Noxa, a BH3-Only Member of the Bcl-2 Family and Candidate Mediator of p53-Induced Apoptosis

Eri Oda, et al.

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seals. Although the two harbor seals with proven influenza B virus infection displayed respiratory symptoms during their rehabilitation period, this occurred at a time when many of the admitted juvenile seals suffered from lungworm (Oostronculus circumlitus and Parafilaroides gymnurus) infections (17). Association of lungworm infections in pigs with influenza A virus pathogenesis and transmission has been described (22), but the evidence was considered weak (26).

The combined serological and virological data obtained from seal 99-012 indicate that shedding of influenza B virus in seals was prolonged as compared to shedding in humans (27, 28) and that IgG antibody responses to NP and HA/NA were delayed. Possible explanations for this apparent suboptimal immune response upon infection may be associated with xenobiotic-related immunosuppression (11) or the therapeutic use of corticosteroids to combat the lungworm infections (17). Prolonged virus shedding in addition to the limited spreading of influenza B virus among seals (as shown in the SRRC and indicated by the limited seroprevalence of specific antibodies in the wild) may explain why little or no genetic and antigenic drift of influenza B virus is observed in seals.

Our data not only highlight the fact that influenza B virus infections can emerge in seal populations but also show that seals may constitute an animal reservoir from which humans may be exposed to influenza B viruses that have circulated in the past.

R E P O R T S

References and Notes
18. RNA was isolated by means of a high pure RNA isolation kit (Boehringer-Mannheim). RNA was used for RT-PCR analysis to amplify a 240–base pair fragment of the influenza B virus NS gene segment using primers 5'-ATG GCC ATC GGA TCC TCA AC-3' and 5'-TGT CAG CTA TTA TGA AGC TG-3', and AMV reverse transcriptase, AmpliTaq DNA polymerase, recombinant ribonuclease inhibitor (Promega) in the presence of 50 mM tris-HCl, 50 mM NaCl, 2 mM dithiothreitol, 7 mM MgCl2, 1 mM dNTP, and 400 mM each of primer. Cycling parameters were 30 min at 42°C, 4 min at 95°C, 1 min at 45°C, and 3 min at 72°C once; and then 1 min at 95°C, 1 min at 45°C, and 3 min at 72°C, repeated 39 times. PCR fragments were sequenced with a DyeCycle-ET terminator cycle sequencing premix kit (Amersham) on an ABI-373A apparatus (Perkin Elmer).
19. Seed kidney cells were plated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% l-glutamine, penicillin, and streptomycin at 1 x 105 cells per well in 24-well plates. Cells were inoculated with 1 x 104 TCID50 of influenza virus B/Seal/Netherlands/1/99 in DMEM supplemented with 4% bovine serum albumin, 1% l-glutamine, penicillin, and streptomycin. Influenza B virus infection was detected by immunofluorescence with influenza B NP-specific antibodies, which were labeled with fluorescein isothiocyanate (IMAGEN influenza A+B, DAKO Diagnostics) after 24 hours. Cryptotachy and HA activity (titer = 32) were detected in the culture cell after 48 hours.
21. Antibody titers were determined with a recombinant fusion protein between maltose-binding protein (MBP) and NP or with HA/NA proteins purified from virions (both proteins were derived from B/Harbin/7/94). IgG antibody levels to HA/NA and NP proteins were determined by an indirect ELISA with antigen-coated plates and peroxidase-labeled protein A for detection. IgM antibody levels to NP were determined by means of an antibody-capture ELISA with goat anti-dig IgM-coated plates and peroxidase-labeled MBP-NA antigen for detection. The goat anti-dog IgM antibody preparation specifically captures seal IgM, as was shown in routine serological tests for PDV and PHV.
23. The sequences of influenza B virus HA and NS genes are available at www.flu.lanl.gov.
24. Analysis of the H1T sequences from human influenza B virus strains that are available from the influenza sequence database showed that between 1991 and 1999, the proportion of strains with >98% nucleotide identity to B/Seal/Netherlands/1/99 was 0, 17, 27, 46, 62, 29, 0, 0, and 0% of all epidemic strains for each successive year, respectively.
29. HAI titres were amplified with M13-tagged primers P1 (5'-29M13-GAC TTA CCT TAT AAT CAC AA-3') and P4 (5'-21M13-TTT GGG AAG CCA CCA ATG TG-3') or primers P3 (5'-29M13-CCT ATA ATG CAC GAC AGA AC-3') and P6 (5'-21M13-AAA CCA GCA ATG CGG AA-3'), followed by sequencing with primers 29M13 (5'-CAG GAA ACA GCT ATG ACC-3') and 21M13 (5'-TGT AAA ACG GGC AGT AGT-3') using a DyeNamic ET terminator cycle sequencing premix kit (Amersham) and an ABI-373A sequencing apparatus (Perkin Elmer).
30. We are grateful to L. van der Kemp, G. de Mutsert, and M. van der Bildt for technical assistance; J. Habova for electron microscopy; Solvay Pharmaceuticals, Weesp, the Netherlands, for providing HA/NA proteins from B/Harbin/7/94 and the SRRC staff for taking care of and samples from seals. R. F. is a fellow of the Royal Netherlands Academy of Arts and Sciences.
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Noxa, a BH3-Only Member of the Bcl-2 Family and Candidate Mediator of p53-Induced Apoptosis

