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Tables S1 to S3
Dataset S1

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Habitat Split and the Global Decline of Amphibians

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The worldwide decline in amphibians has been attributed to several causes, especially habitat loss and disease. We identified a further factor, namely "habitat split"—defined as human-induced disconnection between habitats used by different life history stages of a species—which forces forest-associated amphibians with aquatic larvae to make risky breeding migrations between suitable aquatic and terrestrial habitats. In the Brazilian Atlantic Forest, we found that habitat split negatively affects the richness of species with aquatic larvae but not the richness of species with terrestrial development (the latter can complete their life cycle inside forest remnants). This mechanism helps to explain why species with aquatic larvae have the highest incidence of population decline. These findings reinforce the need for the conservation and restoration of riparian vegetation.

Amphibian populations are declining worldwide (*1*, *2*). Among the factors determining the amphibian declines are habitat loss and fragmentation, which affect amphibians just as they affect any other organisms: through population isolation, inbreeding, and edge effects (*3*–*5*). Another important factor is the fungus *Batrachochytrium dendrobatidis*, a highly virulent pathogen that attacks many amphibian species and has been responsible for the decline of many populations even in undisturbed environments (*6*, *7*). Amphibians can also be threatened by climate shifts (*7*), ultraviolet-B radiation (*8*), introduction of exotic species (*9*), and agrochemical contaminants (*10*). We investigated the role of a further factor, which we define as "habitat split."

Amphibian species with aquatic larvae typically undergo a major ontogenetic niche shift, whereby tadpoles and adults occupy two distinct habitats (*11*). In pristine environments, the aquatic habitat of the tadpoles and the terrestrial habitat of the postmetamorphics grade into each other. However, in landscapes occupied by humans, land use has often resulted in a spatial separation between remnants of terrestrial habitat and breeding sites (*12*). Adults of species with aquatic larvae, in order to breed, are obliged to abandon forest remnants to reach water bodies, and at the end of the reproductive season, both

scriptional 3′ end processing of transcripts from snRNA and protein-coding templates, whereas five CTD repeats (Δ5) do not (*2*, *4*) (fig. S2, A and B).

Mutation of Ser^7 to the non-phosphoacceptor alanine (S7A) in a background of 25 repeats reduces the level of properly processed U2G transcripts (Proc) and increases the ratio of unprocessed transcripts (Unproc). However, this mutation affects neither the level nor 3′ end processing of hnRNPK transcripts (Fig. 1A and fig. S1B). Mutation of Ser^7 to alanine in a background of 48 consensus repeats [$(Con)^{48}$] (*10*) has a similarly strong effect on U2G transcripts, without affecting hnRNPK transcripts. This mutation does not affect expression of Rpb1 or Ser^2/Ser^5 phosphorylation (fig. S1C). Thus, Ser^7 is specifically required for efficient production of properly 3′ end–processed transcripts from an snRNA template.

Mutation of Ser^7 to the phosphomimic glutamic acid (S7E) has a strong effect on the level and 3′ end processing of transcripts from both the U2G and hnRNPK templates (Fig. 1A), possibly resulting from a defect in Pol II recruitment or association caused by charged residues. The increase in −111Unproc may reflect a defect in termination of transcription of U2G resulting from the defect in 3′ end processing (*2*, *11*, *12*). The S7E mutation abolishes recognition by antibodies to phosphoserine 2 (Ser^2-P) (fig. S1C), reflecting a drop in Ser^2 phosphorylation and/or interference with antibody recognition. A drop in Ser^2 phosphorylation would account for the defect in 3′ end processing and indicate that mutations can have secondary effects. Rpb2 is not affected by α-amanitin treatment in the same way as Rpb1 (fig. S1C), indicating that not all Pol II–specific subunits are subject to α-amanitin–induced turnover in HEK 293 cells.

