Coordinate Regulation of Transcription and Splicing by Steroid Receptor Coregulators

Didier Auboeuf,[1] Arnd Hönig,[2]* Susan M. Berget,[2] Bert W. O'Malley[1]†

Recent observations indicating that promoter identity influences alternative RNA-processing decisions have created interest in the regulatory interactions between RNA polymerase II transcription and precursor messenger RNA (pre-mRNA) processing. We examined the impact of steroid receptor–mediated transcription on RNA processing with reporter genes subject to alternative splicing driven by steroid-sensitive promoters. Steroid hormones affected the processing of pre-mRNA synthesized from steroid-sensitive promoters, but not from steroid-unresponsive promoters, in a steroid receptor–dependent and receptor-selective manner. Several nuclear receptor coregulators showed differential splicing effects, suggesting that steroid hormone receptors may simultaneously control gene transcription activity and exon content of the product mRNA by recruiting coregulators involved in both processes.

Alternative splicing can yield multiple mRNAs per gene, and because most of transcripts expressed from human genes are alternatively spliced (~60% of the genes), this process is more a rule than an exception and plays an essential role in expanding protein diversity (*1*). Regulation of alternative splicing depends on cis-acting sequences that are localized in the pre-mRNAs and on trans-acting factors that recognize these sequences and control exon choice (*2*). The way that transcription and splicing are coordinated and tightly regulated remains poorly understood.

The concept of functional coupling between transcription and splicing was suggested by the findings that RNA processing occurs on RNA polymerase II, that promoter structure contributes to splice site selection, and that modifications in transcription elongation rate affect splicing decisions (*3*–*7*). A number of transcriptional coregulators that act with the ligand-dependent transcriptional factors of the steroid receptor family are related to factors involved in pre-mRNA processing (table S1).

To study the impact of steroid-mediated transcriptional regulation on alternative splicing, we positioned steroid-sensitive promoters {mouse mammary tumor virus [MMTV], progesterone response element [$(PRE)_2$-TATA], or estrogen response element [$(ERE)_2$-TATA]} or promoters that do not respond to steroid hormones [cytomegalovirus (CMV) or herpes simplex virus–thymidine kinase (HSV-TK)] upstream of two different splicing reporter minigenes whose products are subject to alternative splicing decisions. Each minigene produced spliced RNA products that differed in size, permitting the determination of the relative amount of splicing (*8*). To assess the splicing of alternative cassette exons, we inserted the variable exons v4 and v5 of the human CD44 gene, along with their surrounding intron sequences, into an intron of the β-globin gene (*9*). The minigene gives rise to two major spliced RNA products, containing either both variable exons (inclusion) or none of these exons (skipping), and a minor product with only one variable exon (Fig. 1A). When the HeLa cells were transfected with progesterone receptor (PR), activation by progesterone (Pg) of the steroid-sensitive MMTV promoter driving the CD44 minigene resulted in the expected increase in the amount of total mRNA produced. Pg treatment also caused a 2.6-fold (± 0.1, $n = 4$) increase in the skipping/inclusion ratio of spliced mRNAs as compared with the ratio obtained in the absence of Pg (Fig. 1A, lanes 3 and 4). However, Pg had no effect on the splicing of the CD44 minigene products synthesized from the steroid-unresponsive HSV-TK or CMV promoters (Fig. 1A, lanes 1 and 2) (*10*). When endogenously expressed glucocorticoid receptor (GR) was used in HeLa cells, activation by the synthetic glucocorticoid dexamethasone (dex) of the MMTV promoter driv-

