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Cell-type-specific epigenetic marking of the *IL2* gene at a distal *cis*-regulatory region in competent, nontranscribing T-cells

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ABSTRACT

T-cells retain cell-type-specific programming for IL-2 inducibility through many rounds of division without being stimulated to transcribe the locus. To understand the layering of controls needed to poise this gene heritably for activation, we have used chromatin immunoprecipitation to map histone modifications across the murine *IL2* locus, from −10.2 through +0.25 kb, in induction-competent and incompetent cells. In highly inducible EL4 T-lineage cells, stimulation with PMA/A23187 induced strong acetylation of histone H3 and H4, in parallel with transcriptional activation, from −4.6 through +0.25 kb. However, dimethylation of histone H3/K4 was already fully elevated across the same restricted domain before stimulation, with little change after stimulation. RNA polymerase II binding, in contrast, was only found at the known promoter region after stimulation. Similar patterns of histone modifications were seen also in normal IL-2-inducible T-lineage cells. However, neither acetylated histone H3, H4 nor dimethylated histone H3/K4 marking was detected, with or without stimulation, in expression-incompetent cells (NIH/3T3 or Scid.adh). These results identify a discrete new domain of *IL2* regulatory sequence marked by dimethylated histone H3/K4 in expression-permissive T-cells even when they are not transcribing *IL2*, setting boundaries for histone H3 and H4 acetylation when the *IL2* gene is transcriptionally activated.

INTRODUCTION

Mammalian development makes extensive use of stem-like cells for different tissues, in which a specific range of developmental potentials is programmed for future use without being overtly expressed, a condition that can persist for long periods of time (1). Epigenetic chromatin marking could be involved in establishment and long-term maintenance of the latent gene expression capabilities that must exist in these cells. However, for most mammalian systems of developmental gene regulation in which chromatin configurations have been studied closely, changes in potential for expression of a gene have been tightly linked to changes in actual transcriptional activation of that gene, making the mechanisms difficult to disentangle. Chromatin modifications, such as acetylation of histones H3 and H4, and methylation of H3 on Lys-4 (H3/K4), are known to be correlated with 'open' chromatin, but in most cases studied this is also transcriptionally active chromatin [reviewed in (2,3)]. Epigenetic marking strictly correlated with latent activity has been analyzed only rarely (4–6). Here, we propose that the murine *IL2* gene can shed light on this more general problem in developmental molecular biology.

IL-2 is one of the important cytokines produced by mature T-cells in response to antigenic stimuli. *IL2* gene regulation is subject to dual control, since it acutely depends on combinatorial signaling through particular combinations of pathways, and can only be activated in a very limited spectrum of cell types [reviewed in (7–9)]. Although strongly induced by combinations of Ca^{2+}, Ras/MAP kinase and costimulatory signals, IL-2 transcription is transient and returns to an 'off' state in expression-competent cells between bouts of stimulation. For the most part, competence to express *IL2* is restricted to T-lymphoid cells and a few other hematopoietic cell types, and its expression is observed in certain immature stages (10,11) as well as in the antigen responses of mature T-cells. The regulatory basis for the activation dependence of *IL2* gene expression in T-cells is well-understood, with key roles for NF-AT, NF-κB/c-Rel and AP-1 factors interacting with constitutive regulators such as Oct-1 in a ∼300 bp promoter-proximal region [reviewed in (7,9,12,13)]. Additional regulators such as HMG box factors and the Ets factor

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GABPα may also play a role, through highly conserved sites just 100–200 bp further upstream (14,15). The binding of these factors in response to activation is correlated with changes in DNA methylation at a series of sites extending from the promoter to about −800 bp (16). However, the basis for *IL2* cell-type specificity is still obscure, since all of the known transcription factors used for its induction are expressed broadly in many cell types that cannot express the gene. Meanwhile, the great majority of naïve T-cells retain the potential to activate *IL2* expression even after long periods when the gene is transcriptionally silent. The cell-type-specific regulation of IL-2 presents one of the clearest known cases of dissociation between developmentally programmed competence to express a gene, and actual expression of that gene for the great majority of a cell's lifetime.

Transgenic studies have shown that the highly conserved 600 bp region that contains the proximal promoter-enhancer is not enough for *IL2* regulatory sequence activity in transgenic mice (17,18), raising the possibility that additional positive regulatory functions may be encoded elsewhere. Addition of less-characterized sequences to −2.0 to −2.7 kb (with respect to the *IL2* transcriptional start site) permit correct expression in a fraction of transgenic founder lines (19,20). While ectopic expression of these constructs is rare, the locus appears to require additional sequences to counteract silencing. Experiments with hybrid regulatory sequence constructs show that one of the functions needed can be supplied by the locus control region of the human *CD2* gene (21). We have therefore hypothesized that the basis of cell-type specificity might reside in a discrete regulatory function that controls *IL2* chromatin accessibility (7,14). If so, then the sequences through which this effect is mediated could be expected to improve the frequency and efficiency of expression of *IL2* regulatory sequence transgenes if these sequences were included.

Previously, we reported that a GFP transgene driven with 8.4 kb of *IL2* 5′-flanking sequence (8.4 kb *IL2*-GFP) displayed efficient, lineage-specific expression in transgenic mice, much improved in frequency and fidelity as compared to a 2.0 kb *IL2*-GFP transgene (20). Thus, the additional 6.4 kb upstream sequence contains important regulatory regions for conferring accessibility to induction on the *IL2* gene. Here, we use analyses of the chromatin structure in these *IL2* upstream regions to investigate the nature of the regulatory function that they confer.

To analyze features of the distal sequence, we performed chromatin immunoprecipitation (ChIP) assays to define the nature and extent of any associated histone modifications. Here, we report significant levels of histone acetylation (AcH3, AcH4) and histone H3 lys-4 dimethylation (diMeH3/K4) in a discrete region from −4.6 kb through the promoter and first exon of the *IL2* gene in IL-2 producing T-cells. Whereas acetylation was seen only in cells transcribing the gene in response to stimulation, diMeH3/K4 is maintained at a high level across the same region in cells competent to express *IL2*, even before stimulation. These results indicate the presence of transcriptionally permissive state which is marked by diMeH3/K4 without histone acetylation over a discrete 5′-flanking domain of the *IL2* gene, and which is restricted to competent IL-2-inducible cells.

MATERIALS AND METHODS

Cell culture and stimulations

EL4.F4 cells (22,23), Scid.adh cells (24), NIH/3T3 cells and splenocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, South Logan, UT), 50 μM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acids (Invitrogen, Carlsbad, CA), 10 mM HEPES, 1 mM sodium pyruvate (Sigma–Aldrich, St Louis, MO), 2 mM L-glutamine and penicillin/streptomycin.

