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Tcra Enhancer Activation by Inducible Transcription Factors Downstream of Pre-TCR Signaling

Beatriz del Blanco,* Alberto García-Mariscal,* David L. Wiest,† and Cristina Hernández-Munain*

The Tcra enhancer (Eα) is essential for pre-TCR–mediated activation of germline transcription and V(DJ) recombination. Eα is considered an archetypical enhansome that acts through the functional synergy and cooperative binding of multiple transcription factors. Based on dimethylsulfate genomic footprinting experiments, there has been a long-standing paradox regarding Eα activation in the absence of differences in enhancer occupancy. Our data provide the molecular mechanism of Eα activation and an explanation of this paradox. We found that germline transcriptional activation of Tcra is dependent on constant phospholipase Cγ2, as well as calcineurin- and MAPK/ERK-mediated signaling, indicating that inducible transcription factors are crucially involved. NFAT, AP-1, and early growth response factor 1, together with CREB-binding protein/p300 coactivators, bind to Eα as part of an active enhansome assembled during pre-TCR signaling. We favor a scenario in which the binding of lymphoid-restricted and constitutive transcription factors to Eα prior to its activation forms a regulatory scaffold to recruit factors induced by pre-TCR signaling. Thus, the combinatorial assembly of tissue- and signal-specific transcription factors dictates the Eα function. This mechanism for enhancer activation may represent a general paradigm in tissue-restricted and stimulus-responsive gene regulation. *The Journal of Immunology, 2012, 188: 000–000.

The generation of αβ T-lymphocytes requires the construction of a TCR complex through a highly ordered series of somatic rearrangement events at the TCRα and TCRβ loci (Tcra and Tcrb) during T cell development. Thymocytes mature through a series of stages that are identified by the expression of surface receptors. Most immature thymocytes, known as double-negative (DN) thymocytes, are CD4−CD8−. DN thymocytes can be classified into four populations (DN1–4) based on the expression of CD25 and CD44. Tcrb rearrangements are completed at the DN3 stage. Based on the expression of CD27, DN3 thymocytes can be subdivided into two populations; those that have not yet undergone TCRβ-selection (DN3a) and those that have (post-TCRβ–selected DN3b) (1). DN3a thymocytes that have successfully rearranged a Tcrb allele differentiate into DN3b, DN4, and CD4+CD8+ double-positive (DP) thymocytes in a process known as β-selection. This process is driven by signaling through the pre-TCR, which is composed of TCRβ and the invariant pre-Toα, and through cooperating NotchRs. Pre-TCR signaling is sufficient for Tcrb allelic exclusion and the activation of Tcra transcription and rearrangement but not for DN to DP differentiation (2). Among the transcription factors induced by pre-TCR signaling, it has been well established that NFAT, AP-1, and early growth response factors (Egr) are essential for β-selection (3–7). NFAT2 and Egr-1/3 factors are especially interesting because they are sufficient for traversal of the β-selection checkpoint (5, 8–10) and functionally collaborate to perform this function (11). At present, little is known about the molecular targets of these inducible transcription factors during thymocyte differentiation.

Tcra germline transcription is required for Vα-to-Jα recombination (12–15). All VαJα rearrangements, with the exception of a few Vα2 rearrangements that occur in DN cells by the action of the Tcra enhancer (Eβ) (16), depend on the Tcra enhancer (Eα) (17). Eα influences chromatin structure across a 500-kb region that includes the 65-kb Jα array and the proximal third part of the 1.5-megabase Vα array (18). Initial Tcra germline transcription depends on the T early-α promoter (TEA) and the Jα49 promoter (Jα-49p), which are activated by the action of Eα and required for the activation of primary Vα-to-Jα recombination (19). A notable aspect of Eα as a critical regulator of Tcra locus rearrangements is its strict regulation during thymocyte development. Eα is inactive in DN1 to DN3a thymocytes when they attempt to successfully rearrange their Tcra locus; its activity is first detected after pre-TCR signaling in DN4 thymocytes, coinciding with the detection of Tcra germlinal transcripts (1, 20, 21). Because the Tcra locus is...
positioned between the Vα and Jα gene segments at the combined Tcra locus. Vα-to-Jα gene rearrangements cause the Tcra locus to be deleted from the chromosome. Hence, Eα-dependent developmental control of Tcra rearrangement is a critical component of αβ and γδ T cell lineage commitment.

Eα, defined as a 275-bp fragment containing four protein-binding elements (Tcrl–Tc4), is the minimal fragment required for proper developmental regulation (22). Tcrl–Tcro2 is considered the core enhancer because it is the smallest fragment with transcriptional activity in vitro (23), and it is thought to be controlled by a compact nucleoprotein structure, the enhancosome, formed by functional synergy and cooperative binding to enhancer DNA among specific transcription factors (24, 25). Known factors bound to Tcrl–Tc4 comprise lymphoid-specific factors that are constitutively present during thymocyte development (Fig. 1). Previous genomic footprinting experiments using dimethylsulfate (DMS) have shown that the occupancy of Eα is indistinguishable between DN3a and DP thymocytes, indicating that proteins bound in both cell stages must be identical or very closely related (21, 26, 27). However, an

In vitro and in vivo cell stimulation
Sci.adh cells (1 × 10^6 cells/ml) were stimulated in culture with human CD25 (TAC) mAb from the hybridoma for 24 h at 37°C with 5% CO₂, (9, 30) or stimuli such as PMA (50 ng/ml), ionomycin (1 μg/ml), PMA (20 ng/ml) and ionomycin (0.5 μg/ml), or thapsigargin (50 nM) for 3.5 or 6 h at 37°C with 5% CO₂. For inhibition assays with cyclosporine A (CsA; 0.5–1 μg/ml), ETA (10 mM), U0124 (10 μM), U0126 (10 μM), or PD98059 (30 μM) (Calbiochem), the inhibitors were added 1 h before stimulation. For the inhibition assays using U73122, 10 μM inhibitor was added in cultures at 0.2–0.5 × 10^6 Rbx thymocytes/ml at 37°C with 5% CO₂. For in vivo thymocyte stimulation, Rag2^−/− mice were injected i.v. with 50 μg purified CD3ε (2C11) mAb as described (31) and sacrificed after 16 h.

RT-PCR
RNA was isolated using TRIzol (Invitrogen) from unstimulated or 6-h stimulated cells. A total of 200 U M-MLV RT (Invitrogen) and 300 ng hexa primers was used to synthesize cDNA from 100–400 ng total RNA. The PCR conditions were performed as described (9, 12). The amplified fragments were detected using radiolabeled probes. For quantitative RT-PCR, templates (equivalent to 20 ng RNA) were assessed with reported primers (1) using IQ SYBR Green Supermix (Bio-Rad) in a Bio-Rad iCycler thermocycler (Bio-Rad). PCR conditions were: 3.5 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 20 s at 72°C, followed by a final extension step of 5 min at 72°C. Expression levels of the transcripts were normalized to levels of Actb in each sample. Paired sample t tests were used to determine statistical significance between values. The p values are represented as follows: *p < 0.05, **p < 0.005–0.0005, and ***p < 0.0005.

EMSA
rDNA-binding domains of NFAT1 (rNFAT-DBD) was purified as previously described (32), and recombinant Egr-1 (rEgr-1) was obtained from Enzo Life Sciences. For mapping the Egr binding sites in Eα, extracts from HEK-293T cells transfected with a human Egr-1 expression plasmid (pEFPBOST7/Egr-1) were used. To generate this plasmid, human Egr-1 cDNA was obtained by PCR with the oligonucleotides 5′-CTCATGATC-CTCAAGTATGCAGCTCGCTGCTGGAGAG-3′ (containing an XbaI site) and 5′-GCT- GAGTTTCAATAGCAAATTTACAATGCTCCGGAGG-3′ (containing a BstBI site), using the pCMVXLS–Egr-1 plasmid obtained from Enzo Life Sciences as a template. Egr-1 cDNA was then subcloned into the XbaI- and BstBI-digested expression vector pEFPBOST7 (33). The pEFPBOST7/Egr-1 was transfected into ~6 × 10^5 cells seeded 20 h before at 60–70% confluence using calcium phosphate in 100-mm diameter plates. Approximately 48 h after transfection, the cells were harvested, washed in PBS, and lysed in 1 ml 20 mM HEPES (pH 7.9), 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, and protease inhibitors (Complete; Roche), and Egr-1 expression was analyzed by Western blotting with Egr-1 Ab (Santa Cruz Biotechnology) and T7-epitope Ab (Bethyl Laboratories).

