Intergenic Transcription Is Not Required in Th2 Cells to Maintain Histone Acetylation and Transcriptional Permissiveness at the \textit{II4-II13} Locus

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Intergenic Transcription Is Not Required in Th2 Cells to Maintain Histone Acetylation and Transcriptional Permissiveness at the \textit{Il4-Il13} Locus

Aurelie Baguet,$^{2,\ast}$ Xizhang Sun,* Thomas Arroll,$^{3,\ast}$ Anton Krumm,$^{\dagger}$ and Mark Bix$^{4,*}$

Noncoding RNA transcripts mapping to intergenic regions of the \textit{Il4-Il13} locus have been detected in Th2 cells harboring transcriptionally permissive \textit{Il4} and \textit{Il13} genes but not in Th1 cells where these genes are repressed. This correlation has given rise to the idea that intergenic transcription may be involved in maintaining the “open” chromatin structure of the \textit{Il4-Il13} locus in Th2 cells. We present evidence from real-time RT-PCR, nuclear run on, chromatin immunoprecipitation and 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside-mediated transcriptional inhibition analyses that argue against this hypothesis. Instead, our results are consistent with an alternative role for intergenic transcription in the maintenance of transcriptional silence in Th1-primed cells.


A daptive immune responses are tailored to each confronting pathogen class’s unique survival strategy. For the CD4 T cell arm of the immune system this involves the development of Th1 and Th2 cells, specialized for the control of intracellular and extracellular pathogens, respectively. For example, Th1 cells, through their production of the immunoregulatory cytokine IFN-γ are crucial in the control of the intracellular protozoal parasite \textit{Leishmania major} (1). Th2 cells, through their production of the cytokines IL-4 and IL-13, are important for the expulsion of the extracellular helminth \textit{Nippostrongylus brasiliensis} (2). Inappropriate Th1 and Th2 development can fatally impair effective host immune responses and cause autoimmunity and atopic disease (3).

\textit{Il4} and \textit{Il13}, adjacent chromosome 11 genes encoding IL-4 and IL-13, undergo transcriptional silencing and enhancement during Th1 and Th2 development, respectively. How these dissimilar transcriptional states are developmentally specified is an area of intense investigation, both due to its intrinsic immunological importance as well as its experimental tractability as a physiological model of developmentally regulated transcriptional control. A number of studies have shown that within Th2 cell clones individual gene copies of \textit{Il4} and \textit{Il13} can adopt dissimilar yet mitotically heritable transcriptional states, clearly demonstrating an epigenetic element to the transcriptional regulation of these genes (4–6). This conclusion is supported by a host of chromatin structure-related features that correlate with the development of Th1 and Th2 cells. Thirteen distinct clusters of DNase I hypersensitive sites (HS)$^5$ occur at the Th2 cytokine locus of Th2 cells (7, 8), and depending upon lineage specificity and activation dependence, these fall into three groups: Th2-specific/constitutive, Th2-specific/activation-dependent, and naive/Th1/Th2-shared/constitutive. Targeted deletion of several HS clusters (HS$_{V/N_a}$ and HS$_{S1/S2}$) has been shown to impair expression of both \textit{Il4} and \textit{Il13} in Th2-primed cells (9, 10). More recently, a second set of HSs have been described at the 3’ end of the flanking \textit{Rad50} gene that together appears to comprise a locus control region, perhaps coordinating the expression of \textit{Il4} and \textit{Il13} (11). During effector T cell development, nucleosomal histones at the Th2 cytokine locus undergo a highly stereotyped dynamic sequence of N-terminal covalent modifications (12–15). Similarly, DNA methylation, a marker of transcriptional silence, undergoes coordinated changes that correlate with Th1 and Th2 development (16, 17). Finally, several groups have described the existence in Th2 but not Th1 cells of a functionally enigmatic class of RNA transcripts mapping to noncoding intergenic regions of the Th2 cytokine locus, suggesting possible involvement in \textit{Il4} and \textit{Il13} gene regulation (13, 18, 19).