Cells were stimulated for 3 h with 10 nM PMA and 180 nM A23187 (Sigma–Aldrich). Cell concentrations were 10^6 cells/ml of non-adherent cells and were semi-confluent of adherent cells. For stimulations of TCR-negative SCID thymocytes, IL-1α (10 ng/ml; Biosource, Camarillo, CA) was also required in the stimulation cocktail (25–27), but IL-1α has no effect on responses of $CD4^+$ $CD8^+$ cortical thymocytes, as previously described (28).

Purification of $B220^-$ splenocytes and thymocytes from SCID and MHC-deficient mice

Red-blood cell depleted splenocytes from C57BL/6 mice were stained with biotin-conjugated anti-B220 Ab (Immunotech, Marseille, France), and then incubated with streptavidin-conjugated MACS microbeads (Miltenyi Biotech, Auburn, CA). Cells were passed through MACS columns to remove $B220^+$ cells. Purity of separated cells was determined by staining with FITC-conjugated anti-B220 Ab (BD PharMingen, San Diego, CA). Thymocytes were prepared by mincing the tissue followed by filtration through a wire mesh.

Chromatin immunoprecipitation assay

Antibodies for ChIP assays were purchased from the following companies. Anti-acetyl-histone H3 (06-599), anti-acetyl-histone H4 (06-866) and anti-dimethyl-histone H3 (Lys-4) (07-030) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Pol II (N-20) (sc-899) and normal rabbit IgG (sc-2027) were from Santa Cruz Biotechnology (Santa Cruz, CA).

ChIP assays were performed by the protocol of Upstate Biotechnology with modifications. Briefly, samples of 2×10^7 cells were subjected to protein–DNA crosslinking with formaldehyde at a final concentration of 0.4% for 10 min at room temperature. Cells were lysed in 1 ml of lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris–HCl, pH 8.0) and DNA was sheared by sonication (Labsonic 1510, B. Braun, Melsungen, Germany) at 100 W for 1 min, to lengths between 200 and 1000 bp.

One hundred microliters of sonicated sample was kept as an input sample, and the remainder was diluted with 8.1 ml of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0 and 167 mM NaCl). Diluted samples were precleared with 100 μl of salmon sperm DNA/protein A agarose (Upstate Biotechnology) for 1 h, then divided into three tubes (3 ml each).

Ten micrograms of antibodies were added to each tube and incubated for 3–4 h. After that, 60 μl of salmon sperm DNA/protein A agarose was added and incubated overnight at 4°C. Agarose was washed once with 1 ml of low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl,

pH 8.0 and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0 and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA), and twice with TE buffer (pH 8.0). Immune complexes were eluted twice with 250 µl of elution buffer (1% SDS and 0.1 M $NaHCO_3$). Protein–DNA crosslinking was reversed by incubation at 65°C for 4 h with 0.2 M NaCl, and then protein was digested at 45°C for 1 h in the presence of 10 mM EDTA (pH 8.0), 40 mM Tris–HCl (pH 6.5) and 40 µM proteinase K. The DNA was purified by phenol–chloroform extraction followed by ethanol precipitation.

Quantitative PCR analysis

Quantitations of chromatin immune precipitates were performed by real-time PCR using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primers were designed by the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (29) to amplify 100–250 bp amplicons. PCR analyses were performed in 25 or 30 µl reactions with SYBR Green (PE Applied Biosystems). All primer sets used gave no signal ($C_T > 40$) in control reactions without template. Dissociation curve analyses showed that single products with expected T_m values were generated by each primer set. A second peak of lower T_m was detected only in products of one primer set (−8.9 to −8.7), or as a shoulder in products of one other set (−3.2 to −3.0).

Standard curves were determined for each primer set by dilution of input DNA in a 0.1–100 ng range, and only primer sets yielding slopes close to the theoretical values for 1.9- to 2.0-fold amplification per cycle were used. The amounts of each sequence in the input and precipitated DNA were calculated from the cycle thresholds (C_T) for each primer set by using the standard curves. Relative units recovered for each primer set were determined by dividing the calculated amounts of precipitated sequence by the amount of that sequence in the input DNA. Due to lower cell recovery of primary thymocytes, results in Figure 7 were further normalized by the results of the S16 ribosomal protein gene.

RNA purification and real-time quantitative RT–PCR

Total RNA was extracted from the cells by RNAzol (Leedo Medical, Houston, TX) following the manufacturer's instructions. One microgram of RNA was treated with RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. After that, DNase was inactivated by the incubation for 5 min at 75°C. Then, first-strand synthesis reactions were performed with Super-script II reverse transcriptase (Invitrogen, Carlsbad, CA) and 500 ng oligo dT primers (Pharmacia, Uppsala, Sweden) following standard protocols.

Real-time PCR was performed by using ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Taqman probes and primers for murine IL-2 (FAM labeled) and GAPDH (VIC labeled) were purchased from PE Applied Biosystems. To normalize the samples, control GAPDH C_T values were subtracted from IL-2 C_T values of each sample (ΔC_T). Then, ΔC_T of the unstimulated NIH/3T3 sample (reference sample) was subtracted from ΔC_T of each sample ($\Delta\Delta C_T$). Relative levels of IL-2 mRNA were calculated as $2^{-\Delta\Delta C_T}$.

RESULTS

Stimulation with PMA/A23187 induces histone acetylation at the *IL2* locus in IL-2 producing cells only

Several lines of evidence suggested that regulatory elements important for cell-type-specific accessibility of the *IL2* gene might reside on the 5′ side of the well-characterized promoter-enhancer region. First, a series of T-cell-specific DNase I hypersensitive sites were found between −10 and −2 kb from the transcriptional start site of the *IL2* gene, including a cluster of cell-type-specific sites from about −2.8 to −4.5 kb which were hypersensitive even in resting cells (20). Second, a GFP reporter transgene controlled by an extended 8.4 kb upstream region of the *IL2* gene, which includes most of the new hypersensitive sites, showed more efficient, lineage-specific expression in transgenic mice than a GFP transgene controlled by a 2.0 kb *IL2* regulatory region, which does not include any of the new hypersensitive sites (20). Most recently, comparative genomics have made it possible to identify three islands of mouse–human sequence similarity upstream of the *IL2* gene, as indicated in Figure 1 (for full three-way alignment of murine, human and rat sequences, see Supplementary Figure 1). Two of these islands lie within the region included in the 8.4 kb *IL2* promoter transgene and one lies just beyond it.

