26. P. F. Policastro, U. G. Munderloh, E. R. Fischer, T. E. D. Hackstadt, *J. Med. Microbiol.* **46**, 839 (1997).
27. S. F. Hayes, W. Burgdorfer, *Infect. Immun.* **37**, 779 (1982).
28. The repeated elements in the *R. conorii* genome were initially identified on the basis of the direct self-comparison of the genomic DNA by the BLASTN program (*E* value $< 10^{-4}$). The BLASTN result was then analyzed by the repeat identification program Mocca (*49*). An exhaustive analysis (types, locations, insertion in ORFs, etc.) of the repeats identified in the *R. conorii* genomic sequence is available under the *Rickettsia* database section at http://igs-server.cnrs-mrs.fr/RicBase/.
29. Ten repeat families include the previously identified *Rickettsia* palindromic element described in H. Ogata *et al.*, *Science* **290**, 347 (2000), and H. Ogata, S. Audic, J.-M. Claverie, *Science* **291**, 252 (2001).
30. D. R. Leach, *Bioessays* **16**, 893 (1994).
31. P. M. Sharp, D. R. Leach, *Mol. Microbiol.* **22**, 1055 (1996)
32. D. Raoult *et al.*, *Emerg. Infect. Dis.* **7**, 73 (2001).
33. The presence of transcripts associated with all *R. conorii* split genes was assessed by RT-PCR with primers designed from their predicted coding regions. In addition, we tested five genes (RC0873, RC0426, RC0888, RC0374, and RC0909) of *R. conorii* with no functional counterparts in *R. prowazekii*. Primer sequences and expected amplicon sizes are listed in Web table 2 (*10*). RNA was prepared as follows: A suspension of fresh *R. conorii* was adjusted to 10^8/ml and the bacteria were separated from cells with a sucrose gradient. RNA was extracted with the RN easy Midi kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. RT-PCR was performed on the resulting RNA with the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. Reverse transcription and amplifications were performed in PTC-200 thermocyclers (MJ Research, Watertown, MA) with 40 PCR cycles. RT-PCR products were run in 1% agarose gels, stained with ethidium bromide, and revealed on an ultraviolet box. All amplicons exhibited the expected size. Once RT-PCR was performed, all primer pairs were validated by positive PCR reactions on genomic DNA. The absence of genomic DNA contamination in the RNA preparation was controlled by attempting PCR (with the Gibco-Brl Elongase polymerase) with the primers for ORF RC0909 (WASP-like gene) and ORF RC0843 (comF gene). The PCR assay was negative in both cases.
34. The divergence time was estimated on the basis of 16*S* rRNA sequences, assuming a relatively constant rate of evolution of 1 to 2% per 50 million years (*50*).
35. E. J. Feil *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 182 (2001).
36. V. Roux, D. Raoult, *J. Bacteriol.* **175**, 4895 (1993).
37. B. Ewing, L. Hillier, M. C. Wendl, P. Green, *Genome Res.* **8**, 175 (1998).
38. B. Ewing, P. Green, *Genome Res.* **8**, 186 (1998).
39. S. F. Altschul *et al.*, *Nucleic Acids Res.* **25**, 3389 (1997).
40. S. Audic, J.-M. Claverie, *Proc. Natl. Acad. Sci. U.S.A* **95**, 10026 (1998).
41. M. Kanehisa, S. Goto, *Nucleic Acids Res.* **28**, 27 (2000).
42. A. Bairoch, R. Apweiler, *Nucleic Acids Res.* **28**, 45 (2000).
43. A. Bateman *et al.*, *Nucleic Acids Res.* **28**, 263 (2000).
44. K. Hofmann, P. Bucher, L. Falquet, A. Bairoch, *Nucleic Acids Res.* **27**, 215 (1999).
45. J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* **22**, 4673 (1994).
46. H. Ogata, W. Fujibuchi, S. Goto, M. Kanehisa, *Nucleic Acids Res.* **28**, 4021 (2000).
47. W. R. Pearson, D. J. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 (1988).
48. X. Huang, W. Miller, *Adv. Appl. Math.* **12**, 373 (1991).
49. C. Notredame, *Bioinformatics* **17**, 373 (2001).
50. H. Ochman, S. Elwyn, N. A. Moran, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12638 (1999).

