An Immune Evasion Mechanism for Spirochetal Persistence in Lyme Borreliosis

Fang Ting Liang, Mary B. Jacobs, Lisa C. Bowers, and Mario T. Philipp

Department of Parasitology, Tulane Regional Primate Research Center, Tulane University Health Sciences Center, Covington, LA 70433

Abstract

*Borrelia burgdorferi*, the Lyme disease spirochete, persistsently infects mammalian hosts despite the development of strong humoral responses directed against the pathogen. Here we describe a novel mechanism of immune evasion by *B. burgdorferi*. In immunocompetent mice, spirochetes that did not express ospC (the outer-surface protein C gene) were selected within 17 d after inoculation, concomitantly with the emergence of anti-OspC antibody. Spirochetes with no detectable OspC transcript that were isolated from immunocompetent mice reexpressed ospC after they were either cultured in vitro or transplanted to naive immunocompetent mice, but not in OspC-immunized mice. *B. burgdorferi* persistently expressed ospC in severe combined immune-deficient (SCID) mice. Passive immunization of *B. burgdorferi*-infected SCID mice with an anti-OspC monoclonal antibody selectively eliminated ospC-expressing spirochetes but did not clear the infection. OspC-expressing spirochetes reappeared in SCID mice after the anti-OspC antibody was eliminated. We submit that selection of surface-antigen nonexpressers is an immune evasion mechanism that contributes to spirochetal persistence.

Key words: *Borrelia burgdorferi* • OspC • chronic infection • immune evasion • immune selection

Introduction

Lyme disease is a complex multi-system disorder with both early and late manifestations. Early signs and symptoms include erythema migrans, acute meningitis, and Bell’s palsy, whereas in the late phase of the disease, arthritis, chronic neurologic abnormalities, and acrodermatitis chronica atrophicans are manifest (1–3). The manner whereby *Borrelia burgdorferi* can chronically infect both humans (4) and animals (5–7) in the face of a vigorous and specific immune response continues to be an enigma. Especially intriguing is the long-term survival of the spirochete despite the presence of a strong antibody response that is directed against surface antigens (6).

Several defense stratagems are potentially available to *B. burgdorferi*, including antigenic variation (8), seclusion into immune privileged sites (9), and suppression of harmful immune responses (10, 11). Like other vector-borne pathogens, such as the protozoan *Trypanosoma brucei* (12), and the spirochete *Borrelia hermsii* (13), *B. burgdorferi* expresses a surface protein (VlsE) that undergoes antigenic variation (8). While the variable surface glycoprotein of *T. brucei* appears to be the only antigenic structure exposed at the surface of this organism (12), and the variable major protein of *B. hermsii* dominates the antibody response to this relapsing fever spirochete, a large body of evidence indicates that in addition to VlsE, several other surface proteins, e.g., outer surface protein C (OspC)* (14), decorin-binding protein A (DbpA) (15), the porin P66 (16), and the fibronectin-binding protein BBK32 (17) are immunogenic, and thus expressed, during a natural (tick-transmitted) *B. burgdorferi* infection. Moreover, antibodies to at least some of these surface proteins are able to partly or fully protect mice from a challenge infection with *B. burgdorferi* administered either via ticks (BBK32 [17], OspC [18]) or by implantation of skin biopsy tissue containing host-adapted spirochetes (P66 [19]). Thus, VlsE antigenic variation cannot be the only mechanism that makes it possible for *B. burgdorferi* spirochetes to establish a chronic infection. As for selection of the spirochete into classical immune privileged sites such as the central nervous system, this indeed occurs, yet *B. burgdorferi* also is readily cultivable from nonimmune-privileged organs such as the urinary bladder, skin, and...
heart (20). It also must be available for transmission in intradermal blood pools generated by blood feeding ticks (21). Finally, *B. burgdorferi* lipoproteins induce in monocytes the production and secretion of the anti-inflammatory cytokine IL-10 (10), but IL-10 knockout mice do become infected with *B. burgdorferi*, albeit with a lower spirochetal burden than wild-type mice (11). Thus, neither immune exclusion nor suppression are essential for persistence of a *B. burgdorferi* infection.

