11-1-1997

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Published version. *Nucleic Acids Research*, Vol. 25, No. 21 (November 1997): 4296-4300. DOI.

4296–4300 *Nucleic Acids Research, 1997, Vol. 25, No. 21*

Expression of the thyroid hormone receptor gene, erbAα, in B lymphocytes: alternative mRNA processing is independent of differentiation but correlates with antisense RNA levels

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Received July 8, 1997; Revised and Accepted September 10, 1997

ABSTRACT

The erbAα gene encodes two α-thyroid hormone receptor isoforms, TRα1 and TRα2, which arise from alternatively processed mRNAs, erbAα1 (α1) and erb α2 (α2). The splicing and alternative polyadenylation patterns of these mRNAs resemble that of mRNAs encoding different forms of immunoglobulin heavy chains, which are regulated at the level of alternative processing during B cell differentiation. This study examines the levels of erbAα mRNA in eight B cell lines representing four stages of differentiation in order to determine whether regulation of the alternatively processed α1 and α2 mRNAs parallels the processing of immunoglobulin heavy chain mRNAs. Results show that the pattern of α1 and α2 mRNA expression is clearly different from that observed for immunoglobulin heavy chain mRNAs. B cell lines display characteristic ratios of α1/α2 mRNA at distinct stages of differentiation. Furthermore, expression of an overlapping gene, Rev-ErbAα (RevErb), was found to correlate strongly with an increase in the ratio of α1/α2 mRNA. These results suggest that alternative processing of erbAα mRNAs is regulated by a mechanism which is distinct from that regulating immunoglobulin mRNA. The correlation between RevErb and erbAα mRNA is consistent with negative regulation of α2 via antisense interactions with the complementary RevErb mRNA.

INTRODUCTION

Thyroid hormone receptors (TRs) mediate the cellular response to thyroid hormone (T3) by regulating target gene transcription (1–3). In all vertebrates TRs are the products of two different genes, erbAα and erbAβ (4). The mammalian erbAα gene produces two mRNAs, erbAα1 (α1) and erbAα2 (α2), through alternative processing of the 3′-end of its pre-mRNA transcript (5–7). These mRNAs give rise to receptor isoforms with antagonistic functions. α1 codes for the α-thyroid hormone receptor (TRα1), whereas α2 codes for an orphan nuclear receptor (TRα2) which does not bind T3 (6,7). TRα2 competes with TRα1 and TRβ for specific DNA binding sites, thereby antagonizing T3 action (8). Because the erbAα gene produces both a transcriptional activator (TRα1) and its specific inhibitor (TRα2), regulation of the alternative processing of α1 and α2 mRNA may provide an important mechanism for determining the cellular response to thyroid hormone.

Alternative processing of the 3′-end of erbAα RNA transcripts involves competition between splicing and polyadenylation. Polyadenylation at an upstream site yields α1 mRNA, whereas processing from a 5′ splice site (ss) within the final exon of α1 to a downstream 3′ ss produces α2 mRNA. The levels of α1 and α2 vary in different tissues and at different developmental stages (9,10). However, the mechanisms which regulate expression of the alternatively processed mRNAs are not well understood.

Two general models have been described for the regulation of alternative splicing and polyadenylation (11). In some cases alternative processing is regulated by transcript-specific factors which alter the efficiency of either splicing or polyadenylation. In other instances, the activity of one or more constitutive components of the mRNA processing apparatus is altered. In the latter case, the processing of many unrelated transcripts may be affected.

The organization of the erbAα gene and the alternative mRNA processing of its transcripts appear similar to that of the immunoglobulin (Ig) heavy chain genes. The Ig heavy chain gene produces two functionally distinct mRNAs which encode heavy chains for the membrane-bound (mb) and secreted (sec) forms of Ig. Like α1, Ig sec mRNA processing utilizes an upstream polyadenylation site. Similar to α2, Ig mb mRNA processing utilizes a 5′ ss in the last common exon to splice to a downstream exon. Regulation of Ig heavy chain mRNA processing in B lymphocytes has been well characterized. In early stages of B cell

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development downstream splicing of Ig mb mRNA is equal to or greater than that of Ig sec mRNA processing. At later stages upstream polyadenylation of Ig sec mRNA predominates (12). Several studies suggest that the balance between splicing and polyadenylation required for processing of Ig mb and sec mRNAs is regulated by a change in the level of a general polyadenylation factor (13–16). In view of these findings, it is possible that erbAα mRNA processing may parallel that of Ig heavy chain mRNA during B cell development.

