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Borrelia burgdorferi genes selectively expressed in the infected host

(Lyme disease/microbial pathogenesis/antibody/gene induction)

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ABSTRACT **An immunological screening strategy was used to select microbial genes expressed only in the host. Differential screening of a *Borrelia burgdorferi* (the Lyme disease agent) expression library identified a gene (*p21*) encoding a 20.7-kDa antigen that reacted with antibodies in serum from actively infected mice but not serum from mice immunized with heat-killed *B. burgdorferi*. Selective expression of *p21* in the infected host was confirmed by Northern blot analysis and RNA PCR. Further differential screening of the expression library identified at least five additional *B. burgdorferi* genes are selectively expressed *in vivo*. This screening method can be used to identify genes induced *in vivo* in a wide variety of pathogenic microorganisms for which a gene transfer system is not currently available.**

Identification of virulence factors is crucial for a better understanding of the mechanisms that pathogenic microorganisms use to evade host defenses, cause disease, and survive within the host. A number of virulence factors are not expressed or the level of expression remains very low in *in vitro* cultured pathogens, and their expression is often induced by various environmental signals (1). A similar induction of gene expression may occur in the infected host where specific external signals are present. Thus, in an attempt to search for potential virulence factors, it is important to identify microbial genes that are expressed only in the host and not in *in vitro* culture and then to study the functional role of the gene products in pathogenesis. One way of identifying such genes is to differentially screen an expression library of the pathogen with two types of antibodies, one from animals immunized with killed cultured pathogens and the other from infected hosts. The genomic clones that react with antibody from infected animals—but not from immunized animals—are likely to contain the genes that are expressed only in the host.

A genetic system using *Salmonella typhimurium* as a model pathogen has been developed to identify bacterial genes induced *in vivo* (2). However, this system may not be applied to pathogenic microorganisms for which a gene transfer system and well-defined auxotrophs are not available. In contrast, the differential screening method described here can be widely used for diverse microbial pathogens. Because of the unavailability of such a genetic system in *Borrelia burgdorferi*, the causative agent of Lyme disease (3), we employed a differential immunological screening method to identify *B. burgdorferi* genes whose expression is specifically induced in infected mice.§

MATERIALS AND METHODS

Antibodies. Sera were collected from mice infected or immunized with *B. burgdorferi*. To initiate infection, three C3H/HeJ mice were intradermally challenged with 10^4 *B. burgdorferi* spirochetes and sacrificed after 9 months. Infection was documented by culturing spirochetes from the spleen, bladder, and skin of the challenged mice and histopathologic examination of the joints and heart for evidence of inflammation. For immunization, three C3H/HeJ mice were subcutaneously injected with 10^7 heat-killed (1 hr at 60°C) *B. burgdorferi* spirochetes in complete Freund's adjuvant and given booster injections at 2 and 4 weeks with the same dose of *B. burgdorferi* in incomplete Freund's adjuvant. All the heat-inactivated spirochetes were killed, as mice could not be infected with this *B. burgdorferi* preparation, and spirochetes were not cultured when this preparation was placed in BSK II medium. Sera were first incubated with immobilized *Escherichia coli* lysate to remove antibodies that react with *E. coli* proteins (4). Remaining sera were appropriately diluted and used for screening of the library.

Library Screening. A λZAPII (Stratagene) genomic expression library of *B. burgdorferi* N40 was previously constructed (4). In brief, genomic DNA of *B. burgdorferi* was mechanically sheared, methylated, ligated to *Eco*RI linkers, digested with *Eco*RI restriction endonuclease then ligated to λZAPII arms before *in vitro* packaging. Packaged phages were amplified once and plated with *E. coli* XL1-Blue (Stratagene) for screening. The plated phage library was differentially screened with sera from infected mice and sera from immunized mice. Mouse antibodies were detected by sequential incubation with goat anti-mouse antibody conjugated to alkaline phosphatase, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (*pico*Blue immunoscreening kit; Stratagene).

Determination and Analysis of DNA Sequence. Nucleotide sequence was determined by the Circumvent thermal cycle dideoxy DNA sequencing kit (New England Biolabs). Conditions for denaturation, annealing, and extension were 94°C for 20 sec, 55°C for 20 sec, and 72°C for 20 sec, respectively. A GenBank search was conducted with the Genetics Computer Group program (University of Wisconsin Biotechnology Center, Madison).