Although indicating some functional significance for the region between −2.0 and −8.4 kb, the transgenesis results did not determine whether the sequences in this region act as auxiliary stimulation-dependent enhancers, amplifying the response upon induction, or whether they mark the locus stably as accessible to induction by a separate mechanism uncoupled from transcription. To determine which regions of the extended *IL2* upstream region might participate in control of IL-2 expression, we used ChIP with quantitative real-time PCR quantitation. Although regions enriched for simple-sequence repeats needed to be excluded from analysis, we designed 17 primer sets to amplify segments from the first transcribed exon up to −10 kb of the *IL2* gene (Figure 1 and Table 1), including amplicons bridging all three of the conserved regions. To correlate chromatin structure with competence to express IL-2, the murine thymoma cell line EL4, the immature pro T-cell line Scid.adh and the non-hematopoietic cell line NIH/3T3 were subjected to ChIP assay with or without PMA/A23187 stimulation. Of these, EL4 is the only one which expresses IL-2 in response to stimulation (Figure 2A). However, in unstimulated EL4 cells, the *IL2* gene is virtually silent, with levels of RNA $>10^5$-fold lower than after 3 h of stimulation (note log scales in Figure 2).

In resting EL4 cells, acetylation of histones H3 and H4 was not substantially elevated across the *IL2* locus, and the recovery of all *IL2* flanking sequences was low compared to the positive control. However, acetylation was greatly increased after 3 h of stimulation with PMA/A23187, in a defined interval across the sequences from −4.6 through +0.25 kb. By this time point, specific transcription factor occupancy of target sites in the enhancer is already maximal, as we have reported previously (30). The acetylated region included two islands of mouse/rat/human conserved sequence, at about −2.2 and at about −3.9 kb, but excluded the third, more distal conserved region at about −8.6 kb and all other regions tested out to −10.2 kb.

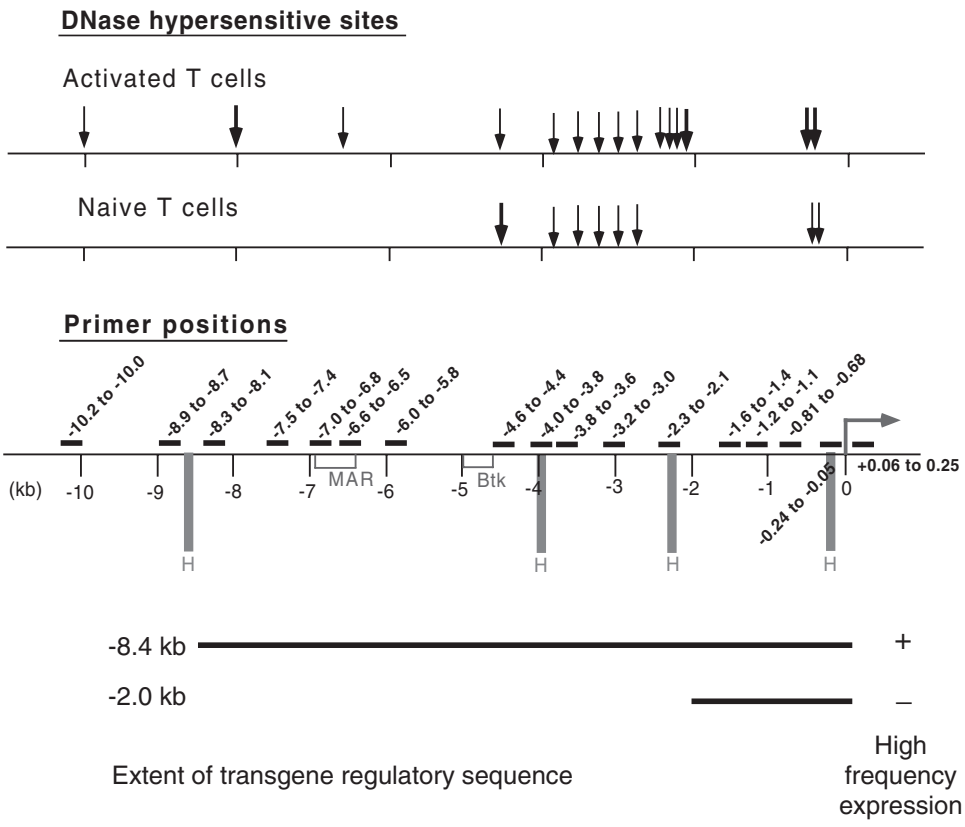

Figure 1. Diagrams of DNase hypersensitive sites and primer positions on the IL2 locus. DNase hypersensitive sites in stimulated and unstimulated purified splenic T-cells are shown by arrows (↓). Regions of high homology with an upstream sequence in the human *IL2* gene were determined by Blast search (H). Putative matrix attachment region (MAR) and a region of high homology with an intronic sequence in the Bruton's thymidine kinase gene (Btk) are also indicated. For full alignment of these murine *IL2* sequences with human and rat orthologues using FamilyRelations and SeqComp software (46), see Supplementary Figure 1. Bottom: the functional importance of these sequences is demonstrated by the distinct behavior of *IL2* regulatory sequence transgene constructs previously reported by this group (20). The sequences to −8.4 kb, but not the sequences to −2.0 kb, are sufficient to direct *IL2*-like expression of the reporter gene in a high frequency of independent integration sites (20).

Unexpectedly, within the region from −4.6 through +0.25 kb, the sequence that was least enriched in acetylated H3 and H4 immune precipitates after stimulation was the amplicon from the *IL2* proximal promoter region itself (−0.24 to −0.05 kb) (Figure 3A; top). Since the proximal promoter is known to be a core region for IL-2 transcription factor binding which undergoes dramatic changes in nuclease accessibility upon stimulation (14,30–34), the regulatory protein complexes that are assembled there upon induction might exclude nucleosomes or mask epitopes of acetylated histone. A similar result has been reported for active promoters in the β-globin and prostate-specific antigen loci (6,35). In accordance with our results, a recent report has demonstrated directly that stimulation causes specific loss of histone protein from the proximal promoter region of the *IL2* gene (36).

Acetylation at the *IL2* locus appears to be regulated normally by an equilibrium between histone acetyltransferase and histone deacetylase (HDAC) activities, because exposure of the cells to HDAC inhibitor trichostatin A increases acetylation of H3 and especially H4, even in the absence of stimulation (Supplementary Figure 2A). This increased acetylation is not associated with transcriptional induction; in fact, trichostatin A decreases the induction of IL-2 in response to stimulation under the conditions used here (37) (Supplementary Figure 2B). Even so, most increased acetylation induced by trichostatin A is seen in the region between about −5 kb and the promoter region, where the stimulation-inducible acetylation occurs (Supplementary Figure 2A).

The IL2-nonproducing cell lines, Scid.adh and NIH/3T3, maintained very low levels of acetylation of the *IL2* gene flanking sequences with no increase upon stimulation (Figure 3A; middle and bottom); however, the acetylation of a sequence from the S16 ribosomal protein promoter was comparable between all these three cell lines (Figure 3B). Controls without antibody or with normal IgG precipitation did not detectably precipitate *IL2* sequences in any of these cell lines (Supplementary Figure 3). Thus, there is a specific *IL2* upstream region extending to −4.6 kb, i.e. >4 kb beyond the well-characterized proximal promoter-enhancer, which is subject to histone acetylation only upon stimulation and only in IL-2 induction-competent cells.

