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Complementation by SR Proteins of Pre-mRNA Splicing Reactions Depleted of U1 snRNP

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Individual small nuclear ribonucleoproteins (snRNPs) U1, U2, and U4/U6 were removed from nuclear extracts of HeLa cells by antisense affinity depletion. Addition of a highly purified preparation of SR proteins fully restored splicing activity in reactions depleted of U1 snRNP but did not reconstitute splicing in reactions depleted of the other snRNPs. Affinity selection experiments revealed that spliceosomes lacking U1 snRNA formed in the U1 snRNP–depleted reactions reconstituted with SR proteins. Thus, high concentrations of SR proteins facilitate the assembly of precursor messenger RNA (pre-mRNA) into a spliceosome in the absence of interactions with U1 snRNP.

Pre-mRNA splicing takes place within a large complex termed the spliceosome, which contains four snRNP particles (U1, U2, U4/U6, and U5) and also many non-snRNP protein factors (*1*). SR proteins belong to a family of non-snRNP splicing factors that are highly conserved from *Drosophila* to primates and contain extensive repeats of the diamino acid sequence serine-arginine (*2*). Proteins of the SR family have been implicated both in constitutive splicing and in the regulation of alternative splicing (*3*). Recent work suggests that SR proteins function during an early step in the commitment of a substrate to splicing (*4*), facilitate the binding of U1 snRNP to the 5′ splice site (*5*), and also bridge interactions between the 5′ splice site and the branch site (*6*). Here, we show that high concentrations of SR proteins circumvent the requirement for U1 snRNP in the pre-mRNA splicing reaction.

Antisense 2′-O-methyl oligoribonucleotides were used to deplete HeLa cell nuclear extracts of either U1, U2, or U4/U6 snRNPs (*7*). These extracts were not active for the splicing of added pre-mRNA, but complemented each other in any pairwise combination (Fig. 1D) (*7*, *8*). Depleted nuclear extracts were assayed for the presence of snRNAs by Northern (RNA) hybridization (Fig. 1A). In the reaction depleted of U1 snRNP, the amount of this snRNA was reduced approximately to a thousandth of the previous amount, whereas the levels of the nontargeted snRNAs were not greatly affected.

Fractions containing SR proteins complemented splicing of a β-globin substrate in reactions depleted of U1 snRNP (Fig. 1D). Preparations of purified SR proteins were derived from nuclear extracts of HeLa cells as described (*2*) (Fig. 1B). A typical SR preparation also contained a small amount of contaminating snRNAs that were degraded by digestion with micrococcal nuclease. The resulting preparations contained no detectable snRNAs when analyzed by Northern hybridization (Fig. 1C).

After digestion with micrococcal nuclease, SR preparations retained the ability to restore splicing to reactions depleted of U1 snRNP (Fig. 1D). This resistance contrasted with the sensitivity of both the nuclear extract and the U2 snRNP–depleted extract, which did not restore splicing to a U1 snRNP–depleted reaction upon nuclease treatment (Fig. 1D). Nuclear extract and U2 snRNP–depleted extract probably complement the absence of U1 snRNP by contributing functional U1 snRNPs; degradation of the U1 snRNA would be expected to render these preparations inactive. This suggests that the activity of the SR preparation is not the result of residual U1 snRNA. The effects of SR proteins were specific, because their addition had little effect on the extent of splicing of mock-depleted reactions and, in addition, did not restore splicing to either U2 snRNP– or U4/U6 snRNP–depleted reactions (*8*). In titration experiments, the concentration of SR proteins required to complement the U1 snRNP–depleted reactions was approximately 10-fold greater than that of the endogenous concentration (*8*).