Northern Blot Analysis. Total RNA was isolated from cultured *B. burgdorferi* by acid guanidinium thiocyanate/phenol/chloroform extraction (5). Twenty micrograms of isolated RNA was electrophoresed and blotted onto Hybond-N membrane (Amersham). Hybridization and signal detection were conducted with a Phototope chemiluminescent kit (New England Biolabs). In brief, the blotted membrane was prehybridized for 1 hr at 68°C and then hybridized with biotinylated probes for *p21* or *ospA* at 68°C overnight. The membrane was washed at a final stringency of 0.1× standard saline citrate (SSC)/0.1% SDS at 68°C. Biotin-labeled probe was detected by a series of incubations with streptavidin, biotinylated alkaline phosphatase, and lumigen-PPD. Biotinylated probes were

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Abbreviation: BSA, bovine serum albumin.

§The sequence reported in this paper has been deposited in the GenBank database (accession no. L32797).

generated with a Phototope random-primer biotin-labeling kit (New England Biolabs), and the *p21* and *ospA* probes contained the entire *p21* and *ospA* sequences, respectively. Amplified PCR products of *p21* or *ospA* (6) were used as templates for the random octamer-primed labeling reaction.

Dot Blot Analysis and PCR. For dot blot analysis, 2 μg of denatured λ phage or *B. burgdorferi* DNA was spotted on Hybond-N membrane and probed for *p21* or *ospA* as described for Northern blot analysis. Conditions for PCR of *B. burgdorferi* DNA were 30 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2 min. The 5′ and 3′ primers for PCR were 5′-AGATCCTCGAGAAGATTCATACTTTATCTATG-3′ and 5′-TGTACAAGCTTCTATTTTAAATTTTTTTTAAGATC-3′, respectively. The 3′ regions of these primers are distinct from *ospE*, and under the conditions used for PCR, these primers are specific for *p21* and do not amplify *ospE*. For RNA PCR, total RNA was isolated from spleens of the mice infected with *B. burgdorferi* or from *in vitro* cultured *B. burgdorferi* by acid guanidinium thiocyanate/phenol/chloroform extraction. Ten micrograms of pooled RNA was treated with RNase-free DNase (Promega) for 3 hr at 37°C, and cDNA was synthesized by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Stratagene) and 3′ primers for either *p21* (murine tissue and cultured *B. burgdorferi*), γ-actin (7) (murine tissue), or *ospA* (4) (cultured *B. burgdorferi*). Subsequently, the reverse transcriptase was inactivated by heating for 5 min at 95°C, 5′ primer for *p21*, γ-actin, or *ospA* was added, and PCR was carried out for 45 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min.

Immunoblot Analysis. Three micrograms of bovine serum albumin (BSA), synthetic peptide coupled to BSA, or *B. burgdorferi* lysate was spotted on nitrocellulose membranes, and the dried membranes were incubated with either serum from *B. burgdorferi*-immunized mice or serum from infected mice. Then, bound antibody was detected by incubation with the secondary antibody conjugated to horseradish peroxidase (ECL Western blot detection system; Amersham). Finally, the membranes were stained with amido black to demonstrate that an equal quantity of proteins was present on all the test samples. Based on amino acid sequence comparison between P21 and OspE, a unique region of P21 comprising amino acids 31–40 was chosen, and the peptide coupled to BSA was synthesized by Quality Control Biochemicals (Hopkinton, MA). The peptide was coupled to BSA for the efficient binding of the peptide to nitrocellulose. The amino acid sequence of the peptide was CNNELKVKQSN. A cysteine was added to the amino terminus of the peptide for the BSA coupling reaction.

