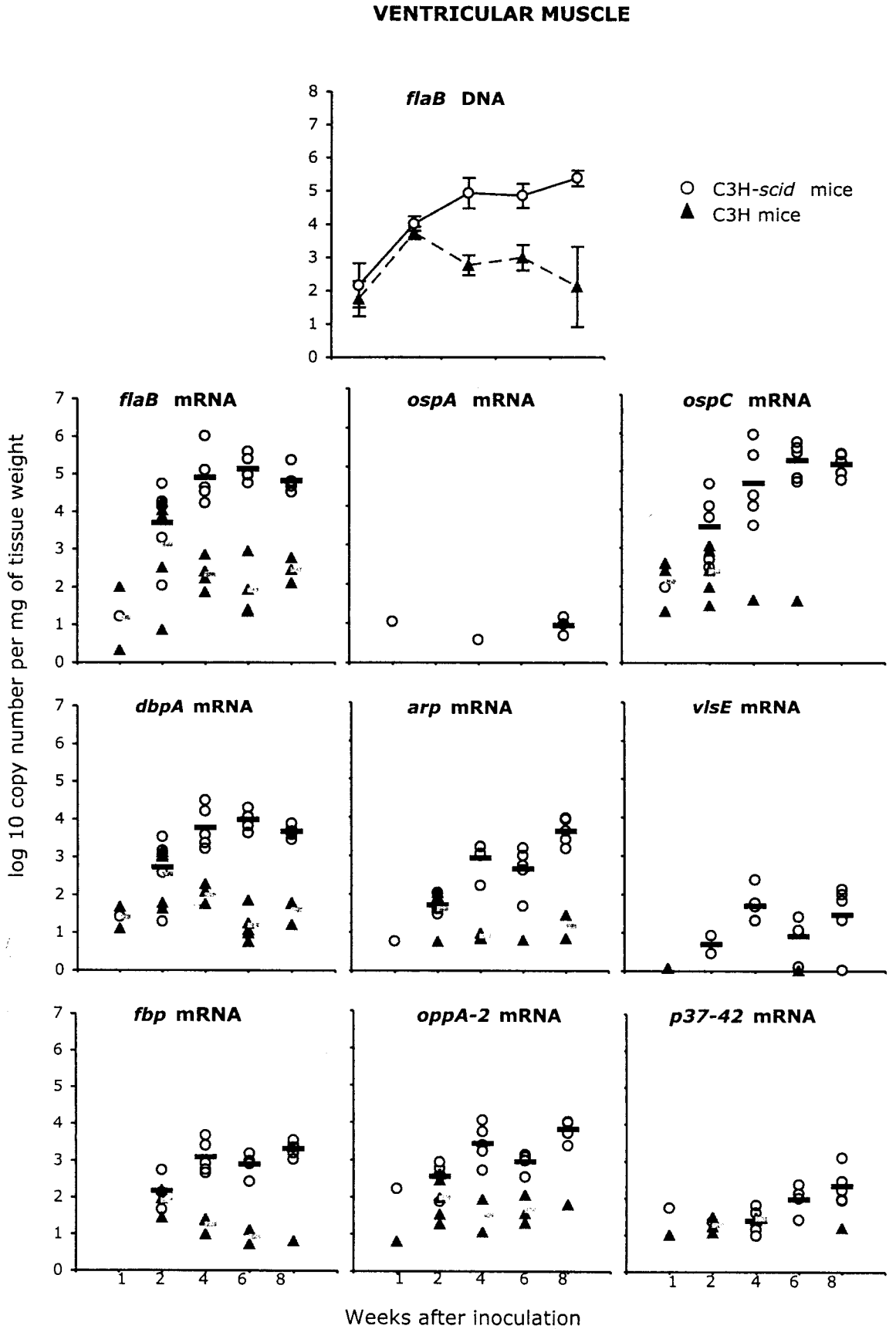

FIG. 5. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42* RNA in cardiac ventricular muscle at 1, 2, 4, 6, and 8 weeks after inoculation in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

