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9. To determine the presence of diarrhea, we examined each mouse pup every 1 to 2 hours for the first 8 hours and at 24 hours after inoculation by gently pressing the abdomen. Diarrhea was noted and scored from 1 to 4, with a score of 1 reflecting unusually loose yellow stool and a score of 4 indicating completely liquid stool. A score of 2 (mucous with liquid stool, some loose but solid stool) and above was considered diarrhea. A score of 1 was noted but was not considered as diarrhea. The scoring was done by a single person and the pups were coded during analysis of diarrhea. Other symptoms monitored included lethargy, coldness to the touch, and ruffled coats in older animals.
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17. Short-circuit currents ($I_{sc}$) were measured with an automatic voltage clamp across unstripped intestinal mucosal sheets from CD1 mice 19 to 22 and 35 days old (Bioengineering, Univ. of Iowa) as described [C. L. Sears, R. L. Guerrant, J. B. Kaper, in *Infections of the Gastrointestinal Tract*, M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, R. L. Guerrant, Eds. (Raven, New York, 1995), chap. 44; A. P. Morris, S. A. Cunningham, A. Tousson, D. J. Benos, R. A. Frizzell, *Am. J. Physiol.* **266**, C254 (1994)]. The midileum of the mouse intestines was used. The mucosal sheets taken from the intestine were placed into modified Ussing chambers with 0.12-cm$^2$ apertures (machine shop, Univ. of Texas Health Science Center) and transepithelial potential ($V_t$) was registered by 3 M KCl agar bridges connected to balanced calomel half-cells. The transepithelial current required to clamp $V_t$ to 0 was passed through Ag-AgCl electrodes connected to the 3 M KCl bridges. All experiments were performed at 37°C in bicarbonate Ringer solution gassed with 95% $O_2$ and 5% $CO_2$ by airlift circulators as previously described (*ibid.*). The mucosal bath contained sodium-free (*N*-methyl-D-glutamine) substituted Ringer solution to minimize the effects on $I_{sc}$ of cAMP-stimulated electrogenic $Na^+$-glucose co-transport across the small bowel [B. R. Grubb, *Am. J. Physiol.* **268**, G505 (1995)]. After temperature and ionic equilibration, basal $I_{sc}$ measurements were taken and intestinal mucosal sheets were challenged with peptide, Cch, or FSK. Bumetamide sensitivity was tested and confirmed the chloride secretory response.
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# Protective Effect of Rotavirus VP6–Specific IgA Monoclonal Antibodies That Lack Neutralizing Activity

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Rotaviruses are the leading cause of severe gastroenteritis and dehydrating diarrhea in young children and animals worldwide. A murine model and "backpack tumor" transplantation were used to determine the protective effect of antibodies against VP4 (an outer capsid viral protein) and VP6 (a major inner capsid viral protein). Only two non-neutralizing immunoglobulin A (IgA) antibodies to VP6 were capable of preventing primary and resolving chronic murine rotavirus infections. These antibodies were not active, however, when presented directly to the luminal side of the intestinal tract. These findings support the hypothesis that in vivo intracellular viral inactivation by secretory IgA during transcytosis is a mechanism of host defense against rotavirus infection.

Mucosal IgA is a secretory antibody that forms a first line of defense against many pathogens. It is synthesized as an oligomeric molecule that can be transported via transcytosis across certain epithelial cell types lining mucosal surfaces and then released into the mucosal environment (*1*). Several mechanisms by which secretory IgA provides protection have been proposed (*2*). Recently, Mazanec *et al.* described an in vitro model in which transcytosing IgA molecules form complexes with certain viruses that have entered the cell and thereby inhibit viral replication intracellularly (*3*). To determine whether this can occur in vivo and whether non-neutralizing antibodies can mediate this intracellular effect, we studied the effects of IgA monoclonal antibodies (mAbs) on rotavirus infection in mice.