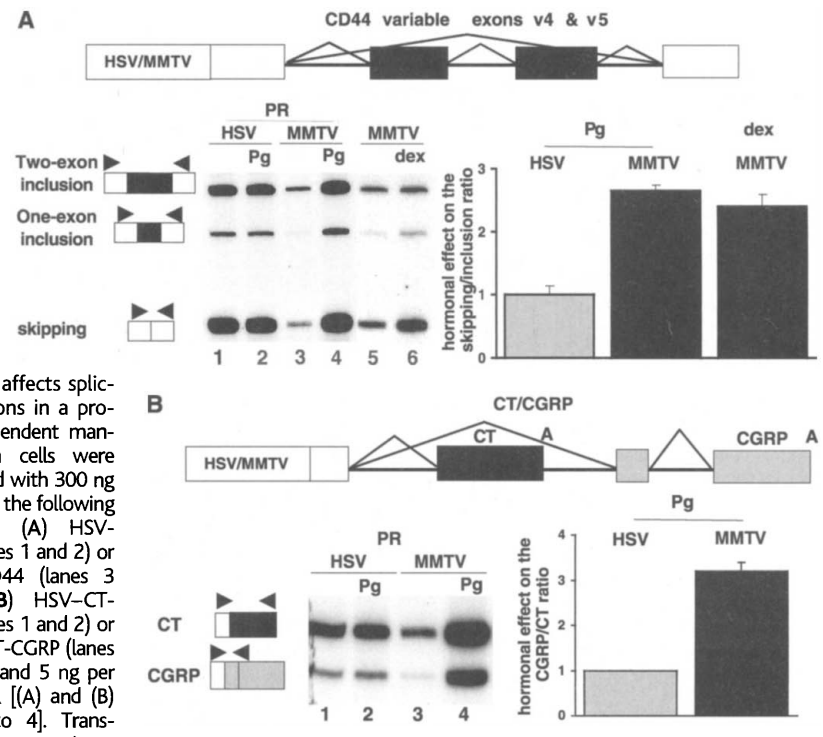

Fig. 1. Pg affects splicing decisions in a promoter-dependent manner. HeLa cells were transfected with 300 ng per well of the following minigenes: (A) HSV-CD44 (lanes 1 and 2) or MMTV-CD44 (lanes 3 to 6); (B) HSV–CT-CGRP (lanes 1 and 2) or MMTV–CT-CGRP (lanes 3 and 4), and 5 ng per well of PR [(A) and (B) lanes 1 to 4]. Transfected cells were incubated for 24 hours in the absence [(A), lanes 1, 3, and 5; (B), lanes 1 and 3] or presence of Pg [(A) and (B), lanes 2 and 4] or dex that can stimulate endogenously expressed GR in HeLa cells [(A) lane 6]. (Left) Autoradiographic films of the radiolabeled–polymerase chain reaction (PCR) products obtained in a representative experiment. (Right) Mean (± SD, $n = 4$) quantifications of the hormonal effects; the values shown are the ratios of processing in pairs in the presence or absence of hormone: (A) CD44 skipping/inclusion, (B) CGRP/CT. For example, on the right panel of (A), the CD44 skipping/inclusion ratio obtained from lane 4 (ratio in presence of Pg) was divided by the skipping/inclusion ratio from lane 3 (ratio in absence of Pg) to yield the MMTV histogram bar on the right representing the fold hormonal effect on splicing.

[1]Department of Molecular and Cellular Biology, [2]Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

*Present address: Department of Obstetrics and Gynecology, University of Tubingen, 72076 Tubingen, Germany.

†To whom correspondence should be addressed. E-mail: berto@bcm.tmc.edu

ing the CD44 minigene also caused a 2.4-fold (± 0.2, n = 4) increase in the skipping/inclusion ratio (Fig. 1A, lanes 5 and 6).

Another reporter that we tested contained an alternative 3′-terminal exon derived from the human gene encoding calcitonin (CT) or calcitonin gene–related peptide (CGRP) (Fig. 1B). The CT-CGRP pre-mRNA is processed either to include exon 4 with use of the exon 4 polyadenylation site or to exclude exon 4 with use of the exon 6 polyadenylation site, to produce the CT or CGRP peptides, respectively (*11*). Pg caused a 3.2-fold (± 0.2, n = 4) increase in the CGRP/CT mRNA ratio as compared with the ratio obtained in the absence of Pg, when the CT-CGRP minigene is driven by the steroid-sensitive MMTV promoter (Fig. 1B, lanes 3 and 4), but had no effect when the same minigene was driven by the steroid-unresponsive HSV-TK promoter (Fig. 1B, lanes 1 and 2). These results demonstrate that Pg affects different splicing decisions as a consequence of hormonal action at the promoter level. Promoter-dependent hormonal effects on splicing were not a consequence of an increase in transcription rate or of a saturation of the splicing machinery, because splicing of the minigene products was not affected by the amount of pre-mRNA synthesized, the density of RNA polymerase II, or the strength of the promoter driving the minigene (figs. S1 and S2).