A distinguishing feature of the mammalian erbAα gene locus is the presence of a third gene, Rev-ErbAα (RevErb), encoded on the DNA strand opposite erbAα (17,18). The 3′ exon of RevErb overlaps with the α2-specific 3′ exon but not with α1 sequence. The unusual organization of these genes results in α2 and RevErb mRNAs which are complementary at their 3′-ends allowing the possible formation of antisense/sense RNA duplexes. Such basepairing interactions between the RevErb and α2 mRNAs could negatively regulate α2 mRNA levels, and therefore offer a transcript-specific mechanism by which α1 and α2 mRNA levels are regulated.

In this study, we examine the expression of α1 and α2 mRNA from B cells representing different stages of differentiation. We find that the thyroid hormone receptor, TRα1, and the orphan receptors TRα2 and RevErb are expressed at all stages of differentiation but at varying levels in the different cell lines. Our results indicate that the regulation of alternative RNA processing of α1 and α2 is distinct from that of Ig sec and mb RNA processing regulation. However, changes in the relative levels of α1 and α2 correlate strongly with variations in levels of RevErb mRNA.

MATERIALS AND METHODS

Cell lines

All cell lines are from the mouse B cell lineage and were grown as previously described (13,14,19,20). 70Z/3.12 represents a pre-B cell stage line with equal amounts of sec and mb IgM mRNA (20). WEHI-231 is an early B cell with equal amounts of sec and mb IgM (20). The M12 cell line represents an early B cell which has lost its endogenous heavy chain but expresses approximately equal amounts of sec and mb IgM mRNA when transfected with an IgM gene (13). The A20 and 2PK3 cell lines are memory B cells and produce about equal quantities of sec and mb IgG heavy chain mRNA (14). 4T001 is a plasmacytoma cell line which secretes large amounts of γ2b, κ molecules of IgG (14). S194 is a plasmacytoma cell line which has lost its endogenous heavy chain but produces a large excess of sec over mb mRNA when transfected with an IgM gene (13). J558L is a plasmacytoma cell line which has lost its endogenous α heavy chain but when transfected with an IgG gene sec mRNA is expressed in excess over mb mRNA (19).

Recombinant plasmids and RNA probes

The erbAα probe used for northern analysis was prepared from a 600 nt *Xba*I–*Sac*I DNA fragment excised from plasmid pα2HN. pα2HN contains an *Eco*RI–*Hin*cII fragment which is common to both α1 and α2. This fragment was isolated from plasmid pα2ΔC-Sac/stop (21) and subcloned in pBluescript KS$^+$ (Stratagene). The probe was uniformly labeled with [α-^{32}P]dCTP by random oligonucleotide-primed synthesis (22). For RNase protection assays, a single-stranded antisense riboprobe of α1 and α2 common sequence was prepared from pB3EOP which contains the 162 nt *Pst*I–*Eco*0109I erbAα DNA fragment cloned between the *Apa*I–*Pst*I sites of pBluescript KS$^+$. This fragment of the erbAα gene spans the α2-specific 5′ ss within the 3′-most exon of α1. pB3EOP was linearized with *Xba*I and transcribed with T3 RNA polymerase to produce a 209 nt probe. Of this probe, 162 nt are complementary to α1 mRNA and 135 nt are complementary to α2 mRNA. The RevErb riboprobe was made from pB4E6 which contains 303 nt of a *Bgl*II–*Bsu*36I fragment from pB4-1 (17) cloned into pBluescript KS$^+$. pB4E6 was linearized with *Xba*I and transcribed with T3 RNA polymerase to produce a 360 nt probe, 130 nt of which are complementary to RevErb exon 6. RNA probes were uniformly labeled with [α-^{32}P]UTP and purified by electrophoresis (22). Plasmids pα2ΔC-Sac/stop and pB4-1 were generously provided by M.A. Lazar, University of Pennsylvania.

RNA isolation and northern analysis

Cytoplasmic RNAs were isolated by detergent lysis and phenol/chloroform extraction as previously described (19). The RNA was passed over oligo(dT) columns to isolate poly(A)$^+$ RNA. Electphoresis of 1–3 μg of poly(A)$^+$ RNA on a 1.0% agarose–0.22 M formaldehyde gel was run in buffer containing 0.22 M formaldehyde. The RNA was transferred by capillary action to Nytran (Schleicher and Schuell, Inc.) after which the RNA was UV-irradiated and hybridized to labeled probes (23). RNA size markers (GIBCO-BRL) were included on the gel and stained with methylene blue after transfer to Nytran (22).