Spirochete dynamics and gene expression in muscle and joint tissues. Two adjacent musculoskeletal tissues were evaluated: tibiotarsal joint (Fig. 6) and nearby quadriceps muscle (Fig. 7). The tibiotarsal joints develop progressively severe fibrinopurulent and proliferative arthritis in C3H-*scid* mice (11), and the quadriceps femoris muscle develops mild or no nonsuppurative inflammation in C3H-*scid* mice (15, 38). Spirochetal DNA was not consistently detected in tibiotarsi until week 2 in either C3H or C3H-*scid* mice. Analysis of *flaB* DNA revealed significantly lower numbers of spirochetes in tibiotarsal joints of C3H mice compared to those in C3H-*scid* mice at weeks 2, 6, and 8 postinoculation ($P = 0.0008$, 0.0007, and 0.017, respectively). Spirochetes peaked at week 4 in C3H mice and then declined slightly at weeks 6 and 8, whereas in C3H-*scid* mice, spirochete numbers peaked at week 6.

Levels of *flaB*, *dbpA*, and *arp* mRNA in tibiotarsal joints of C3H mice compared to C3H-*scid* mice at 2, 4, 6, and 8 weeks paralleled *flaB* DNA quantitative dynamics. As in other tissues, *ospA* mRNA was detected in many (but not all) C3H-*scid* mice, but none of the C3H mice. Transcription of *ospC* was inconsistently detected in joints of C3H mice, but its transcription was commonly detected in all C3H-*scid* mice at every time point. There was a notable lack of *p37-42* transcription in joints of C3H mice at any time point, in contrast to C3H-*scid* mice. Transcription of *fbp* and *oppa-2* was detected in nearly all joint samples of C3H-*scid* mice and some C3H mice, and *vlsE* mRNA was either absent or detected at low levels in C3H mice, but was consistently present in C3H-*scid* mice.

Spirochetes were detected in quadriceps muscle as early as 1 week after inoculation in C3H-*scid* mice and at week 2 in C3H mice. Comparison of mean numbers of spirochetes per milligram of tissue weight revealed significantly lower numbers in C3H mice compared to C3H-*scid* mice at weeks 2, 4, 6, and 8 ($P = 0.017$, 0.013, 0.011, and 0.005, respectively). Examination of gene expression in quadriceps muscle of C3H mice revealed that transcriptional activity was lower than that in other tissues in the same group of mice. *flaB* was consistently transcribed in both C3H and C3H-*scid* mice, while transcription of *ospC*, *dbpA*, *arp*, and *oppA-2* mRNA was at low or undetectable levels in C3H mice. Even in C3H-*scid* mice, the level of transcriptional activity was lower than those in the other tissues from the same animals, and only one sample contained detectable *ospA* RNA.

DISCUSSION

This study examined *B. burgdorferi* population dynamics and expression of selected prototype genes in target tissues during disease evolution, disease resolution, and persistent phases of infection in the well-characterized C3H mouse model (3, 10). C3H and C3H-*scid* mice were used to assess the role of host immunity as a modulator of spirochete populations and gene expression, since infected C3H-*scid* mice undergo progressive arthritis and persistent active carditis, whereas disease of the joints and heart resolves in immunocompetent mice (3, 11, 47, 48).

This study selected several prototype genes for analysis for a number of specific reasons. First, this laboratory has cloned and sequenced all of these genes from an N40 genomic library and has found that nonlipidated recombinant proteins of all (except OspA) are reactive with serum from mice infected for 60 to 90 days (unpublished observations). OspA was selected because it has become an important "gold standard" in Lyme disease research, and it is generally believed that it is not expressed during mammalian infection (9, 18). In contrast to OspA, OspC is generally regarded to be reciprocally expressed during tick feeding and mammalian infection (16, 17, 49). OspC has been shown to elicit protective (41, 43) as well as therapeutic (58, 59) immunity, but such immunity is *B. burgdorferi* strain specific, and neither can be demonstrated with OspC immunity in *B. burgdorferi* N40 infection (8, 12).