RESULTS

First, duplicate plaque lifts of 4×10^4 *B. burgdorferi* library clones were screened with two different sera. One set of plaque lifts was incubated with pooled sera from mice hyperimmunized with heat-killed *B. burgdorferi*, and the other set with sera obtained from mice infected with *B. burgdorferi* (8). Both sera had similar high titers of antibodies to *B. burgdorferi* whole cell lysates: antibodies in sera from immunized and infected mice were detectable at a dilution of 1:15,000 and 1:10,000 by immunoblot, and 1:6400 and 1:3200 by ELISA, respectively. Moreover, both sera recognized many *B. burgdorferi* antigens by immunoblot, with varying degrees of intensity (Fig. 1*C*). One hundred and seventy-two clones showed reactivity with sera from infected mice, and 169 of these clones bound with antibodies in sera from immunized animals. The 3 phage clones that showed differential reactivity with the two sera were selected and subjected to another round of differential screening, with identical results. In addition, these 3 phage clones bound antibodies in the sera of two additional mice that had been infected with *B. burgdorferi* for 9 months, and did not react with sera from two mice that had been immunized with heat-killed spirochetes. After secondary screening, we performed a detailed analysis of one of the clones (no. 1) that strongly reacted with sera from infected mice but not with sera from immunized mice.

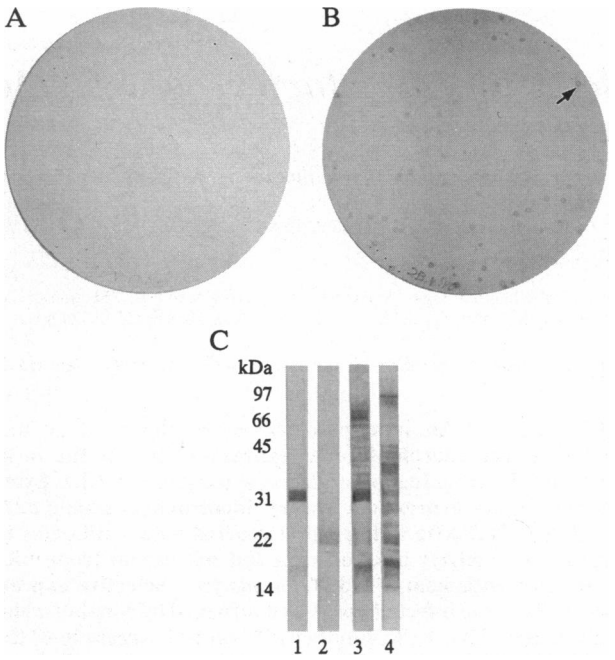

FIG. 1. Differential screening of the *B. burgdorferi* expression library. The plated phage library was differentially screened with sera from infected mice and sera from immunized mice. A representative clone with different reactivity at the primary screening was plaque purified and plated, and the screening was repeated. Duplicate plaque lifts of the clone were similarly incubated with sera from immunized (*A*) or infected (*B*) mice. The clone marked with an arrow was further analyzed. (*C*) *B. burgdorferi* lysate probed with pooled sera from mice immunized with recombinant OspA, a 31-kDa protein (control) (lane 1); recombinant OspC, a 22-kDa protein (control) (lane 2); or lysate of heat-killed *B. burgdorferi* (lane 3); or with sera from mice infected with *B. burgdorferi* for 9 months.

The pBluescript plasmid harboring the *B. burgdorferi* genomic insert was rescued by *in vivo* excision from clone 1 that was obtained from differential screening. From sequence analysis, we found a complete open reading frame and a partial open reading frame, which were believed to be the two cistrons of an operon (Fig. 2*A*). Since the expression of both open reading frames was under the same control, we focused on the first open reading frame, designated *p21*. The partial second open reading frame was designated *k2*. The deduced amino acid sequence of P21 contained a typical prokaryotic signal sequence for posttranslational processing by cleavage and lipidation, suggesting that the gene product is likely to be a lipoprotein of 20.7 kDa (4, 8). A search of GenBank (December 1994) revealed that *p21* is a novel gene, and P21 has 71% amino acid sequence identity to *B. burgdorferi* OspE (Fig. 2*B*) (4). Moreover, the deduced sequence of the first 11 aa at the amino terminus of K2 had 64% homology with OspF. The control regions of transcription and translation of p21 are compared with those of other known *B. burgdorferi* genes in Table 1.