Over the last five years it has become apparent that *B. burgdorferi* is able to regulate the expression of several lipoproteins in response to environmental cues such as changes in temperature, pH, or cell density. The spirochete also differentially expresses lipoproteins in the tick and mammalian hosts (for a review, see reference 22). We hypothesized that, as it infects the mammalian host, *B. burgdorferi* might be able, perhaps in response to cues provided by tissue microenvironments, to downregulate the expression of surface antigens that otherwise would be targeted by bactericidal antibodies gradually elicited during infection. These antibodies would thus select surface-antigen nonexpressers, and these organisms, if viable, would continue to infect the mammalian host. We tested this hypothesis by assessing OspC mRNA expression in the skin of naive and OspC-immunized mice as well as in SCID mice both in the presence and absence of passively transferred anti-OspC bactericidal antibody. Here we present the results of these studies.

**Materials and Methods**

**Spirochete Strain.** *B. burgdorferi* sensu stricto clonal isolate B31 5A3, low passage (reference 8; a gift from Steven Norris, University of Texas, Houston, TX) was cultivated in BSK-H medium supplemented with 10% rabbit serum (Sigma-Aldrich). Spirochetes grown to either mid-logarithmic or stationary phase were supplemented with 10% rabbit serum (Sigma-Aldrich). Spirochete Strain. *B. burgdorferi sensu stricto* clonal isolate B31 5A3, low passage (reference 8; a gift from Steven Norris, University of Texas, Houston, TX) was cultivated in BSK-H medium supplemented with 10% rabbit serum (Sigma-Aldrich). Spirochetes grown to either mid-logarithmic or stationary phase were supplemented with 10% rabbit serum (Sigma-Aldrich). Spirochetes grown to either mid-logarithmic or stationary phase were supplemented with 10% rabbit serum (Sigma-Aldrich). Spirochetes grown to either mid-logarithmic or stationary phase were supplemented with 10% rabbit serum (Sigma-Aldrich).

**Mouse Infection and Biopsy.** Both C3H/HeN (C3H) and C3H-SCID mice (6 to 8-wk-old; Charles River Laboratories and The Jackson Laboratory, respectively) were given one single intradermal injection of 10⁴ spirochetes that were grown to stationary phase. Mouse infection and biopsy. Both C3H/HeN (C3H) and C3H-SCID mice (6 to 8-wk-old; Charles River Laboratories and The Jackson Laboratory, respectively) were given one single intradermal injection of 10⁴ spirochetes that were grown to stationary phase.

**Mouse Immunization.** Three C3H mice were given two subcutaneous injections at 3-wk intervals of 5 µg purified recombinant OspC (a gift from Robert Gilmore, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO) emulsified with the MPL plus TDM adjuvant (Sigma-Aldrich). As controls, an additional three mice received two doses each of the adjuvant. 6 d after the last injection, all six animals were inoculated with host-adapted spirochetes.

**Mouse Inoculation with Host-adapted Spirochetes.** A C3H mouse that had been infected with *B. burgdorferi* by needle-inoculation was killed 2 wk postinoculation (PI). One ear was used to prepare RNA for PCR analysis. The remaining ear was cut into small pieces and homogenized in a 1.7-ml plastic centrifuge tube with a plastic piston. The homogenate was suspended in 550 µl BSK-H medium and immediately digested, subjected to the six C3H mice that had received either rOspC plus adjuvant or adjuvant alone. The mice were killed at 14 to 18 d PI. Mouse ears were used to prepare RNA for PCR analysis and blood samples to assess the anti-OspC antibody titer.

**Anti-OspC Monoclonal Antibody.** The hybridoma cell line was kindly provided by Robert Gilmore (National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO). B5, the anti-OspC IgG2a monoclonal antibody secreted by this cell line was able to passively protect mice against a tick-transmitted infection with *B. burgdorferi* B31 (23). B5 was purified from mouse ascites fluid using a protein-G column (Pierce Chemical Co.). B5 purity and concentration were assessed using SDS-PAGE and the Bio-Rad protein assay kit (Bio-Rad Laboratories), respectively.