Materials and Methods

Cell lines and mice
The Sci.adh cells have been described previously (20) and were maintained in RPMI 1640 supplemented with 10% FBS and standard amounts of glutamine, penicillin, streptomycin, nonessential amino acids, sodium pyruvate, and 50 μM 2-ME. Jurkat cells were maintained in RPMI 1640 supplemented with 10% FBS and standard amounts of glutamine, penicillin, and streptomycin. HEK-293T cells were maintained in DMEM supplemented with 10% FBS and standard amounts of glutamine, nonessential amino acids, penicillin, and streptomycin.

Rag2^−/− and Rbx mice (28, 29) were purchased from Taconic Farms and maintained in pathogen-free conditions within the Animal House at the Instituto de Parasitología y Biomedicina “López-Neyra.” Five- to 8-wk-old mice were used in all experiments. Animal use adhered to Instituto de Parasitología y Biomedicina “López-Neyra” and Consejo Superior de Investigaciones Científicas Bioethical Guidelines.
Human Tα1-Tα2 and Tα3-Tα4 fragments were obtained as reported (22). The consensus binding site (CS) and mutated CS oligonucleotide sequences were obtained from Santa Cruz Biotechnology. The Eco-derived oligonucleotides used in the EMSAs are listed in Supplemental Table I.

For EMSAs, Scid.adh nuclear extracts (2 μg), HEK-293T cell extracts (3 μg), NFAT-DDB, or rEgr-1 proteins were incubated with 1 μg poly (deoxyinosinom-deoxyctydilic) acid sodium salt carrier and 1 μg BSA in a 30 μl reaction containing 50 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM EDTA, and 5% glycerol for 30 min at 4°C in presence or absence of 12.5-fold excess of unlabeled CS or mutated CS competitors. Some of the reactions also included 1 μg indicated Ab and were incubated for 30 min at room temperature. All Abs were purchased from Santa Cruz Biotechnology except for the c-Jun Ab (BD Biosciences). The 3P-radio labeled probes (80 fmol, 5 × 10^10 cpm) were added for an additional 20 min of incubation at 4°C. The samples were electrophoresed through a 4% PAGE gel containing 22.5 mM Tris-borate and 0.5 mM EDTA at 4°C. The abundance of DNA/protein complexes was quantified using a phosphor-imager (Storm 820; Molecular Dynamics).

Chromatin immunoprecipitation experiments
Chromatin immunoprecipitation (ChIP) experiments were conducted as previously described with modifications (27). Unstimulated Scid.adh cells or Scid.adh cells (2 × 10^6/ml) stimulated with PMA-ionomycin for 3.5 h at 37°C or thymocytes (3–5 × 10^9/ml) were cross-linked in culture medium by the addition of 1% formaldehyde (v/v) and incubated for 10 min at room temperature. After fixation, the nuclei was collected by centrifugation and permeabilized by a cocktail of ice-cold buffers (containing protease inhibitors): 1) buffer A; 2) 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1); 3) 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1). The nuclei were then washed twice in buffer A and resuspended in 1% SDS, 1 mM PMSF, and protease inhibitors. Five percent of the starting material (250 μl) was saved as input for PCR detection. Chromatin was precleared by incubation for 2 to 3 h at 4°C with 150 μl 50% salmon sperm DNA/protein A-agarose slurry, prepared as recommended by the manufacturer (Upstate Biotechnology). Precleared chromatin corresponds to 5 × 10^9 Scid.adh cells or 7 × 10^8 thymocytes (∼2.3 ml) was used for ChIP. For ChIP, the 3P-specific probe (equivalent to 1–Tcra DNA) eluted from the protein A-agarose slurry, 600 μg proteinase K at 45°C for 1 h, extracted once with phenol/chloroform and twice with chloroform, and precipitated with 30% (v/v) ethanol. The DNA/protein complexes were eluted twice from the protein A-agarose slurry by heating at 65°C for 2 min, centrifuged for 15 min at room temperature in 250 μl 50 mM NaHCO3 and 1% SDS. The complexes were treated with 20 μg RNase A at 37°C for 1 h and 20 μg protease K at 45°C for 1 h, extracted once with phenol/chloroform and twice with chloroform, and precipitated with ethanol to purify the DNA. The DNA was resuspended in 30 μl water for subsequent PCR analysis as a template to evaluate the presence of Eμ and Oε2 sequences. For semiquantitative PCR analysis, 4 μl Ab-bound DNA (equivalent to 13.3% of the total Ab-bound material) or 4 μl 100K, 1200, and 1400 diluted input DNA (equivalent to 0.0033–0.0008% of the starting material used for each ChIP) was amplified using previously described primers (35). The PCR conditions were as follows: 5 min at 95°C, followed by 25 cycles of 25 s at 94°C, 25 s at 55°C, 20 s at 72°C, and a final extension step of 2 min at 72°C. The PCR products were resolved on 1.5% agarose gels, blotted, hybridized with 3P-labeled probe, and quantified using a phosphor-imager (Storm 820; Molecular Dynamics). For quantitative PCR analysis, 3–5 μl Ab-bound DNA was amplified using LightCycler FastStart DNA Masterplus SYBR Green I (Roche) in a Roche LightCycler 1.5 thermocycler (Roche). The primer sequences used to amplify Eμ are 5′-CCCTGAAATGGTGACTGG-3′ and 5′-TGTCAGCAACCGCTAG-3′, and the primer sequences used to amplify Oε2 are 5′-CCGGTGTGAGGGTGTTG-3′ and 5′-CGAGTCTGAGG-CAAGCCGAT-3′. The PCR conditions were: 3 min at 95°C, followed by 55 cycles of 5 s at 95°C, 20 s at 55°C, and 1 s at 72°C. Paired sample t tests were used to determine the statistical significance between values. The p values are represented as follows: *p < 0.05, **p = 0.005–0.0005, and ***p < 0.0005.

Construction of reporter plasmids and luciferase assays
The Jα49-flirefly luciferase (LUC), LUC-Eμ, and Jα49-LUC-Eμ (referred to as Jα49p, Eμ386, and Jα49p-Eμ386, respectively) pXPG plasmids have been previously described (19). The Eμ440 fragment was obtained by PCR with the primers 5′-GTCTCCGAATTCAGCAGTATCGCCTAG-3′ and the same 3′ primer previously used to clone Eμ386 into pXPG (19), which includes the introduced EcoRI sites. After digestion with EcoRI, Eμ440 was cloned into the unique EcoRI site downstream of LUC in pXPG and in Jα49p to generate LUC-Eμ440 and Jα49p-LUC-Eμ440 (in this study called Jα49p-Eμ440). The TEA fragment was obtained by PCR with the primers 5′-CTGATGTCCGGCGCCATCCTCG-3′ (with an introduced SmaI site) and 5′-GACAGTCAAGCCTAGTCTGCTC-3′ (with an introduced HindIII site). To generate TEA-LUC (called TEA), TEA-LUC-Eμ386 (called TEA-Eμ386), and TEA-LUC-Eμ440, the 673-bp HindIII fragment was cloned into the unique SmaI and HindIII sites upstream of LUC instead of pXPG. To generate the V61 promoter (V61p-LUC, V61p-LUC-Eμ386, and V61p-LUC-Eμ440 (called V61p, V61p-Eμ386, and V61p-Eμ440, respectively), the 1.6-kb V61p fragment was excised from the V61/CTAT construct (36) by digestion with Scal and HindIII and cloned into the Scal and HindIII sites of pXPG or derivative plasmids. The structures of all the PCR products were confirmed by sequencing. The 3XNFAT (37) and 9XNFAT (38) have been previously described. The 3XNFAT-LUC contains three copies of the NFAT-AP-1 site of the murine IL-2 promoter (37), and the 9XNFAT-LUC contains nine NFAT sites from the IL-4 promoter (38). Jurkat cells (4 × 10^6) were transfected with 5 μg LUC reporter plasmid plus 10 ng pRL-TK (renilla luciferase plasmid; Promega) by electroporation in 300 μl ice-cold RPMI 1640 medium at 260 V, 80 Ohms, and 1500 μF. After 6 h, the cells were lysed, and luciferase activity (firefly/renilla) was measured with a Dual Luciferase Kit (Promega). The results were expressed as a ratio of firefly and renilla luciferase activity (firefly/renilla). In some experiments, the 3XNFAT-LUC construct was cotransfected with 10 ng of a reporter plasmid, pGL3-Control (Promega). The 3XNFAT-LUC pGL3-Control construct contains three copies of the NFAT-AP-1 site of the murine IL-2 promoter, and the 9XNFAT-LUC pGL3-Control construct contains nine NFAT sites from the IL-4 promoter. The Jurkat cell lines (4 × 10^6) were transfected with 5 μg LUC reporter plasmid plus 10 ng pRL-TK (renilla luciferase plasmid; Promega) by electroporation in 300 μl ice-cold RPMI 1640 medium at 260 V, 80 Ohms, and 1500 μF. After 6 h, the cells were lysed, and luciferase activity (firefly/renilla) was measured with a Dual Luciferase Kit (Promega). Paired sample t tests were used to determine statistical significance between values. The p values are represented as follows: *p < 0.05, **p = 0.005–0.0005, and ***p < 0.0005.