Mutation of Ser^2 to alanine (S2A) affects 3′ end formation of U2G and hnRNPK transcripts, whereas transcript levels are strongly reduced by mutation to glutamic acid (S2E) (fig. S2, A, B, and D) (*6*). S2A accumulates to a higher level than $(Con)^{25}$ and S2E is undetectable (fig. S2C), suggesting that phosphorylation of Ser^2 is involved in Rpb1 turnover. Introduction of alanine at position 5 (S5A) reduces steady-state U2G and hnRNPK transcript levels and processing (fig. S2, A, B, and D). This likely reflects the requirement for Ser^5 phosphorylation for addition of the 5′ cap, which protects the RNA and activates 3′ end processing (*5*, *13*). Although introduction of glutamic acid at position 5 restores RNA levels, suggesting that capping now occurs, 3′ end processing is still inefficient, demonstrating that a charged amino acid does not fully compensate for the lack of a serine. The increase in −111Unproc accompanies loss of processing of U2G transcripts in all cases (fig. S2A) and likely reflects a termination defect. In contrast to mutations in Ser^7, mutations in Ser^2 and Ser^5 affect production of snRNAs and mRNAs in largely the same way.

Mutation of Ser^2 to alanine and Ser^5 to alanine does not reduce recognition by antibodies to Ser^5-P and Ser^2-P, respectively (fig. S2C), suggesting that both phosphorylation events can occur independently. Mutation of Ser^5 to glutamic acid reduces recognition by the antibody to Ser^2-P, reflecting a drop in Ser^2 phosphorylation and/or interference with antibody recognition. A drop in Ser^2 phosphorylation would again account for the defect in 3′ end processing (fig. S2, A and B).

To determine whether Ser^7 is required for expression of endogenous snRNA genes, we used cells with stably integrated α-amanitin–resistant Rpb1 genes controlled by a tetracycline-regulated promoter (*10*) (Fig. 1B). U2 pre-snRNA (pre-U2) and stable mature U2 snRNA (U2) are readily detected in RNA from cells expressing α-amanitin–resistant Rpb1 with $(Con)^{48}$. A third minor protection product corresponds to transcripts that have escaped 3′ box–directed processing (U2Unproc) (*2*). Because fully processed snRNAs are very stable (*14*), there is little change in U2 levels when Ser^7 is mutated to alanine. However, accumulation of pre-U2 and U2Unproc is severely impaired, although expression of the α-amanitin–resistant Rpb1 is unaffected. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of the RNA indicates that this mutation decreases the level of pre-U2 to less than 30%, whereas mRNA encoding the transcription factor hElf-1 is unaffected. Chapman *et al.* (*15*) have independently determined that this