RNase protection assays

Labeled probes were hybridized to 10–30 μg target RNAs as previously described (22). The resulting hybrids were digested with 1.5 μl RNase (1 mg/ml RNase A, 20 000 U/ml RNase T1, Ambion, Inc.) at 30°C for 1 hr. Protected RNAs were denatured in formamide and resolved on 5.5% polyacrylamide–urea gels. Band intensities were quantified by radioanalytic scanning with an AMBIS 100 Image Analyzer. Background was subtracted by using regions of identical size located immediately above each experimental band. RevErb/α2 ratios were computed from experiments in which probes were prepared in parallel using identical mixes of labeled and unlabeled nucleotides to ensure identical specific activities. The specific activity of the probes ranged from 9×10^8 to 2×10^9 c.p.m./nmol between experiments. Molar ratios were calculated after correcting for length and composition of the protected RNA fragments.

RESULTS

Structural organization and expression of erbAα mRNAs in B cell lines

In mammals, a single locus codes for the two alternatively processed erbAα mRNAs and the overlapping RevErb mRNA (Fig. 1A). To determine if the erbAα mRNAs are expressed in B lymphocytes, we assayed α1 and α2 mRNA levels in two mouse B cell lines representing different stages of differentiation. The cell line 70Z/3 is a tumor line arrested in the pre-B cell stage (20) and J558L is a myeloma cell line representing a late-stage B cell or plasma cell line (19). The C6 rat astrocytoma cell line, which is well-characterized for erbAα mRNA levels (9), was also

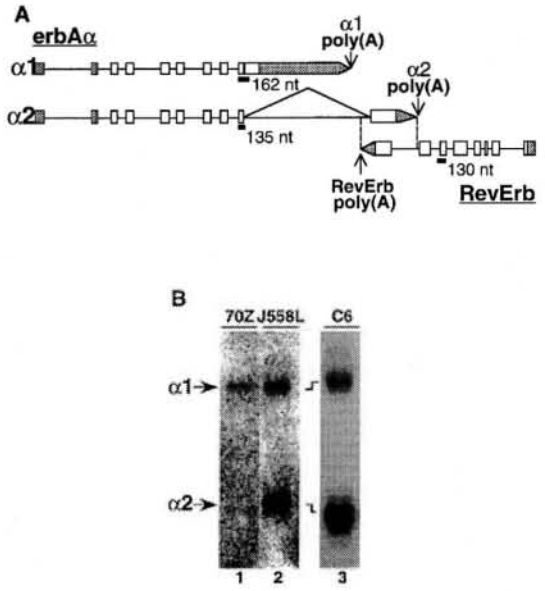

Figure 1. Expression of erbAα and RevErb in B cell lines. (**A**) Structural organization of the overlapping c-erbAα locus. Arrows indicate the direction of transcription, boxes represent exons, lines represent introns. Shaded boxes represent 5′ and 3′ untranslated regions. Vertical, dotted lines demarcate RevErb and α2 complementary sequence. The α1, α2 and RevErb poly(A) sites are noted. The alternative splicing of the α2-specific exon is represented by the angled line above the map. Probes used in RNase protection assays are indicated as bars beneath the sequence with length of the protected fragments indicated in nt. (**B**) Northern blot analysis of 1 μg 70Z/3 (lane 1), 2 μg J558L (lane 2) and 3 μg C6 (lane 3) polyA$^+$ cytoplasmic RNA. Blots were hybridized with a probe from the region common to α1 and α2.

