Inhibition of c-erbA mRNA Splicing by a Naturally Occurring Antisense RNA*

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The rat erbAa locus encodes two overlapping mRNAs, α1 and α2, which are identical except for their most 3' exons. α1 mRNA encodes a thyroid hormone receptor, while α2 encodes an altered ligand binding domain of unknown function. Previous studies have shown that the ratio of α1 to α2 is highest in cells expressing a high level of a third RNA, Rev-ErbAa mRNA, which is transcribed in the opposite direction and is complementary to α2 but not α1 mRNA. It was hypothesized that base pairing with Rev-ErbAa blocks splicing of α2 mRNA, thereby favoring formation of the non-overlapping α1. To test this model, a system was developed in which α2 pre-mRNAs were accurately spliced in vitro. Splicing was inhibited by the addition of a 5-fold excess of antisense RNAs containing the 3' end of Rev-ErbAa mRNA. Both an antisense RNA extending across the 3' splice site and a shorter RNA complementary only to exon sequences efficiently blocked splicing. However, splicing was only inhibited by complementary RNAs. These observations are consistent with a mechanism in which base pairing with a complementary RNA regulates alternative processing of α1 and α2 mRNAs.

The diverse biological effects of thyroid hormone (T3)' are mediated by nuclear receptor proteins which regulate transcription of target genes (1, 2). The presence of these receptors and their binding to specific sequences within target genes determines the primary response of cells to thyroid hormone which is regulated in a tissue- and stage-specific manner. In many tissues multiple mRNAs specify different forms of the receptor, while not α1, mRNA (Fig. 1A). Like α1 and α2, Rev-ErbAa encodes a protein belonging to the steroid/thyroid hormone receptor superfamily (14, 23), but of unknown function. However, previous studies have shown that in tissues where Rev-ErbAa mRNA levels are high, the ratio of α2/α1 mRNA is relatively low (16, 23). Furthermore, conditions which increase transcription of Rev-ErbAa mRNA also decrease the ratio of α2/α1, both by increasing α1 and decreasing α2 mRNA levels (25). These changes are due to an alteration in post-transcriptional processing of erbAα pre-mRNA, since neither transcription nor stability of these two mRNAs is altered (25).

While alternative processing of some mRNAs is known to be mediated by RNA-binding proteins (26), the unusual organization of the c-erbAα locus and the reciprocal relationship between Rev-ErbAα levels and the α2/α1 ratio suggested the possibility that base pairing of Rev-ErbAα mRNA with the 3' end of α2 pre-mRNA may directly inhibit splicing of α2 mRNA, thereby favoring expression of the non-overlapping α1 mRNA (23, 25). In order to test this mechanism, a model system for studying α2-specific splicing in vitro has been developed. The present work shows that erbAα2 transcripts are accurately spliced in nuclear extracts. Furthermore, RNAs derived from the 3' portion of Rev-ErbAα efficiently and specifically block splicing of exons unique to α2 provided that complementarity is maintained, as predicted by the antisense mechanism outlined above.

MATERIALS AND METHODS

Plasmids.—The construction of plasmids pHBS500 and pHBS has been described previously (27). pHB-α2L was constructed by inserting the 775-nt HincII/EcoRI fragment (A in Fig. 1A) which includes the α2-specific 3' splice site from the rat erbAα locus (16, 23) into the same sites of pHBS, downstream of the β-globin insert. pHB-α2S was constructed by deleting the 473-nt HincIII/Sspl fragment from the α2 intron sequences of pHB-α2L. The resulting insert is labeled B in Fig. 1A. poL-2 is a minigene in which the 3' and 5' splice sites are identical to those in the rat erbAα gene. This plasmid was constructed by replacing the globin insert of pHB-α2L with the 510-nt PstI/XbaI fragment (C in Fig. 1A) from α1 cDNA (16). This fragment contains 150 nt of exon sequence common to α1 and α2 mRNAs, including the entire length of the last common exon, and 360 nt of contiguous sequence specific for α1 mRNA (but spliced out of α2). poL-2 includes a short region of polylinker from pBluescript (Stratagene) between the HindIII site of the vector (pGEM3, Promega) and a PstI site at the end of the α1 cDNA insert as well as a short region of pGEM4 between the XbaI site of the insert and the SplI site of the vector. poL-2 was constructed by replacing fragment...
The structure of these transcripts is shown in Fig. 1H. Rev-470 and A22084 polymerase from pErhAtrP/E cut with PoulI and pHH500 with pre-mRNAs required 1.6 mM clear extract and subsequent analysis of the resulting products were carried out. 1.6-SN was similarly transcribed from fragment from pHH-cr2L ligated to the same sites in pGEM4. The longest transcript tested, n2-SN, contains both 3' and 5' splice sites bounding the final exon of n2 mRNA and adjacent exon and intron sequences. The longest transcript tested, α2-L, retained 360 and 530 nt of intron sequence adjacent to the 3' and 5' splice sites, respectively, including the putative polyadenylation site of α1 mRNA and sequences complementary to the polyadenylation site of Rev-ErhAa mRNA. Two chimeric transcripts were also tested which include the first exon and 5′ splice site of β-globin pre-mRNA upstream of the α2-specific 3′ splice site (Fig. 1B). Each of these transcripts was efficiently spliced in the presence of HeLa cell nuclear extract. Following incubation of both α2-L and α2-S for 45–120 min, a prominent band of 430 nt appeared, corresponding to the size expected for splicing of the α2 exons (Fig. 2A, lanes 2–4 and 6–9). Additional bands corresponding to expected products and intermediates of splicing were observed. Lariat forms of the intron and intron/exon intermediate were identified on the basis of their size and characteristic shift in mobility on gels of varying polyacrylamide concentrations (results not shown). To confirm the identity of the putative spliced products, the 430-nt band was eluted and analyzed by RNase protection assays following hybridization to an unlabeled RNA probe complementary to the 3′ α2-specific exon and adjacent intron sequences. The 430-nt band from both α2-L and α2-S yielded a RNase-resistant fragment of 250 nt, corresponding to the length of the accurately spliced 3′ exon.