In addition to OspC, OspA, DbpA, and Fbp have been shown to elicit some degree of protective immunity (22, 24, 28). Furthermore, DbpA is one of only a few proteins that are reactive with serum from infected T-cell-deficient mice, and such serum contains protective and disease-resolving activity (36). DbpA and Fbp were also selected because of their substrate-binding properties, which are believed to be involved in tissue invasion (27, 42). Arthritis-related protein (Arp, BBF01) is the only protein that has been incriminated as an antigenic target for arthritis-resolving, but not protective, immunity (21). VlsE was selected because it undergoes recombination during infection as a potential mechanism for immune evasion (57). FlaB (flagellin) was selected as an immunogenic, but nonprotective antigen (23) that is presumably constitutively expressed. OppA-2 (oligopeptide permease protein) is encoded by a gene that belongs to a family of three genes (*oppA-1*, *oppA-2*, and *oppA-3*) that encode peptide transport and is conserved among different species of Lyme borreliosis spirochetes (33). P37-42 has no known function, but immunity to this protein may also modulate arthritis (21).

Our experience with optimizing the real-time PCR assays has revealed highly reproducible quantitative data from replicate assays of the same sample, with a high degree of detection sensitivity and virtually no false-positive results. We had initially intended to relate quantitative RNA to the quantitative DNA results by homogenizing the tissue and then processing split aliquots of the same sample for DNA or RNA extraction. Despite the reproducibility of amplification results, we found considerable variation in quantitative data from the split aliquots, thus negating the ability to relate RNA levels directly to DNA levels. This variation is likely due to our morphological findings with immunohistochemistry, in situ hybridization, and/or silver stains that have shown that spirochetes in tissues tend to "congregate" in foci and are thus not evenly distributed within a sample (3, 7, 10, 11). We also elected not to relate quantitative spirochete results to host tissue standards, such as actin, because the cellularity of tissues varies among tissues and also varies within tissues whether inflammation is present or not. Furthermore, we found that RNA levels were quite variable among different tissues and mice and were therefore best illustrated as scatter plots that could be compared with quantitative *flaB* DNA levels (Fig. 1 to 7).

Our selected panel of biologically and immunologically relevant prototype genes should provide insight into differential gene expression within the host, within specific tissues, and at defined intervals of infection. Comparison between C3H and C3H-*scid* mice allowed examination of the effects of acquired immunity on these events. Unexpectedly, we found frequent *ospA* expression in all tissues of C3H-*scid* mice at all intervals,