The activity of SR preparations complementing a U1 snRNP–depleted reaction was dependent on the particular pre-mRNA. The β-globin substrate was the most active under these conditions, whereas an adenovirus pre-mRNA substrate, Ad1, was also spliced (Fig. 2A), but at approximately 10% of the level observed in the mock-depleted reaction. In the case of the Ad1 substrate, addition of SR proteins to the mock-depleted reaction resulted in the activation of a cryptic 5′ splice site that is located 125 nucleotides downstream of the normal splice site (Fig. 2B). Both sites were also active in the U1 snRNP–depleted reaction supplemented with SR proteins. A third substrate, pPIP85A, which is largely based on sequences in the Ad1 pre-mRNA but with many sequence variations, was not active for splicing upon addition of SR proteins to U1 snRNP–depleted reactions (*8*). Thus, there is a sequence specificity for splicing in the absence of U1 snRNP and in the presence of high concentrations of SR proteins.

To investigate further the role of SR proteins in the facilitation of splicing in U1 snRNP–depleted reactions, spliceosomes were formed in reactions containing 0.2 μg of substrate RNA that had been synthesized with biotinylated uridine triphosphate (*9*). This amount of substrate corresponded to at least a 100-fold excess over the minute amounts of U1 snRNA in the depleted reactions. Biotinylated β-globin pre-mRNAs were incubated under splicing conditions for 40 min in mock-depleted, U1 snRNP–depleted, and U2 snRNP–depleted reactions either with or without added SR proteins; the extent of splicing was assayed by addition of a trace amount of labeled substrate (Fig. 3A).

The spliceosomes from these reactions were recovered by chromatography on streptavidin agarose beads; and the bound RNA was analyzed by Northern blot analysis (Fig. 3B). All six snRNAs were associated with the spliceosome in reactions containing mock-depleted extract. The addition of excess SR proteins had a small stimulatory effect on splicing and also on the amount of snRNAs in spliceosomes. Trace amounts of U2, U4, U5, and U6 snRNAs

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were affinity-selected in the U1 snRNP–depleted reactions (Fig. 3B), which is consistent with the very low level of splicing that was observed under these conditions. Addition of excess SR proteins, which restored splicing to the levels found in the mock-depleted reactions (Fig. 3A), enhanced the binding of U2 snRNA to levels found in the mock reactions. Furthermore, U4, U5, and U6 snRNAs were also enriched in complexes formed under these conditions. No U1 snRNA above the background was selected in the U1 snRNP–depleted reaction upon addition of high concentrations of SR proteins (Fig. 3B).

Analysis of the U2 snRNP–depleted reactions revealed that a small amount of U1 snRNA was associated with the substrate in the absence of additional SR proteins and that this amount increased upon addition of SR proteins (Fig. 3B). However, consistent with the lack of splicing under these conditions (Fig. 3A), the addition of SR proteins did not promote the association of the other snRNAs (Fig. 3B). Results similar to those shown in Fig. 3B were obtained from selection experiments carried out with the Ad1 pre-mRNA substrate (*10*).

Pre-mRNA splicing is efficient in reactions depleted of U1 snRNP if the concentration of SR proteins is increased 10-fold. We suggest that splicing under these conditions is independent of the activity of U1 snRNP. This conclusion is supported by the absence of detectable U1 snRNA in affinity-selected spliceosomes under conditions in which other spliceosomal snRNAs were clearly present. Furthermore, substrate RNAs that are processed with similar efficiencies in U1 snRNP–dependent reactions have differential activities in reactions depleted of U1 snRNP and supplemented with SR proteins. Even with these strong indications, it is impossible to prove chemically that the minute amount of U1 snRNP present in the depleted reaction is not active in a very transient mode in the promotion of splicing. However, we propose that these results shift the argument to the premise that U1 snRNP is not required for splicing under all conditions.

That high concentrations of SR proteins permit processing of pre-mRNA in reactions depleted of U1 snRNP suggests that U1 snRNA is not essential for catalysis of either of the transesterification steps in splicing. This finding was anticipated by previous studies that show that U1 snRNP is easily displaced from the spliceosome (*11*) and that there is a lack of an obvious analog to U1 snRNA in trans-splicing reactions (*12*). Further evidence that a spliceosome can form without U1 snRNP is suggested by recent studies showing that spliceosome-type complexes containing U2, U5, and U4/U6 snRNAs assemble on short