Dimethylation of histone H3/K4 at the *IL2* upstream region before stimulation in EL4 cells

To assess whether there is any other kind of modification at the *IL2* locus that might mark it as accessible, we monitored

Table 1. Sequences of the primers

−10.2 to −10.0	5′-ACCTTGGGAGCTGAAATCCT
	5′-TTTTGAGGGATCGCTAATGG
−8.9 to −8.7	5′-TGTTTTAGCCCACAAGATTT
	5′-GAGTGGCAATAGAAAAATGC
−8.3 to −8.1	5′-TGACAATGCTGGCTTCTGAC
	5′-CAAGCAAAACGATCCAAGAG
−7.5 to −7.4	5′-TACCTATGGAAGAAGTTACA
	5′-AATCTTGTTGGCATCTGCAA
−7.0 to −6.8	5′-GGAGGGATAGGGGATTTTCA
	5′-TGGTTCTTACAAAACCACTGAGC
−6.6 to −6.5	5′-GTGCTCTTAAGTGATCAGTA
	5′-GAAGTGCATATGTGTATGTA
−6.0 to −5.8	5′-TTAGGCCACCCATGGTACTC
	5′-ACAAAGAGGCCCATTCTCCT
−4.6 to −4.4	5′-TGGGCTAGATTTCCATAAGAACA
	5′-TGTCAAAAGCACCCTTTTCC
−4.0 to −3.8	5′-CCACCACACCCAGCTTTTAT
	5′-CCTTTCAAGCCCTGAGGATT
−3.8 to −3.6	5′-CTCAGGGCTTGAAAGGACAG
	5′-TGCCTCAGGACATTCCTACA
−3.2 to −3.0	5′-CAGATGGGACGAGAGAGAGG
	5′-ACAGCTGACTCCGACTTGGT
−2.3 to −2.1	5′-AGCCCATGAGGACATCAAAG
	5′-CGTGAGGGGCATCTACCTAA
−1.6 to −1.4	5′-ACCCACAGTGTGCATGTAGC
	5′-AGCCTCACCACACACCTACC
−1.2 to −1.1	5′-CACGGAGGATAAGGAATCCA
	5′-GCCTTTACCACTGAGCCATC
−0.81 to −0.68	5′-CACGCACATGCACATACTCA
	5′-TCTCAGGAAGGGTTGCATCT
−0.24 to −0.05	5′-GCCACCTAAGTGTGGGCTAA
	5′-ATATGGGGGTGTCACGATGT
+0.06 to 0.25	5′-CTCGCATCCTGTGTCACATT
	5′-GGATGGCTGTGCACTTACCT
S16 ribosomal protein promoter	5′-ATTCACACGTCCCCTCTCAG
	5′-ATAGCTCCGAGCACAAGCAC

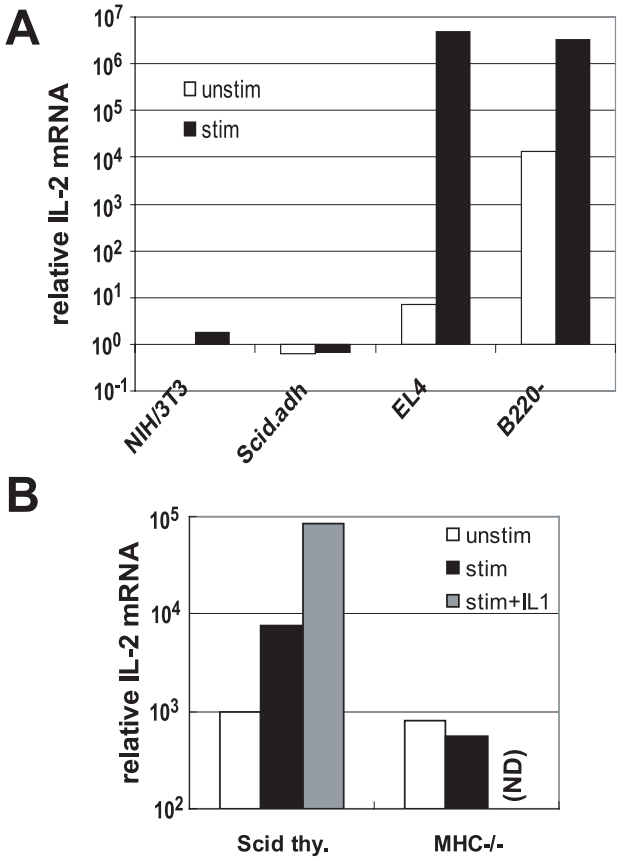

Figure 2. Quantitative RT–PCR of IL-2 mRNA induction in different cell types. RNA was extracted from the indicated cell types, either unstimulated or stimulated with PMA/A23187 for 3 h. IL-2 mRNA levels were determined in the samples by real-time PCR following reverse transcription, and normalized to equal GAPDH levels. Units of IL-2 mRNA expression were then calculated as $2^{-\Delta\Delta C_T}$ relative to an unstimulated NIH/3T3 sample ($2^0 = 1$). (**A**) Cell lines NIH/3T3 (fibroblasts, nonhematopoietic), Scid.adh (pro-T-like thymic lymphoma), and EL4 (mature T-cell thymic lymphoma), compared with primary $B220^-$ splenic lymphocytes (mature T-cells). Levels of IL-2 in stimulated EL-4 and $B220^-$ splenic T cell samples are within 2- to 3-fold of the levels of GAPDH (data not shown). (**B**) Primary immature pro-T cells from SCID thymus, compared with primary $CD4^+$ $CD8^+$ TCR-low cortical thymocytes from MHC-deficient thymus. For SCID thymocytes, stimulation was only for 2 h, in the presence or absence of IL-1α as indicated. Levels of IL-2 in maximally stimulated SCID thymocytes are ∼1% of the level of GAPDH. Note that all results are displayed on a log scale. ND = not determined.

dimethylation of histone H3/K4, which is also associated with active chromatin and often accompanies acetylation (6,38–41). Like AcH3 and AcH4, diMeH3/K4 was highly associated with sequences from −4.6 through +0.25 kb in EL4 cells after stimulation (Figure 4A; top). However, unlike acetylation, a significant level of diMeH3/K4 was observed from −4.6 through +0.25 kb before stimulation as well, comparable to its level after stimulation. In unstimulated EL4 cells, this modification could also be seen to extend through the proximal promoter (−0.24 to −0.05 kb), although it appeared somewhat decreased after stimulation. In the Scid.adh and NIH/3T3 cells, in contrast, which cannot express IL-2, no association of diMeH3/K4 with the *IL2* gene was observed, with or without stimulation (Figure 4A; middle and bottom). These differences were specific to the *IL2* gene, because diMeH3/K4 modification of chromatin associated with the S16 ribosomal protein promoter was comparable in the three cell lines (Figure 4B).