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A critical function of tumor suppressor p53 is the induction of apoptosis in cells exposed to noxious stresses. We report a previously unidentified pro-apoptotic gene, Noxa. Expression of Noxa induction in primary mouse cells exposed to x-ray irradiation was dependent on p53. Noxa encodes a Bcl-2 homology 3 (BH3)–only member of the Bcl-2 family of proteins; this member contains the BH3 region but not other BH domains. When ectopically expressed, Noxa underwent BH3 motif–dependent localization to mitochondria and interacted with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9. We also demonstrate that blocking the endogenous Noxa induction results in the suppression of apoptosis. Noxa may thus represent a mediator of p53-dependent apoptosis.

The mechanism of p53-induced apoptosis has been extensively studied in the context of tumor suppression (7). p53-dependent apoptosis is regulated, at least in part, by transcriptional activation of its target genes (8), and this process is dependent on the Apaf-1/caspase-9 activation pathway (2). Among the identified target genes of p53, Bax encodes a pro-apoptotic Bcl-2 family of proteins that can activate this pathway (3). However, in Bax-deficient mice, DNA damage–induced apoptosis occurs normally in thymocytes, and apoptosis induced by treatment with anticancer drugs is only partly inhibited in mouse embryo fibroblasts (MEFs) expressing the adenovirus oncoprotein E1A (4). Furthermore, thymocytes from p53-defi-
Fig. 1. Primary sequence of Noxa and its expression. (A) Predicted amino acid sequence of Noxa (28). Two putative BH3 motifs are underlined (regions A and B). (B) Alignment of the Noxa BH3 motifs with the BH3 domains of Bcl-2 family proteins; anti-apoptotic Bcl-2 subfamily proteins (human Bcl-2, GenBank accession number M14745; human Bcl-XL, GenBank accession number Z23115; human Mcl-1, GenBank accession number Q07820), pro-apoptotic Bax subfamily proteins (human Bax, GenBank accession number U23765), and pro-apoptotic BH3-only subfamily proteins (mouse Bad, GenBank accession number L22473; human Bak, GenBank accession number L37296; human Bik, GenBank accession number U75506). Amino acids identical to Noxa’s BH3 motifs are shaded. (C) Expression of Noxa mRNA following x-ray irradiation in MEFs. Noxa mRNA was analyzed by RNA blotting with RNAs (5 μg in each lane) isolated from wild-type (WT) and p53−/− MEFs following x-ray irradiation [20 grays (Gy)] time after radiation is indicated in hours. The same filter was probed with MDM2 or β-actin cDNA. (D) Expression of Noxa protein following x-ray irradiation. Noxa protein was determined by immunoblot analysis with antibody to Noxa in WT thymocytes following x-ray irradiation (5 Gy); time after radiation is indicated.

Fig. 2. Activation of the Noxa promoter by p53. (A) The Noxa promoter and luciferase reporter gene constructs. Putative p53-recognition sequence and p53-consensus binding sequence (p53 CBS) are shown. The following reporter plasmids using this assay are also indicated: Noxa-luc containing Noxa promoter and putative p53-recognition sequence, Noxa-ΔLuc lacking putative p53-recognition sequence, and Noxa-mt-luc in which four critical nucleotide residues for p53 binding were altered (indicated by lowercase letters). (B) Transient cotransfection analysis of p53. p53 expression vector (pEF-p53) (0.05 μg) was transfected into 8 × 10^4 p53−/− MEFs with 0.2 μg of each reporter plasmid. Luciferase activity was measured 24 hours after transfection. RGC-luc (29) containing synthetic p53-binding sequences was used as a positive control. Histogram shows the mean of three independent experiments, and error bars show standard deviations. The assay was repeated three times, and the results were reproducible.