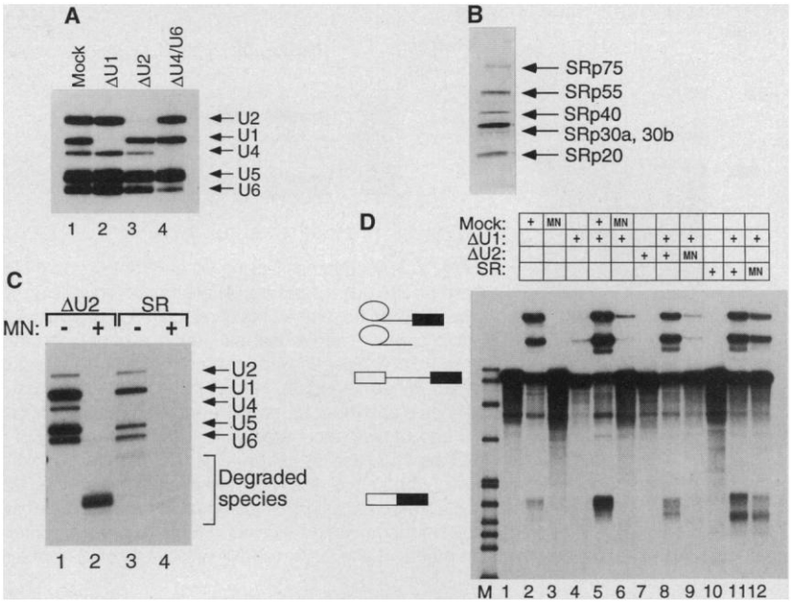
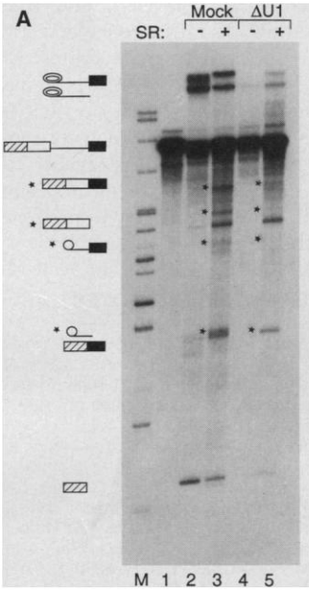


Fig. 1. SR proteins restore splicing to U1 snRNP–depleted reactions. (**A**) Antisense affinity depletion of snRNPs from HeLa cell nuclear extracts. RNA recovered from snRNP-depleted nuclear extracts was analyzed by Northern hybridization with snRNA-specific riboprobes. Lane 1, mock-depleted nuclear extract. Lanes 2, 3, and 4, extracts depleted of U1, U2, and U4/U6 snRNPs, respectively. (**B**) SR proteins were isolated from HeLa cells, separated by SDS–polyacrylamide gel electrophoresis, and detected with Coomassie blue. (**C**) The SR protein preparation was treated with micrococcal nuclease to degrade endogenous snRNAs (*20*). RNA recovered from the digested SR preparation was analyzed with snRNA-specific riboprobes as in (A) (lane 4). Lane 2 shows a micrococcal nuclease (MN)–digested U2 snRNP–depleted nuclear extract. Lanes 1 and 3 show mock nuclease-treated, U2 snRNP–depleted extract and SR protein preparation, respectively. (**D**) Splicing of β-globin pre-mRNA is reconstituted in U1 snRNP–depleted reactions supplemented with micrococcal nuclease–treated SR proteins (*21*). Reactions containing U1 snRNP–depleted extract were incubated with mock nuclease-treated SR proteins (lane 11) or nuclease-treated SR proteins (lane 12). Lanes 2, 4, and 7 show reactions with the mock-depleted, U1 snRNP–depleted, and U2 snRNP–depleted nuclear extracts, respectively. Reactions containing different combinations of nuclear extracts or SR proteins are as indicated. SR, HeLa cell SR proteins; MN, pretreated with micrococcal nuclease. Splicing intermediates and products are indicated by icons on the left. The 5′ exon species is not shown, although it was detected in all active splicing lanes. Lane M shows end-labeled Msp I fragments of pBR322. Lane 1 shows a reaction containing a mock-depleted nuclear extract that was incubated on ice.