RNA polymerase II is not bound to the distal region of the *IL2* gene

Both AcH3, H4 and diMeH3/K4 modifications extend >4 kb from the known promoter-enhancer of the *IL2* gene. We considered possible explanations for the relatively sharp 5′ border and the broad extension of the modified region all the way through the promoter-enhancer. In some cases, pol II itself is recruited to a distal regulatory region (42), from which it can spread acetylated histone in a 5′ to 3′ polar fashion as it moves along the gene [e.g. (41,43)]. Therefore, we analyzed pol II binding on the *IL2* gene in EL4 cells by ChIP assay. As expected, pol II immune precipitation of stimulated EL4 cell samples enriched for promoter proximal sequences (amplified with primers from −0.24 to −0.05 kb), with further enrichment of sequences immediately downstream of the transcriptional start site (+0.06 to +0.25 kb). Pol II recruitment was correlated with transcriptional activation, as little or no enrichment was seen in samples from unstimulated cells. However, even in stimulated EL4 cells, only minimal binding was found from −4.6 through +0.25 kb, or in more distal 5′ sequences (≤20% of promoter level) (Figure 5). Thus, no evidence for specific pol II recruitment was found in any of the upstream regions examined.

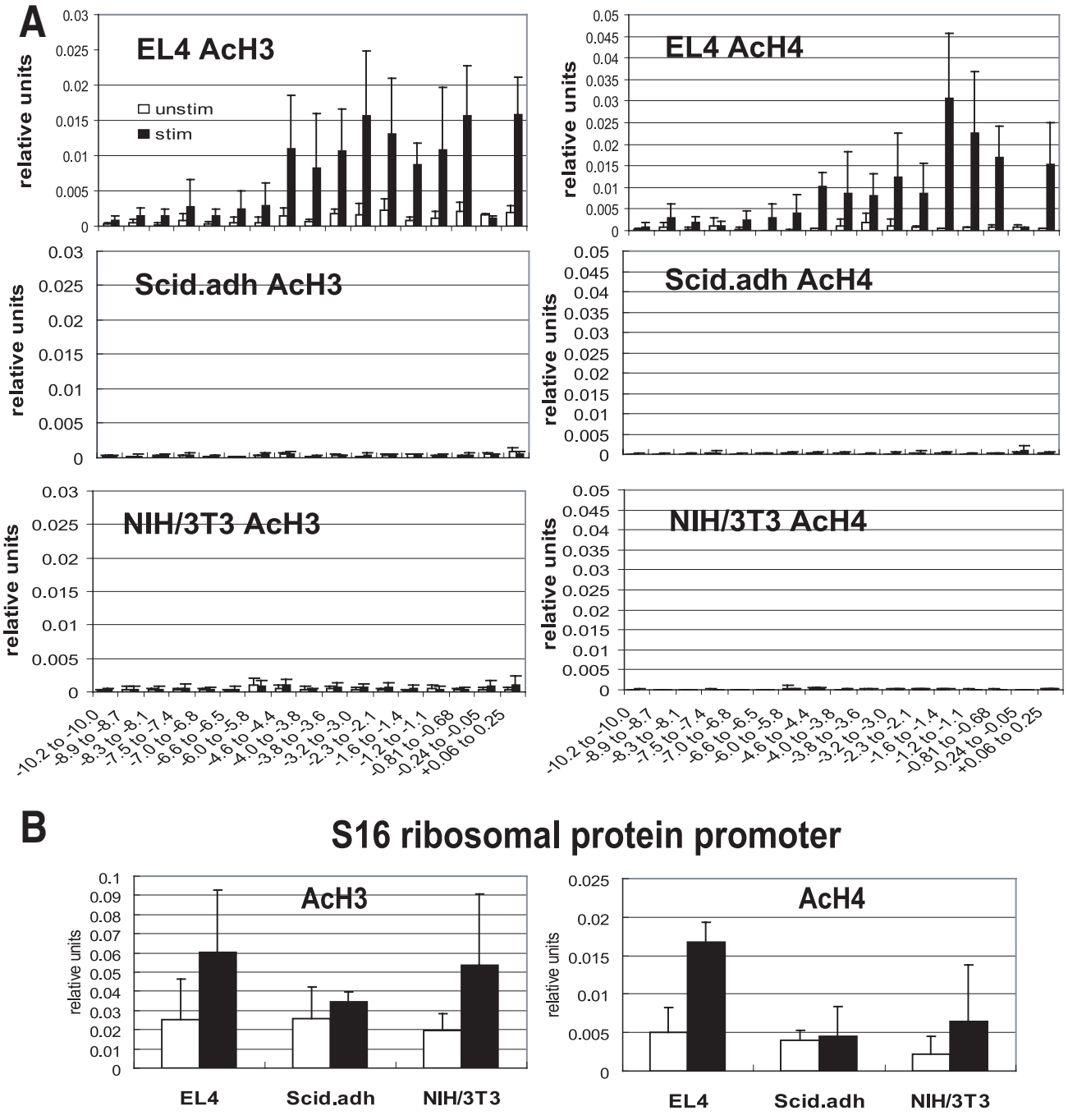

Figure 3. Histone acetylation pattern of the *IL-2* gene. (**A**) Acetylated histone H3 and H4 of the *IL-2* gene were analyzed with quantitative ChIP assay. Results of EL4, Scid.adh and NIH/3T3 cells are shown. (**B**) Acetylated histone H3 and H4 on the promoter of S16 ribosomal protein. The results are shown as mean ± SD of three or four independent experiments. Open bars; unstimulated, filled bars; stimulated with PMA/A23187.

Histone modifications at the *IL2* locus in primary T-cells resemble those in EL4 cells

To determine whether the distinctive patterns of chromatin modifications found in EL4 cells could be relevant to normal T-cell control of IL-2 expression, we examined three developmentally distinct populations of primary T-lineage cells: mature, peripheral splenic T-cells; cortical TCR^{low} $CD4^+$ $CD8^+$ thymocytes (>95% of cells in MHC-deficient thymus); and immature TCR^- SCID thymocytes. As previously reported (10,25,44), both mature peripheral T-cells and SCID thymocytes can be stimulated to express IL-2 (Figure 2A, 'B220-'; Figure 2B, 'scid thy'), but the cortical thymocytes, although developmentally intermediate, cannot (Figure 2B, '$MHC^{-/-}$').