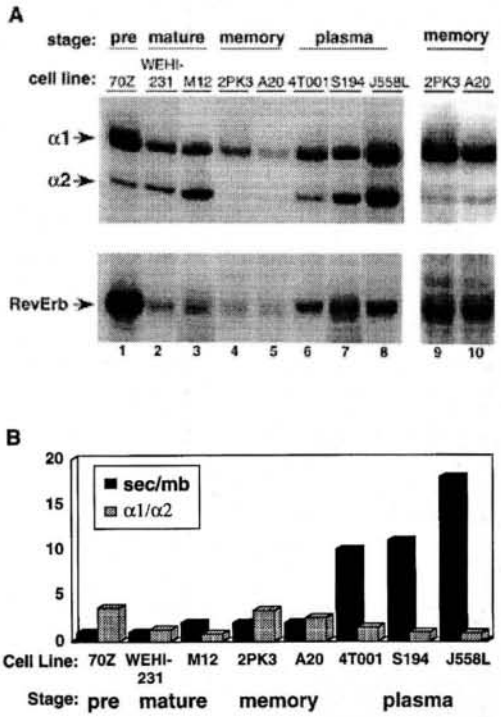

Figure 2. Differential erbAα alternative RNA processing in B cell lines representing various stages of differentiation. (**A**) RNase protection assays of B cell cytoplasmic mRNA using the erbAα and RevErb-specific probes shown in Figure 1A. Assays were carried out with 10 μg J558L RNA, 20 μg 70Z/3, M12, 4T001, S194 RNA and 30 μg WEHI-231, 2PK3, A20 RNA. Assays were carried out in parallel except for those shown in lanes 3 and 7 which were analyzed in a separate experiment. Lanes 9 and 10 show results of the same experiment in lanes 4 and 5 except for a 2-fold increase in exposure time. The specific B cell lines and their representative stage in differentiation is indicated above each lane. The protected fragments corresponding to α1, α2 and RevErb mRNAs are indicated at left. (**B**) Comparison of the ratio of α1/α2 mRNA and Ig sec/mb mRNAs (13,14,19,20) is shown schematically.

analyzed. Northern blot experiments using an erbAα specific probe show the expected 5.5 kb α1 mRNA and 2.6 kb α2 mRNA which correspond to the documented sizes of C6 cell α1 and α2 mRNA (Fig. 1B) (17). These results demonstrate that both erbAα mRNAs are expressed at early and late stages of B cell differentiation.

Alternative mRNA processing of erbAα mRNA

We next asked whether the balance between alternative processing of α1 and α2 mRNA parallels that of Ig sec and mb mRNA at different stages of B cell differentiation. Cytoplasmic mRNA was analyzed from eight mouse cell lines representing four different stages of B cell development. In addition to the pre-B and plasmacytoma cell lines described above, mRNA from two mature B cell lines [WEHI-231 (20) and M12 (13)], two cell lines representing memory B cells [2PK3 and A20 (14)], and two additional plasma cell lines [4T001 (14) and S194 (13)] were examined to determine the levels of expression of α1 and α2 mRNA. RNase protection assays of mRNA from these eight cell lines show that the pre-B cell line, 70Z/3, has the highest ratio of α1/α2 mRNA, 3.6, indicating a predominance of upstream polyadenylation over downstream splicing (Fig. 2A and B). The lowest α1/α2 ratios are seen in the plasma cell lines, S194 and J558L and the mature B cell line, M12, which demonstrates an excess of α2 splicing over polyadenylation. The ratios of α1/α2 mRNA in the different B cell lines are clearly distinct from those seen for sec and mb mRNA from the Ig heavy chain gene (Fig. 2B). For example, the levels of Ig sec (polyadenylated) mRNA levels are nearly equal to those of Ig mb (spliced) mRNA at early stages of B cell differentiation but increase sharply as cells switch almost exclusively to production of Ig sec mRNA in plasma cells. Although changes in the levels of erbAα mRNAs during B cell differentiation are opposite from the Ig mRNA alternative processing pattern, B cell lines representing distinct developmental stages display characteristic ratios of α1/α2 mRNA (Fig. 2B, Table 1).

Expression of RevErbAα mRNA in B cells

The complementary, overlapping organization of the erbAα and RevErb genes may have important implications for the coordinate regulation of these genes. To determine whether the differential expression of erbAα RNA processing during B cell differentiation correlates with changes in expression of RevErb, we assessed the levels of RevErb mRNA in parallel with α1 and α2 mRNA in the same eight B cell lines. RevErb mRNA is expressed

in all of the cell lines but at variable levels (Fig. 2A). The highest expression of RevErb is seen in the 70Z/3 mRNA, which shows an 8-fold excess of RevErb over α2. Conversely, S194, J558L and M12 mRNA, the cell lines with the lowest α1/α2 (1.0–0.8), also have the lowest RevErb/α2 ratio (0.5–0.9) though their absolute levels of RevErb extend over a broad range. In general, increases in the ratio of RevErb/α2 correlate strongly with increases in the ratio of α1/α2 (Fig. 3, Table 1).