Noncoding RNA transcription is known to play an important role in the specification of transcriptional states. For example, for each stage of human erythroid lineage development intergenic transcripts can be detected that map to the nucleosensitive chromosomal domains circumscribing the globin gene isoforms active at that stage (20). A promoter deletion that abolishes adult stage-specific intergenic transcripts causes abnormal expression of adult but not fetal globin gene isoforms (20). Antisense transcription at the Ig H chain locus has been proposed to remodel the V region to facilitate V-to-DJ recombination (21). Recently, at the human HLA-DRA locus, transcription, histone acetylation and chromatin structure were all shown to correlate with bidirectional intergenic transcription from an upstream locus control region (22). Several recent studies of the \textit{Drosophila iab}, a large intergenic region containing regulatory elements that direct segment-specific homeotic gene expression, indicated a role for intergenic transcription in relieving polycomb-mediated epigenetic silencing (23–27). Thus,

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in a variety of contexts, intergenic transcription appears capable of “opening” chromatin, making it accessible for gene rearrangement and transcription.

Here, we investigate the developmental dynamics of intergenic transcription at the Th2 cytokine locus to assess its role in Th2-specific enhancement of Il4 and Il13 transcriptional potential. Using real time RT-PCR, 5,6-dichlorobenzimidazole 1-β-β-ribofuranoside (DRB)-mediated transcriptional inhibition and chromatin immunoprecipitation (ChIP) analyses, we provide evidence inconsistent with the hypothesis that intergenic transcription is required in Th2 cells to maintain elevated levels of nucleosomal histone acetylation and transcriptional permissiveness of Il4 and Il13. Furthermore, using nuclear run-on (NRO) analyses, we show that nascent intergenic transcripts are produced in Th1 cells. Thus, intergenic transcription occurs in both Th2 and Th1 lineages but appears to be selectively processed/degraded in the latter. We discuss our findings in the context of a hypothesis in which intergenic transcription plays a role in specifying Il4 and Il13 transcriptional silence in Th1 cells.

Materials and Methods

Cell lines

The Th2 clone D10.G4.28 (ATCC TIB-224) was reconstituted (no more frequently than once every 15 days) with irradiated AKR/J spleenocytes and conalbumin (100 μg/ml; Sigma C0755). Cells were fed on day 2 after stimulation with 10 U/ml recombinant human IL-2 (rIL-2; Hoffman La Roche). For expansion, cells were split into thIL-2 (10 U/ml)-containing RP10. Cells harvested 5 days after the last rIL-2 split were considered resting. For activation, resting cells were cultured for 4 h with PMA (5 ng/ml) and ionomycin (250 ng/ml).

T cell purification

CD4+ T cells were purified from spleen and lymph nodes by either positive or negative selection. For positive selection (Fig. 4), cells were stained with FITC-conjugated rat anti-CD4 Ab (GK1.5; BD Pharmingen), washed, and stained with anti-FITC MultiSort microbeads (Miltenyi Biotec; 130-058-701) and AutoMACS purified. For negative selection (Fig. 5), the CD4+ T cell isolation kit (Miltenyi Biotec; 130-090-860) was used. Purity of CD4+ T cells as determined by FACS analysis was ≈80% (positive selection) and ≈95% (negative selection). Naïve CD4+CD62Lhi T cells were isolated from CD4+ T cells by staining with anti-CD62L microbeads (Miltenyi Biotec; 130-049-71) followed by AutoMACS purification. For positively selected CD4+ T cells, anti-FITC MultiSort microbeads were enzymatically removed (according to the manufacturer’s instructions) before anti-CD62L staining. Purity of CD4+CD62Lhi T cells as determined by FACS analysis was ≈98%.

Th1 and Th2 cultures

Primary T cell cultures were generated using 4–6 wk BALB/c mice (The Jackson Laboratory) housed under specific pathogen-free conditions at the University of Washington, Seattle, according to IACUC guidelines. Splenic APCs were prepared by complement-mediated lysis using anti-Thy1 Ab (J11; American Type Culture Collection) and a combination of rabbit and guinea pig complement and received 3000 rads of γ-irradiation. T cells were polyclonally stimulated with anti-TCRB (H57-597, American Type Culture Collection) and a combination of rabbit and guinea pig complement and received 3000 rads of γ-irradiation.