**Passive Immunization of SCID Mice with Anti-OspC Monoclonal Antibody.** Six C3H-SCID mice were infected with 10⁴ cultured spirochetes by subcutaneous injection. Each of two of these mice received 2, 5, 10, 50, 100, and 100 µg of monoclonal antibody B5 at 10, 12, 14, 17, 19, and 21 d PI, respectively. Another two animals were each given three 100-µg doses of mAb B5 at 17, 19, and 21 d PI, while the remaining two mice were treated with three 100-µg doses of purified normal mouse IgG2a (Sigma-Aldrich) as a control. The mice were killed at 3–4 d after the last passive immunization. Mouse ears were used to prepare RNA for reverse transcription (RT)-PCR analysis and as a source to culture spirochetes. In a separate experiment, five C3H-SCID mice were inoculated with cultured spirochetes. Four of the animals received three 50-µg doses of antibody B5 at 10, 12, and 14 d PI and the remaining mouse was given mouse IgG2a as a control. 3 d later, the control and one immunized mouse were killed for RT-PCR analysis. The remaining animals were treated with 150 and 100-µg doses of anti-mouse IgG antibody at 5 and 7 d, respectively, after the last passive immunization and killed either 10 d or 2 mo later. Total RNA was purified from mouse ears for RT-PCR analysis.

**RNA Preparation from Cultured Spirochetes and Mouse Ear Tissues.** Spirochetes grown to mid-logarithmic phase were harvested by centrifugation. Mouse ear tissues were frozen in liquid nitrogen and homogenized. Total RNA was purified using RNase Easy Mini Kit (QIAGEN). Purified RNA preparations were further treated with RNase-free DNase (QIAGEN) and monitored for absence of residual DNA by PCR analysis using the primers listed in Table 1.

**Probe Preparation and Southern Blotting.** DNA was purified from spirochetes grown to stationary phase using DNeasy Mini Kit (QIAGEN). Purified DNA was amplified by PCR using Taq PCR Core Kit (QIAGEN) and the primers listed in Table 1. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN). Purified DNA fragments were digested either with restriction endonuclease DraI or MboI (Life Technologies). Agarose gel electrophoresis revealed the expected fragment lengths of the digested PCR products and thus confirmed the identity of the amplified sequences. Southern blotting was performed using ECL direct nucleic acid labeling and detection systems, following the manufacturer’s instructions (Amersham Pharmacia Biotech).

**RT-PCR Analysis.** RNA samples purified from cultured spirochetes or mouse ear tissues were used as templates to prepare cDNA using Omniscript Reverse Transcriptase (QIAGEN). The reverse primers for the genes flab, ospC, and vlsE are listed in Table 1. 2 µg of purified RNA were used in each reverse transcrip-
tion reaction of 20 μl. 5 μl of cDNA preparation were amplified by PCR using Ready-TO-Go PCR beads following the manufacturer’s instructions (Amersham Pharmacia Biotech). RT-PCR products were analyzed on ethidium bromide-incorporated agarose gels and then transferred onto Hybond-ECL membranes for Southern blotting.

**ELISA.** OspC ELISA was performed as described previously (24). Recombinant OspC antigen was cloned from *B. burgdorferi* B31 (a gift from Robert Gilmore). The sequence of peptide Ct and the peptide-based ELISA were described elsewhere (25, 26). The Ct sequence reproduced that of the COOH-terminal invariable domain of VlsE of *B. burgdorferi* B31 (8). Mouse sera were serially diluted two- to fourfold and reacted with recombinant OspC or peptide Ct bound to an ELISA plate. Titer was defined as the highest serum dilution at which the ELISA OD (optical density) was ≥ the mean OD value of the preimmune sera of all of the mice plus 3 standard deviations.

**Results**

**Spirochetes Not Expressing OspC Are Selected Concomitantly with the Appearance of Anti-OspC Antibody.** We first infected a group of five mice of the C3H/HeN (C3H) strain by needle inoculation and killed them individually at 10, 17, 24, 45, and 66 d PI. We purified total ear RNA and subjected it to RT-PCR to amplify the transcripts of the *ospC*, *flaB*, and *vlsE* genes (Table I). As expected, both the *flaB* and *vlsE* gene transcripts were persistently expressed during the whole study (Fig. 1). In contrast, and in agreement with our hypothesis, the *ospC* gene transcript was detected only at day 10 PI but not thereafter (Fig. 1). These results indicated that the infection continued despite the diminished or ceased expression of OspC. We confirmed the identity of all of the RT-PCR amplicons by Southern blot with gene-specific probes (data not shown). A separate set of five mice was infected and processed in the same fashion and essentially identical results were obtained (not shown).