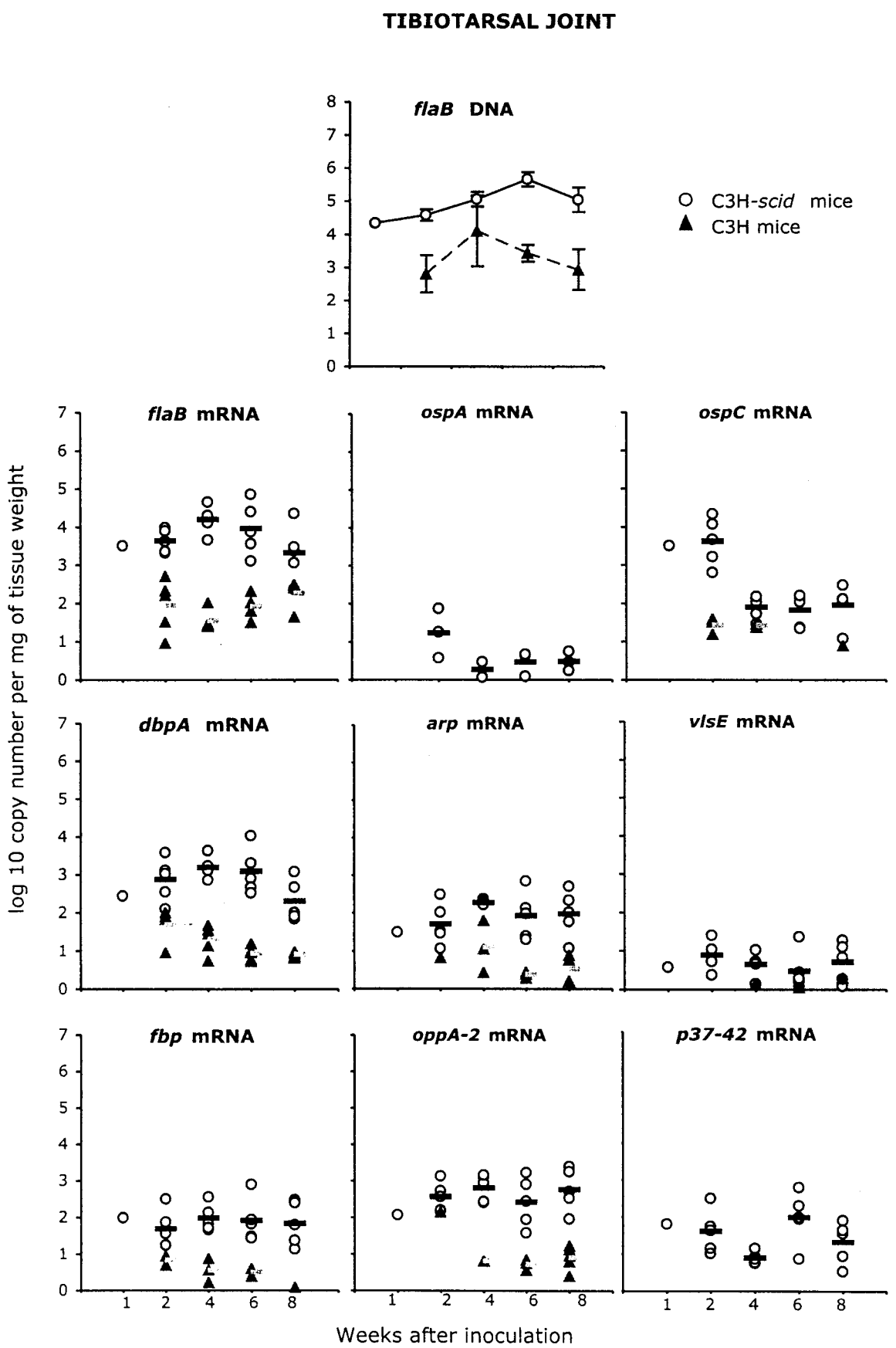

FIG. 6. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42* RNA in tibiotarsal joint tissue at 1, 2, 4, 6, and 8 weeks after inoculation in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