ChIP assays for AcH3 and diMeH3/K4 were performed on samples of stimulated or unstimulated peripheral T-cells, which were highly enriched from C57BL/6 mouse spleens by

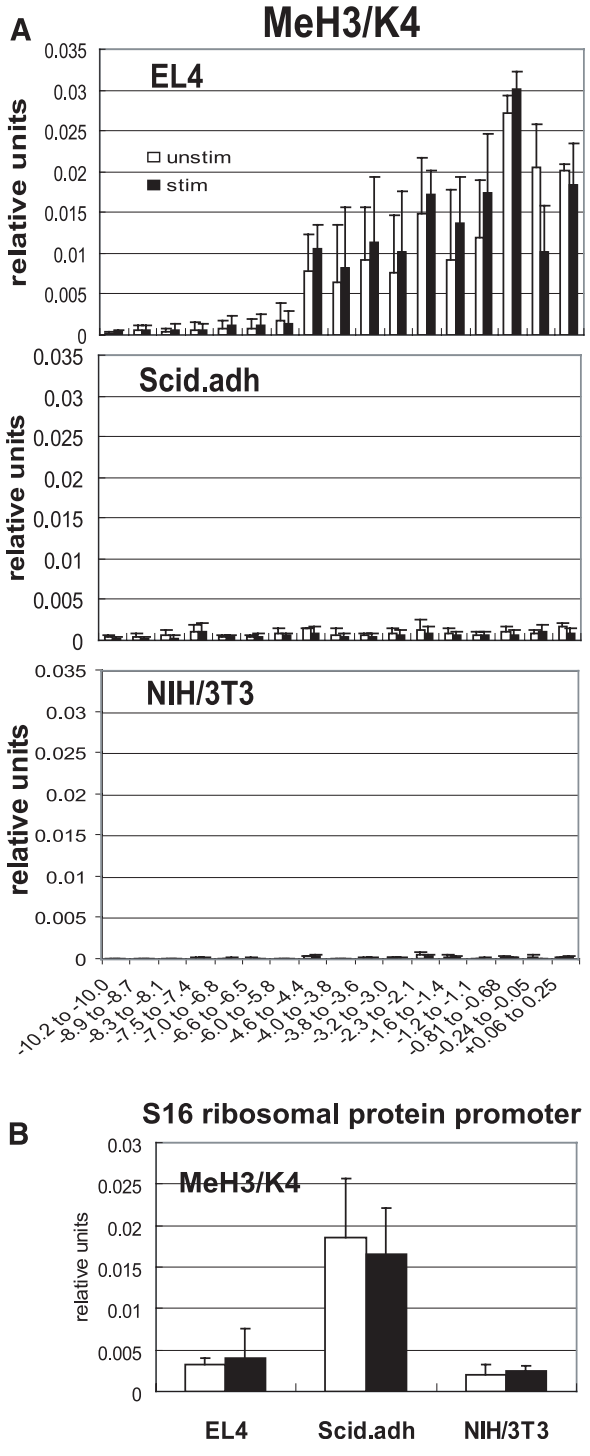

Figure 4. Dimethylated histone H3 at lys-4 is observed before stimulation in EL4 cells. (**A**) ChIP analyses were performed for diMeH3/K4 on the *IL2* locus of EL4, Scid.adh and NIH/3T3 cells. DiMeH3/K4 on the promoter of S16 ribosomal protein is shown in (**B**). The results are shown as mean ± SD of three independent experiments. Open bars; unstimulated, filled bars; stimulated with PMA/A23187.

magnetic bead depletion of $B220^+$ cells, as shown in Figure 6A. In these cells, AcH3 was increased from −4.6 kb through the IL-2 promoter after stimulation, while diMeH3/K4 was high from −4.6 kb to downstream both before and after stimulation

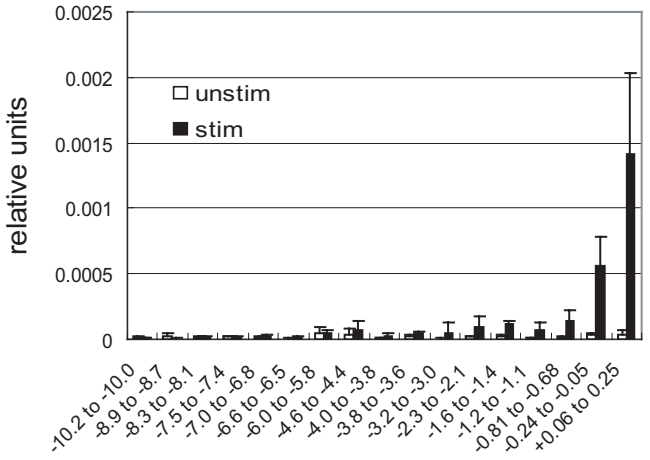

Figure 5. Binding of RNA polymerase II on the *IL2* locus. ChIP analyses were performed for pol II binding on the *IL2* locus of EL4 cells. The results are shown as mean ± SD of three independent experiments. Open bars; unstimulated, filled bars; stimulated with PMA/A23187.

(Figure 6B, $B220^-$ cells). These patterns in stimulated and unstimulated primary T-cells closely match those in EL4 cells. The maximum increase of AcH3 upon stimulation in $B220^-$ cells was only 4- to 5-fold, as compared to >10-fold in EL4 cells (Figure 3A; top), but this may be attributed to known quantitative differences between the frequencies of IL-2 induction in unstimulated primary T-cells and in EL4 cells (Figure 2). The fraction of primary T-cells expressing IL-2 mRNA in a particular response is never as high as the fraction of EL4 cells, which can approach 80% (E. V. Rothenberg, unpublished data), whereas the background level of IL-2 mRNA expression in unstimulated $B220^-$ cells *ex vivo* is often higher than that of unstimulated EL4 cells, possibly due to a low frequency of cells that received some stimulation *in vivo* already (45). Thus, these results indicate that chromatin modification of the *IL2* locus from −4.6 kb through the promoter region may be a stable characteristic of normal mature T-cells.

Dimethyl H3/K4 marking correlates with competence to express in immature T-lineage cells

To determine how early in T-lineage development this chromatin modification might be detectable, SCID thymocytes were analyzed. Although SCID thymocytes cannot rearrange or express T-cell receptor genes and thus remain immature, they are inducible for IL-2 expression (Figure 2B) (up to 20% of the cells capable of expressing IL-2 mRNA in immediate response to stimulation in C.B17-SCID) (26,27). In two independent experiments, even without stimulation, SCID thymocytes showed high association of diMeH3/K4 with the region from −4.6 kb through the promoter region (Figure 7, top graph). Thus, both immature and mature T-cells specifically modify chromatin in a defined region upstream of the *IL2* promoter.