Results
Both the calcineurin/Ca2+ and MAPK/ERK signaling pathways are required for induction of germline Tcra transcription
Tcra transcription and recombination depend on Eox activation by pre-TCR signaling. This enhancer acts through the binding of multiple transcription factors constitutively present in DN3a and DP thymocytes (Fig. 1). To study the molecular mechanisms for the induction of germline transcription at Tcra by pre-TCR signaling, we sought to establish an in vitro system that would allow us to analyze the proximal signaling events involved in this process. Scid.adh (TAC,CD3e) cells (in this study denoted as Scid.adh cells) have been previously established as an excellent cellular system for studying the molecular mechanisms of pre-TCR signaling by stimulation through a chimeric human CD25;CD3e surface protein (20). These

![Figure 1](http://www.jimmunol.org/content/jimmunol/189/5/2874/F1.large.jpg)
cells resemble the phenotype of DN3a thymocytes and express pre-Tcra transcripts. CD3ε-induced changes in these cells include the downregulation of IL2ra (CD25), Rag1, Rag2, and Pre-Tcα transcripts and the upregulation of CD5, CD27, CD28, Egr-1, Egr2, Egr-3, and germline Tcra-Cca transcripts (9, 20). Consistent with previous reports, stimulation of these cells with TAC Ab induced the reported changes, including the induction of Egr-1, Egr-3, and germline Tcra-Cca transcripts (Fig. 2A). These changes are identical to those induced in normal DN3a thymocytes during β-selection (1, 39).

In an attempt to dissect the signaling requirements for this induction during β-selection, we evaluated the total germline Tcra-Cca transcripts after cell stimulation with phorbol esters, such as PMA, and/or Ca2+ ionophores, such as ionomycin or thapsigargin (Fig. 2B). Germline Tcra-Cca transcription was activated by all three stimuli. Quantitative analysis of these transcripts induced by the different treatments revealed that Ca2+-mediated signaling was the major contributor to the activation of germline Tcra transcription, as Ca transcripts were induced much more robustly by ionomycin or thapsigargin treatment than by PMA treatment. As a control for treatment specificity, the induction of Egr-1 and Egr-3 transcripts was also analyzed (Fig. 2C). Egr-1 transcription was specifically induced by sustained protein kinase C activation by PMA treatment, whereas Egr-3 transcription was more dependent upon activation of Ca2+-mediated signaling as expected (40, 41). These data indicate that Ca2+-mediated signaling is by far the most relevant pathway involved in inducing Tcra germline transcription in Scid.adh cells. To investigate whether Ca2+-mediated signaling activates the Cc transcripts through a calcineurin-dependent pathway, we pretreated cells with CsA before stimulation (Fig. 2B, 2C). In agreement with previous studies that found that CsA completely inhibits TAC Ab-induced Egr-3 and Cc transcript induction in Scid.adh cells (11), we found that CsA totally blocked the induction of Egr-3 and germline Tcra-Cca transcripts in cells stimulated with ionomycin or thapsigargin. These results demonstrate that the calcineurin/NFAT signaling pathway is essential for activation of Tcra germline transcription.

To evaluate whether the MAPK/ERK signaling pathway is required in addition to calcineurin/NFAT for the induction of germline Tcra transcripts, we pretreated cells with specific inhibitors of MAPK/ERK or Ca2+-mediated pathways before stimulating the cells with PMA and ionomycin (Fig. 2D). Chelation of extracellular Ca2+ with EGTA, which inhibits the entry of Ca2+ into the cell and the nuclear translocation of NFAT, totally abrogated the induction of germline Tcra-Cca transcripts. In addition to specific inhibitors of Ca2+-mediated signaling, pretreating the cells with PD98059 (MAPK inhibitor) and U0126 (ERK inhibitor), but not with the control U0124, also inhibited the induction of Cc transcripts, suggesting that transcription factors induced through the MAPK/ERK pathways are also involved in the activation of Tcra germline transcription. As expected, the PLCγ inhibitor U73122 did not inhibit germline Tcra-Cca transcripts because stimulation with PMA and ionomycin bypasses PLCγ activation. The induction of Tcra germline transcripts by independent MAPK/ERK or Ca2+-mediated signaling pathways in Scid.adh cells, as seen in Fig. 2B, is likely most to be due to the bioavailability of low levels of endogenous NFAT or Egr factors in some cells before their activation (11). Altogether, our data indicate that both calcineurin/NFAT- and MAPK/ERK-mediated pathways are essential for induction of germline Tcra transcription during β-selection. NFAT, AP-1, and Egr-1 factors bind specifically to multiple sites within Eα

Our functional data suggest an essential role for Ca2+/calcineurin-dependent transcription factors, such as NFAT, together with MAPK/ERK-dependent transcription factors, such as AP-1 and Egr-1, in the germline transcriptional induction of Tcra. By comparing the nucleotide sequences of the human and murine Eα with rVista and MatInspector programs (42–44), we identified 10 conserved putative sites for NFAT (GGA motifs with 3′-adenine tracts), 4 for AP-1 within the 5′ Tcα1 and Tcα1-To2 regions, and 7 for Egr factors within the 5′ Tcα1, Tcα1-To2, and 3′ To4 regions. The concentration of previously undetected NFAT, AP-1, and Egr binding sites present within Tcα1-To2 and the flanking regions is very striking because Tcα1-To2 constitutes the previously reported core Eα, which is extensively occupied by multiple transcription factors during thymocyte development (21, 26, 27) (Fig. 1, Supplemental Fig. 1, Supplemental Table I).

To validate the sites that can readily support NFAT binding, we performed EMSAs with Eα-derived probes and rNFATDBD (Supplemental Fig. 2A). Binding experiments to 5′ Tcα1, Tcα1, and To2 demonstrated highly efficient binding of rNFAT-DBD to the three regions (data not shown). The 5′ Tcα1 can accommodate simultaneous binding of two NFAT molecules to the same DNA molecule (Supplemental Fig. 2A); however, further experiments using sites that can support the formation of NFAT dimers (45) demonstrated noncooperative NFAT binding to 5′ Tcα1 (B. del Blanco and C. Hernández-Munain, unpublished observations). To clearly identify those sites that were actually bound to NFAT from among the potential sites, we performed EMSAs using Eα probes containing single binding sites (Supplemental Fig. 2A). Our dissection of the NFAT sites indicated that the relevant Eα NFAT sites in vivo might include the following four sites: 5′ Tcα1 sites I and II, Tcα1 site III, and To2 site IV (Fig. 3A). Furthermore, incubation of the single site-containing probes with increasing amounts of rNFAT-DBD allowed us to compare the relative affinity among the several binding sites (Supplemental Fig. 2A). Our data indicate that sites I and III can afford very strong NFAT binding, whereas sites II and IV can afford moderate NFAT binding.