To determine whether *p21* transcription occurred *in vitro*, its expression in cultured *B. burgdorferi* was assessed by Northern blot analysis (Fig. 3*A*). Total RNA isolated from cultured *B. burgdorferi* was probed for *p21* or *ospA* (control) expression. In contrast to *ospA*, *p21* was not expressed in cultured *B. burgdorferi*, confirming the results of immunological screening. Because *in vitro* culture of *B. burgdorferi* is often associated with the loss of genes or plasmids (9), we examined the

A

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GGAGAGATTTTTGGGAGTTGGTTAAAATTACATTTGCGTTTGTTATATGTAACAGCTGAATGTAACAAAA
TTATATATTTAAATCTTTCAAAAATTGTAATTATTATGTAATATGGTATGATTAAGATTTATGGAGAAATTT
1
ATG AAT AAG AAA ATG TTT ATT GTT TGT GCT GTT TTT GCA CTT ATA AGT TCT TGC
Met Asn Lys Lys Met Phe Ile Val Cys Ala Val Phe Ala Leu Ile Ser Ser Cys
55
AAG ATT CAT ACT TTA TCT ATG TAT GAT GAG CAA AGT AAT AAT GAG TTA AAA GTT
Lys Ile His Thr Leu Ser Met Tyr Asp Glu Gln Ser Asn Asn Glu Leu Lys Val
109
AAG CAA AGC AAT GGC GAG GTG AAA GTT AAA AAA ATA GAA TTC TCT GAA TTT ACT
Lys Gln Ser Asn Gly Glu Val Lys Val Lys Lys Ile Glu Phe Ser Glu Phe Thr
163
GTA AAA ATA AAA TAT AAA AAA GAC AAT AGC AGT AAT TGG GAA GAC TTA GGA ACT
Val Lys Ile Lys Tyr Lys Lys Asp Asn Ser Ser Asn Trp Glu Asp Leu Gly Thr
217
TTG GTT GTA AGA AAA GAA GTA GAT GGT ATT GAT ACA GGG TTA AAT GTT GGG AAG
Leu Val Val Arg Lys Glu Val Asp Gly Ile Asp Thr Gly Leu Asn Val Gly Lys
271
GGA TAC TCT GCT ACA TTC TTT TCA TTA GAA GAG TCA GAA GTT AAT AAC TTT ATA
Gly Tyr Ser Ala Thr Phe Phe Ser Leu Glu Glu Ser Glu Val Asn Asn Phe Ile
325
AAA GCA ATG ACT AAA GGT GGA ACA TTT AAA ACT AGT TTG TAT TAT GGA TAT AAG
Lys Ala Met Thr Lys Gly Gly Thr Phe Lys Thr Ser Leu Tyr Tyr Gly Tyr Lys
379
GAA GAA CAA AGT GGT GAA AAT GGT ATT CAA AAT AAG AAG ATA ATA ACA AAA ATA
Glu Glu Gln Ser Gly Glu Asn Gly Ile Gln Asn Lys Lys Ile Ile Thr Lys Ile
433
GAA AAA ATT GAT GAT TTT GAA TAT ATT ACA TTT TTA GGA GAT AAA ATT AAG GAT
Glu Lys Ile Asp Asp Phe Glu Tyr Ile Thr Phe Leu Gly Asp Lys Ile Lys Asp
487
TCA GGA GAT AAA GTT GTT GAA TAT GCA ATA CTA CTA GAA GAT CTT AAA AAA AAT
Ser Gly Asp Lys Val Val Glu Tyr Ala Ile Leu Leu Glu Asp Leu Lys Lys Asn
541
TTA AAA TAG AAGTTAGAAGTATAGGGGAGAACAATTATGAATCAAAAAGCATTTATTATTTGCGCTGT
Leu Lys ***
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B

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P21   1    MNKKM--FIVCAVFALISSCKIHTLSMYDEQSNNELKVKQSNGEVKVKKI
           ||||| ||| ||| ||  ||||| | |||||         | || ||||| 
OspE       MNKKMKMFIVYAVFILIGACKIHT-S-YDEQS--------S-GESKVKKI

      51   EFSEFTVKIKYKKDNSSNWEDLGTLVVRKEVDGIDTGLNVGKGYSATFFS
           ||| |||||| | | | || ||| |||||| |||||| | | ||||||
           EFSKFTVKIKNK-DKSGNWTDLGDLVVRKEENGIDTGLNAG-GHSATFFS