**Table 1**

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<tr>
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**Table 2**

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**Table 3**

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**Table 4**

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9–amino acid sequences (A and B) characteristic to the Bcl-2 homology 3 (BH3) motif of the Bcl-2 family of proteins (Fig. 1B) (8).

Noxa mRNA was constitutively expressed in small amounts in the brain, thymus, spleen, lung, kidney, and testis of adult mice (9). X-ray irradiation of wild-type MEFs increased expression of Noxa mRNA about fivefold (Fig. 1C), with kinetics similar to those of the p53-depend-
of Noxa mRNA in p53-deficient MEFs (Fig. 1D). Thymocytes undergo DNA damage–induced apoptosis in a p53-dependent manner (11). Increased expression of Noxa mRNA in response to x-ray irradiation also occurred in wild-type thymocytes (fivefold increase) but not in p53-deficient thymocytes (Fig. 1E). Noxa protein also accumulated in wild-type thymocytes after x-ray irradiation (Fig. 1F) (12).

To determine whether the p53-dependent expression of the Noxa gene involves direct activation of its promoter, we isolated and characterized the mouse Noxa gene (13). This gene contains three exons in which the BH3 motifs A and B are encoded by exons 2 and 3, respectively. The transcription initiation site was determined to be 158 base pairs upstream from the initiator ATG by polymerase chain reaction (PCR)–based primer extension, and one potential p53-recognition sequence, located at −155 to −174, was found in the promoter region (Fig. 2A). The contribution of p53 to the activation of the Noxa promoter was examined by a transient cotransfection assay using a luciferase reporter gene linked to Noxa promoter (Noxa-luc in Fig. 2A) (14). The promoter was activated (on average, sevenfold) by coexpressed p53 in p53-deficient MEFs. In contrast, reporter genes containing a deletion (NoxaΔLuc) or point mutations (Noxa-mt-luc) in the putative p53-recognition sequence were not activated by p53 (Fig. 2B). Collectively, these results lend support to the idea that expression of the Noxa gene in x-ray–irradiated cells involves direct activation of its promoter by p53.

On the basis of its retention of various BH domains, the Bcl-2 family can be divided into three classes: the anti-apoptotic Bcl-2, the pro-apoptotic Bax, and Bcl-2–only subfamilies (8). The BH3 domain of the pro-apoptotic members is critical for association with other Bcl-2 family proteins in the promotion of apoptosis (8). Unlike Bax, whose expression is also regulated by p53 (3), Noxa contains BH3 but not other BH motifs (BH1, BH2, and BH4) or a transmembrane domain; hence, it appears to be a previously unknown member of the BH3-only subfamily. Ectopic expression of Noxa in HeLa cells with an adenovirus-mediated gene expression system caused apoptosis in >90% of the cells 24 hours after virus infection (Fig. 3A) (15). This Noxa-induced apoptosis was also observed in other human cancer cell lines independently of their p53 status (9). Because substitution of the NH2-terminal leucine to alanine in the BH3 domain of another BH3-only member, Bad, is known to cause a loss of pro-apoptotic activity (16), we generated mutant Noxa cDNAs in which one amino acid substitution was similarly introduced in either or both of the leucine residues within the BH3 motif (15). Noxa mutants carrying one substitution (A mt and B mt) had lower pro-apoptotic activities than wild-type Noxa, and the substitution (A mt and B mt) had lower pro-apoptotic activities than wild-type Noxa, and the}