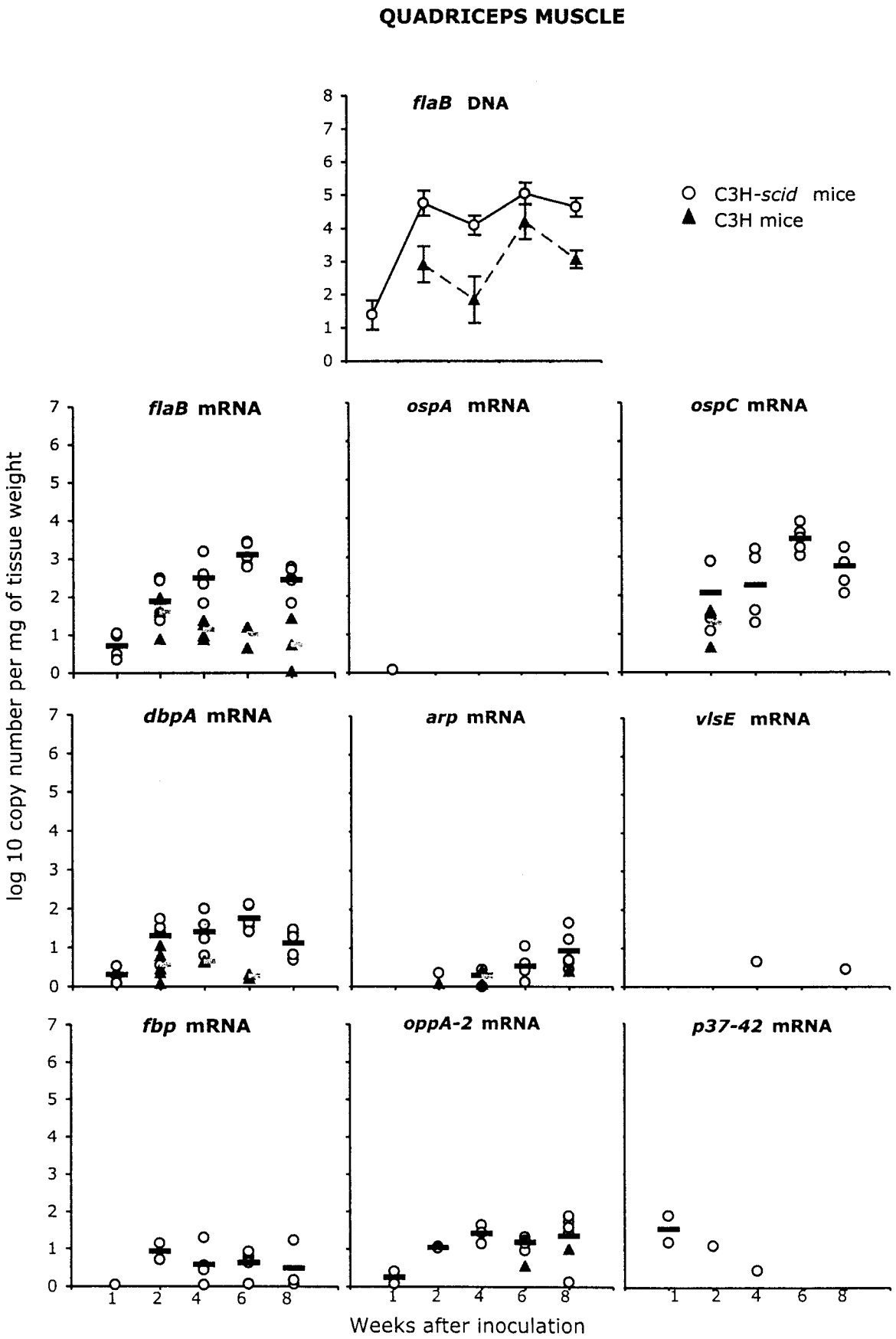

FIG. 7. Quantitative analysis of *B. burgdorferi flaB* DNA (means ± standard deviations [top panel]) and *flaB*, *ospA*, *ospC*, *dbpA*, *arp*, *vlsE*, *fbp*, *oppA-2*, and *p37-42* RNA in quadriceps femoris muscle tissue at 1, 2, 4, 6, and 8 weeks after inoculation in C3H and C3H-*scid* mice. Mean RNA values are represented as horizontal bars (light bars, C3H; dark bars, C3H-*scid*). Values are plotted per milligram of tissue weight.

but not in C3H mice. These results imply that *ospA* transcription is strongly influenced by host immune response.

We selected the inoculation site, noninoculation site, and ear to evaluate the events in different types of skin sites following syringe inoculation with cultured spirochetes, with the caveat that the ear samples contained tissue elements (cartilage) that differ from skin of the thorax. A recent study by our group (31) evaluated spirochete population dynamics and gene expression (*flaB*, *ospA*, *ospC*, *dbpA*, and *arp*) at the site of tick attachment during transmission of infection from tick to host and during acquisition of infection by ticks when feeding upon infected hosts. Most notably, that study revealed that spirochetes entering the uninfected host from infected feeding ticks underwent a local increase in population at the site of tick attachment, but gene expression was minimal in the first 3 days. At 2 weeks of infection, spirochete populations and transcription of *flaB*, *ospA*, *ospC*, *dbpA*, and *arp* were similar to results found in the present experiment in the noninoculation site and ear. The patterns of spirochete populations and gene expression at the site of syringe inoculation, as seen in the present study, were quite different and emphasize the artificial nature of cultured inocula. It is well established that cultured spirochetes are significantly different from spirochetes that enter the host through the vector (9, 19). Cultured spirochetes seem to be cleared from the site of inoculation in both C3H and C3H-*scid* mice. Our present results suggest innate immune clearance mechanisms are effective at clearing cultured spirochetes at the site of inoculation, which is not seen with tick-borne infection. However, the subsequent dissemination of spirochetes to distant skin sites reveals dynamics similar to those seen in tick-borne infections.

We selected heart base and ventricular muscle to examine the difference between regions of the heart that are consistently inflamed (heart base) and minimally affected (ventricular myocardium) during infection of both C3H and C3H-*scid* mice (3, 11, 60). Both sites fostered relatively high populations of spirochetes, and gene expression patterns resembled those seen in skin and joint tissue, except for an apparent relative abundance of *ospC* expression in heart tissues of both types. A difference in gene expression between heart base and myocardium was not obvious.