     101   LEESEV-NNFIKAMTKGGTFKTSLYYGYKEEQSGENGIQNKKIITKIEKI
           ||| || ||| | || || ||||||||||||||  |||||| ||||||||
           LEE-EVVNNFVKVMTEGGSFKTSLYYGYKEEQSVINGIQNKEIITKIEKI

     151   DDFEYITFLGDKIKDSGDKVVEYAILLEDLKKNLK
           |  ||||| ||||| ||||| |||| || |||||| 
           DGTEYITFSGDKIKNSGDKVAEYAISLEELKKNLK
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FIG. 2. (*A*) Nucleotide and deduced amino acid sequences of *p21*. Underlined are −35 and −10 consensus regions and the ribosome binding site. A part of the second open reading frame with the start codon (doubly underlined) and ribosome binding site is also shown. (*B*) Comparison of the amino acid sequences of P21 and OspE. Horizontal lines are included to clarify the sequence alignment.

presence of *p21* in the genome of *B. burgdorferi* whose RNA was analyzed for the expression of *p21*. First, genomic DNA of the spirochete was analyzed by dot blot. The *p21* and *ospA* probes generated for Northern blot analysis were used again, and both probes strongly hybridized to *B. burgdorferi* genomic DNA, but not bacteriophage λ DNA (Fig. 3*B*). Next, PCR of the genomic DNA with *p21*-specific primers generated the

Table 1. The −35 and −10 promoter sequences and ribosome binding sites (RBS) of selected *B. burgdorferi* genes

Gene	Sequence		
	−35 region	−10 region	RBS
ospA	TTGTTA	TATAAT	AAAGGAG
ospB			AAGGAG
ospC	TTGAAA	TATAAA	AAAGGAGG
ospD	TTGATA	TATATT	AAGGAG
ospE	TTGTTA	TATATT	GGAG
ospF			AGGAG
eppA	TTAGTA	TATAAT	AGGAGA
p21	TTGTTA	TATATT	GGAG
k2			GGAG

ospA, ospB, ospD, and *eppA* are from *B. burgdorferi* strain B31; *ospC* is from *B. burgdorferi* strain pKo; *ospE, ospF, p21*, and *k2* are from *B. burgdorferi* strain N40.