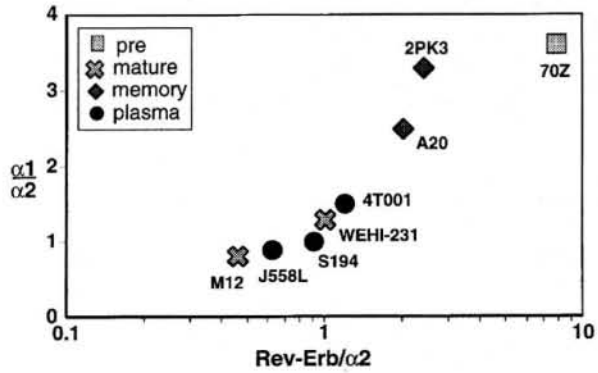

Figure 3. RevErb mRNA expression in B cell lines related to changes in α1/α2 mRNA. The ratio of RevErb/α2 mRNA was quantified and compared to α1/α2 mRNA. Data is presented in a scatter plot with each point representing a specific cell line. The representative developmental stage for each cell line is indicated by specific symbols.

DISCUSSION

B lymphocyte cell lines provide a unique system for investigating the molecular basis of developmentally regulated alternative processing. Such processing has been intensively studied for Ig heavy chain genes during B cell differentiation. In this study we show that erbAα mRNAs are expressed at different levels in B cell lines representing specific stages of development. The ratio of α1/α2 mRNA varies in a manner which suggests stage-specific regulation of mRNA levels, but the pattern of expression is clearly different from the variations in sec/mb mRNA levels. Characterization of mRNA levels from the overlapping RevErb gene shows that expression of RevErb mRNA correlates with changes in the α1/α2 ratio. These observations have important implications both for regulation of alternative processing and for the role of thyroid hormone during B cell development.

Molecular analysis of Ig heavy chain mRNA processing has shown that regulation of polyadenylation and splicing depends on changes in the levels of essential processing factors (14,16). Of particular interest to this study is the observation that unrelated pre-mRNAs which undergo similar 3′-end processing are differentially processed throughout B cell differentiation in the same manner as Ig sec and mb heavy chain mRNAs (13,15). On the basis of these results, erbAα mRNA processing would also be predicted to parallel that of Ig heavy chain mRNAs, with the α1/α2 ratio lowest in pre-B cells, highest in plasmacytoma cells and at intermediate levels in mature and memory B cells. Our results show a different and more complex pattern of variation in the α1/α2 expression than predicted. Mature B cells and plasma cells both have relatively low α1/α2 ratios while higher ratios are observed for memory cells and the pre-B cell line. These results suggest that the balance between alternative splicing and polyadenylation of erbAα mRNAs is regulated in a manner independent of Ig heavy chain alternative mRNA processing.

One possible mechanism for regulating expression of erbAα mRNAs involves interactions with the RevErb mRNA. Since this overlapping mRNA is complementary to α2, but not α1, variations in RevErb expression may differentially affect α1 and α2 expression. The results presented here are consistent with such a relationship. Despite wide variations in the levels at which these mRNAs are expressed in the eight lymphocyte cell lines analyzed (Table 1), a strong correlation is evident between the ratio of RevErb/α2 and the ratio of α1/α2 (Fig. 3). Specifically, when the ratio of RevErb/α2 is between 0.4 and 1.2, the α1/α2 ratio is <1.5, and when the RevErb/α2 is >2.0 the α1/α2 ratio is ≥2.5. Thus, an increase from 1.5 to 2.0 in the RevErb/α2 ratio may represent a threshold associated with a 2- to 3-fold increase in α1/α2. Since the ratio of RevErb/α2 varies over a wider range than the ratio of α1/α2 and there is no evident correlation in the variation of RevErb and α2 levels, interactions between these genes or their mRNA products may be modulated by additional factors.