To evaluate binding of endogenous NFAT factors to Eα during β-selection, we first generated nuclear extracts from unstimulated and PMA+ionomycin-stimulated cells as a source of such inducible factors. The efficient induction of NFAT1, NFAT2, NF-kB, AP-1, and Egr-1/3 factors after cell stimulation was evidenced by binding to their corresponding CsAs in the EMSA experiments (Supplemental Fig. 2B and 2C). Chelation of extracellular Ca2+ with EGTA, which inhibits the entry of Ca2+ into the cell and the nuclear translocation of NFAT, totally abrogated the induction of germline Tcra-Cca transcripts. We then asked whether the endogenous NFAT factors present in PMA+ionomycin-stimulated cell extracts could bind to the NFAT sites present in the Eα-derived probes (Fig. 3B–D). Two specific complexes (marked NFAT1 and NFAT2) based on competition experiments were formed with each of the Eα probes used (data not shown). Inclusion of NFAT1 or NFAT2 Abs resulted in a shift of the correspondent complexes (marked as NFAT1* and NFAT2*, respectively). These experiments demonstrate that endogenous NFAT1 and NFAT2 can bind in vitro to each of their sites within Eα. It is interesting to note that Ets factors also bind to GGA core sequences (46). In fact, the 5′ Tcα1 NFAT sites I and II coincide exactly with previously described binding sites for Ets-1 and Fli-1 (Fig. 1, Supplemental Fig. 1) (26, 47). Both NFAT and Ets factors require intact GG nucleotides within their binding sites in 5′ To1, indicating that these factors share the same contacts with DNA (B. del Blanco and C. Hernández-Munain, unpublished observations). To further discriminate between complexes containing NFAT and Ets-1 factors formed with the 5′ To1 probes, EMSAs in the presence of an Ets-1 Ab were performed (Fig. 3E). These
Both the calcineurin/Ca\textsuperscript{2+} and MAPK/ERK-mediated signaling pathways are required for the induction of germline Tcra transcription. (A) Analysis by RT-PCR of germline Tcra-Ca, Egr-1, and Egr-3 transcripts in unstimulated (−) Scid.adh cells or in cells stimulated with TAC mAb or control Ab (control). These results are representative of three experiments. (B and C) Analysis by quantitative RT-PCR of germline Tcra-Ca, Egr-1, and Egr-3 transcripts in unstimulated cells (NS) or cells stimulated with the indicated stimuli in the presence or absence of CsA. The effect of the DMSO vehicle control was also analyzed. (B) Transcript levels were normalized to Actb in each sample and expressed as the percentage of the level of germline Tcra-Ca transcripts in ionomycin-treated cells. Data represent the mean ± SEM of three determinations from two independent experiments. Paired sample t tests were used to determine the statistical significance between the levels of transcription present in the unstimulated cells versus cells stimulated with ionomycin, thapsigargin, or PMA (∗∗∗p < 0.0005, ∗p = 0.045, and ∗∗p = 0.0244, respectively) and to determine the statistical significance of the effect of CsA treatment on the expression of germline Tcra-Ca transcripts in ionomycin-, PMA+ionomycin-, and thapsigargin-stimulated cells (∗∗∗p < 0.0005, ∗p = 0.0161, and ∗∗p = 0.0445, respectively). (C) Expression levels of the transcripts were normalized to Actb in each sample and are expressed as the percentage of the level of Egr-1 transcripts in the PMA+ionomycin-treated cells. Data are represented as the mean ± SEM of three determinations from two independent experiments. (D) Analysis by real time RT-PCR of germline Tcra-Ca transcripts in stimulated cells in the presence or absence of the indicated inhibitors. Paired sample t tests were used to determine the statistical significance of the effect of EGTA versus untreated cells. 

Figure legend continues...
experiments identified a complex containing Ets-1 for which formation was inhibited in the presence of the Ets-1 Ab. The Ets-1-containing complex can be easily discriminated from complexes containing NFAT1 or NFAT2 due to its increased mobility. These experiments revealed that in contrast to the induction of NFAT-containing complexes, the formation of the Ets-1-containing complex was inhibited upon cell stimulation. These results are consistent with a previously reported Ca\(^{2+}\)-dependent and protein kinase C-mediated phosphorylation of Ets-1, which inhibits its binding to DNA (48).

We next analyzed the binding of AP-1 to its putative Eox sites. We asked whether AP-1 complexes present in the nuclear extracts of stimulated cells could bind to the putative Eox AP-1 sites. Our experiments showed that only one site present in T\(\alpha\)1 was bound efficiently by AP-1 (Fig. 3A, 3F, 3G, Supplemental Fig. 2C). This complex (marked AP-1) was judged to be specific based on competition experiments (Supplemental Fig. 2C). Inclusion of Jun Abs inhibited complex formation, confirming the identity of this complex (Fig. 3F). The JunB Ab was more efficient than JunD and c-Jun Abs in inhibiting the formation of this complex (Fig. 3F and data not shown). These results are consistent with the relative expression of Jun proteins upon Scid.adh cell stimulation: JunB transcripts are more abundant than JunD and c-Jun transcripts in stimulated cells (B. del Blanco and C. Hernández-Munain, unpublished observations). This site coincides with a previously described essential site for CREB (24). In agreement with this, we have detected a CREB-containing complex in nonstimulated and stimulated cells, which was supershifted in the presence of a CREB Ab (Fig. 3F). CREB is mostly present in an unphosphorylated form in resting thymocytes, and its phosphorylation is induced by different pharmacological stimuli that activate different signaling pathways resembling TCR engagement (49). Consistent with this, we have detected a p-CREB–containing complex bound to this sequence (p-CREB\(^*\)) in stimulated cells (Fig. 3G) as previously reported (22, 26). Interestingly, the abundance of the induced AP-1 complex exceeds that of the p-CREB\(^*\) complex, suggesting the possibility that AP-1 might outcompete p-CREB for binding to T\(\alpha\)1.

To verify whether the putative Egr binding sites present at Eox can support Egr-1 binding, we performed EMSAs using Eox-derived probes. Because the use of nuclear extracts did not allow us to clearly discriminate the specific binding of Egr factors to these probes, we performed these experiments with rEgr-1 (Fig. 3H) and extracts from HEK-293T cells overexpressing Egr-1 (Supplemental Fig. 2). Analysis of rEgr-1 binding to 5' T\(\alpha\)1, T\(\alpha\)1-T\(\alpha\)2, and 3' T\(\alpha\)4 revealed the formation of complexes of identical mobility to the complex formed with the CS (data not shown). To verify that Egr-1 is present in these complexes, we used an Egr-1 Ab (Fig. 3H). Inclusion this Ab resulted in a supershift of the Egr-1-containing complex (Egr-1\(^*\)). As expected, we did not observe any binding of rEgr-1 to T\(\alpha\)3-T\(\alpha\)4 demonstrating the specificity of the assays (B. del Blanco and C. Hernández-Munain, unpublished observations). Our dissection of the Egr binding sites present in Eox indicated that the relevant sites in vivo might include five sites: 5' T\(\alpha\)1 site I, T\(\alpha\)1 site II, T\(\alpha\)2 sites III and IV, and 3' T\(\alpha\)4 site V (Fig. 3A, Supplemental Figs. 1, 2D, 2E). Our comparison of Egr-1 binding to Eox probes containing a single Egr-binding site with respect to that to the CS allowed us to compare the relative affinities of the different Egr binding sites (Supplemental Fig. 2E). Our data indicate that Egr-1 has a very low affinity for binding to the Egr binding sites present in Eox. Among the five confirmed Egr binding sites, only the T\(\alpha\)1 site II affords the strongest Egr-1 binding to Eox, and it represents 11.725 ± 1.421% of the binding of this factor compared with that of the CS. The capability of Egr-1 to bind to the other Eox Egr binding sites is much weaker compared with Egr site II, and each of them represents <1% of the binding of Egr-1 to the CS. The percent of Egr-1 binding to these individual sites versus to the CS is as follows: 3' T\(\alpha\)4 Egr site V (0.820 ± 0.139%), T\(\alpha\)2 Egr site III (0.203 ± 0.035%), 5' T\(\alpha\)1 Egr site I (0.079 ± 0.016%), and T\(\alpha\)2 Egr site IV (0.044 ± 0.008%). We conclude that Egr-1 can bind in vitro specifically to several sites present in 5' T\(\alpha\)1, T\(\alpha\)1-T\(\alpha\)2, and 3' T\(\alpha\)4 with low affinity (T\(\alpha\)1 Egr site II) or very low affinity (5' T\(\alpha\)1 site I, T\(\alpha\)2 sites III and IV, and 3' T\(\alpha\)4 site V). These results suggest that the T\(\alpha\)1 Egr site II might be the only relevant Egr binding site present in Eox. Taken together, our data suggest that NFAT, AP-1, and Egr proteins are inducible factors that might function in the activation of Eox during β-selection. A summary of the confirmed binding sites for these factors present at Eox is presented in the Fig. 3A and Supplemental Fig. 1.