The promoter-dependent effect of Pg on splicing was also PR dependent, because Pg had no effect on splicing in the absence of PR (Fig. 2, lanes 1 and 2) and increasing the amount of transfected PR enhanced the Pg-mediated effect on splicing in a dose-dependent manner (fig. S2). The PR activation domain AF-2 was dispensable for mediating this effect. A PR mutant (PR/9) that lacks the AF-2 domain but binds promoters in the absence of hormone was constitutively active for both transcription and splicing (Fig. 2, B and C, lanes 9 and 10). Although this mutant showed less than 10% of the full-length PR transcriptional activity, it demonstrated ~80% of the full-length PR splicing effect. The deletion of the PR NH_2-terminal domain in one mutant decreased its transcriptional activity but only slightly affected its splicing effect (Fig. 2, B and C, lanes 5 and 6), whereas complete deletion of the AF-1 domain abolished the splicing effect (Fig. 2B, lanes 7 and 8).

Estrogen receptors ERα and ERβ, which differ essentially in the AF-1 domain (*12*), did not mediate the same splicing effects on the minigene products synthesized from the estradiol-sensitive $(ERE)_2$-TATA promoter. When HeLa cells were transfected with ERα, estradiol (E2) caused a 1.8-fold (± 0.1, n = 5) increase in the skipping/inclusion ratio when the CD44 minigene was driven by an $(ERE)_2$-TATA promoter as compared with the ratio obtained in the absence of E2 (Fig. 3A, lanes 3 and 4), but E2 had a minimal effect when activating ERβ (Fig. 3A, lanes 7 and 8). In contrast, when the cells were transfected with the ERE–CT-CGRP reporter, ERβ induced a 2.2-fold (± 0.6, n =5) increase in the CGRP/CT ratio in the presence of E2 as compared with the ratio obtained in the absence of E2 (Fig. 3B, lanes 7 and 8), but E2 had no effect when activating ERα (Fig. 3B, lanes 3 and 4).

Because steroid receptors, notably estrogen receptors, have different tissue-specific biological functions and because alternative splicing is a tissue-specific regulated process (*2*, *12*), we compared E2 splicing effects in different cell lines. E2 affected splicing when transfected ERα in Cos-1 cells or endogenously expressed ERα in T47D cells were used (Fig. 3C, lanes 3 and 4) (*10*), whereas E2 did not alter the splicing pattern when endogenously expressed or transfected ERα was used in the breast cancer MCF-7 cells (Fig. 3C, lanes 5 to 8). These results implicate additional cellular-specific factors in the steroid receptor effects on splicing.

Steroid receptors mediate the transcriptional effects of steroid hormones by recruiting transcriptional coregulators to target promoters (*13*, *14*). To test whether coactivators affect CD44 splicing, we compared their effects on E2-mediated splicing. Among the growing list of steroid receptor coregulators potentially involved in RNA processing, we selected those involved in ER transcriptional activation, including the DEAD-box RNA helicase p72 and the heterogeneous nuclear ribonucleoprotein-like proteins TLS and CoAA (*15*–*17*). p72 is in a complex containing ER and the RNA coactivator SRA that binds the RNA-binding protein YB-1 (*10*) through a C/A-rich sequence similar to the splicing enhancer found in the CD44 v4 exon regulated by YB-1 (*9*, *17*). CoAA, along with the coactivator of activating protein-1 and estrogen receptors, which contains arginine-serine–rich domains characteristic of the SR splicing factors, participates in steroid receptor–mediated transcriptional regulation by interacting with the thyroid hormone receptor–binding protein (TRBP) coregulator (*16*, *18*).

HeLa cells were cotransfected with one of these coregulators, along with the ERE-CD44 reporter minigene and either ERα or ERβ activated by E2. In the experimental condi-