Cells (20–30 × 106) were washed with PBS containing 2% FBS and cross-linked by addition of 1% formaldehyde. Cross-linking was allowed to proceed at room temperature for 5 min and was terminated with 0.125 M glycine. Cells were rinsed several times with ice-cold PBS containing 2% FBS and resuspended in 2 ml of buffer 1 (1% SDS, 5 ml EDTA, 50 ml Tris-HCl (pH 8) 200 pmol antibodies, phosphate buffer, and histone deacetylase (HDAC) inhibitors), incubated on ice for 10 min and sonicated (on/off 1:15, 0.5-μm transducer amplitude 30%) to produce DNA fragments with an average size of 500 bp. A small portion of chromatin was set aside for use as the “input” fraction by processing as described below for immunoprecipitated chromatin, beginning with the final 1% SDS, 0.1 M NaHCO3 extractions. The remaining chromatin was diluted 1:10 in 1% Triton X-100, 2 mm EDTA, 150 mm NaCl, 20 ml Trit-HCI (pH 8), phosphate antibodies, phosphate buffer, and HDAC inhibitors, pre-cleared with beads, and input DNA was analyzed by real-time PCR as described above (Fig. 7A) by qPCR and rotated overnight at 4°C with 2 μg of anti-H3K9/14Ac Ab (60-599, Upstate Biotechnology). Immune complexes were recovered, and pellets were washed sequentially for 10 min at room temperature; once in 0.1% SDS, 1% Triton X-100, 2 ml EDTA, 20 ml Tris-HCI (pH 8), 150 mm NaCl, phosphate antibodies, phosphate buffer, and HDAC inhibitors; once in 0.1% SDS, 1% Triton X-100, 2 mm EDTA, 20 ml Tris-HCI (pH 8), phosphate antibodies, phosphate buffer, and HDAC inhibitors; and once in 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mm EDTA, 10 ml Tris-HCI (pH 8), phosphate antibodies, phosphate buffer, and HDAC inhibitors. Immunoprecipitates were then washed three times with Tris/EDTA and extracted twice with 1% SDS, 0.1 M NaHCO3. Eluates and input chromatin were heated at 65°C overnight to reverse formaldehyde cross-linking and DNA fragments purified (QIAamp PCR purification kit 28106; Qiagen). Total DNA (input fraction) and immunoprecipitate DNA samples were quantified using picoGreen (Molecular Probes) fluorescence. Equivalent mass of immunoprecipitate and input DNA was analyzed by real-time PCR as described above for RT-PCR with the following modifications. Hot start Taq polymerase was from Qiagen and cycling conditions were as follows; 94°C for 15 min followed by 40 cycles of 94°C for 20 s, 61°C for 1 min and 72°C for 40 s. Data are presented as the ratio of immunoprecipitate to input Ct values. Fold enrichment is thus enrichment relative to the genome average for a particular modification. Thus, modifications that are widespread in the genome are subject to a smaller dynamic range of enrichment than are those that are relatively rare. Differences in the efficiency and specificity of Abs will also markedly influence the observed dynamic range of enrichment.

Transcriptional inhibition

DRB (1 mg/ml; Calbiochem 287891) was dissolved in Dulbecco’s PBS (DPBS) by heating at 75°C for ~1 h with occasional vortexing. A 2× working stock (DRB, 200 μg/ml) was prepared by dilution into prewarmed RP10; 1 ml of 2× prewarmed DRB or carrier (DPBS) was added to quiescent D10.G4 cells. Following 0, 0.5, 2, and 3 h of culture, cells were recovered and processed for RNA and ChIP. For DRB washout experiments, cells were incubated with DRB or carrier for 2 h, washed three times, reactivated in RP10 for an additional 2 h, and processed for RNA.

NRO assays

NRO assays were performed as described (30). Briefly, nuclei from 2 to 5 × 107 resting Th1- and Th2-primed cells were harvested and frozen.