NFAT, AP-1, and Egr-1 factors bind transiently to Eox to assemble an active enhanceosome, together with GATA-3, E47, Ets-1, CREB, and CREB-binding protein/p300 factors, during thymocyte development

To confirm the presence of NFAT factors, AP-1, and Egr-1 at the Eox enhanceosome in vivo in PMA+ionomycin-stimulated versus unstimulated Scid.adh cells, we performed ChIP experiments and analyzed them by quantitative PCR (Fig. 4) or semiquantitative PCR (Supplemental Fig. 3). As a control for gene specificity, factor binding to a presumably negative control sequence present in the Oct2 gene (35) was analyzed in all experiments.

GATA-3 was originally identified as a positive regulator of Eox (50), which is required for β-selection (51). We have previously shown that it participates in the formation of the Eox enhanceosome assembled in vitro (22) and in vivo (27). Because the expression of GATA-3 is upregulated in thymocytes by TCR signaling as a result of the additive inputs from the Ras/MAPK and calcineurin pathways (52, 53) and because binding of GATA-3 to Eox depends on its bioavailability in cell nuclei (54), we decided to evaluate possible changes in GATA-3 binding to Eox in unstimulated and stimulated Scid.adh cells. As is shown in Fig. 4A, GATA-3 binding to Eox was induced by ∼3-fold in stimulated versus unstimulated cells. These results are consistent with previous reports of GATA-3 inducibility upon cell stimulation and suggest that this factor might have an important role in the assembly of a functional Eox enhanceosome.

We next analyzed binding of NFAT, AP-1, and Egr-1 factors to Eox upon cell stimulation (Fig. 4B–D). As shown in Fig. 4B, NFAT2 binding to Eox was induced by ∼5-fold in stimulated cells but not in unstimulated cells. In fact, NFAT2 was not present in the Eox enhanceosome in unstimulated cells. Our analysis of NFAT4 binding to Eox revealed that the NFAT4 binding pattern parallels that of NFAT2 upon cell stimulation (Supplemental Fig. 3A), whereas we did not find clear evidence of NFAT1 binding to Eox (data not shown). These results suggest that NFAT2 and NFAT4 are the relevant NFAT factors present on an active Eox enhanceosome.

Analysis of the AP-1 components revealed that the binding of JunB and JunD to Eox was induced upon cell stimulation (Fig. 4C), which involved significant differences in their binding to

(**p = 0.0069). Paired t tests were also used to analyze the effect of the PU0126 and PD98059 treatments compared with the effect of U0124 treatment on the expression of germline Tcra-Cx transcripts (***p < 0.0005 and *p = 0.0152, respectively).
FIGURE 3. NFAT, AP-1, and Egr-1 factors bind specifically to several sites within the Tα1-Tα2 and flanking regions. (A) The diagram depicts the NFAT, AP-1, and Egr binding sites present within Ea and in the flanking regions. Analysis of the binding of NFAT to 5′ Tα1 (B), Tα1 (C), and Tα2 (D) by EMSAs. (E) Analysis of the Ets-1 binding to 5′ Tα1 by EMSA. (F) Analysis of AP-1 and CREB binding to Tα1 by EMSA. (G) Analysis of AP-1 and p-CREB binding to Tα1 by EMSA. (H) Analysis of Egr-1 binding to 5′ Tα1, Tα1-Tα2, and 3′ Tα4 by EMSAs. Radiolabeled probes were incubated with nuclear extracts from unstimulated (NS) or PMA+ionomycin-stimulated (S) cells (B–G) or rEgr-1 (H) in the presence of control or specific Abs. The complexes containing NFAT1-, NFAT-2, Ets-1, JunB, JunD, CREB, and Egr-1 are indicated. NFAT1*, NFAT-2*, CREB*, p-CREB*, and Egr-1* denote complexes formed in the presence of specific Abs.
the enhancer in unstimulated and stimulated cells. Analysis of c-Jun binding also highlighted an increase in c-Jun binding to Eα upon cell stimulation with binding kinetics that are initiated 3.5 h after cell stimulation and increase for 6 h after cell stimulation (Supplemental Fig. 3B). These results suggest AP-1 complexes containing different Jun components can all be part of an active Eα enhanceosome.

Analysis of the presence of Egr-1 on the Eα demonstrated that binding of this factor was dramatically increased upon cell stimulation (Fig. 4D). Egr-1 Ab binding was enriched by ~16-fold with respect to the control Ab, and its binding was induced by ~6-fold in stimulated cells compared with unstimulated cells. No clear binding of Egr-3 to Eα was detected, probably due to its low expression compared with Egr-1 in these cells (data not shown).

**FIGURE 4.** NFAT2, AP-1, Egr-1, and p300 factors, together with GATA-3, E47, Ets-1, and CREB, are part of the active Eα enhanceosome in Scid.adh cells. Chromatin preparations from unstimulated cells (NS) or cells stimulated with PMA+ionomycin (S) for 3.5 h were immunoprecipitated with the indicated Abs: GATA-3 (A), NFAT2 (B), JunB and JunD (C), Egr-1 (D), p300 (E), E47 (F), Ets-1 (G), and CREB (H). DNA purified from the Ab-bound fractions was used as a template for quantitative PCR to evaluate the presence of the Eα and Oct2 sequences. The values obtained from the Eα and Oct2 signals were normalized to that of the input and expressed as the relative enrichment of the indicated factor binding versus the signals obtained from the control Abs. Data represent the mean ± SEM of three determinations from two to three independent experiments. Paired sample t tests were used to determine the statistical significance between the values of the ChIPs from unstimulated versus stimulated cells (GATA-3, *p = 0.0101; NFAT2, **p = 0.0054; JunB, **p = 0.0073; Egr-1, ***p < 0.0005; p300, **p = 0.0036; and Ets-1, **p < 0.0005).
Furthermore, consistent with the dependence of the proper Eo-mediated functional activation of both transcription and V(DJ) recombination on the recruitment of histone acetylases (HATs) (27, 35), we found that the levels of p300 and CREB-binding protein (CBP) in the Eo enhanceosome were induced upon cell stimulation (Fig. 4E and data not shown). Binding of p300 to Eo was increased by ~3-fold in stimulated cells compared with unstimulated cells. These results are consistent with the fact that the binding of p300 constitutes a common marker of activated enhancers (55).

All transcription factors assayed exhibit increased binding to Eo in stimulated versus unstimulated cells. To formally exclude potential biases with regard to specific Ab binding in stimulated cells, we analyzed the behavior of a transcription factor for which binding does not change before and after early stimulation. For that purpose, we performed ChIP experiments analyzing the binding of E-protein E47 to Eo (Fig. 4F), which is present at the Eo enhanceosome in DP thymocytes (27). Our ChIP experiments indicated that the binding of E47 to Eo is comparable in unstimulated and stimulated cells (Fig. 4F). Although E2A-binding site occupancy generally decreases during β-selection, a recent genome-wide analysis of E2A occupancy revealed that 247 out of 939 of those sites are shared in DN3a and DN4 cells, suggesting that occupancy of ~25% of E-boxes is not altered by pre-TCR signaling (56). Similar data were obtained in regard to CREB binding; there were no obvious differences in binding between unstimulated and stimulated cells (Fig. 4H, see below). These results validate our ChIP data and demonstrate the specific recruitment of inducible transcription factors to Eo upon DN3a cell stimulation.