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Role of Nonimmune IgG Bound to PfEMP1 in Placental Malaria

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Infections with *Plasmodium falciparum* during pregnancy lead to the accumulation of parasitized red blood cells (infected erythrocytes, IEs) in the placenta. IEs of *P. falciparum* isolates that infect the human placenta were found to bind immunoglobulin G (IgG). A strain of *P. falciparum* cloned for IgG binding adhered massively to placental syncytiotrophoblasts in a pattern similar to that of natural infections. Adherence was inhibited by IgG-binding proteins, but not by glycosaminoglycans or enzymatic digestion of chondroitin sulfate A or hyaluronic acid. Normal, nonimmune IgG that is bound to a duffy binding–like domain β of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) might at the IE surface act as a bridge to neonatal Fc receptors of the placenta.

Malaria infection with *P. falciparum* during pregnancy is an important cause of maternal morbidity and mortality. It may induce premature delivery, spontaneous abortion, or lead to a low birth weight (*1, 2*). Infections often cause more severe symptoms in primiparous than in multiparous women. The incidence of placental malaria similarly diminishes with increasing parity (*3, 4*), probably due to the acquisition of immunity to the infecting parasites (*5, 6*).

IEs are not passed from the mother to the fetus, but accumulate in the placenta which can experience high parasite densities (>50% IEs) while the peripheral circulation is almost free of IEs. Placental malaria may thus be caused by IEs that are selected for and expanded on receptors only present in the placenta (*7–10*), as opposed to those in other vascular beds.

Certain strains of *P. falciparum* bind nonimmune immunoglobulins onto the surface of the host erythrocyte, a fact that made us investigate their role in sequestration, in particular the possibility that IgG could bridge the IEs to Fc-receptors present in the placenta. We thus examined the frequency of IgG- (and IgM-) binding IEs accumulated during pregnancy in the placenta. Small pieces of snap-frozen placental tissues were obtained from six malaria-infected Cameroonese women after approved consent. The parasitemia of the placentas ranged from three to 23% (Fig. 1A), and all of them were classified as having active or active-chronic infections (*11*). IgG-binding IEs (Fig. 1B) were found in all of the placentas (10 to 75% IgG positive, mean 44%), whereas IgM-binding IEs (Fig. 1C) were more rare (2 to 34%, mean 18%) (*12*). IEs attached to the syncytiotrophoblasts bound only IgG (20 to 80%, mean 50%), except those of placenta CP42DJ, where the number of IgG-binding IEs was equal to that of the IgM-binding IEs. By studying the Ig-binding phenotype of IEs eluted from the placentas (*13, 14*), we confirmed that a majority of parasites causing active placental infection bound IgG (Table

Table 1. The phenotype of *P. falciparum* infecting the human placenta. IEs were eluted from the infected placentas and scored for their immunoglobulin binding.

Placenta	IgG-binding IE/IE tested*	Percent
CP24	18/23	78
CP42	14/14	100
CP42DJ†	–	–
CP193	205/276	74
CP939	32/247	13
CP940†	–	–

*Fractions of the IEs were also studied for their capacity for binding to Sca1D cells including inhibition with soluble CSA (100 μg/ml) and treatment with CSAnase ABC (0.5 U). About 50% of the IEs were specific for CSA (*11*). The populations of parasites studied for CSA-binding were not identical to those studied for immunoglobulin-binding since not all eluted IEs were scored in the CSA-assays. †The number of eluted parasites obtained from the placentas CP42DJ and CP940 were not sufficient for the assay.

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1). Fractions of IEs eluted from the same placentas also bound to chondroitin sulfate A (CSA) (Table 1). IgG binding was very infrequent among the small number of IEs available from the corresponding peripheral venous blood (1/15, 7%, mother 42; 0/4, mother 193; 0/21, mother 939). The accumulation of IEs carrying IgG in the placenta and their absence from the peripheral circulation suggests that *P. falciparum* IEs with an IgG-binding phenotype are selected for in the placenta.