The shared pattern of epigenetic marking in mature and very immature populations could be strictly correlated with competence to express IL-2, or it could be a lineage marker for all normal T-lineage cells. To distinguish these possibilities, we

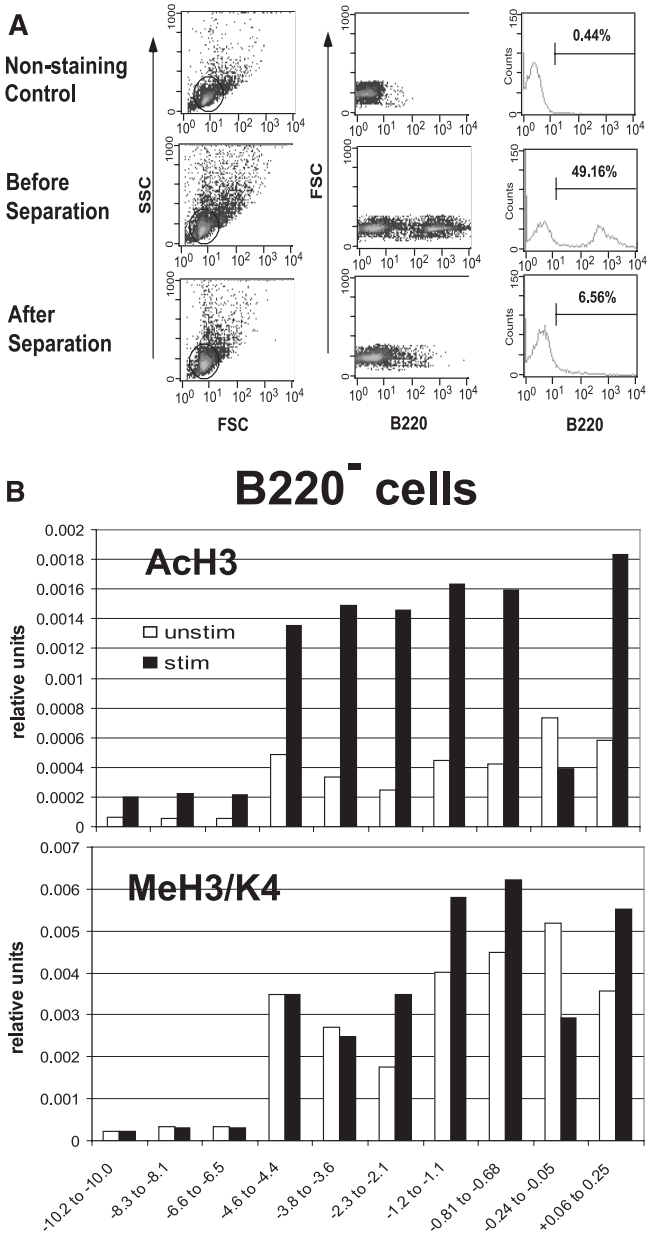

Figure 6. Pattern of acetylated histone H3 and dimethylated histone H3 at lys-4 at the *IL2* locus in an enriched population of mature splenic T-cells. B220^{+} cells were depleted from C57BL/6 mouse splenocytes by magnetic columns (**A**), and the remaining B220^{-} cells (T-enriched) were subjected to ChIP assay (**B**). Two independent experiments were done with similar results. Open bars; unstimulated, filled bars; stimulated with PMA/A23187.

analyzed intermediate CD4^{+} CD8^{+} TCR-low thymocytes, which constitute >95% of the cells in the thymus of major histocompatibility complex-deficient mice (MHC$^{-/-}$). These cells are more advanced than SCID thymocytes developmentally, but have lost or suspended all competence to express IL-2 (10,28,44). As shown in Figure 7 (bottom graph), these cells show much lower levels of diMeH3/K4 modification over the *IL2* flanking sequences, relative to S16 positive control sequences, than EL-4 cells, mature T-cells or SCID thymocytes. The pattern is also flat, with little if any increase in modification over the region from −4.6 kb to the promoter.

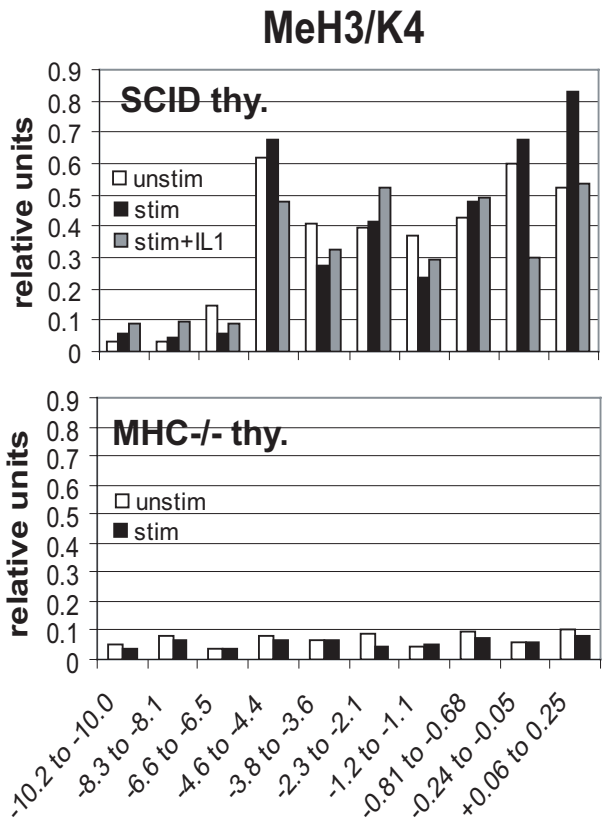

Figure 7. Dimethylated histone H3/K4 association with *IL2* 5′-flanking region is correlated with IL-2 inducibility in immature thymocytes: ChIP analysis of the *IL2* locus in thymocytes from SCID and MHC-deficient mice. Cells from MHC-deficient mice, >95% CD4^{+} CD8^{+} TCR-low, were cultured with or without PMA/A23187 for 3 h. Cells from SCID mice, >95% CD4^{-} CD8^{-} TCR-negative, were cultured with or without stimulation for 2 h, and IL-1α was also added as indicated. To correct for differences in efficiency of immune precipitation between these samples due to the extensive differences in cell physiology, ChIP analysis results for diMeH3/K4 at the *IL2* locus for each sample were normalized by results at the S16 ribosomal protein locus. Two independent experiments were done with similar results.

Thus, diMeH3/K4 modification over the region from −4.6 kb to the *IL2* promoter is not universal among all T-lineage cells, but is tightly linked to the ability to express IL-2.

In summary, as shown in Figure 8, both diMeH3/K4 and AcH3 types of modifications of the *IL2* flanking sequences are cell-type specific. Both are restricted to T-lineage cells that are competent to express IL-2 and not found in fibroblasts or T-lineage cells that lack this competence. However, histone acetylation is tightly linked with the recruitment of active transcription complexes to the *IL2* promoter. Only diMeH3/K4 marking is a stable differentiated characteristic of these competent cells that is retained at the locus as long as they retain their developmental state, *in vivo* and *in vitro*, even in the absence of activating signals and in the complete absence (<10^{-5} of the induced levels) of IL-2 mRNA expression.