Because our in vitro binding data suggest that the binding of Ets-1 to 5′ Tcra might be outcompeted by the binding of NFAT factors and that the binding of CREB to Tcra might be outcompeted by the binding of AP-1 factors upon cell stimulation, we evaluated these possibilities by performing ChIPs to analyze Ets-1 and CREB binding to Eo before and after cell stimulation (Fig. 4G, 4H). In agreement with our previous analysis that demonstrated the presence of both Ets-1 and CREB in the Eo enhanceosome assembled in DP thymocytes in vivo (27), we confirmed the binding of these factors to Eo in both unstimulated and stimulated Scid.adh cells. In contrast to our expectations, we found that Ets-1 binding was increased by ~3-fold upon cell stimulation (Fig. 4G), whereas CREB binding was unaffected (Fig. 4H). Hence, the results obtained from the ChIP analysis of Ets-1 and CREB binding to Eo in stages prior to small resting DP thymocytes are blocked at the small resting DP thymocytes (B. del Blanco and C. Hernández-Munain, unpublished observations). Consistent with this notion, the in vivo binding of E47 to Eo in Rxβ thymocytes was robust and increased by ~5-fold with respect to the levels in Rag2−/− thymocytes (Fig. 5B).

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Our ChIP analyses demonstrated no significant differences in NFAT1 and NFAT2 binding to Eo in Rxβ thymocytes compared with that in Rag2−/− thymocytes (Fig. 5C, 5D). In the analysis of AP-1 binding, we did not detect a significant induction of JunD, JunB, or c-Jun binding in Rxβ thymocytes compared with in Rag2−/− thymocytes (Fig. 5E and data not shown). Similarly, there was no apparent increase in Egr-1 binding in Eo versus Rag2−/− thymocytes (Fig. 5F). However, in vitro stimulation of Rxβ thymocytes induced the further binding of Egr-1 to Eo (B. del Blanco and C. Hernández-Munain, unpublished observations); this is probably related to a transient induction in synchronously stimulated cells (40). These results are consistent with a possible transient recruitment of inducible factors to Eo in stages prior to small resting DP thymocytes. In contrast, we found a strong increase in Ets-1 binding to Eo in Rag2−/− versus Rxβ thymocytes (Fig. 5G).

These results are in agreement with the correlation between the occupancy of E2A sites and the enrichment of Ets-1 binding sites reported recently in a genome-wide analysis during β-selection (56), supporting a collaborative functional interaction between these factors. In agreement with the data obtained in Scid.adh cells, we found that pre-TCR signaling stimulated the recruitment of coactivators, such as p300 and CBP, to the Eo enhanceosome, as evidenced by their stronger binding in Rxβ thymocytes than in Rag2−/− thymocytes (Supplemental Fig. 3E and data not shown). These results are consistent with the requirement for Eo to recruit HATs to induce V(DJ) recombination (35).

To evaluate whether the formation of an active Eo enhanceosome containing inducible transcription factors is indeed transient during DN3a to DP thymocyte differentiation, we performed ChIP experiments using thymocytes from Rag2−/− mice that were induced to differentiate to DN4 in response to CD3 stimulation by
FIGURE 5. Inducible transcription factors are transiently recruited to Eκ during thymocyte development. (A) Analysis of Cα transcripts by quantitative RT-PCR in Rxβ thymocytes in the absence (none) or presence of U73122 or DMSO control. Expression levels of Cα transcripts were normalized to that of Actb. Data represent the mean ± SEM of three determinations from three independent experiments. Paired sample t tests were used to determine the statistical significance between the transcription levels present in the DMSO- and U73122-treated cells. *p = 0.0243. Chromatin preparations from total Rag2^{−/−} (R) or Rxβ thymocytes were immunoprecipitated with the indicated Abs: E47 (B), NFAT1 (C), NFAT2 (D), JunD and JunB (E), Egr-1 (F), and Ets-1 (G). DNA purified from the Ab-bound fractions was used as a template for quantitative PCR to evaluate the presence of Eκ and Oct2 sequences. The Eκ and Oct2 signal levels were normalized to the input and expressed as the relative enrichment of the indicated factor binding to signals obtained with control Abs. Data represent the mean ± SEM of three determinations from two to four independent experiments. Paired sample t tests were used to determine the statistical significance between the ChIP values obtained using Rag2^{−/−} and Rxβ thymocytes (E47, ***p < 0.0005; and Ets-1, ***p < 0.0005). Chromatin preparations from total Rag2^{−/−} (R) or CD3ε mAb-injected Rag2^{−/−} (R/CD3ε) thymocytes were immunoprecipitated with the indicated Abs: E47 (H) and JunD (I). Data represent the mean ± SEM of three to four determinations from two independent experiments. Paired sample t tests were used to determine statistical significance between the values of ChIPs for JunD obtained in Rag2^{−/−} and CD3ε Ab-injected Rag2^{−/−} thymocytes (*p = 0.0284).
i.v. injections with CD3ε mAb. CD3ε mAb injection of Rag2−/− mice mimics pre-TCR signaling because it stimulates the proliferation and differentiation of DN3a to DP thymocytes and promotes allele exclusion at the Tcrb locus and transcription at the Tcra locus (62–64). Previous experiments have demonstrated that at 12 h after CD3ε mAb injection, approximately half of the DN3a thymocytes have differentiated into DN4 thymocytes (4, 60). We have analyzed the injection, approximately half of the DN3a thymocytes have

**FIGURE 6.**

Ea-transcriptional induction is dependent on calcineurin-mediated signaling

All of our data indicate that Tcra germline transcription is dependent on signaling pathways mediated by calcineurin and MAPK/ERK, which culminate in the binding of NFAT2/4, AP-1, and Egr-1 factors to the Ea enhanceosome after β-selection. To formally investigate whether Ea is the element through which these signaling pathways activate germline Tcra transcription, we evaluated Ea inducibility after Jurkat cell stimulation in transient transfection experiments (Fig. 6). In addition, we also evaluated the functional contribution of NFAT sites I and II and Egr site I within 5' To1 on Ea activation. To this end, we employed reporter constructs in which Ea-dependent TEA or Jo49p is controlled by Ea variants containing (Ea440) or lacking (Ea386) these sites (Fig. 6A). We note that these types of experiments, with Ea positioned within the context of artificial reporter constructs, allow the analysis of Ea function in the absence of cell stimulation and therefore do not recapitulate the in vivo situation, in which transcriptional activation of Tcrb, with Ea positioned at large distances from its responding promoters, is cell stimulation dependent. However, this in vitro system allowed us to evaluate some essential aspects of Ea inducibility. Transient transfection of these constructs revealed that, consistent with previous results (19, 65), in the absence of Ea, neither TEA nor Jo49p was able to activate the transcription of the reporter gene, whereas Ea efficiently activated transcription from each of these promoters (Fig. 6B). The high basal transcriptional activity observed in the Jo49p/