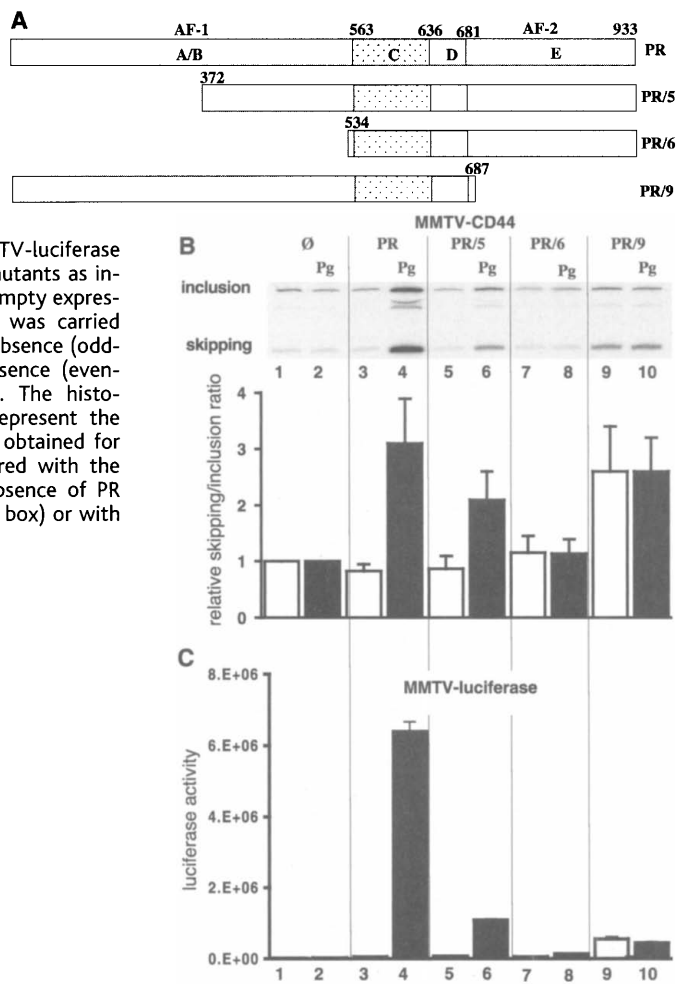

Fig. 2. Assessment of the PR domains involved in the Pg-mediated splicing effect. (**A**) Schematic representation of the PR mutants used. A to E indicate domains of PR. HeLa cells were transfected with (**B**) MMTV-CD44 or (**C**) MMTV-luciferase and with the different mutants as indicated (∅ denotes the empty expression vector); incubation was carried out for 24 hours in the absence (odd-numbered lanes) or presence (even-numbered lanes) of Pg. The histograms in (B) and (C) represent the skipping/inclusion ratios obtained for each PR mutant compared with the ratio obtained in the absence of PR either without Pg (white box) or with Pg (black box) (n = 3).

tions used, TLS had no effect on splicing (Fig. 4, lanes 1 and 2 or 6 and 7), whereas p72 increased exon inclusion [Fig. 4, lanes 1 and 3 or 6 and 8; p72 decreased the skipping/inclusion ratio 13-fold (± 2.4, n = 5) and 11-fold (± 0.8, n = 5), respectively]. In contrast, CoAA increased the skipping/inclusion ratio 6.8-fold (± 1.5, n = 5) in the presence of ERβ (Fig. 4, lanes 6 and 9). The CoAA effect was more pronounced with ERβ than ERα (6.8-fold ± 1.5 versus 1.7-fold ± 0.4). In contrast to CoAA, a natural spliced variant of CoAA called CoAM, which lacks the TRBP-interacting domain required for the recruitment of CoAA by activated nuclear receptors to the transcriptional machinery (*16*), did not stimulate exon skipping (Fig. 4, lanes 11 to 13). The slight increase in exon inclusion mediated by CoAM could potentially be due to a dominant negative effect of CoAM on endogenous CoAA as it was proposed in terms of transcription (*16*). The steroid receptor coactivator SRC-1 had no effect on CD44 splicing (Fig. 4, lanes 5 and 10). Although all the tested coregulators coactivated ERE-mediated transcription as measured with a luciferase assay (*16*, *17*), distinct steroid receptor coregulators promoted differential, and even opposite, effects on CD44 splicing. Finally, although the steroid hormone–mediated effect on splicing was less affected when we used chromosomal integrated CD44 minigenes, the coregulators tested led to similar effects on splicing, suggesting that the mechanisms observed in transient transfection exist in the context of chromatin (*10*).