Fig. 4. Role of Noxa in p53-induced apoptosis. (A) Comparison of the amino acid sequences of human and mouse Noxa (28). Human Noxa is identical to APR (25). Identical amino acids are indicated with an asterisk. BH3 motifs are also indicated. (B) Induction of Noxa mRNA in Saos2 cells by p53. Cells were infected with adenovirus expressing p53 (Ad-p53) for the indicated time periods in hours, and RNA blotting was performed. The same filter was probed with β-actin cDNA. (C) Reduction of endogenous Noxa protein by transfection with Noxa antisense oligonucleotide. Noxa protein was determined by immunoblot analysis with antibody to human Noxa in Saos2 cells 20 hours after infection with Ad-p53 following transfection with 4 μM antisense (Ad-p53 + antisense) or control (Ad-p53 + control) oligonucleotide for 4 hours. Noxa protein was also determined before (−) and after infection with Ad-p53 (Ad-p53) for 20 hours without oligonucleotides. (D) Effect of Noxa antisense oligonucleotide in p53-induced apoptosis. Saos2 cells were transfected with antisense (open circles) or control (solid circles) oligonucleotide as in (C) and infected with Ad-p53 for the indicated times. Viable cells were determined by trypan blue exclusion and calculated as the percentage of survival in relation to the number at the start of trial. Error bars represent standard deviations from two independent samples. (E) Effect of Noxa antisense oligonucleotide in x-ray irradiation–induced apoptosis of BAF-3 cells. BAF-3 cells were incubated with 10 μM antisense or control oligonucleotide for 12 hours and were subjected to x-ray irradiation (4 Gy). Noxa protein was determined by immunoblot analysis with antibody to mouse Noxa 16 hours after x-ray irradiation (+) (−, before infection) in the absence or presence of indicated oligonucleotides (left). Viable cells were determined after irradiation in the presence of antisense (open circles) or control (solid circles) oligonucleotides by trypan blue exclusion and calculated as percentage of survival (right). Error bars represent standard deviations from two independent samples. (F) Reduction of endogenous Noxa protein by transfection with Noxa antisense oligonucleotide. Noxa protein was determined by immunoblot analysis with antibody to human Noxa in Saos2 cells 20 hours after infection with Ad-p53 following transfection with 4 μM antisense (Ad-p53 + antisense) or control (Ad-p53 + control) oligonucleotide for 4 hours. Noxa protein was also determined before (−) and after infection with Ad-p53 (Ad-p53) for 20 hours without oligonucleotides. (D) Effect of Noxa antisense oligonucleotide in p53-induced apoptosis. Saos2 cells were transfected with antisense (open circles) or control (solid circles) oligonucleotide as in (C) and infected with Ad-p53 for the indicated times. Viable cells were determined by trypan blue exclusion and calculated as the percentage of survival in relation to the number at the start of trial. Error bars represent standard deviations from two independent samples.
epitope-tagged Noxa by immunohistochemical analysis in HeLa cells. Noxa was colocalized with a mitochondrial marker, CMTMRos (Fig. 3B) (17). Immunoblot analysis of subcellular fractions also showed that most of the ectopically expressed Noxa protein was located in the mitochondria-rich heavy membrane fraction and a small amount was detected in the low-speed pellet, which contains residual mitochondria together with nuclei (Fig. 3C) (18). On the other hand, the Noxa mutant lacking functional BH3 motifs (AB mt) was found in all fractions (Fig. 3C), indicating that the selective localization of Noxa to mitochondria is contingent on its functional BH3 motifs. Bax is known to accumulate in mitochondria in response to death signals (19, 20). Therefore, the function of Noxa is likely to be independent of that of Bax. In fact, BH3-only subfamily members are known to induce apoptosis by association with anti-apoptotic Bcl-2 family members or by stimulating other apoptosis-promoting factors (8). Noxa indeed coimmunoprecipitated with coexpressed Bcl-XL or Bcl-2, and this coimmunoprecipitation was dependent on the BH3 motifs of Noxa (Fig. 3D). We also found that endogenous Noxa, induced in irradiated thymocytes, also coimmunoprecipitated with Bcl-XL (9). Such an interaction was also observed with another Bcl-2 member, Mcl-1 (20), collectively suggesting the selective interaction of Noxa with the anti-apoptotic Bcl-2 subfamily of proteins.

Because p53-dependent apoptosis is dependent on the activation of Apaf-1 and caspase-9 (21), we also examined whether Noxa affected these events. Cytochrome c release, which induces Apaf-1 activation (21), and caspase-9 activation were also observed in these cells (Fig. 3E). The mitochondrial permeability change is also induced during the process of p53-dependent apoptosis (22). A decrease in mitochondrial membrane potential (ΔΨm), which is mediated by the opening of the mitochondrial permeability transition pore (17, 23), was detected 12 hours after infection of HeLa cells with the Noxa-expressing adenovirus (Fig. 3F).