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Rotaviruses cause 130 million cases of gastroenteritis and diarrhea worldwide (*4*, *5*). A rotavirus's protein-coated RNA core is surrounded by a second protein layer composed of VP6 (42 kD). VP6 makes up about 50% of the virion mass and is highly immunogenic; however, antibody to VP6 does not have neutralizing activity in vitro (*4*, *6*). Two viral proteins of the outermost protein coat, the viral hemagglutinin VP4 (85 to 88 kD) and the viral glycoprotein VP7 (37 kD), have been directly implicated as targets of serotype-specific neutralization in vitro and protection in vivo (*6*). However, the presence of neutralizing antibody to rotavirus in serum may not correlate with protection (*7*, *8*), and in some studies of humans and animals, protection against rotavirus infection appears to be serotype-independent (*7*). There is a strong correlation between virus-specific intestinal IgA amounts and protection in vivo (*8*–*10*).

We have isolated and characterized a library of IgA-secreting mAbs directed at several rotavirus proteins and evaluated them in the murine model of rotavirus infection (*8*, *9*, *11*). Monoclonal antibodies of the IgA isotype were generated to the murine rotavirus strains EC, EHP, or EW (*12*). A "backpack tumor" model (*13*) was used for analysis, in which hybridoma cells were injected subcutaneously into the upper back of histocompatible BALB/c mice. At the injection site, the mice secreted mAbs that were subsequently transported in the circulatory system. If the secreted IgA antibody molecules were oligomeric, they could be physiologically transcytosed to mucosal surfaces (*14*).

Multiply cloned IgA or IgG hybridoma cell lines (Table 1) reactive with either VP4 or VP6 were transplanted into 2-month-old, rotavirus-naïve, histocompatible BALB/c mice at a dose of $10^6$ cells per mouse. All the IgA monoclonals were oligomeric as measured by polyacrylamide gel electrophoresis (PAGE) migration (*15*). Three out of four IgA mAbs to VP4 had in vitro neutralizing activity against the challenge virus (EC), whereas none of the three mAbs to VP6 demonstrated neutralizing activity in vitro (Table 1). A high-titer, serotype G3–specific, neutralizing, anti-VP7 IgG hybridoma, 4F8, was also included because investigators have reported that large amounts of serum-neutralizing antibody correlate with protection against subsequent rotavirus infection (*16*). Between 14 and 16 days after transplantation, when the tumors were visible and hybridoma-produced antibodies could be detected in sera and stools, the backpack mice were orally challenged with $10^4$ shedding dose 50s of wild-type EC murine rotavirus (serotype G3P16), and daily stool samples were collected as described (*8*). Control animals and animals transplanted with IgA mAbs to VP4, IgG mAbs to VP7, or IgG mAbs to VP6 were not protected from challenge (Fig. 1A and Table 1). However, two of three IgA antibodies to VP6 (7D9 and 10C10) completely blocked infection (Table 1 and Fig. 1A), whereas one IgA mAb to VP6 (8D3) had no effect. Four clonings of the 7D9 hybridoma did not alter its antiviral activity. The two positive antibodies to VP6 (7D9 and 10C10) were isolated from independent fusions. Both 7D9 and 10C10 react exclusively with the trimeric form of VP6 (*15*) and neither has detectable neutralizing activity in vitro (Table 1).

All animals that received the 7D9 backpack tumor transplants had detectable amounts of IgA in their stool by day 12 (Fig. 2); however, the amount of IgA varied in different animals as previously described (*13*). Similar studies with IgA mAbs to VP4 demonstrated that these antibodies were also transported into the small intestine by the time of challenge (*15*). IgG mAbs were not detected in feces before challenge (*15*). The amount of IgA antibody in the feces at the time of challenge (Fig. 2) was less than or

**Table 1.** Specificity and reactivity of selected IgA and IgG mAbs. ND, not determined.

| mAb | Isotype | Protein specificity | In vitro neutralization* | Protection in vivo†‡ | Resolution in SCID mice†‡ |
|---|---|---|---|---|---|
| 6D10 | IgA | VP4 | <40 | 0/3 | 0/3 |
| 8D6 | IgA | VP4 | 40,960 | 0/4 | 0/2, 0/2 |
| 4G10 | IgA | VP4 | 2,560 | 0/2, 0/4 | ND |
| 8D2 | IgA | VP4 | 10,240 | 0/3 | 0/3 |
| 10C10 | IgA | VP6 | <20 | 3/3 | 2/2, 2/2 |
| 7D9 | IgA | VP6 | <20 | 2/2, 3/3, 5/5 | 3/3, 3/3 |
| 8D3 | IgA | VP6 | <20 | 0/2, 0/3 | 0/3, 0/2 |
| 5F6 | IgG2b | VP6 | <20 | 0/3 | ND |
| 4F8 | IgG1 | VP7 | 163,840 | 0/4 | 0/2 |

*Neutralization titer determined by focus reduction assay with EC murine rotavirus. †Protection and resolution indicate absence of rotavirus shedding. ‡Shown below is the number of protected or resolved mice versus the total number tested in a specific experiment.