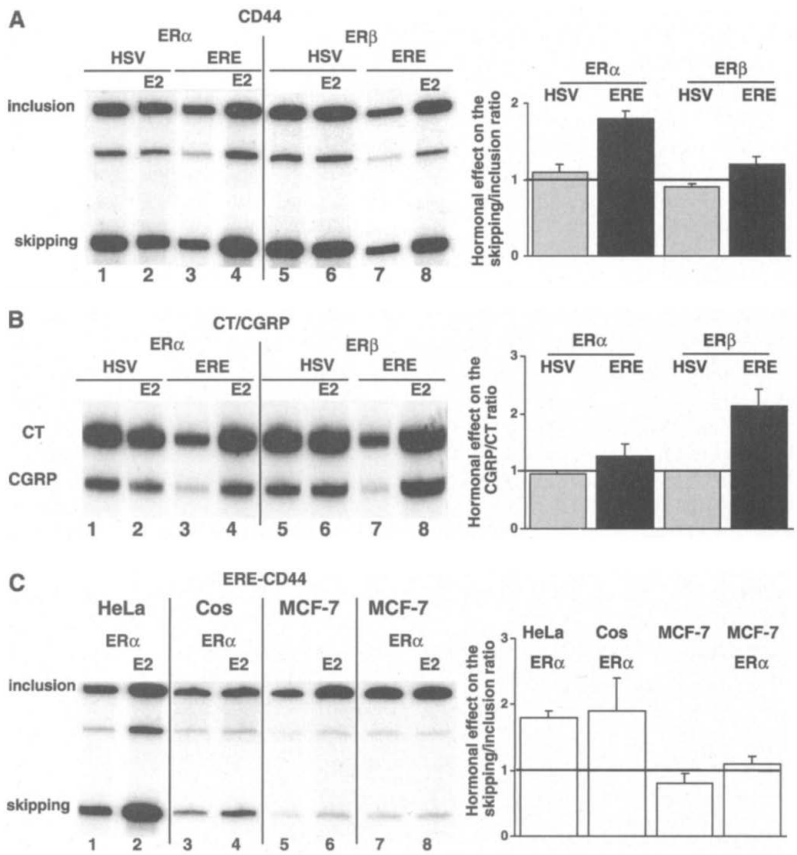

Fig. 3. The E2-mediated splicing effect is ER-subtype selective and cell specific. (**A** and **B**) HeLa cells were transfected with 5 ng per well of ERα (lanes 1 to 4) or ERβ (lanes 5 to 8) and 300 ng per well of the following minigenes: HSV-CD44 [(A) lanes 1, 2, 5, and 6] or ERE-CD44 [(A) lanes 3, 4, 7, and 8); HSV–CT-CGRP [(B) lanes 1, 2, 5, and 6) or ERE–CT-CGRP [(B) lanes 3, 4, 7, and 8). Transfected cells were incubated for 24 hours in the absence (odd-numbered lanes) or presence (even-numbered lanes) of E2. The values shown are the ratios of processing in pairs either with or without E2 as described in Fig. 1. (**C**) HeLa cells (lanes 1 and 2), Cos cells (lanes 3 and 4), or MCF-7 cells (lanes 5 to 8) were transfected with ERE-CD44 minigene and with ERα (except lanes 5 and 6). (Left) Autoradiographic films of the radiolabeled-PCR products obtained in a representative experiment. (Right) Mean (± SD, n = 4) quantification of the hormonal effect. The values shown are the ratio of processing (CD44 skipping/inclusion ratio) in pairs, either with or without E2 as described in Fig. 1.

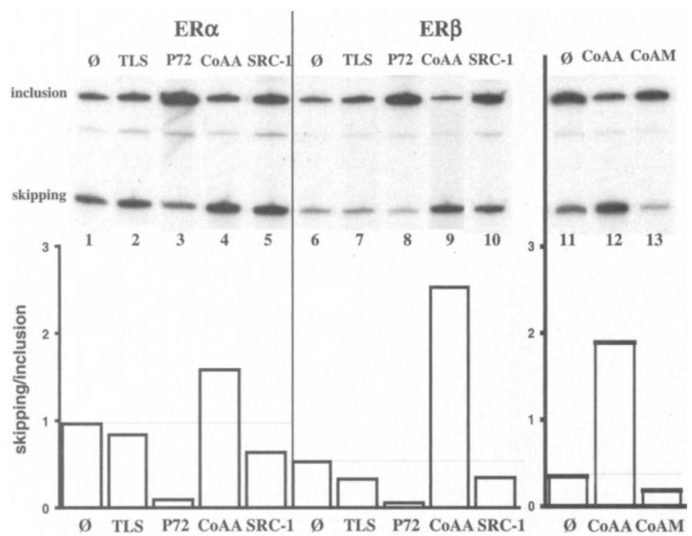

Fig. 4. Differential splicing effects in the presence of distinct steroid receptor coregulators. HeLa cells, transfected with 300 ng of ERE-CD44 minigene, 5 ng of ERα (lanes 1 to 5) or ERβ (lanes 6 to 13), and 200 ng of different coregulators as indicated (∅ denotes the empty expression vector) were incubated for 24 hours after transfection in the presence of E2.