B

Cryptic CAUCUG / GUCAGAAAAGACAAU

WT GUUGGG / GUGAGUACUCCCUCU

Fig. 2. SR proteins restore splicing to an adenovirus pre-mRNA in U1 snRNP–depleted reactions and also activate a cryptic 5′ splice site. (**A**) Splicing reactions containing the Ad1 pre-mRNA substrate supplemented with SR proteins pretreated with micrococcal nuclease (*21*). Lanes 2 and 3 show reactions in a mock-depleted nuclear extract with (lane 3) or without (lane 2) added nuclease-treated SR proteins. Lanes 4 and 5 show the corresponding reactions in a U1 snRNP–depleted nuclear extract. Icons indicate the migration of splicing intermediates and products generated by selection of either the wild-type or cryptic splice sites. Species marked with a star are those of the cryptic splicing pathway. (**B**) Comparison of the wild-type (WT) and cryptic 5′ splice sites in Ad1 pre-mRNA (*22*). The cryptic 5′ splice site region shares sequence similarity with a consensus sequence, although it has a few differences.

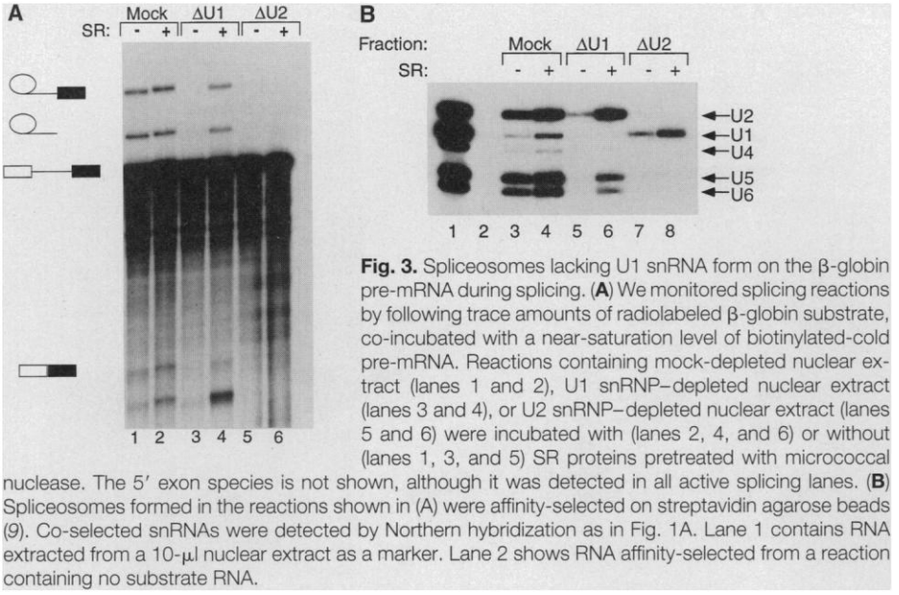


Fig. 3. Spliceosomes lacking U1 snRNA form on the β-globin pre-mRNA during splicing. (**A**) We monitored splicing reactions by following trace amounts of radiolabeled β-globin substrate, co-incubated with a near-saturation level of biotinylated-cold pre-mRNA. Reactions containing mock-depleted nuclear extract (lanes 1 and 2), U1 snRNP–depleted nuclear extract (lanes 3 and 4), or U2 snRNP–depleted nuclear extract (lanes 5 and 6) were incubated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) SR proteins pretreated with micrococcal nuclease. The 5′ exon species is not shown, although it was detected in all active splicing lanes. (**B**) Spliceosomes formed in the reactions shown in (A) were affinity-selected on streptavidin agarose beads (*9*). Co-selected snRNAs were detected by Northern hybridization as in Fig. 1A. Lane 1 contains RNA extracted from a 10-μl nuclear extract as a marker. Lane 2 shows RNA affinity-selected from a reaction containing no substrate RNA.

oligoribonucleotides encompassing a 5′ splice site (*13*). Formation of these complexes was dependent on the recognition of the 5′ splice site sequence but did not depend on the presence of an intact U1 snRNA. Consistent with this, it was recently reported that SR proteins reconstituted splicing reactions in which U1 snRNP was debilitated by site-specific blockage of the 5′ end of U1 snRNA. In these reactions, no U1 snRNA was detected interacting with the 5′ splice site by psoralen cross-linking (*14*).