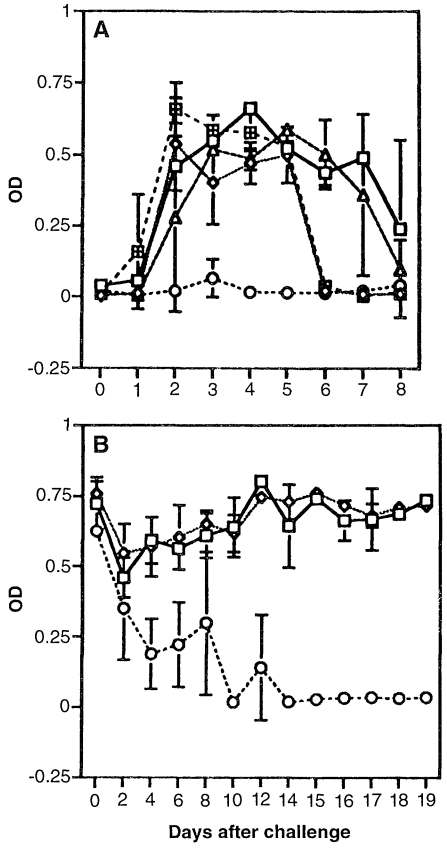


**Fig. 1.** (**A**) Rotavirus antigen shedding profiles from BALB/c mice bearing hybridoma backpacks. Groups of three rotavirus-naïve BALB/c mice (6 to 8 weeks old) were injected in the scruff of the upper back with $10^6$ hybridoma cells. Fourteen to sixteen days after injection (day zero), all mice were orally inoculated with $10^4$ shedding dose 50s of wild-type EC murine rotavirus (G3P16) as previously described (*9*). Daily stool samples were collected from each mouse for the duration of the experiment (8 days). Ten percent stool suspensions (w/v) were prepared in tris-buffered saline [10 mM tris, 100 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4)] containing 5% fetal bovine serum, 0.05% Tween 20, 5 mM sodium azide, and 1% aprotinin (stool diluent). Rotavirus stool antigen shedding was measured by ELISA (*8*). Each point represents the daily average of a group of three mice; error bars represent ±1 SD. OD, optical density. Squares, 4G10 IgA mAb to VP4; diamonds, 5E6 IgG mAb to VP6; circles, 7D9 IgA mAb to VP6; triangles, 8D3 IgA mAb to VP6; squares with crosses, control. (**B**) Rotavirus antigen shedding profiles from chronically infected SCID mice bearing hybridoma backpacks. SCID BALB/c mice were chronically infected with the wild-type EW strain of murine rotavirus as described (*29*). Two to three months after becoming chronically infected, groups of three mice were injected with $10^6$ hybridoma cells as described (*13*). Daily stool samples were collected before hybridoma transplantation on day zero and afterward for 19 days. Ten percent stool suspensions were prepared, and antigen shedding was measured by ELISA as described (*8*). Each point represents the daily average of a group of three mice; error bars represent ±1 SD. OD, optical density. Squares, 4F8 IgG mAb to VP7; diamonds, 6D10 IgA mAb to VP4; and circles, 7D9 IgA mAb to VP6.

comparable to the amount seen after primary rotavirus infection, which indicates that the protective effect observed was not due to massive amounts of IgA mAb to rotavirus entering the gastrointestinal tract lumen (*8*, *9*).