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**FIGURE 6.** Ea-transcriptional induction is dependent on calcineurin-mediated signaling. (A) The diagram depicts the structure of Ea440 and Ea386, indicating the location of the NFAT and AP-1 binding sites present and the location of the To1, To2, To3, and To4 regions. (B and C) The indicated enhancers, Ea440 or Ea386, were cloned into enhancer-dependent test constructs in which LUC is transcribed from the indicated promoters. The 3xNFAT/LUC and 9xNFAT/LUC reporter constructs were used as controls. The constructs were electroporated into Jurkat cells together with a reference plasmid containing the renilla luciferase reporter. Cells were unstimulated (NS) or PMA+ionomycin stimulated (S) for 24 h. The LUC activity levels were normalized to the renilla luciferase levels from the control plasmid. Data represent the mean ± SEM of 6–11 determinations. Paired sample t tests were used to determine for statistical significance between the levels of relative firefly/renilla luciferase activity in the unstimulated versus stimulated Jurkat cells (Jo49p-Ea386, *p = 0.0119; Jo49p-Ea440, **p = 0.0061; TEA-Ea386, *p = 0.0189; TEA-Ea440, *p = 0.0347; 3xNFAT, **p = 0.0013; 9xNFAT, *p = 0.0336; and V61-Ea440, *p = 0.0407) and between relative the firefly/renilla luciferase activity present in untreated and CsA-treated stimulated Jurkat cells (Jo49p-Ea386, *p = 0.0493; Jo49p-Ea440, *p = 0.0374; TEA-Ea386, *p = 0.0479; TEA-Ea440, *p = 0.0148; 3xNFAT, *p = 0.0463; and 9xNFAT, *p = 0.0295).
Eo386 construct compared with that in the Jeq49p/Eo440 construct supports the presence of a negative regulatory site within 5’ To1 involved in this collaboration. The opposite situation was found in the comparison between the TEA/Eo386 and TEA/ Eo440 constructs, indicating that Eo440 was a more efficient transcriptional activator of transcripts derived from TEA than Eo386. Cell stimulation with PMCA-ionomycin further activated Eo440- and Eo386-dependent transcription from both promoters, demonstrating Eo inducibility in this system (Fig. 6B). It is interesting to note that both the Jeq49p/Eo386 and Jeq49p/Eo440 constructs had the same transcriptional activity, suggesting that the 5’ To1 NFAT sites I and II are dispensable for enhancer induction driven by Jeq49p. This result is consistent with the lack of any observed effect on transcription driven by either Jeq49p or TEA after the introduction of a point mutation at the 5’ To1 NFAT site I, the major NFAT site in the 5’ To1 region, that affects NFAT binding without disturbing Ets-1 binding (GGAA to GGTT instead of GGAA to TTAA) (data not shown). In contrast to data obtained from the Jeq49p/Eo constructs, when using the TEA/Eo constructs, the Eo440-dependent transcriptional induction was twice as high as the Eo386-dependent transcriptional induction. The fact that Eo440 was also a more efficient transcriptional activator for transcripts derived from TEA than Eo386 in unstimulated cells suggests that the binding of noninducible factors might be involved in the functional collaboration between TEA and the 5’ To1 region. As a control for transcriptional induction upon cell stimulation, the activities of two reporter constructs containing several NFAT sites in tandem, 3xNFAT and 9xNFAT (37, 38), were analyzed (Fig. 6B). The transcriptional activity of both positive control constructs was efficiently induced upon cell stimulation (Fig. 6B). Pretreatment of cells with CsA before stimulation totally abrogated the ability of both Eo440 and Eo386 to activate cell stimulation-dependent transcription (Fig. 6B), suggesting a specific role for calcineurin and NFAT factors in this stimulation. The transcriptional activity of both the 3xNFAT and 9xNFAT control constructs was efficiently inhibited upon CsA treatment (Fig. 6B). Because it is possible that cell stimulation might also be involved in TEA and Jeq49p function, we assessed whether Eo is specifically induced by cell activation. To address this possibility, Eo386- and Eo440-dependent transcriptional induction was assayed from the Vβ1p, which is an active promoter in DN3a cells prior to β-selection (Fig. 6C). Vβ1p alone or directed by E6 or Eo386 did not respond to cell stimulation, whereas it strongly responded when directed by Eo440. (Fig. 6C and B. del Blanco and C. Hernández-Munain, unpublished observations). These results indicate that Eo itself responds to cell stimulation and that calcineurin-mediated signals are crucially involved in this function. Altogether, our data indicate that pre- TCR signals induce Tεrα germine transcription through calcineurin- and MAPK/ERK-dependent signaling pathways that activate Eo through the assembly of a functional Eo enhanceosome formed by multiple inducible factors.

**Discussion**

Previous experiments have established Tεr1-Toa2 as a paradigm of an enhanceosome created by stereospecific interactions among activators bound to the enhancer (24, 25). This view has also been supported by previous genomic footprinting experiments using DMS that revealed that Tεr1–Toa4 and the flanking areas are extensively occupied without major differences between DN3a and DP thymocytes from Rag22Δ/Δ and RxB mice, respectively (21, 26, 27). However, this notion has been challenged by the creation of a mutant version of Eo, EoMC, in which E6 Myb and Runx binding sites were substituted for the To2 Runx and Ets binding sites (27). EoMC was a highly potent enhancer, indicating that the stereospecific interactions among proteins that form an Eo enhanceosome are rather flexible. These experiments suggested the possibility of the assembly of distinct sets of proteins on Eo that might represent a more flexible form of information processing during thymocyte development. In support of this hypothesis, analysis of DNase I sensitivity at the nucleotide resolution revealed that Eo chromatin is generally much more sensitive to digestion in DN3a than in DP thymocytes from Rag22Δ/Δ and RxB mice, respectively (22). This suggests a more compact enhanceosome structure in the latter cell population due to the assembly of a different multiprotein complex or as a consequence of physical interactions between Eo and its associated promoters, such as TEA (13, 66). In addition to these general changes in the Eo DNA structure, striking differences were also found at specific nucleotides, including the 5’ To1 and To1-Toa2 regions (26), suggesting that a new complex is formed in these regions by direct contact with Eo or be indirectly recruited through enhancer/promoter interactions. Therefore, Eo undergoes general chromatin structural changes at the DN3a to DP transition at specific regions that coincide with the regions where the NFAT, AP-1, and Egr-1 factors bind, suggesting that a new complex containing these inducible factors is formed at the DN4/early proliferating DP Eo enhanceosome to establish specific contacts with specific promoters. Based on these observations, together with the locations of the NFAT, AP-1, and Egr-1 factor binding sites described in this study, the requirement for an intact To1 for the assembly of an Eo enhanceosome in vivo (25), and our observation that in vivo Ets-1 binding seems not to be outcompeted by NFAT binding, we favor a scenario in which these inducible factors occupy their sites in DN3b, DN4, and early proliferating DP thymocytes, without disturbing the general nucleoprotein structure established by the binding of LEF-1/TFF-1-Runx1 and Ets-1 to Toa2 and of the Ets-1/ FlI-1 factors to 5’ To1 in DN3a thymocytes (Fig. 7). In fact, the complex formed by the factors bound to Toa2 seems to be very stable based on in vitro (24) and in vivo studies (25, 47) because the binding sites at Toa2 facilitate cooperative protein binding to DNA (24, 25, 67). We propose that the binding of these lymphoid-restricted factors to Eo prior to its activation in DN3a thymocytes would constitute a regulatory landscape for recruiting factors induced by pre-TCR signaling (Fig. 7). In DN3b/DN4/early proliferating DP thymocytes, the binding of inducible transcription factors, such as NFAT, AP-1, and Egr-1, together with GATA-3, Ets-1, CREB, and E-proteins, to Eo in cells results in the recruitment of CBP/p300 coactivators to assemble an active enhanceosome. This enhanceosome is in turn able to activate TEA and Jeq49p by intrachromosomal Eo/Eo physical promoter interactions to trigger first Tεrα germine transcription and then primary Vc to Jα rearrangements (13, 66). Interestingly, specific interactions between inducible factors have also been demonstrated to be involved in other specific inducible enhancer/promoter interactions (68–70). In the later stages of β-selection, these inducible factors are not present in small resting DP nuclei (39, 71, 72), and the Eo enhanceosome composition presumably changes to an active DN3a-like state (Fig. 7). The active DP Eo enhanceosome differs from the inactive DN3a Eo enhanceosome by enhanced binding of Ets-1 to Toa2 and E-G7 to the E-box III and the recruitment of HATs. The assembly of different transient pre- and post-β-selection enhanceosomes in DN3a, DN3b/ DN4/early proliferating DP cells, and small resting DP cells (Fig. 7) provides a new explanation for the long-standing paradox regarding the activation of this enhancer without obvious differences in the footprint of bound proteins by DMS between the DN3a and small resting DP cells but accompanied by general changes in Eo chromatin structure (21, 22, 26, 27).