The 5′ splice site sequence is recognized by components of the spliceosome other than U1 snRNA. Recent experiments have revealed an interaction between U6 snRNA and the 5′ splice site by detection of allele-specific complementation between mutations in the 5′ splice site and mutations in U6 snRNA and also by ultraviolet light–induced cross-links between these two RNAs (*15*). Consistent with specific recognition of the 5′ splice site sequence by spliceosome factors other than U1, we have found that mutations at either +1, +2, +5, or +6 in the 5′ splice site region have similar effects on reactions containing either a mock-depleted extract or U1 snRNP–depleted extract reconstituted with SR proteins (*16*). Because bases at these positions are certainly recognized in a rate-limiting step by U1 snRNA under normal conditions, the same bases must also be recognized in another potentially rate-limiting step after interaction by U1 snRNP, probably by U6 snRNA.

The mechanism by which SR proteins bypass the requirement for U1 snRNP is unclear. U1 snRNP recognizes the 5′ splice site by sequence complementarity (*17*) and promotes commitment to the splicing pathway by stabilizing the binding of U2 snRNP to the branch region (*7*, *18*). The SR subdomain of the U1 snRNP 70-kD protein may interact either directly or indirectly with the SR domain of the U2AF protein, which is required for the stable binding of U2 snRNP (*6*). We propose that this U1 snRNP–mediated signal for U2 snRNP binding and spliceosome formation is not essential with high concentrations of SR proteins, because the SR proteins bind the substrate directly and stabilize the subsequent association of U2 snRNP. This suggestion is consistent with the observed differences in splicing activity of specific substrates in the U1 snRNP–depleted reactions with high SR protein concentrations. SR proteins are thought to bind RNA in a sequence-specific fashion (*19*), and thus substrate pre-mRNAs might be expected to have differential activity in reactions dependent on recognition by SR proteins. Further studies on the sequence specificities of reactions in the presence of high concentrations of SR proteins will probably be informative in determining the mechanisms by which SR proteins function to promote splicing in the absence of U1 snRNP.

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9. Biotinylated RNA was transcribed as described [P. J. Grabowski and P. A. Sharp, *Science* **233**, 1294 (1986)]. Splicing reactions (100 μl) containing 0.2 μg of β-globin substrate were incubated for 40 min at 30°C. Immediately before incubation at 30°C, a small amount of labeled pre-mRNA was added to 10 μl of each splicing reaction. The remaining 90 μl of each reaction was incubated at 30°C and then mixed for 1 hour at 4°C with pre-blocked streptavidin agarose beads (Sigma). The beads were washed several times with a 350 mM KCl buffer as described [U. Ryder, B. S. Sproat, A. I. Lamond, *Nucleic Acids Res.* **18**, 7373 (1990)]. Affinity-selected RNA was released by proteinase K treatment, extracted with phenol chloroform, then recovered by ethanol precipitation for analysis by Northern hybridization.
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20. SR proteins were prepared from HeLa cells as described (*2*) but with a second $MgCl_2$ precipitation. To remove endogenous snRNAs, we treated SR proteins and nuclear extract with micrococcal nuclease for 30 min at 30°C, in the presence of 1 mM $CaCl_2$. The extent of degradation of the snRNAs was assayed by Northern hybridization with snRNA-specific riboprobes. The amounts of U2 snRNP–depleted extract and SR proteins digested corresponded to the amounts required to obtain equivalent levels of complementing activity, as assayed by the ability to restore splicing to a U1 snRNP–depleted extract. After the incubation with nuclease, EGTA was added to a final concentration of 3 mM.
21. Splicing reactions were performed essentially as described [P. J. Grabowski, R. A. Padgett, P. A. Sharp, *Cell* **37**, 415 (1984)]. Splicing-complementation assays contained 30% nuclear extract, supplemented with 20% of a second extract or 1 μg of SR proteins. In complementation controls lacking extract or SR proteins, buffer D [J. D. Dignam, R. M. Lebowitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)] containing 5% glycerol was added instead. Splicing reactions were incubated for 2 hours.
22. RNA species from splicing reactions with Ad1 pre-mRNA were extensively characterized by comparison of the mobility of the RNA products and by complementary DNA (cDNA) cloning of the exon product RNAs after elution from polyacrylamide gels. The cDNA and second-strand synthesis was performed with rTh polymerase (Perkin-Elmer Cetus) in coupled reverse transcription–polymerase chain re-