An IgG-binding parasite (TM284, 30% IgG-positive IEs) was cloned by micromanipulation and the progeny were used to further study the role of IgG for the binding of IEs in the placenta. One of the cloned lines (TM284S2) had an up-regulated capacity to bind normal, nonimmune immunoglobulins with ≈80% of the IEs being IgG- (and IgM-) positive. It also formed giant rosettes (≈80% of IEs) composed of mixed rosettes and autoagglutinates. TM284S2 IEs were found to have very low or no binding to common endothelial receptors such as CD36 (28 ± 1 IE/100 transfectants), CD31/PECAM-1 (6 ± 1 IE/100 transfectants), or ICAM-1 (0 ± 0 IE/100 transfectants). TM284S2 did not bind to CSA spotted on plastic (0 IE/mm^2 ± 0) (*15*), whereas FCR3^{CSA}—a parasite enriched in vitro for CSA binding (*16*) studied in the same experiment—avidly did so (434 ± 31 IE/mm^2). Further, late-stage IEs of TM284S2 adhered massively to fresh cryosections of normal human placental tissues in a pattern similar to the in vivo localization of IE (Fig. 1, D and E). The binding occurred at the syncytiotrophoblast surfaces and (70 ± 1%) at syncytial bridges with a mean of 224 ± 88 IEs per mm^2 without any prior panning or enrichment steps. IEs of two sibling clones that lacked the IgG-binding phenotype (TM284S25, TM284S27) did not bind to the placental sections.

Preincubation of the placental sections with IgG (5 to 20 mg/ml) before the adhesion assay increased the binding for TM284S2 to around 200% (Fig. 1F), whereas preincubation of the IEs with the same amounts of IgG increased the binding to 300%. A similar effect was seen when whole human serum was added. However, oversaturation with IgG reduced the binding to around 30% compared to the control (≈70 IEs/mm^2; Fig. 1F). In order to verify the importance of IgG, an independent IgG-binding protein, protein A of *Staphylococcus aureus*, was used as an inhibitor. A strong and dose-dependent inhibition of the placental binding of TM284S2 was obtained using *Staphylococcus* protein A (Fig. 1G). No inhibition was seen at equal (or higher) concentrations of an unrelated protein, bovine serum albumin (BSA).

Treatment of the placenta sections with the enzymes hyaluronidase as well as chondroitinase ABC prior to IE binding did not affect the binding of TM284S2-IE (Fig. 1H). Binding was only marginally affected by CSA or hyaluronic acid (HA); when incubated at concentrations as high as 100 μg/ml, they reduced the binding by 30% at most. Thus, the IEs of TM284S2 adhere to the syncytiotrophoblasts via Fc receptors bridged by IgG whereas CSA or HA probably play minor roles in the sequestration in this parasite. In contrast, FCR3^{CSA} readily adhered in a CSA-dependent manner to the placental sections (125 ± 23/mm^2) without forming auto-agglutinates or giant rosettes, or binding IgG to the IE surface (Fig. 1H).

Antibodies in normal, nonimmune human sera were found to bind to a ≈300,000 M_r polypeptide having the characteristics of PfEMP1 from an extract of [^{125}I]lacto-peroxidase–labeled IEs of TM284. The corresponding *var* gene in TM284S2 was identified with reverse transcription polymerase chain reaction (RT-PCR) using degenerate primers and it was subsequently cloned and sequenced (*17*). A 7582-bp sequence was identified equivalent to an open reading frame of 2527 amino acids composed of an NH_2-terminal head-structure (NTS, a DBL1α, CIDR1α), three additional duffy binding–like (DBL) motifs (β, γ, δ), one additional CIDRγ domain and an acidic terminal segment (ATS) (Fig. 2A). Thus, the EMP1 of this placental-binding parasite presents a similar architecture (including a DBL3γ-domain) to that of the PfEMP1s expressed by previously identified placental-binding parasites (*18, 19*).