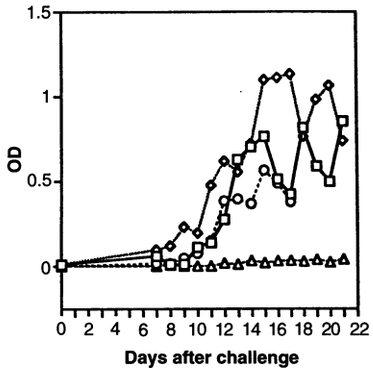


**Fig. 2.** Presence of IgA antibody in stool of mice transplanted with the 7D9 hybridoma. Three rotavirus-naïve BALB/c mice were injected with $10^6$ 7D9 hybridoma cells as a backpack tumor on day zero. Stool samples were collected for 21 days, and 7D9 IgA amounts were determined by ELISA as described previously (*8*). Mouse number 3 died on day 17. Squares, mouse number 1; diamonds, mouse number 2; circles, mouse number 3; and triangles, control.

To determine if antibody was capable of resolving ongoing infection, BALB/c mice with severe combined immunodeficiency disease (SCID) were infected as sucklings with wild-type EW (G3P16) murine rotavirus. These mice developed chronic rotavirus infection and persistently shed detectable amounts of viral antigen in their stool. After the SCID mice had been chronically infected for at least 2 months, hybridoma cell lines were transplanted subcutaneously into these animals ($10^6$ cells per mouse), and daily stools were assayed for the disappearance of viral antigen (Table 1 and Fig. 1B). The animals given the 7D9 cell line showed no detectable antigen shedding 12 days after cell transfer and remained free of virus until the experiment was terminated 8 days later (Fig. 1B and Table 1). The SCID mice transplanted with the other hybridoma lines (with the exception of 10C10 IgA mAb to VP6) continued to shed rotavirus (Table 1). In other studies involving more than 60 chronically infected SCID mice, we have never observed spontaneous resolution of rotavirus infection.

In initial studies, we investigated the ability of passively transferred IgA ascites to reduce illness in 5-day-old suckling mice. Intraperitoneal administration of 7D9 IgA ascites, but not of 8D3 control antibody (100 μl per day for 7 days), delayed the onset and shortened the duration of diarrheal illness by 3 days in more than 50% of animals. Diarrhea lasted 7 days in control mice.

A potential confounding factor in interpretation of the passive cell transfer experiments was the possibility that the 7D9 and 10C10 VP6-specific mAbs were forming immune complexes with rotavirus in the intestinal lumen, thereby blocking enzyme-linked immunosorbent assay (ELISA) reactivity in the shedding assays. However, RNA gene segments were not detected in the stool of the 7D9-treated mice, whereas typical rotavirus double-stranded RNA gene segments were apparent in the stool of mice treated with other hybridomas (Fig. 3). In additional experiments, we demonstrated that mixing of murine rotavirus with 7D9 ascites in vitro did not block our ability to detect virus by solid phase immunoassay (*15*).

We next carried out a passive feeding study with high-titer ascites ($3 \times 10^5$ dilution positive in ELISA) containing the 7D9 antibody or control ascites containing a non-neutralizing IgG mAb to VP7 (*17*). Feeding of 100 μl of antibody 7D9 in ascites form did not alter the shedding of EC virus in three mice, indicating that orally administered mAb 7D9 to VP6 is not capable of inhibiting rotavirus infection (*15*).

We used a ligated intestinal loop model to better characterize the site of action of mAb 7D9. No intestinal cell staining was observed when closed intestinal loops (*18*) of mice were inoculated with EC virus mixed with high-titer IgG neutralizing antibodies to VP4 and VP7 (*15*), whereas loops inoculated with EC virus alone, with EC virus mixed with 7D9 mAb, or with EC plus IgA myeloma (an irrelevant antibody) all showed staining of rotavirus-infected intestinal epithelial cells (Fig. 4, A through C, respectively). On the other hand, isolated loops of small intestine in mice carrying 7D9 backpack tumors were fully resistant to primary infection with the EC strain of murine rotavirus (Fig. 4D) (*19*). Thus, IgA antibody was not able to inhibit primary rotavirus replication when directly administered on the luminal side of the intestine, even at very high concentration, but was effective if delivered to isolated loops from the circulation.