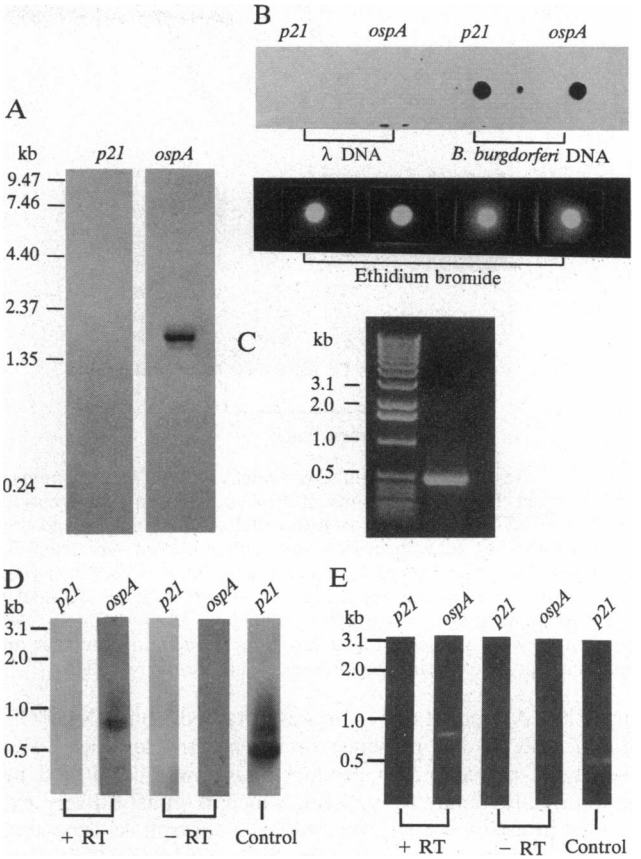

FIG. 3. Assessment of *p21* transcription in cultured *B. burgdorferi*. (*A*) Expression of *p21* in *in vitro* cultured *B. burgdorferi* was evaluated by Northern blot analysis. The blotted membrane was first probed for *p21* and then reprobed for *ospA*. (*B*) Denatured λ DNA (2 μg) or *B. burgdorferi* DNA (2 μg) was spotted on Hybond-N membrane. The dried membrane was first stained with ethidium bromide to confirm that an equal amount of DNA was present and then hybridized with *p21* or *ospA* probes as described for Northern blot analysis. (*C*) *B. burgdorferi* DNA (10 ng) was subjected to PCR to generate a 513-bp product of the *p21* coding region. (*D*) Assessment of *p21* mRNA in cultured *B. burgdorferi* by RNA PCR. Electrophoresed RNA PCR products were denatured, transferred to Hybond-N membrane, and probed for *p21* or *ospA*. The control lane contains PCR-amplified *p21* from *B. burgdorferi* DNA hybridized with a *p21* probe. RT, Moloney murine leukemia virus reverse transcriptase. (*E*) Electrophoresed RNA PCR products shown in *D*, stained with ethidium bromide.

appropriate product, further indicating that *p21* was present in the cultured spirochetes used for Northern blot studies (Fig. 3*C*).

We next confirmed the expression of *p21* in infected mice by demonstrating the presence of antibodies against P21 in sera from two infected animals. Dot immunoblot analysis was performed with sera from *B. burgdorferi*-infected mice and a peptide of 10 aa derived from P21 (Fig. 4). Because of the potential antibody crossreactivity between P21 and OspE based on sequence homology, the synthetic peptide was derived from a unique P21 region that does not have any sequence homology to OspE or other known *B. burgdorferi* antigens. Infected, but not immunized, murine sera reacted with the peptide, reflecting the selective *in vivo* expression of *p21*.

Expression of *p21* in infected mice was further demonstrated by RNA PCR (Fig. 5). Total RNA isolated from two infected mouse spleens was subjected to RNA PCR to detect *p21* RNA. Although RNA samples were treated with DNase, RNA PCR was conducted with or without reverse transcriptase in order to exclude the possibility that residual DNA might be also

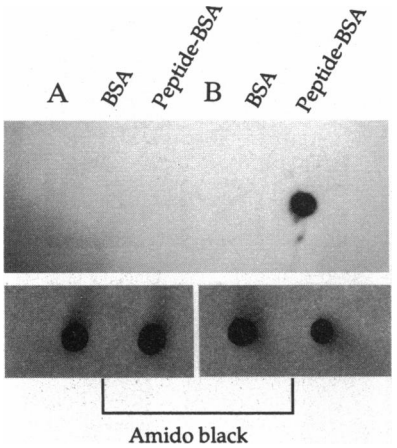

FIG. 4. Presence of the antibody against P21 in infected mouse sera. (*Upper*) Three micrograms of BSA or the synthetic peptide coupled to BSA was spotted on nitrocellulose membranes, and the dried membranes were incubated with either pooled sera from *B. burgdorferi*-immunized mice (*A*) or pooled sera from infected mice (*B*). Then, bound antibody was detected by incubation with the secondary antibody conjugated to horseradish peroxidase. (*Lower*) Finally, the membranes were stained with amido black to demonstrate that an equal quantity of proteins was present on all the test samples.

amplified. A product of 513 bp was obtained from RNA PCR of *p21* only in the presence of reverse transcriptase. The identity of the amplified product as *p21* was confirmed by Southern blot analysis. γ-Actin, which is constitutively expressed in mouse tissues, was used as a control. As expected, a PCR product could not be amplified with the *p21*-specific primers from RNA prepared from uninfected mice (data not shown). In contrast, *p21* expression could not be detected by RNA PCR from *B. burgdorferi* cultured *in vitro* (Fig. 3 *D* and *E*).