To map the immunoglobulin-binding site of TM284S2, PfEMP1 constructs corresponding to the six extracellular domains and

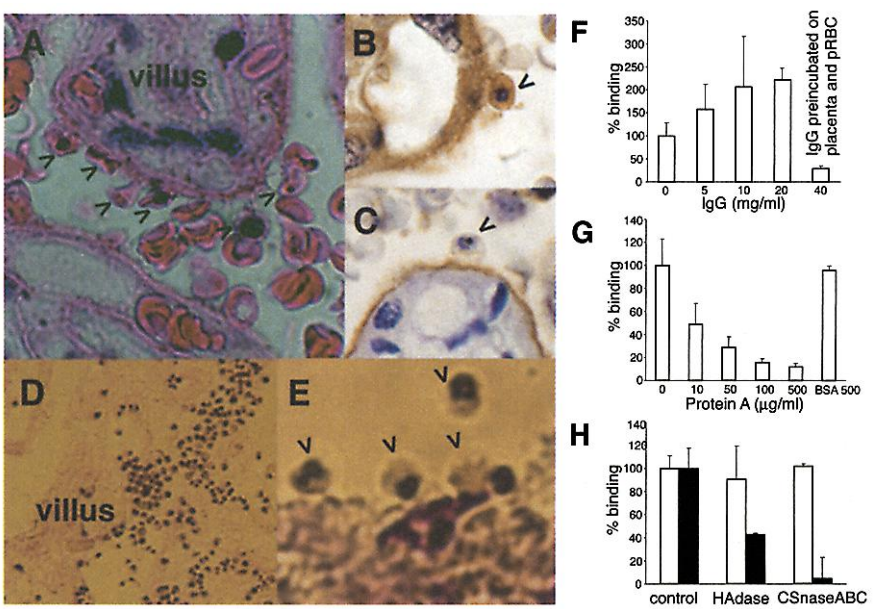

Fig. 1. The binding of *P. falciparum*–infected erythrocytes in the human placenta. (A through C) Sections of the Cameroonese placenta ICP193 stained with different methods. Paraffin sections of placenta tissue were fixed immediately after partus, dehydrated, and paraffin-embedded according to standard procedures. (**A**) Binding of IEs (arrowheads) to the syncytiotrophoblast surface, hematoxylin/eosin-staining. (**B**) IgG staining with peroxidase (brown) and DNA counterstaining with Mayers hematoxylin (blue). The IgG staining is seen at the surface of the syncytiotrophoblasts and within them. Staining is further seen in the stroma, the endothelial cells and the capillaries, reflecting the complex transport pathway of IgG from the maternal to the fetal blood. Arrowhead: IgG-binding IE. (**C**) IgM staining with peroxidase and counterstaining as in (B). Note that in contrast to IgG, IgM is found only at the rim of the villi, because IgM is not transported from the maternal to the fetal side of the circulation. Arrowhead: bound IEs. (**D** and **E**) Binding of TM284S2 late-stage IEs to cryosections of normal Swedish placentas. IEs enriched by Percoll-separation were incubated on the placenta sections for 1 hour at 37°C, nonadherent cells were washed off. The sections were fixed and stained with 5% Giemsa. Numbers were obtained by counting at least 25 fields in 400× magnification and expressed per mm^2. IE bound to placenta tissue in means in numbers of 224 ± 88 IE/mm^2. Arrowheads: bound IEs. (F through H) Binding of TM284S2-IE to normal Swedish placenta cryosections under the influence of various molecules. Binding is expressed as a percentage compared to a control without treatment. Error bars represent SE. (**F** and **G**) The placental sections were incubated with increasing concentrations of nonimmune IgG (5 to 40 mg/ml) or protein A (10 to 500 μg/ml) for 1 hour at 37°C prior to the IE binding. Adhesion assays were thereafter carried out as described in (D). An unrelated protein (BSA) is shown as negative control. (**H**) Placenta sections were treated twice with hyaluronidase (10 μg/ml) or chondroitinase ABC (0.5 U/ml) for 20 min at 37°C prior to IE binding. Open bars represent the binding of TM284S2 and solid bars show the binding of FCR3^{CSA}.

seven subfragments of DBL2β were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* and tested for binding (Fig. 2, A and B). Amino acids 214 through 365 of the DBL2β were found to carry multiple immunoglobulin-binding domains. IgG of 11 other, distanct animal species also bound to DBL2β.

To investigate whether PfEMP1 is involved in the binding to IgG in the placenta, all seven DBL2β-GST fusion proteins were tested for their ability to adhere to normal placental tissues. Only those fusion proteins which showed binding activity to IgG in enzyme-linked immunosorbent assay (ELISA) were found to bind (Fig. 2C), and preincubation of the sections with IgG strongly reduced their binding. Furthermore, the adherence of IEs to cryosections of the placenta could be reduced by preincubation with the immunoglobulin-binding fusion proteins (DBL2B, DBL2C; Fig. 2D). Thus, DBLβ is the critical domain of PfEMP1 that binds monomeric nonimmune IgG through the F(ab′) part, leaving the Fc-γ domain free for binding to Fc-receptors in the placenta.