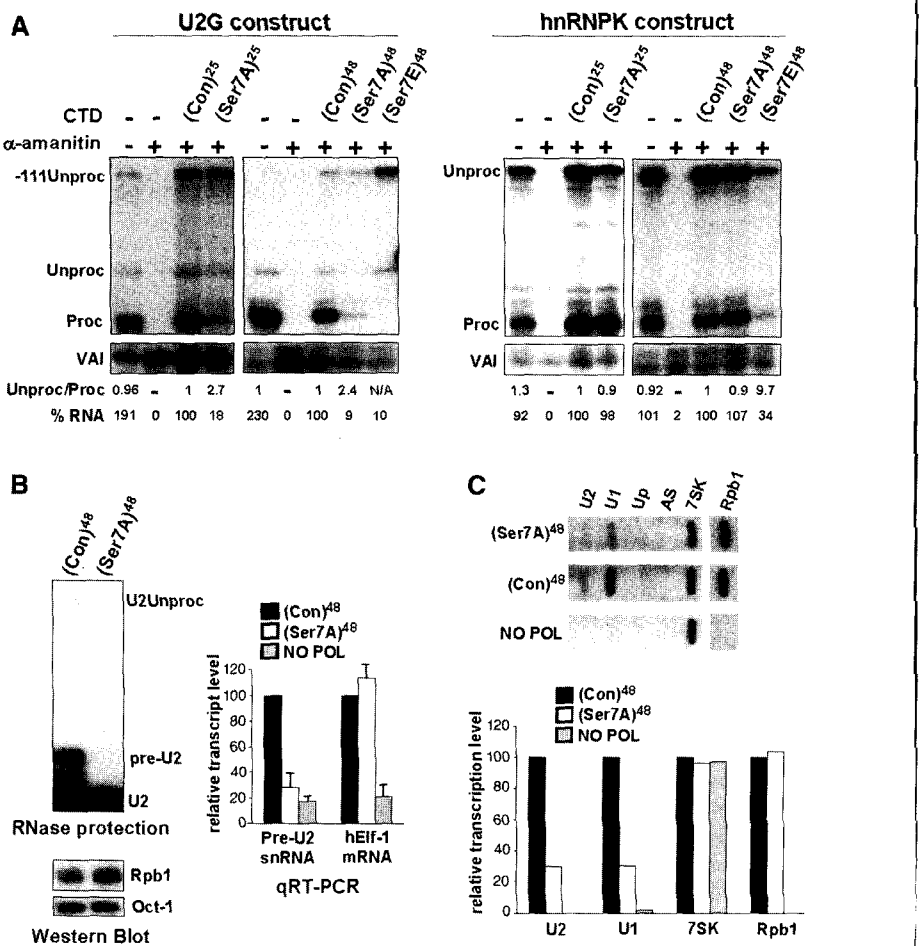

Fig. 1. Ser^7 is required for expression of snRNA but not protein-coding templates. (**A**) Ribonuclease (RNase) protection analysis of RNA transcribed from U2G or pCMV-hnRNPK constructs after ectopic expression of α-amanitin–resistant Rpb1 (fig. S1A). (Con) designates consensus CTD heptapeptides. Ser7A, S7A; Ser7E, S7E; VAI, virus-associated RNA I. (**B**) RNase protection analysis of transcripts from endogenous U2 genes in cells stably expressing α-amanitin–resistant Rpb1 and Western blot analysis of Rpb1 expression. qRT-PCR analysis of U2 pre-snRNA and hElf-1 mRNA in total RNA normalized to 7SK RNA with α-amanitin–treated cells expressing no Rpb1 as negative control (NO POL). Error bars indicate the range of values from three independent experiments. (**C**) Run-on analysis of endogenous U1 and U2 snRNA genes in cells transfected with α-amanitin–resistant Rpb1s. AS and Up are negative controls (*12*). Quantitation of this data is shown in the bar graph.

mutation does not have a general effect on expression of protein-coding genes. Mutation of Ser^7 to alanine also reduces transcription of U1 and U2 genes to less than 30%, as measured by nuclear run-on analysis (Fig. 1C), whereas transcription of the transfected CMV promoter-driven Rpb1 template and the Pol III–dependent 7SK gene is unaffected.

Taken together, these results indicate that Ser^7 is required for endogenous snRNA gene expression. Mutating Ser^7 to alanine does not affect the level of Rpb1 associated with γ-actin and glyceraldehyde phosphate dehydrogenase (GAPDH) protein-coding genes, as measured by chromatin immunoprecipitation (ChIP) (Fig. 2A), indicating that Pol II is recruited efficiently. Unexpectedly, the mutant Pol II is also recruited efficiently to U1 and U2 genes, indicating that transcription of snRNA genes is affected at a post-recruitment step.

The transcription factor PTF/PBP/$SNAP_C$ (PSE-binding transcription factor/PSE-binding protein/snRNA-activating protein complex), which recognizes the PSE (*1*, *16*), and the Integrator complex, which plays a role in 3′ end processing of snRNAs (*17*), are the only known factors specifically involved in expression of Pol II–transcribed snRNA genes. Because Integrator interacts with the CTD (*17*), we analyzed the effect of Ser^7 mutation on recruitment of this complex. When the CTD contains 48 consensus repeats, tandem affinity purification (TAP)–tagged Integrator subunit 9/RC-74 (TAP-Int9) (*17*, *18*) is clearly detectable on snRNA genes but not on γ-actin and GAPDH genes (Fig. 2A and fig. S3A). Association with snRNA genes is lost when Ser^7 is mutated to alanine. In contrast, association of PTF with the promoters of snRNA genes is Pol II–independent (fig. S3B).