RESULTS

Splicing of erbAα2 Transcripts in Vitro—Fig. 1B shows a number of erbAα transcripts which contain variable lengths of exons and introns. One set of transcripts (α2-L, α2-S, and α2-SN) contain both 3′ and 5′ splice sites bounding the final exon of α2 mRNA and adjacent exon and intron sequences. The longest transcript tested, α2-L, retained 360 and 530 nt of intron sequence adjacent to the 3′ and 5′ splice sites, respectively, including the putative polyadenylation site of α1 mRNA and sequences complementary to the polyadenylation site of Rev-ErhAa mRNA. Two chimeric transcripts were also tested which include the first exon and 5′ splice site of β-globin pre-mRNA upstream of the α2-specific 3′ splice site (Fig. 1B). Each of these transcripts was efficiently spliced in the presence of HeLa cell nuclear extract. Following incubation of both α2-L and α2-S for 45–120 min, a prominent band of 430 nt appeared, corresponding to the size expected for splicing of the α2 exons (Fig. 2A, lanes 2–4 and 6–9). Additional bands corresponding to expected products and intermediates of splicing were observed. Lariat forms of the intron and intron/exon intermediate were identified on the basis of their size and characteristic shift in mobility on gels of varying polyacrylamide concentrations (results not shown). To confirm the identity of the putative spliced products, the 430-nt band was eluted and analyzed by RNase protection assays following hybridization to an unlabeled RNA probe complementary to the 3′ α2-specific exon and adjacent intron sequences. The 430-nt band from both α2-L and α2-S yielded a RNase-resistant fragment of 250 nt, corresponding to the length of the accurately spliced 3′ exon.

Under conditions optimized for splicing of α2 pre-mRNAs, splicing of α2-L was substantially slower than that of α2-S. Both RNAs were spliced more slowly than a well characterized 500-nt globin pre-mRNA, HB500 (28). However, the chimeric transcripts, which include the 5′ exon of HB500 and the 3′ exon of α2, were spliced more efficiently. Both HB-α2S (Fig. 2B) and HB-α2L (not shown) were spliced more rapidly than α2-S. Both yielded the expected products and intermediates, including a band which comigrated exactly with the 5′ β-globin exon (lanes 2, 3, 5, and 6, Fig. 2B). The relative rate of splicing of α2 transcripts increased with decreasing intron length. In fact, HB-α2S (total intron length 118 nt) was spliced more rapidly than globin pre-mRNA (intron length 130 nt) under conditions optimized for HB500 splicing (compare lanes 2 and 5 in Fig. 2B). This result demonstrates that

**Fig. 1. Structure of c-erbAα locus and minigene transcripts.** A, relative orientation and structure of three mRNAs from the c-erbAα locus. Large arrows indicate direction of transcription, filled boxes represent exons, and open boxes represent introns. The 5′ ends of these mRNAs have not been fully characterized with respect to intron/exon structure. Lines labeled A–E mark regions present in minigene transcripts. B, structure of minigene transcripts containing α2 or β-globin mRNA splice sites. Open boxes represent exons of α2 mRNA, hatched boxes represent the first exon of β-globin mRNA, and thin lines connecting exons represent introns. The stippled region of α2 exons represents vector sequence at the 5′ end of transcript. Numbers represent lengths (in nt) of exon and intron sequences, while breaks within introns indicate deletions where 3′ and 5′ segments are joined.