There is a sharp increase in the transport of maternal IgG to the fetus during the second trimester, resulting in serum levels of IgG that are higher in the child than in the mother at the end of pregnancy (*20*). The second trimester is also the time when the placenta goes through a series of important morphological changes, and the frequency of placental malaria peaks. The high prevalence of maternal malaria at this time can be due to an extended availability or higher expression of IE receptors such as CSA or HA but it may also depend on the up-regulation of maternal Fc receptors. No matter what, it is likely that multiple rather than few receptors mediate sequestration of IEs, because the capacity to bind to alternate structures in the placenta will ensure the survival of the parasite.

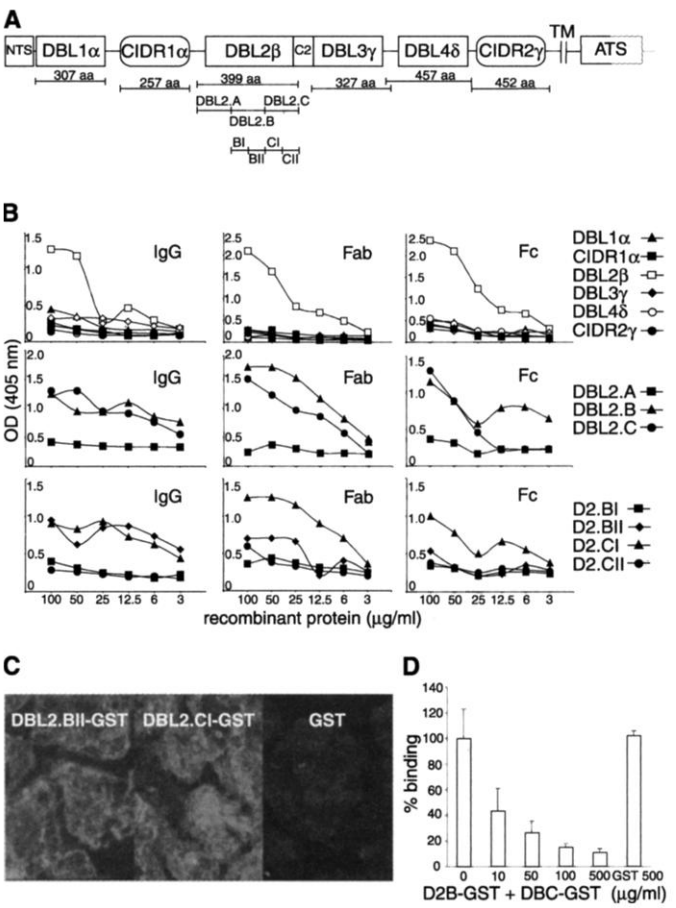

Fig. 2. Structure and function of the PfEMP1 of the placenta-binding *P. falciparum* clone TM284S2. (**A**) The clone TM284S2 expresses a PfEMP1, of which the extracellular part consists of four duffy-binding–like and two cysteine-rich interdomain regions. Six GST fusion proteins each representing one of the domains were constructed and expressed in *E. coli* (constructs indicated as lines). Seven additional GST-constructs representing different fragments of the DBL2β were created to map the Ig-binding part of the molecule. (**B**) Mapping of the Ig-binding domain on PfEMP1 by ELISA: All GST fusion proteins were tested for their ability to bind to IgG as well as the Fab- and Fc-regions of the molecule. ELISA plates were coated with 5 μg/ml of the fusion protein overnight at 4°C and blocked with 3% BSA. The GST-fusion proteins were added in a series of double dilutions from 3 to 100 μg/ml. Binding was detected using an anti-GST antibody and an ALP-coupled antibody. The DBL2β was identified as the Ig-binding domain of the PfEMP1 in this parasite clone. Binding could be observed to IgG as well as the Fab and Fc regions of the molecule [and in a similar pattern to IgM, the Fab and the Fc region of IgM (*21*). Three constructs representing one-third of the DBL2β each indicated the binding sites to Ig to lie in the middle and COOH-terminal part of DBL2β. Four additional constructs allowed to map the binding to two independent regions within the DBL2β and DBL2.BII corresponding to amino acids 912 to 1055 and DBL2.CI to amino acids 1050 to 1109 of the PfEMP1. (**C**) Binding of GST fusion proteins to placenta cryosections of normal Swedish placentas. The fusion protein was added to placental cryosections in concentrations of 25 μg/ml and incubated at 37°C for 1 hour. After washing, the binding was detected with an anti-GST antibody. Only those DBL2 fusion proteins adhered to the placenta tissue, which have been previously shown to bind to IgG in the ELISA assays (results shown for DBLBII and DBLCI). GST only is shown as a negative control. (**D**) Binding of TM284S2-IE to normal Swedish placenta cryosections under the influence of the PfEMP1 fragments DBL2B-GST and DBL2C-GST. Placenta sections were incubated with increasing concentrations of both GST fusion proteins prior to IE binding. Adhesion assays were carried out as described in Fig. 1D. Binding is expressed as a percentage compared to a control without treatment. GST alone is shown as a control. Error bars represent SE.