Our observations support the hypothesis that the antirotaviral effect of the non-neutralizing IgA mAbs is occurring during IgA transcytosis rather than as an extracellular event in the gut lumen. This conclusion is reinforced by the finding that transplantation of a mAb-secreting tumor that produced large amounts of neutralizing IgG antibody (4F8, Table 1) did not prevent or resolve rotavirus infection, presumably because the IgG antibody did not gain access

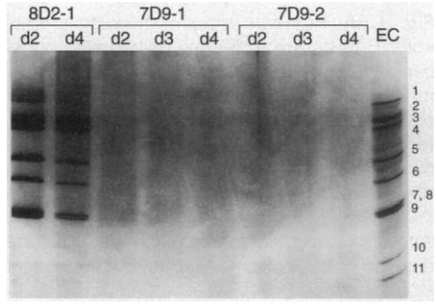


**Fig. 3.** Electrophoretic profiles of RNA extracted from stool suspensions obtained from hybridoma-bearing BALB/c mice. RNA extraction and electrophoresis and silver staining were carried out as described (*9*). Data from one mouse carrying the anti-VP4 IgA hybridoma backpack and two mice carrying 7D9 hybridoma backpacks are shown. Days 2 and 4 after infection (d2 and d4) represent the peak of rotavirus antigen shedding in naïve mice orally infected with wild-type murine rotavirus, as detected by ELISA. Lane EC contains RNA from a preparation of tissue culture–adapted EC murine rotavirus extracted in parallel as a control. The locations of the 11 genome segments are marked at the right.

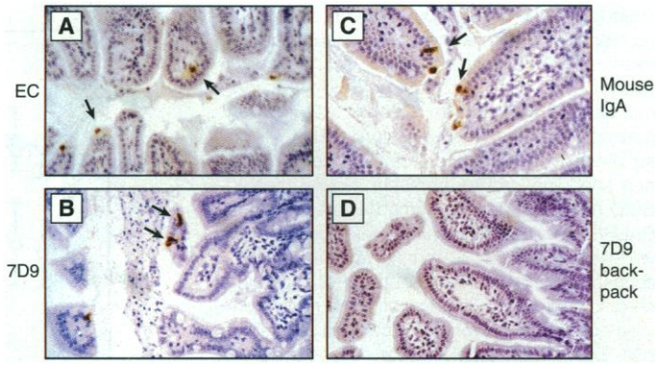


**Fig. 4.** Photomicrograph of sections of mouse small intestine immunostained for EC rotavirus. Approximately 1 cm of ileal loops were removed from mice after 12 hours of exposure to activated EC and to different antibodies as indicated and described (*18*). Four sections were obtained from each end and four from the middle portion of each loop. A total of 12 sections were immunostained for EC with a rabbit hyperimmune serum against rotavirus (1:1000 dilution), according to the protocol recommended by Vector Laboratories (Vectastain ABC kit, Vector Laboratories, Burlingame, California). Loops were inoculated with (**A**) EC alone, (**B**) EC plus mAb 7D9, (**C**) EC plus mouse IgA (irrelevant antibody), and (**D**) EC alone in 7D9 backpack animals (magnification, ×400). Arrows indicate rotavirus-infected cells. All experiments carried out on animals were in accordance with National Institutes of Health and institutional guidelines and approved by the VA animal welfare committee.

to the gut lumen. Further studies with other mucosal pathogens are needed to determine the general applicability of this protective mechanism.

The mAbs 7D9 and 10C10 inhibited replication of at least two separate murine rotavirus strains (EC and EW; Fig. 1, A and B) and reacted with virtually all other mammalian strains in ELISA (*15*). Immunization with VP6-encoding DNA has also been shown to protect mice from rotavirus challenge in recent studies (*20*). If VP6-specific IgA antibodies with similar protective activity are generated after natural rotavirus infection or vaccination, they are likely to play a role in the heterotypic immunity observed in a variety of vaccine field trials and epidemiologic studies.