**Fig. 2. In vitro splicing of α2 transcripts.** Panel A shows the time course of splicing of α2-L and α2-S in the presence of 68 mM KCl, 1.5 mM MgCl2. Panel B shows time course of HB-α2S and HB500 splicing in 3.2 mM MgCl2 and 48 mM KCl. Hatched boxes represent globin exons and open boxes the erbAα2 exon. Pre-mRNA (P), intron lariat (I), intron/exon lariats (I/E), and 5′ exon (E1) are indicated schematically.
the α2-specific 3′ splice site is utilized very efficiently, even in combination with a heterologous 5′ site.

Antisense Inhibition of erbAα2 Splicing by Rev-ErbAα RNA—Having demonstrated accurate splicing of the α2 pre-mRNA in vitro, the proposed role of Rev-ErbAα mRNA in blocking this process was explored. Fig. 3 shows that splicing of the α2-L pre-mRNA was strongly inhibited in the presence of Rev-470 RNA, which consists of 470 nt from the 3′ end of the Rev-ErbAα mRNA; 246 nt at the 5′ end of Rev-470 are complementary to the 3′ α2 exon, and the 3′ end is 10 nt upstream of the Rev-ErbAα polyadenylation site. Of note, a 5- or 10-fold molar excess of Rev-470 blocked the α2-L splicing by more than 70% (compare lanes 5 and 6 with lane 2 in Fig. 3). In contrast, the same molar excess of an RNA complementary to the β-globin pre-mRNA (E2-130) had no effect on α2-L splicing (lanes 2 and 3) but did block splicing of HB500 to which it is complementary (compare lanes 8 and 9 with lane 7). Importantly, however, the inhibitory effect of Rev-470 was specific for the α2-containing pre-mRNA. It had no effect on splicing of β-globin pre-mRNA (lanes 11 and 12).

The region of complementarity between Rev-470 RNA and α2-L includes the 3′ splice site of the α2 pre-mRNA. In order to determine whether base pairing in this region is required for inhibition of splicing, a shorter RNA, Rev-165, was synthesized which is complementary to 165 nt at the 3′ end in the α2 pre-mRNA. This RNA does not include a sequence which hybridizes to the 3′ splice site. Fig. 4 shows that Rev-165 is approximately equal to Rev-470 in its ability to inhibit the splicing of α2-S (lanes 4 and 5), indicating that inhibition of α2 mRNA splicing does not require base pairing with the 3′ splice site. However, the complementary nature of the Rev-165 is necessary for its inhibitory properties in this system, because Rev-165 has no effect on the splicing of α2-SN, a truncated α2 pre-mRNA lacking sequences complementary to Rev-165 (lane 9) while Rev-470, which contains sequences complementary to this pre-mRNA, does efficiently inhibit its splicing (lane 10).

DISCUSSION

The present studies show that a naturally occurring RNA, Rev-ErbAα mRNA, can inhibit splicing of α2 pre-mRNA to which it is complementary. These results are formally similar to those of earlier experiments in which antisense RNAs were observed to block splicing of globin pre-mRNA (27), although in the latter case complementary mRNAs do not exist in vivo. Results demonstrate that inhibition of globin mRNA splicing is associated with base pairing between complementary regions of the pre-mRNA and antisense RNA molecules. Inhibition of globin mRNA was observed with RNAs which anneal.
either directly to splice sites or exclusively to exon sequences as far as 170 nt from the nearest splice site (27). Studies of globin mRNA splicing also demonstrated that a number of proteins facilitate or disrupt base pairing in HeLa cell nuclear extract (27). The potential involvement in antisense inhibition of factors which modulate RNA-RNA base pairing remains to be established.