Enhanced binding of Ets-1 to Toa2 in DP thymocytes with respect to that in DN3a thymocytes is supported by the higher sensitivity of
Figure 7. Model for the assembly of different Eox enhanceosomes during thymocyte development. The described constitutively bound factors are indicated by white ovals, the potentially bound NFAT factors are indicated by black ovals, and the putatively bound AP-1 factors are indicated by gray ovals, and the Egr binding sites are indicated by black lines. The locations of Tα1, Tα2, Tα3, and Tα4 are indicated. In DN3α thymocytes, only the constitutive transcription factors occupy their binding sites at the Eox. After β-selection during the first proliferative phase, the induced NFAT, AP-1, and Egr-1 factors occupy their sites within the enhancer in DN3b, DN4, and early proliferating DP thymocytes. This results in the recruitment of the HATs, CBP, and p300 and productive Eox-TEA/Jsap9p interactions that activate germline transcription. During the second nonproliferative phase in small resting DP thymocytes, when V(D)J recombination at Tcra occurs, Eox is occupied by the constitutive factors, and it remains active through the assembly of a new enhanceosome characterized by strong E-47 binding to E-box-III and Ets-1 binding to Tα2 and the recruitment of CBP/p300.

To α2 chromatin to DNase I digestion detected in DN3α thymocytes and by an increased stability of the Ets-1/Runx-containing complex formed in vitro observed with Toα2- and DP-derived nuclear extracts (22, 26). These data suggest an increased stability in the formation of the DP-derived complex compared with that of the DN3α-derived complex in Toα2. In addition to this, it is possible that enhanced Ets-1 binding might be related with induced binding to specific GGA sequences present at the 5’ of Toα1. Further experiments are required to clearly establish the differential occupancy of Eox E-boxes and 5’ Toα1 GGA sequences during thymocyte development.

The scenario proposed in this study is supported by the description of two distinguishable temporally distinct periods during pre-TCR signaling (39, 59). During the first 36-h period, or proliferative phase, defined by DN3b, DN4, and the early proliferating DP stages, there is a rapid activation of NFAT and AP-1 factors that drives the transient expression of Egr factors. This triggers expression of Id3 and prevents the induction of the E12/E47-dependent transcription factor retinoic acid-related orphan receptor γt and controls the expression of the antiproliferative gene mCPEB4 (11, 39, 60, 61). During the second 36–96-h period, or nonproliferative phase, defined in the small resting DP stage, the activity of the Egr factors declines, whereas E-protein activity increases and drives the induction of retinoic acid-related orphan receptor γt expression, triggering cell quiescence and the activation of Tcra locus rearrangement (39). Essentially all Rxα thymocytes are blocked at the small resting DP stage, which is equivalent to thymocytes that have reached the nonproliferative phase. Consistent with this notion, the in vivo binding of E47 to Eox in Rxα thymocytes was strong, whereas binding was not detected in Rag2−/− thymocytes. The reason why E47 binding to Eox is not detected in Rag2−/− thymocytes is unclear at present, but it might be related to the fact that different combinations of E-proteins seem to occupy the different E-boxes present in Eox (27). Eox contains three E-boxes: 5’ Toα1 E-box-I, Toα3 E-box II, and Toα4 E-box-III. The pattern of DMS in vivo footprints found that the Toα4 E-box III was affected when HEB−/− thymocytes were compared with Rxα thymocytes (27). In fact, the pattern of occupancy of this E-box observed in HEB−/− thymocytes resembled that of Rag2−/− thymocytes, with no occupation of the sequence (27). Together, these data suggest that the Toα4 E-box III might be occupied by an HEB/E47 heterodimer, which is the primary E-protein complex found in thymocytes (73, 74), whereas the 5’ Toα1 E-box-I and Toα3 E-box II might be occupied by dimers of E12 and/or E2-2 proteins (75, 76). Alternatively, it is also possible that E47 occupancy of Eox is induced during the DN3α to DP transition, as it has been shown that this occurs in ~10% of E2A-occupied sites when comparing DN3α and DN4 cells by genome-wide analysis of E2A occupancy (56).

Furthermore, this study has also revealed that the occupancy of E-boxes by Eα2 proteins is accompanied by an enrichment of binding sites for Runx-1 and Egr-1, as it is the case for Eox. Because we detected simultaneous robust recruitment of both E47 and Ets-1, our data suggest that collaborative interactions between these factors and presumably Runx-1 might be of particular interest for Eox function in small resting DP thymocytes.

Our model is supported by the fact that most Fos and Jun components, NFAT proteins, and Egr factors decreased dramatically in the transition from DN3b/DN4 (stimulated Scid.adh cells) to small resting DP thymocytes (Rxα thymocytes) (39, 71, 72). Furthermore, the Scid.adh model allows the synchronous induction of signaling in DN3α-like thymocytes, allowing a clear detection of the transient interactions that occur in the DN3α/DN4/early proliferating DP transition that might be missed in Rxα cells. Supporting our hypothesis that the assembly of an active Eox enhanceosome is highly transient during DN3α to DN4 thymocyte development, we found that: 1) in vivo c-Jun and Egr-1 binding to Eox increase upon Scid.adh cell and Rxα thymocytes stimulation, respectively (Supplemental Fig. 3B and B. del Blanco and C. Hernández-Munain, unpublished observations), presumably due to the transcriptional induction of c-jun and Egr-1 in synchronously stimulated cells (40); and 2) the binding of JunD in Scid.adh is dramatically increased in thymocytes from injected Rag1−/2− mice compared with noninjected Rag1−/2− mice or Rxα mice (Fig. 5I). Therefore, AP-1, NFAT, and Egr-1 factor recruitment to Eox seems to be highly transient during the DN3α to DN3b/DN4/early proliferating DP transition (Fig. 7). Although our ChIP experiments in Scid.adh suggest that AP-1 complexes containing different Jun components can all be part of an active Eox enhanceosome (Figs. 4C, 5F, Supplemental Fig. 3B), our data obtained both in Scid.adh cells and thymocytes indicate that the main Jun component of these complexes is JunD. These results contrast with the relative abundance of Jun upon Scid.adh cell stimulation, as JunB is more abundant than JunD and c-Jun in stimulated cells (Fig. 3F). Similarly, our ChIP data obtained both in Scid.adh cells and thymocytes indicate that the main NFAT components correlating with active Eox enhanceosomes in vivo are NFAT2 and NFAT4, despite the fact that NFAT1 seems to be the most abundant NFAT.
component in these cells (11). These data indicate that a pressure must exist for the binding of JunD-containing AP-1 complexes, together with NFAT2 or NFAT4, to Eα to form an active multi-protein complex on Eα in DN3b/DN4/early proliferating DP thymocytes.

Although our Egr-1 ChIP experiments indicate strong binding to Eα (Figs. 4D, 5E), our in vitro data indicate that the binding of Egr-1 to Eα is very weak compared with the binding to CS. The strongest Egr binding site located within the Eα (Tα1 site II) exhibited only ~10% of the binding of Egr-1 to CS (Supplemental Fig. 2E). The presence of adjacent efficient binding sites for NFAT within the 5′ Tα1 and Tα1–Tα2 regions might compensate in vivo for weak Egr-1 binding because both factors could interact to form complexes with additional proteins to cooperate in regulating gene expression (77). In fact, NFAT sites I and II are immediately proximal for weak Egr-1 binding because both factors could interact to form complexes in early proliferating DP thymocytes.

Our analysis supporting the requirement for inducible factors for the activation of Eα has important novel implications about the functional regulation of this enhancer during T-lymphocyte development. Although our study has been limited to an analysis of the transcriptional activity of Eα, not its recombinational activity, as an indicator of enhancer function, germline Tcra transcription has an essential role in activating the pre-TCR together with adjacent NFAT sites, as well as the possible contributions of putative Egr binding sites within the Jα promoters, in the formation of an active Eα enhanceosome will be addressed in future experiments.

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Disclosures
The authors have no financial conflicts of interest.

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