action. The cDNAs were inserted into pBluescript−, and the DNAs of several clones were sequenced.
23. The authors thank R. Cook and S. Schultz of the MIT Biopolymers Facility for synthesizing the biotinylated 2′-*O*-methyl oligoribonucleotides. R. Issner and Y. Qiu are thanked for their excellent technical assistance. A. MacMillan, P. McCaw, J. Pomerantz, and C. Query kindly made helpful comments on the manuscript. Many thanks also to M. Siafaca for secretarial support. Supported by U.S. Public Health Service grant RO1-GM34277 and grant RO1-AI32486 from NIH (P.A.S.) and partially by a Cancer Center Support (core) grant P3O-CA14051 from the National Cancer Institute. B.J.B. was supported by a Human Frontiers Science Program Organization Long-Term Fellowship.

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Regulation of IgE Responses to Inhaled Antigen in Mice by Antigen-Specific γδ T Cells

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Indirect evidence implicates γδ T cells in the cross-regulation of CD4 αβ T cell responses. Adoptive transfer of small numbers of γδ T cells from ovalbumin (OVA)-tolerant mice selectively suppressed T_H2–dependent immunoglobulin E (IgE) antibody production without affecting parallel IgG responses. Challenge of these γδ T cells in vitro with specific antigen resulted in production of high levels of interferon γ. The effects of the γδ T cells may be mediated by direct inhibition of OVA-specific $CD4^+$ T_H2 cell proliferation or selection for specific CD4 T_H2 cells.

The "normal" immune response to nonpathogenic soluble protein antigens presented at the body's major mucosal surfaces is the selective suppression of antigen-specific delayed-type hypersensitivity (DTH) and IgE production. The process occurring in the gastrointestinal tract in response to food antigens has been termed oral tolerance (*1*). We have described an analogous tolerance process in the respiratory tract of the rat in response to inhalation of antigen (*2*, *3*) and demonstrated the capacity of purified $CD8^+$ T cells to mediate its adoptive transfer (*4*), a finding consistent with later reports on oral tolerance (*5*, *6*).

The $CD8^+$ T cells that mediate adoptive transfer of IgE-selective antigen-specific "tolerance" in the rat model are major histocompatibility complex (MHC) class I restricted and are responsive to soluble OVA antigen (*7*). Activation of these cells depends on interleukin-2 (IL-2) which, at least in vitro, is provided by MHC class II–restricted $CD4^+$ T cells responding to the same antigen (*8*). The early phase of the response occurs in the regional lymph nodes draining the conducting airways; it involves IgE production (*4*, *7*, *8*) as well as IL-4 and IL-2 secretion by OVA-specific $CD4^+$ T_H2 cells. This phase is halted by the emergence of antigen-specific $CD8^+$ T cells which release high levels of interferon γ (IFN-γ) when cultured with OVA (*8*). The OVA-responsive $CD8^+$ T cells express CD3, but surface αβ T cell receptors (TCRs) were not detected with the single TCR chain–specific antibody available for rat (*9*). Accordingly, the model was reestablished here in the mouse, for more detailed analysis.