Steroid hormones that control gene transcriptional activity can also influence post-transcriptional events, including alternative splicing, although the mechanisms involved are not known (table S2). We propose that activated steroid receptors bind to target DNA response elements and promote the recruitment of coregulators that are involved in both transcriptional and splicing regulation. This would ensure coordination between RNA production and the nature of the final gene product. Depending on the promoter and cellular context, the same transcriptional factor could recruit different coregulators, thereby mediating different effects on transcription and processing. Because most human genes yield different spliced mRNA isoforms that can play different biological roles, several mechanisms might coexist to ensure that the proper mRNA isoform is produced in the correct amount in response to different biological signals and in a tissue-specific manner. Pathologic uncoupling of the coordination between transcription and RNA processing could have a dramatic impact on cellular homeostasis.

References and Notes

1. I. H. G. S. Consortium, *Nature* **409**, 860 (2001).
2. C. W. Smith, J. Valcarcel, *Trends Biochem. Sci.* **25**, 381 (2000).
3. Y. Hirose, J. L. Manley, *Genes Dev.* **14**, 1415 (2000).
4. P. Cramer, C. G. Pesce, F. E. Baralle, A. R. Kornblihtt, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11456 (1997).
5. P. Cramer *et al.*, *Mol. Cell* **4**, 251 (1999).
6. S. Kadener *et al.*, *EMBO J.* **20**, 5759 (2001).

7. G. C. Roberts, C. Gooding, H. Y. Mak, N. J. Proudfoot, C. W. Smith, *Nucleic Acids Res.* **26**, 5568 (1998).
8. Materials and Methods are available at *Science* Online.
9. E. Stickeler *et al.*, *EMBO J.* **20**, 3821 (2001).
10. D. Auboeuf *et al.*, data not shown.
11. H. Lou, D. M. Helfman, R. F. Gagel, S. M. Berget, *Mol. Cell. Biol.* **19**, 78 (1999).
12. S. Nilsson, J. A. Gustafsson, *Crit. Rev. Biochem. Mol. Biol.* **37**, 1 (2002).
13. M. G. Rosenfeld, C. K. Glass, *J. Biol. Chem.* **276**, 36865 (2001).
14. N. J. McKenna, B. W. O'Malley, *Cell* **108**, 465 (2002).
15. C. A. Powers, M. Mathur, B. M. Raaka, D. Ron, H. H. Samuels, *Mol. Endocrinol.* **12**, 4 (1998).
16. T. Iwasaki, W. W. Chin, L. Ko, *J. Biol. Chem.* **276**, 33375 (2001).
17. M. Watanabe *et al.*, *EMBO J.* **20**, 1341 (2001).
18. D. J. Jung, S. Y. Na, D. S. Na, J. W. Lee, *J. Biol. Chem.* **277**, 1229 (2002).
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Supporting Online Material
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Materials and Methods
Figs. S1 and S2
Tables S1 and S2

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Separable Roles for rent1/hUpf1 in Altered Splicing and Decay of Nonsense Transcripts

Joshua T. Mendell, Colette M. J. ap Rhys, Harry C. Dietz*

The mechanism by which disruption of reading frame can influence pre–messenger RNA (pre-mRNA) processing is poorly understood. We assessed the role of factors essential for nonsense-mediated mRNA decay (NMD) in nonsense-mediated altered splicing (NAS) with the use of RNA interference (RNAi) in mammalian cells. Inhibition of rent1/hUpf1 expression abrogated both NMD and NAS of nonsense T cell receptor β transcripts. In contrast, inhibition of rent2/hUpf2 expression did not disrupt NAS despite achieving comparable stabilization of nonsense transcripts. We also demonstrate that NAS and NMD are genetically separable functions of rent1/hUpf1. Additionally, rent1/hUpf1 enters the nucleus where it may directly influence early events in mRNA biogenesis. This provides compelling evidence that NAS relies on a component of the nonsense surveillance machinery but is not an indirect consequence of NMD.