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Frozen nuclei were thawed and labeled with [α-32P]CTP (ICN Pharmaceuticals). Labeled RNA, extracted, precipitated, and purified over Sephadex G-25 was hybridized to filters (Dupont NEN plus) slot-blotted (apparatus from Schleicher and Schuell) with single-stranded RNA probes (1/μg/slot) generated by vitro transcription (Ambion; Megashortscript for T7 RNA polymerase and Megascript for SP6 RNA polymerase) from PCR amplicons containing SP6 and T7 promoters on their flanks (see Table II for primer sequences). Blots were washed and analyzed by PhosphorImager (Storm 820; Molecular Dynamics) using Imagequant software (Molecular Dynamics). All reported signals were within the linear range for this instrument.

Results

We used real time RT-PCR to measure the abundance of RNA transcripts mapping to intergenic and intronic regions spanning ~41 kb of the Th2 cytokine locus, including Il4 and Il13. PCR primer pairs targeted 17 locations (Fig. 1) described in Ref. 29, including those known to be computationally (9, 31), structurally (7, 8), or functionally (9, 31) implicated in cytokine regulation. Since genomic DNA could serve as a template for the PCR primers used in our analysis, RNA samples were digested extensively with DNase before RT and no-RT controls were stringently monitored for residual genomic DNA contamination. Contaminated samples were excluded from analysis. To provide a meaningful baseline for comparison, T cell expression (internally normalized to Hprt) was further normalized to signals obtained in the fibroblast cell line NIH3T3 whose Th2 cytokine locus lacks histone acetylation (32), all known HSs (8) and Il4 and Il13 gene transcription (data not shown).

The Th2 clone D10.G4, a well established model of the fully differentiated Th2 fate (28), displayed a characteristic broadly distributed pattern of intergenic transcripts across the noncoding regions of the Th2 cytokine locus (Fig. 1). The minimum length of intergenic transcripts, inferred from amplicon size, ranged from 196 to 496 nucleotides. Upstream of Il13 just beyond HS 1, we detected a strong peak of transcripts (location N). Between HS IV and the 3′ end of Il4 (locations E and E′) and downstream of Il13 near HSs3 and CNS1 (locations K and L) transcript levels were moderate whereas downstream of Il4 near HSV and just upstream of the Il4 promoter near HS1 and Hs0 (locations B and I) levels were low but reproducible. No transcripts were detected near HSVa (location C). At Il4 intronic locations F, G, and H very high transcript levels, presumably reflecting Il4 pre-mRNA precursors of the mature Il4 transcripts constitutively detected at low levels in resting D10.G4 cells (data not shown).
To gain insight into the relationship between intergenic and Il4 and Il13 genic transcription, we tested the effect of PMA/ionomycin-mediated cellular activation on the induction of different RNA transcript classes. Over a 4-h time course, Il4 and Il13 mRNA levels in D10.G4 increased at least 1000-fold (data not shown and Ref. 29). Similarly, the abundance of Il4 intron-containing RNAs (presumably Il4 pre-mRNA) increased up to 72-fold (Fig. 1, sites F, G, and H; compare resting and activated). Interestingly, despite the dramatic increase in coding transcript abundance, intergenic transcript levels remained constant (Fig. 1). Thus, intergenic transcription in mature Th1 and Th2 clones appears constitutive and nonresponsive to the pathways controlling cytokine gene transcriptional induction. These results indicate that intergenic transcripts are not simply a result of read-through transcription from cytokine promoters but rather arise from a functionally distinct process that is subject to independent regulatory control. This conclusion is supported by the recent demonstration that intergenic transcripts at the Th2 cytokine locus are insensitive to inhibition by the calcineurin inhibitor FK506 (33).