As the *ospC* gene is located on a plasmid (cp26) (27), selection of cp26-negative spirochetes might explain the negative RT-PCR results. However, spirochetes isolated from the ear of mice that had shown no detectable *ospC* gene expression did express the *ospC* transcript after cultivation in vitro (Fig. 1). This result indicated that in the course of infection, spirochetes had been selected whose *ospC* gene was shut down but not lost.

If spirochetes expressing OspC are effectively selected against by the gradual appearance of anti-OspC antibody during the course of infection, the titer of anti-OspC antibody should increase early in this process and perhaps eventually decline, as OspC ceases to be expressed. This turned out to be the case. The anti-OspC IgG antibody titer was 1:1,600 as early as day 10 PI. A peak titer value of 1:6,400 was reached by day 24, and after remaining at that level by day 45 PI, it declined to 1:3,200 at day 66 PI. We also titrated anti-OspC IgG antibody in the second set of five mice and similar results were obtained. For comparison, we titrated anti-VlsE antibody using as antigen a peptide (Ct) whose amino acid sequence reproduces that of the COOH-terminal invariable domain of VlsE (8, 25, 26). Unlike antibodies to the variable regions of VlsE, which may be bactericidal (8), antibodies recognizing the COOH-terminal invariable domain of VlsE are not, for although this domain is immunodominant, it is not exposed

![Figure 1](https://example.com/image1.png)

**Figure 1.** Selection of OspC nonexpressers as infection progresses. Five C3H mice were infected with cultured spirochetes and killed at 10 (mouse QI11), 17 (QI12), 24 (QI13), 45 (QI15), and 66 d (QI16) PI. Total RNA was purified from cultured spirochetes (cultured Bb), mouse ear tissues, or resolated spirochetes (resolated Bb), and analyzed by RT-PCR for expression of the *flaB*, *ospC*, and *vlsE* genes. PCR products were separated by ethidium bromide-incorporated-agarose gel electrophoresis.
on the surface of *B. burgdorferi* (25, 26). Unlike with anti-OspC antibody, the anti-VlsE (Ct) antibody titer increased continually up to the last time point measured, with a value of 1:1,600 at day 10 PI and of 1:638,400 at 66 d PI. This result is consistent with the continuous expression of VlsE during this period (Fig. 1).

**Anti-OspC Antibody Is Both Necessary and Sufficient to Select Spirochetes that Do Not Express OspC during Infection.** As an initial assessment of the role of antibody in selecting ospC nonexpressers, we immunized three C3H mice with purified recombinant OspC and adjuvant. 10 d after the last immunization, a time at which anti-OspC antibody was detectable in the serum of the immunized mice, we infected these animals and another group of three mice that had received adjuvant alone, with host-adapted spirochetes. This was achieved by implanting ear tissue from a donor C3H mouse that had been infected with *B. burgdorferi* for 2 wk. RT-PCR of ear RNA from this mouse showed that the resident spirochetes did not express *ospC* but continued to express the *flaB* and *vlsE* gene transcripts (not shown). We killed the six mice between 14 and 18 d PI. RT-PCR revealed that spirochetes reexpressed *ospC* in each of the three naive mice but in none of the immunized animals (Fig. 2).

In a separate experiment, we infected five naive C3H mice with host-adapted spirochetes that had downregulated *ospC*. This gene was reexpressed in all of the five recipients, as evidenced by the appearance of anti-OspC antibody (determined by OspC-ELISA, data not shown). After 24 d of infection *OspC* expression was no longer detectable, as revealed by RT-PCR (data not shown). This time period was longer than the 17 d recorded in mice that were infected with cultured spirochetes (Fig. 1). The longer *ospC* expression period was probably due to a more slowly developing anti-OspC antibody response in mice infected with host-adapted spirochetes (initially not expressing OspC). We then infected four C3H SCID mice by needle inoculation with cultured spirochetes and killed them individually at 10, 17, 31, and 47 d PI. When we analyzed purified ear RNA by RT-PCR it became apparent that, as with the *flaB* and *vlsE* gene transcripts in normal mice, the *ospC* transcript, as well as the *flaB* and *vlsE* transcripts, were continually expressed throughout the 47-d study period (Fig. 3). These results indicated that anti-OspC antibody was necessary to positively select spirochetes not expressing *ospC*. They indicated, moreover, that OspC+ spirochetes were the more viable phenotype, as they appeared to be positively reselected in the absence, or at lower levels, of anti-OspC antibody.