When transcribing snRNA genes, Pol II is phosphorylated on Ser^7 (Fig. 2B), raising the possibility that CTD phosphorylation plays a role in Integrator recruitment. To investigate this, we performed glutathione *S*-transferase (GST) pull-down analysis using consensus or S7A repeats. Phosphorylation on Ser^2, Ser^5, and Ser^7 is detected after in vitro phosphorylation (fig. S3C). Int11/RC-68 (*17*, *18*), presumably as part of the Integrator complex, interacts strongly with the consensus repeats only after phosphorylation, and the interaction increases with the number of repeats (Fig. 2C). Mutation of Ser^7 to alanine has a strong effect on Integrator binding, although Ser^2 and Ser^5 phosphorylation still occur (fig. S3D), strongly suggesting that Ser^7 phosphorylation participates in this interaction.

Taken together, these experiments suggest that phosphorylation of conserved Ser^7 residues within the CTD is critical for association with the Integrator complex in vivo. Disruption of this interaction would account for the defect in 3′ end processing and may also be responsible for the defect in transcription (Fig. 2D). It was proposed that different combinations of phosphorylation of Ser^2 and Ser^5 and proline isomerization could constitute a CTD "code" (*19*). Ser^7 phosphorylation would provide an additional, important element of this code in mammals.

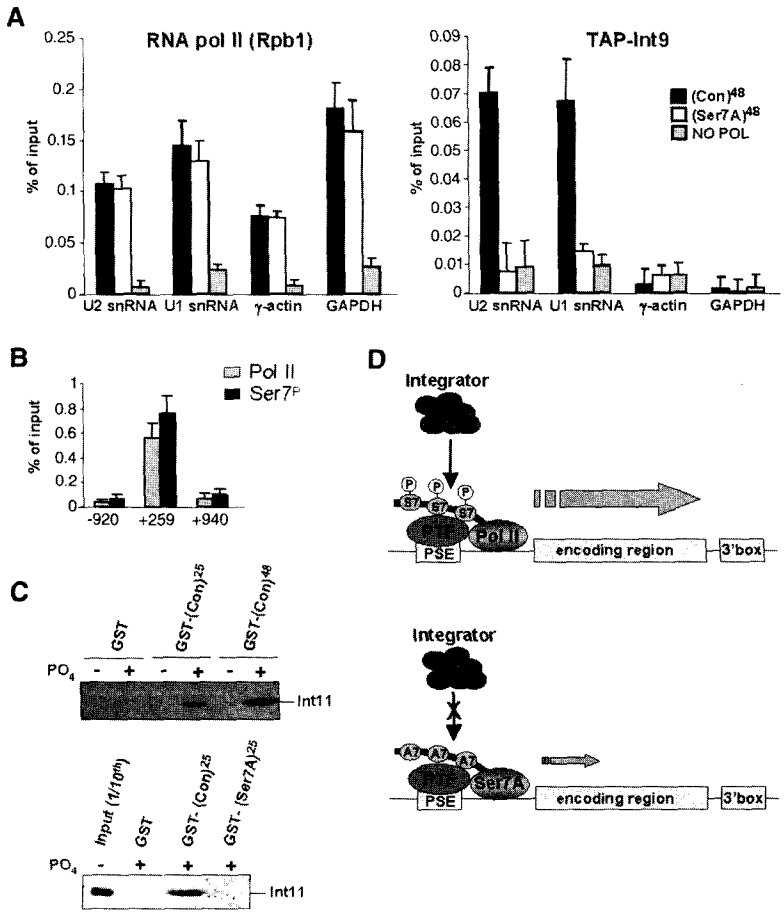

Fig. 2. Mutation of Ser^7 to alanine affects association of Integrator with snRNA genes. **(A)** ChIP analysis of Rpb1 and TAP-Int9 associated with U1, U2, γ-actin, and GAPDH promoters. **(B)** ChIP analysis of endogenous U2 genes with antibodies to Rpb1 (Pol II) or Ser^7-P ($Ser7^P$). Error bars in (A) and (B) indicate the range of values from three independent experiments. **(C)** Western blot analysis of GST-CTD pull down of Integrator with antibodies to Int11 (*18*). **(D)** Phosphorylation of Ser^7 is required for efficient interaction of Integrator with Pol II. Disruption of this interaction may cause a defect in a post-recruitment step of transcription, in addition to affecting 3′ end processing.

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Materials and Methods
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References

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