IgE-selective "tolerance" to inhaled OVA has previously been demonstrated in several strains of mice (*7*, *10*), as has adoptive transfer of the phenomenon by splenic Thy 1.2^+ T cells (*7*). Thus, repeated exposure of C57Bl mice to aerosolized OVA ablated their capacity to mount primary IgE responses to parenteral challenge with OVA plus aluminum hydroxide (AH) adjuvant, without suppressing corresponding IgG responses (*7*). Adoptive transfer of 10^6 unfractionated splenocytes from such tolerized animals (*11*) inhibits IgE (but not IgG) antibody responses to OVA (anti-OVA) in the recipients (Fig. 1). Furthermore, the $CD8^+$ splenocyte subpopulation mediated inhibition of IgE (Fig. 1). The magnitude of the overall IgG anti-OVA response did not change significantly in aerosol-exposed mice (Fig. 1). However, analysis of individual IgG subclasses (*12*) indicated that suppression of the IgE response was accompanied by decreased IgG1 reactivity and a compensatory rise in IgG2a, whereas IgG2b and IgG3 responsiveness remained essentially unaltered.

The capacity of splenocytes to suppress the IgE response was lost by depletion of $\gamma\delta^+$ T cells (Fig. 2). In 10^6 splenocytes there are roughly 3×10^4 γδ T cells. When this number of γδ T cells (purified to ≥98.5% by positive selection) was transferred, suppression of IgE responses was comparable in magnitude to that seen in animals receiving 10^6 unfractionated cells.

Subsequent dose-response experiments (Fig. 3) demonstrated that as few as 5×10^2 positively selected γδ T cells are sufficient for suppression of the IgE component of the anti-OVA response. γδ T cells prepared by negative selection [that is, depletion of αβ T cells (*13*)] from OVA-tolerant donors yielded suppression of the IgE response comparable to that achieved with positively selected cells. We have shown previously that adoptive transfer of splenocytes depleted of $\alpha\beta^+$ T cells from OVA-tolerant rats was capable of mediating antigen-specific tolerance in the IgE isotype (*9*).

To test for the antigen specificity of γδ T

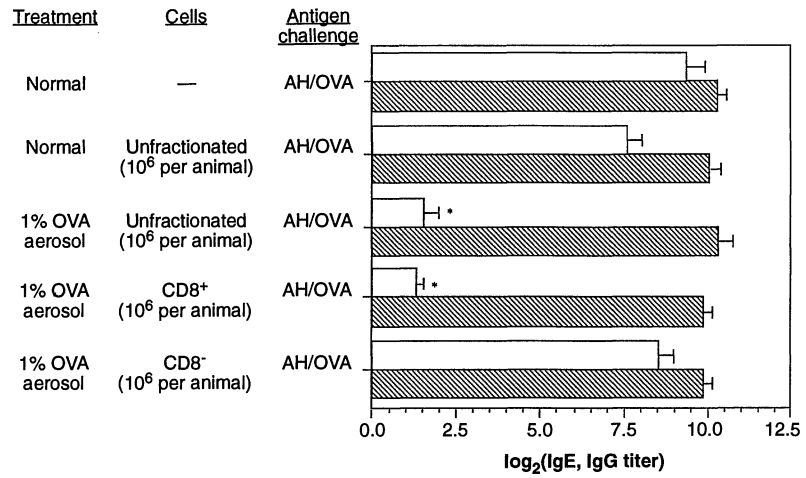

Fig. 1. Selective suppression of IgE anti-OVA responses by adoptive transfer of $CD8^+$ lymphocytes from C57Bl/6J mice rendered "tolerant" to OVA by repeated exposure (*11*). The $CD8^+$ population used here was >99.5% pure, and the $CD8^-$ population contained <0.4% of contaminating $CD8^+$ cells. Data shown are the mean ± SD (n = 5 to 10 per group) at day 21 (peak primary Ig response) and indicate reciprocal log_2(IgE and IgG) anti-OVA titers as determined by standard methods (*8*). Data from day 14 did not alter interpretation of the results of these experiments. Open bars, IgE; hatched bars, IgG (asterisk, <controls; $P < 0.01$).

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