As intergenic transcripts at the Th2 cytokine locus appeared to be regulated independently of Il4 and Il13 mRNAs, we wondered whether their biosynthesis required RNA polymerase II (PolII). To test this, we exploited DRB, a well established reversible inhibitor of PolII-dependent transcriptional elongation (34). Following the onset of DRB-mediated transcriptional inhibition in quiescent D10.G4 cells, we observed a rapid decrease in intergenic transcript abundance, suggesting that PolIII is indeed responsible for generating Th2 cytokine locus-specific intergenic transcripts (Fig. 2). Further, comparison with the turnover rates of other PolII-dependent mRNA species classified intergenic transcripts (Fig. 2, black lines; Table I, \( t_{1/2} = 21–76 \text{ min} \)) with the highly labile Myc (Fig. 2, green line; Table I, \( t_{1/2} = 51 \text{ min} \)) rather than with the slow-turnover housekeeping Hprt (Fig. 2, purple line; Table I, \( t_{1/2} = 250 \text{ min} \)). Cessation of DRB-mediated transcriptional inhibition allowed a minimum estimate of the rate of intergenic transcript accumulation. As shown in Fig. 2, this too was rapid, returning to at least starting levels within 2 h following the cessation of a 2 h pretreatment with DRB. The reason for the transcriptional overshoot observed with several RNA species upon the removal of DRB is not known but has been reported previously (35). Rapid accumulation and turnover of intergenic transcripts suggests that their constitutive invariant level is maintained dynamically.

Histone H3 lysine-9/14-acetylation and intergenic transcription in D10.G4 occur in a similar pattern across the Il4-IIl3 locus (compare Figs. 1 and 3 and see Ref. 29). Moreover, the quantitative level of histone acetylation across the Il4-IIl3 locus has been shown, like intergenic transcription, to remain constant despite cellular activation conditions capable of inducing transcription of Il4 and Il13 (29). These observations, together with the discovery of histone acetyl transferase activity in yeast and human ELP3, a subunit of the elongator complex of actively transcribing PolII (36, 37), provided the basis for speculating that transcription through intergenic regions is responsible for maintaining histone acetylation at the Th2 cytokine locus (13, 19, 38). To test this hypothesis,

![FIGURE 3. Effect of DRB-mediated transcriptional inhibition on histone H3 acetylation. At the top is a map of the Th2 cytokine locus annotated as in Fig. 1. Below this is a plot depicting H3K9/14Ac fold enrichment (mean \( \pm \text{ SEM} \); 0 h, \( n = 5 \); 2 h, \( n = 5 \); 3 h, \( n = 3 \)) at sites A–P in quiescent D10.G4 treated with DRB (100 \( \mu \text{g/ml} \)) for 0 h (black circles), 2 h (blue triangles), and 3 h (green squares) and then processed for ChIP analysis with an Ab to H3K9/14Ac.](image-url)

**Table I. RNA turnover rates**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>71</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
</tr>
<tr>
<td>C</td>
<td>42</td>
</tr>
<tr>
<td>D</td>
<td>27</td>
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<tr>
<td>E</td>
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<td>E'</td>
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<td>F</td>
<td>8</td>
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<tr>
<td>G</td>
<td>9</td>
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<td>I</td>
<td>76</td>
</tr>
<tr>
<td>K</td>
<td>27</td>
</tr>
<tr>
<td>L</td>
<td>25</td>
</tr>
<tr>
<td>M</td>
<td>57</td>
</tr>
<tr>
<td>Myc</td>
<td>51</td>
</tr>
<tr>
<td>Hprt</td>
<td>250</td>
</tr>
</tbody>
</table>