Table 1. Quantitation of α1, α2 and RevErb mRNA in B cell lines

Cell line	α1 amol/μg	α2 amol/μg	RevErb amol/μg	α1/α2 (S.D.)	RevErb/α2 (S.D.)
70Z/3	5.5	1.7	12.9	3.6 (0.3)	7.9 (0.4)
WEHI-231	0.9	0.75	0.65	1.3 (0.2)	1.0 (0.3)
M12	2.3	3.2	1.4	0.82 (0.16)	0.46 (0.04)
2PK3	0.85	0.22	0.5	3.3 (1.0)	2.4 (0.4)
A20	0.6	0.3	0.5	2.5	2.0
4T001	2.4	1.6	1.8	1.5 (0.0)	1.2 (0.5)
S194	2.9	3.1	2.8	1.0 (0.2)	0.91 (0.00)
J558L	8.1	9.6	5.3	0.94 (0.12)	0.63 (0.18)

Levels of erbAα and RevErb mRNAs determined by RNase protection assays as shown in Figure 2. A quantitative determination of the α1 and α2 mRNA levels in each of the cell lines was obtained after correction for mRNA loading and specific activity of the protected fragments. The abundance of both erbAα mRNAs is ~0.1% that of mRNA from the housekeeping gene GAPDH (data not shown). Ratios of α1/α2 mRNA represent the average of three or four experiments. Data from the A20 cell line represents a single experiment. Absolute values and RevErb/α2 ratios are the average of two experiments carried out in parallel with probes of identical specific activity.

The relationship between RevErb expression and erbAα mRNA processing, described above, may reflect either direct or indirect interactions between these genes or their products. Several other studies have suggested that increased expression of RevErb mRNA may result in a decrease in α2 mRNA relative to α1 (24–26). Cycloheximide treatment of a particular pituitary tumor cell line results in a coordinate increase in both RevErb and the α1/α2 ratio (24). Similarly, when adipocyte cells are induced to differentiate *in vitro*, an increase in RevErb mRNA and the α1/α2 ratio is observed (25). The increase in α1/α2 observed in both these studies clearly reflects alterations in the processing of erbAα pre-mRNA, since neither the rate of transcription nor the relative stability of α1 and α2 are altered (24,25). However, it should be noted that in both cases the treatments used cause multiple, widespread alterations in cellular metabolism, in addition to altering levels of RevErb and erbAα mRNA.

In a third study antisense RNA corresponding to the overlapping portion of RevErb mRNA was shown to efficiently block splicing of erbAα pre-mRNA *in vitro* (26). In this case the increasing levels of the antisense RNA clearly block mRNA splicing. However, the relevance of this *in vitro* system as a model for physiological regulation is not clear. Antisense regulation at the level of alternative mRNA processing may provide a novel mechanism for the regulation of functionally antagonistic nuclear receptors. Although artificial antisense transcripts and oligonucleotides have been employed to repress expression of many genes, naturally occurring antisense regulation has not been well characterized in higher eukaryotes (27–29). The clear correlation between RevErb expression and α1/α2 mRNA levels described here for eight lymphocyte cell lines, and the results of previous studies (24–26), support a direct role for RevErb in the regulation of relative levels of erbAα mRNAs. Rigorous demonstration of a physiologically relevant antisense mechanism will require evidence that base-pairing interactions between α2 and RevErb transcripts are essential for the negative regulation of α2-specific splicing *in vivo*.

The differential expression of α1 and α2 mRNAs may have important physiological consequences for B cell differentiation. However, little is known regarding the role of the receptor protein during lymphocyte regulation. This study is the first to report the expression of mRNAs for specific TRs in B cells. Earlier studies have shown that T3 stimulates B cell proliferation and differentiation (30,31), which appears to be a result of changes in T3 reception during differentiation (32). One specific role for TRs in B cell differentiation may involve direct interactions of TRs with the transcription factor AP-1, which regulates stage-specific functions required for B cell proliferation and differentiation (33). The activity of AP-1 is modulated by a number of agents including ligand-activated transcription factors which belong to the TR-related subfamily (34). Both TRα and TRβ have been shown to interfere with AP-1 activated transcription in a T3-dependent manner via direct protein–protein interactions (35,36). Thus, the activity of AP-1 during B cell differentiation may be modulated by alterations in the levels of TRα1 and its antagonist, TRα2.

ACKNOWLEDGEMENTS

We thank Mitch A. Lazar for providing recombinant materials, for helpful discussions and for a critical reading of the manuscript. This research was supported by National Institutes of Health grants #DK48034 to S.H.M. and #GM50145 to C.M. and a National Science Foundation grant #MCB-9507513 to M.L.P. M.L.H. was supported by a pre-doctoral fellowship from the Arthur J. Schmitt Foundation.

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