Mild nonsuppurative myositis has been described in immunodeficient mice infected with *B. burgdorferi* (15, 38). The C3H-*scid* mice in this study had mild perivascular and interstitial infiltrates of mononuclear leukocytes in quadriceps muscle, but not myocardium. Our findings confirm the observation that quadriceps muscle supports relatively large numbers of spirochetes, but comparison of gene expression between quadriceps skeletal muscle and cardiac muscle suggests that, although there are roughly equivalent numbers of spirochetes per unit weight of tissue, there is a higher level of transcription of many of the genes in ventricular myocardium than in skeletal muscle.

Evaluation of the tibiotarsi of C3H and C3H-*scid* mice revealed consistently higher numbers of copies of *flaB* DNA in joints of immunodeficient mice, with similar trends in gene expression. In the comparison of joint tissue with heart as two tissues with significant inflammation, the most striking difference was the higher level of *ospC* expression in hearts than in joints relative to *flaB* DNA copy numbers. There were no clear trends in gene expression that corresponded to disease progression in C3H-*scid* mice or disease resolution in C3H mice. Notably, none of the C3H joint samples tested had detectable *p37-42* transcription, whereas P37-42 was expressed in most of the C3H heart samples.

A recent study (46) has shown major changes in gene expression; the study used DNA microarray analysis of spirochetes under culture conditions that mimic the resting or feeding tick and in spirochetes cultivated in dialysis membrane chambers implanted into rat peritoneal cavities to mimic conditions within the mammalian host. That study reinforced the OspA/OspC paradigm, in which it was shown that OspA expression was low and OspC expression was elevated in spirochetes that were maintained in vivo within dialysis membrane chambers. However, our results suggest that the paradigm is somewhat oversimplified, with frequent *ospA* transcription occurring in immunodeficient mice and *ospC* transcription being quite variable among different tissues and at different time points, reinforcing the complexity of factors that are involved in *B. burgdorferi* gene expression in vivo. Furthermore, global microarray analysis suggested that few differentially regulated genes were exploited in spirochetes maintained in vivo in dialysis membranes, consistent with homeostasis, whereas our results suggest dynamic temporal and tissue-related expression. Such findings mimic the static gene expression found in spirochetes in skin within the first few days after tick transmission (32), but not the patterns seen in the current study within distant tissues at different intervals of infection. Thus, in vivo gene expression is dynamic and complex, influenced by time, tissue, and immune response.

Recently, a customized *B. burgdorferi* lipoprotein DNA microarray was used to examine lipoprotein gene expression during the course of infection of C3H and C3H-*scid* mice. An apparent down-regulation of a number of lipoprotein genes was noted over time in C3H mice compared to C3H-*scid* mice, suggesting that immune response is an additional cue for gene regulation by *B. burgdorferi* (35). Our results support this premise, but for different reasons. In the microarray study, differences in gene expression were not quantified and findings were not related to an index of spirochete numbers, which our study showed were significantly different between C3H and C3H-*scid* mice. Thus, some genes that were concluded to be down-regulated due to immunity were missed due to lack of sensitivity of the assay and lack of correlation with actual spirochete numbers in the tissues assessed.

It is clearly apparent that an "on or off" explanation for gene regulation in different conditions would be nice to explain *B. burgdorferi* biology and pathogenesis, but unfortunately, the story is far more complex. Even the OspA/OspC paradigm is not absolute when examined with sensitive real-time quantitative analysis. Furthermore, the membrane dialysis chamber model as a paradigm for in vivo conditions can also be misleading, because we found considerable variation in gene expression levels in different tissues. The present study suggests that immune competence regulates spirochete numbers in the host, but significant numbers of spirochetes persist in the presence of host immunity. Disease resolution cannot be explained on the basis of down-regulation of the prototype genes examined, including *arp*, whose product has been positively associated with induction of arthritis-resolving immunity (21). Fur-

thermore, transcription may not necessarily relate to protein translation (although more so with bacteria than eukaryotes), protein expression may not necessarily equate with translation, and protein expression can vary among spirochetes and within individual spirochetes. For example, studies have shown that a number of apparently surface-exposed proteins can be found within the periplasm of spirochetes, but exposure on the surface of the spirochete can vary with environmental conditions and among populations of spirochetes (29).

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