Previously it was suggested that levels of the alternatively spliced α1 and α2 mRNAs may be regulated at the level of splicing by a novel antisense mechanism in which Rev-ErbAα transcript base pairs with the complementary pre-mRNA and blocks formation of α2 but not α1 mRNA (23, 25). The results presented here are consistent with this model. Transcripts containing sequences from the 3′ end of Rev-ErbAα mRNA efficiently inhibit splicing of α2 transcripts in HeLa cell nuclear extract (Figs. 3 and 4). This result was observed with Rev-470 mRNA, like endogenous Rev-ErbAα mRNA, extends across the 3′ α2-specific splice site, but also with a shorter antisense RNA complementary only to the 3′ exon of α2 mRNA, indicating that splicing is sensitive to relatively limited base-pairing interactions. The very efficient splicing of α2 mRNA in vitro raises the possibility that formation of α2 mRNA is favored over α1 in the absence of specific regulation. These results, taken together with those of other studies, suggest that inhibition of splicing by a naturally occurring antisense RNA may play a physiologically important role. Since the α1 and α2 proteins are functionally antagonistic (20, 21) relatively modest changes in splice site selection could cause major changes in cellular T3 responsiveness. However, it is possible that other mechanisms acting at the level of polyadenylation or transcriptional termination, as well as splicing, may regulate alternative processing of α1 and α2 mRNAs. Additional genetic and biochemical approaches will be required to ascertain that Rev-ErbAα functions as a physiologically significant antisense regulator in vivo.

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REFERENCES

1. Oppenheimer, J. H., Schwartz, H. L., Mariash, C. N., Kinlaw, W. B., Wong, N. C. & Freahe, H. C. (1987) Endoer. Rev. 8, 288–308

2. Lazar, M. A. & Chin, W. W. (1990) J. Clin. Invest. 86, 1777–1782

3. Hodin, R. A., Lazar, M. A. & Chin, W. W. (1990) J. Clin. Invest. 85, 101–105

4. Lazar, M. A. (1990) J. Biol. Chem. 265, 17474–17477

5. Murakami, T. & Nikodem, V. M. (1989) J. Biol. Chem. 264, 8900–8904

6. Strait, K. A., Schwartz, H. L., Perez-Castillo, A. & Oppenheimer, J. H. (1990) J. Biol. Chem. 265, 10514–10521

7. Glass, C. K. & Holloway, J. M. (1990) Biochim. Biophys. Acta 1032, 157–176

8. Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J. & Evans, R. M. (1986) Nature 324, 641–646

9. Nakai, A., Sakurai, A., Bell, G. I. & DeGroot, L. J. (1988) Mol. Endocrinol. 2, 1087–1092

10. Murray, M. B., Zilz, N. D., McCreary, N. L., MacDonald, M. J. & Towle, H. C. (1988) J. Biol. Chem. 263, 12770–12777

11. Hodin, R. A., Lazar, M. A., Wintman, B. I., Darling, D. S., Koenig, R. J., Larsen, P. R., Moore, D. D. & Chin, W. W. (1989) Science 244, 76–79

12. Thompson, C. C., Weinberger, C., Lebo, R. & Evans, R. M. (1987) Science 237, 1610–1614

13. Nakai, A., Seino, S., Sakurai, A., Sziak, I., Bell, G. I. & DeGroot, L. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2781–2785

14. Miyajima, N., Horiuchi, R., Shibuya, Y., Fukushima, S., Matusbara, K., Toyoshima, K. & Yamamoto, T. (1989) Cell 57, 31–39

15. Mitsuhashi, T., Tennyson, G. E. & Nikodem, V. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5804–5808

16. Lazar, M. A., Hodin, R. A., Darling, D. S. & Chin, W. W. (1988) Mol. Endocrinol. 2, 893–901

17. Koenig, R. J., Warne, R. L., Brent, G. A., Hanney, J. W., Larsen, P. R. & Moore, D. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5031–5035

18. Benbrook, D. & Pfaal, M. (1987) Science 238, 788–791

19. Izuom, S. & Mahdavi, V. (1988) Nature 334, 539–542

20. Lazar, M. A., Hodin, R. A. & Chin, W. W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7771–7774

21. Koenig, R. J., Lazar, M. A., Hodin, R. A., Brent, G. A., Larsen, P. R., Chin, W. W. & Moore, D. D. (1989) Nature 337, 659–661

22. Nakai, A., Sakurai, A., Macchia, E., Fang, V. & DeGroot, L. J. (1989) Mol. Cell. Endocrinol. 72, 145–148

23. Lazar, M. A., Hodin, R. A., Darling, D. S. & Chin, W. W. (1989) Mol. Cell. Biol. 9, 1125–1136

24. Evans, R. M. (1988) Science 240, 889–895

25. Lazar, M. A., Dodin, R. A., Cardona, G. & Chin, W. W. (1990) J. Biol. Chem. 265, 12869–12863

26. Maniatis, T. (1991) Science 251, 33–34

27. Munroe, S. H. (1988) EMBO J. 7, 2523–2532

28. Krainer, A. R., Maniatis, T., Ruskin, B. & Green, M. R. (1984) Cell 36, 993–1005