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12. Four to six days after oral immunization with infectious wild-type murine rotavirus strains EC, EHP, or EW (*9*), Peyer's patch lymphocytes were obtained from 4-month-old mice and fused with FOX-NY myeloma cells (*21*), as previously described (*22*). Rotavirus-positive clones were identified with an immunohistochemical staining procedure and were isolated and characterized as previously described (*23*). The protein specificity of the clones was determined by ELISA with the use of recombinant baculovirus antigen or solid-phase immunoisolation techniques (or both) using radiolabeled rotavirus-infected cell lysates (*22, 24*). The isotype of the individual mAbs was determined as previously described (*22*), and the molecular weight was determined on non-denaturing PAGE gels as previously described (*25*). In vitro neutralization assays of individual mAbs were carried out as previously described (*23*).
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17. Three nonimmune adult BALB/c mice (6 to 8 weeks old) were fed a 1:2 dilution of ascites containing either mAb 7D9 or 60 (*26*) 1 hour before challenge ($10^4$ shedding dose 50s of wild-type EC murine rotavirus) and 1 hour after challenge (100 μl per animal). Subsequently, ascites were fed every 8 hours for 8 days. Stool samples were collected and antigen shedding was measured by ELISA.
18. The closed intestinal loop model was as described by J. L. Wolf *et al.* [*Science* **212**, 471 (1981)]. Closed loops of ileum (approximately 1 cm in length) were constructed in anesthetized BALB/c mice (6 to 8 weeks old) by suture ligation. Animals were anesthetized with equal volumes of xylazine (20 mg per kilogram of body weight) and ketamine (100 mg/kg) diluted in sterile physiologic saline (50 μl per animal). Supplemental anesthesia was administered with halothane inhaled via the open drop method. Fifty microliters of the following solutions were injected into the loops of four mice: (i) 25 μl of activated wild-type EC ($10^6$ shedding dose 50s) plus 25 μl of 7D9 ascites (1:4 dilution); (ii) 25 μl of activated EC plus 25 μl of a cocktail of four high-titer neutralizing IgG antibodies directed at VP4 and VP7 (*27, 28*) (mAbs 4F8, 159, 2G4, and 57-4, each 1:4 diluted); (iii) 25 μl of activated EC plus 25 μl of phosphate-buffered saline (PBS); and (iv) 25 μl of mouse myeloma IgA (undiluted, 1 mg/ml) (Sigma) plus 25 μl of activated EC. After injection, the loops were returned to the abdominal cavity and the abdominal wall was closed. The loops were removed 12 hours later and immunostained for EC with a rabbit hyperimmune serum against rotaviruses (Fig. 4).
19. Twenty-five microliters of activated EC plus 25 μl of PBS were injected into the intestinal loops of a mouse that had received the 7D9 hybridoma transplant 2 weeks before. The loop was removed and immunostained for EC 12 hours after inoculation as described [(*18*) and Fig. 4 legend].
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# Asymmetries Generated by Transcription-Coupled Repair in Enterobacterial Genes


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Although certain replication errors occur at different frequencies on each of the complementary strands of DNA, it remains unclear whether this bias is prevalent enough during chromosome replication to affect sequence evolution. Here, nucleotide substitutions in enteric bacteria were examined, and no difference in mutation rates was detected between the leading and lagging strands, but in comparing the coding and noncoding strands, an excess of C→T changes was observed on the coding strand. This asymmetry is best explained by transcription-coupled repair on the noncoding strand. Although the vast majority of mutations are thought to arise from spontaneous errors during replication, this result implicates DNA damage as a substantial source of mutations in the wild.


One of the fundamental assumptions in molecular evolution is that mutations are equally likely at any site of the genome. Evidence indicates that the probability of a nucleotide substitution may depend on positional factors, including the DNA strand on which the nucleotide is located (*1–4*). Because of the complementary and antiparallel nature of the DNA double helix, each strand is replicated in a very different manner. On one strand, the leading strand, replication proceeds continuously, whereas on the other strand, the lagging strand, replication occurs discontinuously by the synthesis and joining of short Okazaki fragments (*5*). Several experimental systems have revealed that the lagging strand undergoes a higher incidence of certain replication errors, such as mismatches induced by an excess of deoxythymidine triphosphate or deoxyguanosine triphosphate during in vitro replication in human cell extracts (*1, 2*) and deletions during plasmid replication in *Escherichia coli* (*3, 4*). On an evolutionary timescale, a consistent strand-bias in the introduction of mutations would strongly affect the patterns of change in DNA sequences, and such an asymmetry would be detected by reconstructing the substitutions that have occurred among homologous regions (*6*).

Not all replication errors are equally frequent. The introduction of a pyrimidine opposite a template pyrimidine is a very rare event relative to other mismatches (*7*); therefore, most transversions, that is, mutations from a purine (R) to a pyrimidine (Y) or vice versa arise through R:R mismatches. Thus, a Y→R transversion on a given strand of DNA results from an R:R mismatch introduced during the synthesis of


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