References and Notes

1. C. Menendez, *Parasitol. Today* **11**, 178 (1995).
2. I. A. McGregor, *Parassitologia* **29**, 153 (1987).
3. ———, *Am. J. Trop. Med. Hyg.* **33**, 517 (1984).
4. B. J. Brabin, *Bull. WHO* **61**, 1005 (1983).
5. M. Fried, F. Nosten, A. Brockman, B. J. Brabin, P. E. Duffy, *Nature* **395**, 6705 (1998).
6. C. H. Ricke *et al.*, *J. Immunol.* **165**, 6 (2000).
7. C. Robert *et al.*, *Res. Immunol.* **146**, 6 (1995).
8. S. J. Rogerson, S. C. Chaiyaroj, K. Ng, J. C. Reeder, G. V. Brown, *J. Exp. Med.* **182**, 1 (1995).
9. M. Fried, P. E. Duffy, *Science* **272**, 1502 (1996).
10. J. G. Beeson *et al.*, *Nature Med.* **6**, 1 (2000).
11. J. N. Bulmer, F. N. Rasheed, N. Francis, L. Morrison, B. M. Greenwood, *Histopathology* **22**, 3 (1993).
12. Ig binding in the infected placentas was determined by rehydrating and neutralizing the sections, protein cross-links were digested with trypsin (0.1%), endogenous peroxidase activity was removed with 10% H_2O_2, and blocking with rabbit serum was carried out overnight. Biotin-coupled antibodies to human IgG or IgM were added and thereafter samples were incubated with avidin-peroxidase. After three washes with phosphate-buffered saline, the sections were developed with 3,3′-diaminobenzidine and counterstained with Mayers hematoxylin.
13. C. Scholander, C. J. Treutiger, K. Hultenby, M. Wahlgren, *Nature Med.* **2**, 2 (1996). For studying IEs from the peripheral blood of the mothers, parasites were grown for about 20 hours before being scored in the assays.
14. IEs were eluted from the placentas by injecting 0.9% NaCl (plus 5 IU of heparin/ml) and repeated injection of heparinized RPMI 1640. For details, see J. Gysin, B. Pouvelle, N. Fievet, A. Scherf, and C. Lépolard [*Infect. Immun.* **67**, 6596 (1999)].
15. Plates were coated with CSA (50 μg/ml) overnight at 4°C and blocked with BSA (20 mg/ml) for 30 min at room temperature (RT, 20°C), Percoll-enriched late-stage IEs were incubated for 1 hour at RT followed by four washes with RPMI 1640, and bound cells were fixed with methanol and stained with 5% Giemsa.
16. A. Scherf *et al.*, *EMBO J.* **17**, 18 (1998).
17. The sequence reported in this paper has been deposited in the GenBank database (accession no. AF366567).
18. J. C. Reeder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9 (1999).
19. P. A. Buffet *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 22 (1999).
20. D. Gitlin, A. Biasucci, *J. Clin. Invest.* **48**, 8 (1969).
21. K. Flick *et al.*, data not shown.

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