DISCUSSION

The basis for competence to induce transcription of the *IL2* gene in particular cell types has long been obscure, in contrast

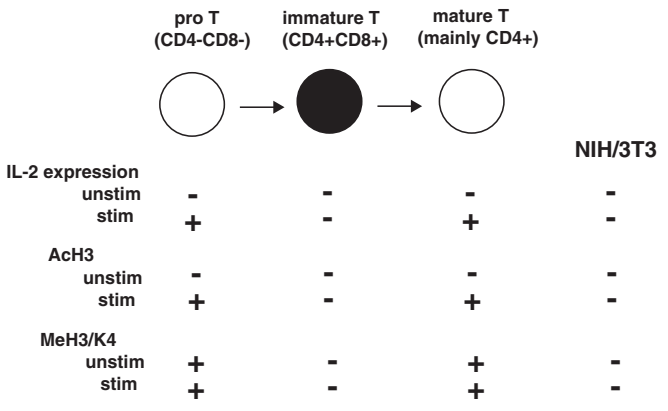

Figure 8. Changes of histone modifications at the *IL2* gene during T-cell differentiation. The correlation between IL-2 expression and histone modifications from −4.6 through +0.25 kb of the *IL2* gene is summarized.

to the detailed knowledge that has been accumulated about the mechanism that actually triggers transcription of this locus in competent cells. Previously, we showed that sequences of the *IL2* gene between −2 kb and −8.4 kb (extended 5′-flanking sequence) could be involved in such a function, since they greatly enhance the likelihood of expression of an *IL2* promoter-GFP transgene in the correct cells (20). The function conferred was not defined, however, and the sequences involved in the function were not mapped. Here, we report that a particular segment of the extended 5′-flanking sequence is a site for epigenetic marking by histone H3/K4 dimethylation that occurs only in IL-2 expression-competent cells. This modification is cell-type specific, tightly linked with competence of the *IL2* locus to be induced by stimulation, and coincides with the region in which increased AcH3 and AcH4 modifications occur when transcription is activated (Figures 3, 4 and 6). However, this H3/K4 methylation is uncoupled from detectable *IL2* transcription, from pol II binding, and from histone acetylation, and it persists independently of stimulation. Our data provide the first direct evidence for the existence of a discrete, developmentally controlled mechanism that may control cell-type-specific accessibility of the *IL2* locus, working through different sequences and different *trans*-acting factors than those that activate transcription of the gene in response to induction.

The relationship between H3/K4 methylation and IL-2 expression supports the model that sequences between −2 and −8.4 kb can have a discrete role in conferring cell-type-specific accessibility on this gene that is separable from any enhancer activity. Indeed, the 8.4 kb *IL2* regulatory region does not drive reporter gene expression any better than the 2.0 kb regulatory region, when tested in transient transfection experiments (20). Two groups of DNase HS sites, centered around different mouse/human homology blocks, suggest that the upstream region may include two types of function. The distal portion of the H3/K4 methylation-marked region, from −4.6 to about −2.8 kb, includes the group of DNase I hypersensitive sites which are most pronounced in resting, not activated, T-cells. Activation of IL-2 transcription induces the appearance of new DNase HS sites, including a cluster around the mouse/human homology block from about −2.2 to −2.3 kb as well as in the promoter-proximal region (14,20). This −2.2 kb region may indeed have cryptic enhancer-like activity. However, activation if anything reduces the prominence of the major DNase hypersensitive site around −4.5 kb (20), at the distal boundary of the MeH3/K4 marked domain. A clear inference would be that any transcription factors binding to regulatory sites in the −2.8 to −4.6 kb upstream region would be distinct from these stimulation-activated transcription factors.

Identification of the critical regulatory elements in this interval is impeded by the remarkably poor sequence conservation between human and murine sequences beyond −2.3 kb in the mouse sequence. When the human, mouse and rat *IL2* flanking sequences are aligned (46) (Supplementary Figure 1), the only well-conserved sequence from −2.8 to −4.6 kb is a ∼64 bp stretch at about −3.9 kb in the murine gene (in the human gene, a LINE element insertion shifts this alignment further upstream). This conserved element should thus provide a useful point of departure for identification of the factors which control the establishment or maintenance of the MeH3/K4 marked domain.

Only minimal histone modifications were seen further upstream of −4.6 kb, with or without stimulation. It remains to be seen whether known boundary-forming transcription factors such as CCCTC-binding factor (CTCF) or the bHLH-ZIP 'Upstream Factor' (USF) play a role in setting these limits. Interestingly, there are several E-box motifs which might bind USF between −8.0 and −8.4 kb, and a predicted double binding site around −6.2 kb. A recent article shows that USF binds to the chicken β-globin 5′HS4 insulator, where it protects the gene from chromosomal silencing by recruitment of histone modification factors (47). It may play a role to keep the *IL2* gene accessible by the same mechanisms. Closer to the region where we see discontinuities in *IL2* chromatin structure, there is an unusually dense clustering of seven CCCTC motifs between −4.5 and −5.3 kb from the transcription start site. Although functional target sites of CTCF can be difficult to predict based on sequence alone (48), these sites will clearly be of interest for further investigation.

The mechanism maintaining the MeH3/K4-marked *IL2* 5′-flanking region in unstimulated cells is clearly distinct from the highly coordinated assemblies of transcription factors, histone acetyltransferases and RNA pol II which are seen associated with 'open chromatin' in other sites (42,43,49,50). In the case of the *IL2* gene, a stable pattern of H3/K4 methylation appeared to be maintained even in the absence of any substantial pol II binding to the region between −4.6 kb and the promoter. The minimal level of binding that was seen could be consistent with co-precipitation by interaction of distal regulatory regions with the proximal promoter via looping, rather than with direct binding. The *IL2* gene thus joins a small number of cases where diMeH3/K4 is associated with accessibility to transcription before transcription itself is induced. In some cases, this modification appears to demarcate the boundaries of a region within which transcription and/or DNA recombination can occur (40,51). Recently, a two-step chromatin modification sequence, with diMeH3/K4 modification preceding histone acetylation, was shown in a variety of genes activated during hybridization between ES cells and somatic cells (52). Even if this sequence is used for regulation of many genes, the regulatory style may differ for each gene.

For example, in the activation of *HNF-4α* gene concomitant with the enterocyte differentiation, diMeH3/K4 is restricted to the promoter region through differentiation while acetylated histones develop from the distal enhancer toward the promoter (49) And at the *IgH* locus in pro B-cells, peaks of diMeH3/K4 are observed at the borders of active DJ regions and inactive V and C regions (51). For immune regulatory cytokines, such as IL-2, maintaining a transcriptionally permissive stage which is marked by long-range diMeH3/K4 modification alone may be important to preserve the potential for immediate gene activation in response to precisely calibrated external stimuli.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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