To ascertain if antibody was also sufficient to modulate the balance between the OspC- and OspC+ phenotypes, we needle inoculated six SCID mice with cultured spirochetes. Four of them received either six (mice QI51 and QI52) or three doses of a protective anti-OspC monoclonal antibody (23) (mice QI53 and QI54). The remaining two animals (QI55 and QI56) received three doses of purified mouse IgG2a (the same isotype as the monoclonal antibody) as control. OspC mRNA was detectable only in the ear biopsy tissue of mice that had received normal IgG2a. No *ospC* transcript was detected in the mice treated with the anti-OspC monoclonal antibody, regardless of whether the animals had received three or six doses of the antibody (Fig. 4). These results confirmed that anti-OspC
antibody was sufficient to effect selection of non-OspC–expressing spirochetes.

To further substantiate this tenet, we examined if *B. burgdorferi* reexpressed *ospC* after the anti-OspC antibody had disappeared. We first passively immunized four infected SCID mice with the anti-OspC monoclonal antibody. After the *ospC* gene was downregulated, we neutralized the antibody by treatment of the mice with anti–mouse IgG. Although *ospC* expression remained undetectable at 10 d after the clearance of specific antibody, 2 mo after this time spirochetes reexpressed the *ospC* transcript (Fig. 5).

**Discussion**

The evidence we have put forward strongly supports the notion that anti-OspC antibody is either able to select OspC-negative phenotypes that coexist with OspC-expressing spirochetes or can induce the downregulation of OspC expression in a manner that is reversible and dependent on antibody titer. While we are not aware that such an inductive mechanism exists, it constitutes a hypothesis that is readily testable in vitro. As for the hypothesis of selection of OspC-negative spirochetes, which we find the more plausible of the two, others have shown that OspC-negative spirochetes are present in the infectious inoculum derived from a tick. Ohnishi et al. analyzed OspC expression in the spirochetes present in the skin attached to the mouthparts of a feeding tick and found both OspC^−/H11002 and OspC^+/H11001 organisms (28). Thus OspC^− organisms might be present and available for selection as of the very onset of infection. In our experiments, by day 17 post-needle inoculation, no OspC transcripts were detectable by RT-PCR in the ears of infected mice.

Although we verified the identity of all of the transcripts we analyzed by Southern blot, we did not attempt to assess by this technique mRNA expression at a level not detectable by ethidium bromide staining. Therefore, our results
only allow us to state that ospC expression is diminished below our detection levels. While this is of course always so, regardless of the sensitivity of the detection technique used, it is a matter of importance in this case, for we cannot state that OspC+ spirochetes were completely eliminated. Hence, the reappearance of detectable OspC+ organisms both in vitro (Fig. 1) and in vivo (Fig. 2 and 5) in the absence of antibody does not automatically entail that OspC+ spirochetes can revert to the OspC+ phenotype. A small surviving population of OspC+ spirochetes may be favorably reselected in the absence of anti-OspC antibody. Furthermore, we do not know if spirochetes do in fact switch between OspC+ and OspC+ phenotypes within the mammalian host.

A B. burgdorferi surface protein whose expression has been described as controlled by the same regulatory network as that of OspC is decorin-binding protein A (DbpA) (29). The alternative sigma factors RpoS and RpoN are both involved in this network (29). When spirochetes are cultivated in vitro in an environment that mimics that of the midgut of an engorging tick, i.e., 37°C, pH 6.8, and increased spirochetal cell density, the expression of both OspC and DbpA is upregulated (30). In vivo the pattern of expression of these two proteins differs somewhat in that, while OspC expression is upregulated in the feeding tick, expression of DbpA is not detected in this environment but only shortly after infection of the mammalian host (15). Interestingly, when mice immunized with DbpA are infected via tick-bite, the anti-DbpA antibody does not protect the host against infection, yet when naive mice are thus infected, anti-DbpA antibody is elicited in the course of infection (15). Thus, on the one hand, DbpA is expressed by B. burgdorferi in the tick-infected mammalian host but on the other, anti-DbpA antibody is not protective regardless of whether it is induced by infection or vaccination. This paradox may be explained by assuming that, as with OspC, DbpA-expressing spirochetes are eventually selected against within the mammalian host.