To ensure the validity of our technique and determine whether additional clones identified by the differential screening approach were likely to encode genes selectively induced within the infected host, we repeated our study using sera from mice infected with *B. burgdorferi* for 90 days. Fourteen phage clones were identified from the *B. burgdorferi* expression library that reacted with antibodies in the sera from mice infected with *B. burgdorferi* for 90 days but not with antibodies

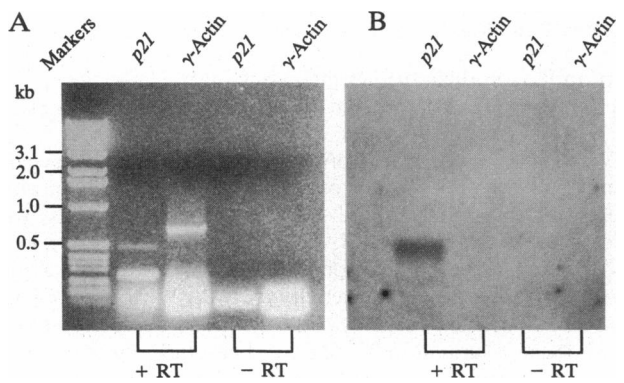

FIG. 5. Detection of *p21* mRNA in infected mice by RNA PCR. (*A*) Total RNA isolated from spleens of infected mice was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (RT) and 3′ primers for either *p21* or γ-actin. Then the RT was heat inactivated, and PCR was performed with additional 5′ primers for *p21* or γ-actin. (*B*) Electrophoresed RNA PCR products were denatured, transferred to Hybond-N membrane, and probed for *p21*. The size markers (kb) for *A* and *B* are the same and the hybridization conditions for *B* are identical to the parameters described for Northern blot analysis in *Materials and Methods*.

in sera of animals immunized with heat-killed spirochetes. The inserts from these 14 individual clones were excised and used to probe the remaining clones by Southern blot. The 14 clones separated in five groups (F1–F5), each of which represented clones that had overlapping DNA segments, indicating that the screening method was sufficiently powerful to isolate redundant clones. Moreover, preliminary sequence data from the 2 additional clones isolated from our initial differential screening (using sera from mice infected for 9 months) revealed overlapping DNA sequences, suggesting that these separate clones encoded the same gene. To determine whether these genes were expressed by *B. burgdorferi* cultured *in vitro*, we performed Northern blot analysis using RNA from spirochetes cultured *in vitro* probed with the *B. burgdorferi* inserts that represented clones from groups F1–F5. RNA from these genes was not detected by Northern blot, further suggesting that these additional genes were not expressed *in vitro* (Fig. 6).

DISCUSSION

We have developed a strategy to identify bacterial genes selectively induced in the infected host, by differentially screening a *B. burgdorferi* expression library with sera from mice infected with live spirochetes for 9 months and sera from mice immunized with killed *B. burgdorferi* in complete Freund's adjuvant. The method is based on the supposition that sera from infected mice contain antibodies that bind antigens expressed *in vivo*, whereas sera from mice immunized with cultured spirochetes that have been killed contain only antibodies to proteins that are expressed by *B. burgdorferi in vitro*. Primary and secondary screening of the library with both sets of sera initially identified 3 clones (from a total of 172) that were recognized only by antibodies in the infected host; clone 1 encoded *p21*. Such differential reactivity could, however,

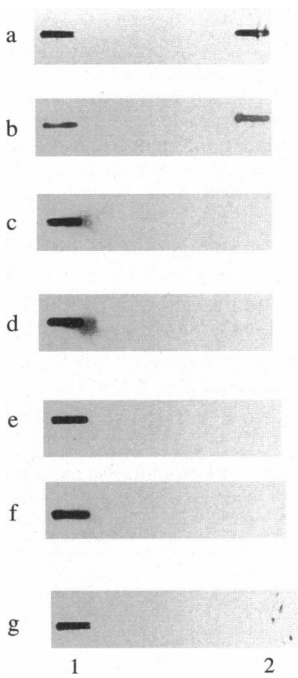

FIG. 6. Identification of five *B. burgdorferi* DNA fragments that encode genes selectively induced in the infected host. Five representative DNA fragments (F1–F5) that encode antigens that bind antibodies in the sera of mice infected with *B. burgdorferi* for 90 days but not in the sera of mice immunized with heat-killed *B. burgdorferi* were used to probe DNA and RNA isolated from *B. burgdorferi* cultured *in vitro*. Column 1, *B. burgdorferi* DNA (5 μg); column 2, *B. burgdorferi* RNA (5 μg). Probes were *ospA*, *ospB*, F1, F2, F3, F4, and F5 in rows a–g, respectively.