To examine the involvement of Noxa in p53-induced apoptosis, we used human Saso2 cells, which lack p53 expression (24). We screened for a human homolog of Noxa cDNA and found that the cloned cDNA showing the highest degree of similarity is identical to the previously identified human gene APR (25). However, the function of this gene is not known, and its regulation by p53 has not been demonstrated. Human Noxa, or APR, encodes 54 amino acids, containing only one BH3 motif at amino acids 29 to 37 (Fig. 4A). This motif probably corresponds to motif B of mouse Noxa, and the human Noxa gene lacks a DNA segment corresponding to the second exon of mouse Noxa (9). Human Noxa also induced apoptosis in various cells, including Saso2 cells in a BH3 motif–dependent manner (9). The promoter region of the human Noxa gene indeed contains one p53-responsive element (9), and increased expression of Noxa mRNA was observed in Saso2 cells infected with adenovirus encoding p53 (Fig. 3B). When an antisense oligonucleotide to Noxa was expressed in Saso2 cells, the increased expression of endogenous Noxa in response to p53 was inhibited. In contrast, control oligonucleotide had no effect (Fig. 4C) (26). Introduction of the antisense oligonucleotide also inhibited p53-induced apoptosis (Fig. 4D). Radiation-induced apoptosis in a hematopoietic cell line, BAF-3, is known to be dependent on p53 (27). Introduction of the antisense oligonucleotide to Noxa also inhibited the induction of Noxa expression and apoptosis (Fig. 4E). These results also support the notion that Noxa is a mediator of p53-induced apoptosis, at least in these assay systems.

Noxa may be an attractive candidate mediator of p53-mediated apoptotic response (1). It is likely that Noxa, and other p53 target genes, functionally cooperate with each other for the efficient induction of apoptosis in various cell types.

References and Notes
8. For isolation of Noxa cDNA, the mRNA differential display method was performed with the mRNA fingerprinting Kit (NipponGene, Tokyo). In this experiment, we cloned Noxa cDNA and two other previously unidentified genes regulated by p53 (R. Ohki, unpublished observation).
11. Primary MEFs and mouse thymocytes were isolated by standard methods and cultured as described (16). A decrease in mitochondrial membrane potential (ΔΨm), which is mediated by the opening of the mitochondrial permeability transition pore (17, 23), was detected 12 hours after infection of HeLa cells with the Noxa-expressing adenovirus (Fig. 3F).
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Stable RNA/DNA Hybrids in the Mammalian Genome: Inducible Intermediates in Immunoglobulin Class Switch Recombination

Robert B. Tracy,1 Chih-Lin Hsieh,2,3 Michael R. Lieber1,2,4,5*

Although it is well established that mammalian class switch recombination is responsible for altering the class of immunoglobulins, the mechanistic details of the process have remained unclear. Here, we show that stable RNA/DNA hybrids form at class switch sequences in the mouse genome upon cytokine-specific stimulation of class switch in primary splenic B cells. The RNA hybridized to the switch DNA is transcribed in the physiological orientation. Mice that constitutively express an Escherichia coli ribonuclease H transgene show a marked reduction in RNA/DNA hybrid formation, an impaired ability to generate serum immunoglobulin G antibodies, and significant inhibition of class switch recombination in their splenic B cells. These data provide evidence that stable RNA/DNA hybrids exist in the mammalian nuclear genome, can serve as intermediates for physiologic processes, and are mechanistically important for efficient class switching in vivo.

Mammalian organisms require two types of DNA recombination to produce functional immunoglobulin (Ig) proteins. The first, called V(D)J recombination, mediates assembly of the variable domains of the Ig heavy and light chains in pre-B cells (J). Downstream of the V, D, and J segments is the region containing the Ig constant domains. In mice, this consists of eight distinct sets of constant domain exons (C\textsubscript{\textbf{H}}), with the following constant organization: 5'-(V(D)-J)-C\textsubscript{\textbf{H}}-C\textsubscript{\textbf{D}}-C\textsubscript{\textbf{J}}-C\textsubscript{\textbf{H}1}-C\textsubscript{\textbf{H}2}-C\textsubscript{\textbf{H}3}-C\textsubscript{\textbf{H}4}-C\textsubscript{\textbf{H}5}-C\textsubscript{\textbf{H}6}-C\textsubscript{\textbf{H}7}-C\textsubscript{\textbf{H}8}-C\textsubscript{\textbf{H9}}-C\textsubscript{\textbf{H10}}-C\textsubscript{\textbf{H11}}-C\textsubscript{\textbf{H12}}-C\textsubscript{\textbf{H13}}-C\textsubscript{\textbf{H14}}-C\textsubscript{\textbf{H15}}-C\textsubscript{\textbf{H16}}-3'. In the second type of recombination, termed “class switch recombination” (CSR), the C\textsubscript{\textbf{H}} exons (and C\textsubscript{\textbf{D}} exons) are replaced by any one of the downstream C\textsubscript{\textbf{H}} exons. This results in a deletion of the intervening genomic DNA as a circular product, which includes the C\textsubscript{\textbf{H}} exons (2). Replacement of C\textsubscript{\textbf{H}} ultimately causes a change from IgM to IgG, IgE, or IgA (3–5).