The most comprehensively studied consequence of a premature termination codon (PTC) is accelerated transcript degradation through NMD (*1*). Despite evidence that NMD requires translation, most nonsense transcripts are degraded in the nuclear fraction of mammalian cells (*2*). Additional evidence that disruption of reading frame can influence intranuclear RNA metabolism stems from the observed effects that PTCs can exert on splicing (*3–5*) and intranuclear trafficking of pre-mRNAs (*6*). In selected examples, altered splicing appears to be specifically dependent on disruption of reading frame rather than isolated inactivation of exonic splicing enhancers (ESEs) (*7–9*). The conclusion that nonsense codon recognition occurs in the nucleus is difficult to reconcile with existing tenets regarding the interpretation of reading frame. A prevailing model posits that recognition and degradation of nonsense transcripts by NMD indirectly influences the processing of pre-mRNAs derived from the same allele through an unknown mechanism (*9*, *10*).

Institute of Genetic Medicine and Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, 858 Ross Building, 720 Rutland Avenue, Baltimore, MD 21205, USA.

*To whom correspondence should be addressed. E-mail: hdietz@jhmi.edu

Substantial insight into the mechanism of mammalian NMD has come from studies of the trans-effectors that mediate the process including rent1/hUpf1, rent2/hUpf2, and hUpf3 (*3*). Assembly of these proteins, collectively referred to as the surveillance complex, on nonsense transcripts initiates NMD (*11*). Here, we sought to determine whether nonsense-mediated perturbations of pre-mRNA metabolism rely on the nonsense surveillance machinery. Additionally, we examined whether these effects are an indirect consequence of NMD or are the result of a distinct mechanism.

RNA interference (RNAi) using synthetic short-interfering RNA (siRNA) duplexes was used to inhibit expression of rent1/hUpf1 and rent2/hUpf2 in HeLa cells (*12*, *13*). Western blotting revealed siRNA sequence-specific and near complete (>10-fold) loss of expression of both targeted transcripts (Fig. 1). Transcripts derived from a previously described T cell receptor β (TCRβ) mini-gene (Fig. 2A) (*14*) were monitored under these conditions. In addition to being well-characterized substrates for the NMD pathway, TCRβ nonsense transcripts show translation-dependent alternative splicing that restores the open reading frame (*9*). In untreated cells or cells treated with siRNA directed against an irrelevant target, the PTC-containing transcript was reduced in abundance to less than 20% of wild-type levels, as assessed using a Northern blot probe specific for the VDJ exon (VDJ probe, Fig. 2, A and B). In contrast, RNAi directed against either rent1/hUpf1 or rent2/hUpf2 increased the level of the mutant transcript to greater than 50% of wild-type levels, extending existing evidence (*15–17*) that these factors are essential for NMD.

To assess the effect of diminished rent1/hUpf1 and rent2/hUpf2 expression on NAS, Northern blot analysis was performed with a probe (LV probe, Fig. 2A) that more efficiently detects the transcripts produced by alternative splicing (designated "TCR alt"). Northern blotting revealed that production of TCR alt was induced by the presence of a PTC, as described by Wang *et al.* (*18*) (Fig. 2C). Direct sequencing of reverse transcriptase–polymerase chain reaction (RT-PCR) products demonstrated that all TCR alt transcripts are generated by use of an alternative splice donor in the VDJ exon. In addition, approximately one-third of TCR alt transcripts use an alternative splice acceptor 22 nucleotides upstream of the bona fide splice acceptor for the VDJ exon (Fig. 2A). Both of these alternative transcripts, which cannot be

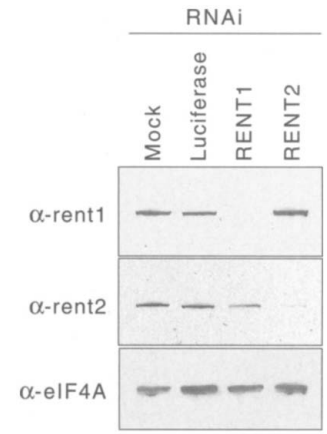

Fig. 1. Sequence-specific inhibition of gene expression with RNAi in mammalian cells. HeLa cells were mock transfected or transfected with siRNA duplexes directed against firefly luciferase (a negative control), rent1/hUpf1, or rent2/hUpf2. Seventy-two hours after transfection, cell lysates were analyzed by Western blotting with antisera specific for rent1/hUpf1 (*17*), rent2/hUpf2 (*11*), or eIF4A as a control for nonspecific effects of RNAi treatment.