reflect several factors unrelated to gene expression, such as the immunogenicity of the protein during natural infection rather than in the cultured preparations of spirochetes, or the cross-reactivity of antibodies to other *B. burgdorferi* antigens. Therefore, we used both molecular and immunologic assays to further demonstrate that *p21* is expressed only *in vivo*. *p21* mRNA could not be detected by Northern blot or RNA PCR in spirochetes grown in culture medium. In contrast, *p21* mRNA was identified by RNA PCR in splenic tissue from mice infected with *B. burgdorferi*. Further, in immunoblot analysis P21-specific peptide that has no homology to other known *B. burgdorferi* proteins, including OspE, reacted with sera from infected mice but not sera from immunized animals. These different approaches show that *p21* is selectively expressed *in vivo*.

Moreover, differential screening with a second set of sera from mice infected with *B. burgdorferi* for 90 days revealed 14 additional clones that express antigens that bind antibodies from infected mice only. Northern blot analysis using 5 representative clones (F1–F5) from the 14 isolates suggested that RNAs from these genes are not expressed *in vitro*. While we have not exhaustively shown that these additional genes are selectively expressed *in vivo*, the reactivity of antibody in the sera of infected mice with the antigens encoded by the DNA fragments and the absence of antibody to these antigens in the sera of mice immunized with heat-killed spirochetes, as well as the absence of RNA specific for these genes in spirochetes cultured *in vitro*, strongly suggest that they are selectively induced in the infected host. Once these genes are cloned, detailed molecular and immunologic analysis similar to the work with *p21* can address this issue further. Nevertheless, these data help validate our screening strategy and indicate that the method is generalizable and sufficiently powerful to identify numerous antigens that are expressed upon infection.

The DNA sequence indicates that *p21* and *ospE* are closely related but distinct genes, with identical −35 and −10 promoter sequences and ribosome binding sites, within the *B. burgdorferi* N40 genome. The deduced amino acid sequence of P21 suggests that it is a putative lipoprotein with 71% identity to OspE. It is of interest that P21 and OspE are expressed differentially, as their potential regulatory sequences are strikingly similar. The 5′ upstream regions of *p21* and *ospE* are completely identical upstream from the −10 sequence to the boundary of the 5′ flanking DNA which has been sequenced (189 nt 5′ of the ATG) (Fig. 2*A*). Only eight nucleotide differences between *p21* and *ospE* are evident in the area between the −10 region and the ATG start codon (upstream of the ATG, the following differences are noted in *ospE*, when compared with p21: −54, G; −45, C; −32, T; −30, G; −24, A; −15, C; −6, T; −3 C, where +1 is the A in the ATG codon. Indeed, all the differences are located in the region likely to contain the 5′ untranslated region of *p21* mRNA. The identity of the 5′ flanking segments of *p21* and *ospE* suggests that a recombinational event has most likely occurred in recent evolutionary time. The function of P21 and the signals that induce gene expression are not known; however, other potentially related *B. burgdorferi* antigens can elicit protective immune responses in the host (OspA, OspB, OspC, and OspF) (6, 10); cause partial destruction of spirochetes within *Ixodes* ticks (OspA, OspB, OspE, and OspF) (11), attachment of spirochetes to endothelial cells (OspA), or spirochete virulence (OspD) (12); or be useful as a serodiagnostic marker expressed during *B. burgdorferi* infection (EppA) (13). It is likely that *B. burgdorferi* genes that are selectively expressed *in vivo*, including *p21* and other genes identified by this differential screening method, will have roles in spirochete survival, pathogenicity, or evasion of the host immune response.

Although further study is required to understand the relationship between specific induction of *p21* in the host and its role in *B. burgdorferi* infection, the validity of the immunological screening technique for our purpose was proved by the current work. A limitation of this selection method is its dependence on the antigenicity of target proteins. Therefore, in contrast to the genetic selection system developed by Mahan *et al.* (2), this immunological selection method may not be used to identify all the genes of a specific pathogen that are induced *in vivo*. Nevertheless, selection and identification of antigenic proteins differentially expressed *in vitro* versus *in vivo* by this method could provide important information on virulent determinants of various pathogens, such as *B. burgdorferi*, that lack well-defined gene transfer systems and mutants in biosynthetic genes. In turn, this will lead to a better understanding of microbial pathogenesis and therapies targeted to the identified virulence factors.

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