CSR from the IgM isotype to one or more of the downstream isotypes takes place any-

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where within the several-kilobase G-rich (non-template strand) regions of repetitive DNA, termed “switch regions,” which are located 5’ to each set of C\textsubscript{\textbf{H}} exons (6). Immediately upstream of each mammalian switch region are intron promoters, which direct direct sterile transcripts into the switch and constant regions upon activation by particular cytokines (4, 7). Targeting of CSR to a given constant gene is considered to be tightly correlated with transcription from the corresponding upstream promoter (3, 4, 8, 9). Although the germ line transcripts appear to be required for CSR (10, 11) and in substrate studies (12, 13), their exact role in the targeting of class switch is unknown. Previous in vitro data showed stable RNA/DNA hybrid formation after transcription through switch sequences (14, 15). The RNA forms a hybrid with the DNA only when it is transcribed in the physiological direction (i.e., generation of G-rich RNA).

On the basis of this circumstantial evidence for RNA/DNA hybrid formation at switch sequences in vitro, we attempted to isolate RNA/DNA hybrids at several different murine switch sequences (S\textsubscript{\textbf{H}}, S\textsubscript{\textbf{Y}}, S\textsubscript{\textbf{Y}1}, S\textsubscript{\textbf{Y}2}, S\textsubscript{\textbf{H}}, and S\textsubscript{\textbf{Y}1}) in the genome of B cells that are actively undergoing CSR [see supplementary Web material (16) for details]. The experimental design involved (i) enriching for small resting B cells from the spleens of wild-type C57Bl/6 mice, (ii) inducing the cells to undergo CSR by adding lipopolysaccharide (LPS) and appropriate cytokines, (iii) allowing the cells to proliferate for 2.5 days, (iv) isolating the genomic DNA, (v) treating the DNA with an excess of ribonuclease (RNase) A, and (vi) digesting all of the genomic DNA with deoxyribonuclease I. At this stage, the only nucleic acid remaining should be RNA that was stably hybridized to genomic DNA and, hence, protected from RNase A treatment.

Initially, we attempted to detect RNA/DNA hybrids at S\textsubscript{H} and S\textsubscript{Y} by reverse transcription–polymerase chain reaction (RT-PCR) on RNA purified from B cells stimulated with LPS and interleukin-10 (IL-10) (17). When we examined the 5’ end of S\textsubscript{H} and S\textsubscript{Y}, we found that an RT-PCR product of the correct size is generated from an RNase A–resistant RNA species at both loci (Fig. 1B, lanes 1 and 4, respectively). The RNA species is also present at the 3’ end of S\textsubscript{Y}, as evidenced by the generation of the correct-sized RT-PCR product (Fig. 1B, lane 7). Lanes 3 and 6 show that these bands are undetectable in the absence of RT, eliminating the possibility that the observed PCR product is a consequence of genomic DNA contamination (Fig. 1B). To confirm that the RNA is involved in hybrid formation, we treated the genomic DNA with RNase H (which only hydrolyzes RNA involved in hybrid formation) and RNase A simultaneously. Upon treatment with RNase H, the RT-PCR products disappear (Fig. 1B, lanes 2, 5, and 8).

To confirm that RNA/DNA hybrid formation is a general property of mouse switch sequences, we examined hybrid formation at S\textsubscript{Y} and S\textsubscript{H} following stimulation of the B cells with IL-4 rather than IL-10. RT-PCR products of the expected sizes were produced in the absence (lanes 9 and 11) but not in the presence of RNase H (lanes 10 and 12) (Fig. 1B), thus confirming the presence of a hybrid at these sequences. For S\textsubscript{H} and S\textsubscript{Y}1, we verified that the RNA in the hybrid was the G-rich RNA being generated in the physiologic direction by performing RT-PCR with strand-specific primers during RT (RTB49, RTB80, and RTB14 in Fig. 1A) [see Web fig. 1 (16)].

To confirm the RT-PCR data, we attempted to detect hybrids formation at the S\textsubscript{H} and S\textsubscript{Y} genomic loci by Northern blot analysis (Fig. 2) (18). As a positive control, we tried to detect germ line transcripts by Northern blot