That the adaptive immune response is involved in the selection (or induction) process leading to the predominance of the OspC− phenotype was clearly demonstrated by the persistence of OspC+ organisms in SCID mice (Fig. 3). The sufficiency of antibody to effect the selection of OspC+ spirochetes was demonstrated by the reconstitution of this process in SCID mice that had been given anti-OspC antibody (Figs. 4 and 5). As we analyzed the antibody response to OspC in C3H mice, we noticed that, in contrast to the titer of anti-VlsE (Ct) antibody, which increased continually, the anti-OspC antibody titer peaked early and then began to decrease, following closely the pattern of expression of the OspC transcript. In SCID mice, the disappearance of passively transferred anti-OspC antibody eventually resulted in the reappearance of spirochetes expressing ospC (Fig. 5). We would therefore predict that if in immunocompetent mice the anti-OspC antibody titer were to decline sufficiently, the ospC transcript might become detectable again, as spirochetes of the OspC+ phenotype again predominate. This should lead to a renewed increase in anti-OspC antibody. In fact, when Fung and coworkers (31) serially studied the humoral immune response to OspC in humans, they noticed that the anti-OspC antibody level oscillated over time, perhaps reflecting the phenomenon we observed in mice. The anti-VlsE antibody response pattern we observed in mice, albeit for only 66 d, also echoes our observations made both in dogs and in monkeys. In these animals, anti-VlsE antibody, as measured by the response to the invariable region (IK)6 of VlsE, remained essentially unchanged for years (24, 32). And in humans, the anti-IR6 response was detected with very high sensitivity in patients with late Lyme disease (24).

Recently, Zhong and colleagues communicated that while active immunization of disease-susceptible AKR/N mice with OspC led to prevention but not resolution of disease and infection (33), passive transfer of OspC immune serum resulted in a dose-dependent resolution of fully established arthritis and carditis as well as infection in needle-challenged C.B-17 SCID mice (33, 34). On the face of it the latter result contradicts our findings. However, experimental protocol differences such as, among many others, type and dose of anti-OspC antibody, spirochetal and mouse strains, and infectious dose precludes a meaningful interpretation of result differences.

OspC is an antigenically diverse molecule (35, 36). In fact, the ospC variation of B. burgdorferi sensu stricto alone within a local population (Shelter Island, NY), as assessed by single-strand conformation polymorphism, was shown to be almost as great as the variation of a similarly sized sample of the entire species (35). However, this diversity appears to be entirely unrelated to the ability of B. burgdorferi to establish a chronic infection. Barthold and coworkers have assessed whether OspC diversity could be arrived at by immune selective pressure imposed by anti-OspC antibody during infection in mice (37, 38). In a most recent series of experiments, mice were infected with clonal B. burgdorferi sensu stricto N40 or B. afzelii PK0 and then were hyperimmunized with homologous recombinant OspC. After 6 mo, a comparison of gene sequences among 4 B. burgdorferi sensu stricto N40 and 9 B. afzelii PK0 isolates from OspC-immunized mice revealed no ospC variation from input inocula. The authors concluded that variation in ospC among B. burgdorferi isolates and species during chronic infection is not likely to be an important mechanism of immune evasion (38).

Sequencing of the B. burgdorferi genome has uncovered no less than 161 paralogous gene families (39, 40). At least one of these families of genes, ospE ospF (a.k.a. erp, p21, pG, elpA, elpB, bbk2.10, and bbk2.11) encodes molecules likely to be exposed on the spirochete surface (41, 42). Mutation and recombination of ospE-related genes result in the development of new antigenic variants and it has been suggested that this process contributes to immune evasion during a B. burgdorferi infection (43). This is a plausible mechanism that may contribute, together with VlsE antigenic variation to the establishment of chronic infection. The mechanism we propose, selection of nonexpressers, should apply preeminently to surface antigens that are not
part of a polymorphic gene family and are not themselves the subject of an antigenic variation mechanism, e.g., OspC, DbpA, BBK32, and P66.

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