* RNA abundance in quiescent D10.G4 cells cultured with DRB for 0 (\( n = 4 \)), 30 min (\( n = 3 \)), 2 h (\( n = 4 \)), and 3 h (\( n = 3 \)) was normalized to the carrier-only control. Normalized abundance was then plotted over time, and the data for each locus were fit to an exponential decay curve from which \( t_{1/2} \) values were interpolated. Hprt and Myc turnover rates agree well with published reports (1,2).
we reasoned that blockade of PolIII-dependent intergenic transcription would lead to a decrease in histone acetylation at the Th2 cytokine locus. Accordingly, we performed kinetic ChIP analysis for H3K9/14Ac levels in resting D10.G4 cells treated with DRB over a 3-h time course. Given that the steady-state half life of acetylated histone H3 in higher eukaryotic cells (including animal cells) is on the order of minutes rather than hours (39–43), it seemed reasonable to expect that a 3-h time course of transcriptional inhibition should suffice to reveal changes in H3 acetylation levels resulting from inhibition of a transcription-dependent maintenance mechanism. However, as shown in Fig. 3, levels of histone acetylation across the Th2 cytokine locus were largely unaffected by DRB-mediated inhibition of intergenic transcription, decreasing at most ~2-fold at HS\textsubscript{V\_10}, CNS1, and the II13 promoter region (locations B, K, and M). By contrast, intergenic transcript levels decreased as much as 15-fold. Thus, these results suggest that (at least over the time course studied) intergenic transcription is not the principal mechanism responsible for maintaining H3 acetylation across the Th2 cytokine locus in Th2 cells.

Seeking additional clues to the function of intergenic transcripts at the Th2 cytokine locus, we decided to investigate their ontology. We began with the progenitors of Th1 and Th2 cells, using MACS to isolate naive CD62L\textsuperscript{Hi}CD4\textsuperscript{+} cells. By contrast, in Th1-primed cells intergenic transcripts were no longer detectable (Fig. 4). Attesting to the quality of the Th1 cells, restimulation 48 h after the onset of Th1 priming induces II4 and II13 mRNA expression at levels comparable to those of activated naive CD4\textsuperscript{+} T cells (data not shown and Ref. 29), demonstrating that transcriptional repression has not yet occurred at this early stage of development. Together, these results indicate that II4 and II13 transcriptional permissiveness does not require intergenic transcription.

### Table II  NRO primers

<table>
<thead>
<tr>
<th>Site</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>ATTTAGTTGACACTATAGGCCAGACATCAGGGTTAGTT</td>
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<td>B</td>
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<td>O</td>
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</table>

*PCR primers used to make templates for synthesizing single-stranded NRO probes using in vitro transcription with SP6 and T7 RNA polymerase. Forward and reverse primers for each site (A–O) at the Th2 cytokine locus are flanked by SP6 and T7 promoters (bold), respectively.
FIGURE 5. NRO analysis of nascent transcripts at the Th2 cytokine locus of Th1 and Th2 cells. A, The PhosphorImager output (contrast-enhanced in Adobe Photoshop) revealing hybridization intensity of radiolabeled Th1 and Th2 RNA to filter-bound single-stranded sense and antisense RNA probes corresponding to locations A–P at the Th2 cytokine locus. Rightward and leftward pointing arrows depict antisense and sense RNAs, respectively, that bind to these probes. Naive CD4 T cells were primed in Th1 or Th2 conditions for 4 days and rested in neutral conditions for 2 days before labeling and isolation of RNA. B, Log plots of relative intensity (in actin-normalized arbitrary units) vs map location quantifying the data depicted in A. The horizontal red line in each plot represents each blot’s detection limit, determined by averaging signals obtained from 10 different locations across each blot, sampling regions where no probe had been bound. The relative intensity for Th2 probe H is 520 (closed square) and for Th2 probe O is 249 and 210 (closed and open squares, respectively). Actin signals were obtained using a dsDNA probe. At the top is a map of the Th2 cytokine locus annotated as in Fig. 1. Leftward and rightward pointing arrows and closed and open symbols represent sense and antisense RNA transcripts, respectively, relative to the polarity of Il4 transcripts.

Seeking an alternative explanation for the lineage-specific detection of intergenic transcription, we took note of the recent finding in fission yeast that the heterochromatin transcriptionally silent state of centromeres, telomeres and the silent mating type locus requires bidirectional noncoding transcriptions produced from repetitive elements encoded within these loci (44, 45). In wild-type (WT) yeast with intact heterochromatin, although these transcripts undergo constitutive biosynthesis, engagement with the RNA interference (RNAi) pathway leads to their rapid processing into 21- to 22-bp-sized double-stranded RNAs, undetectable by RT-PCR. When heterochromatized loci controlled by this RNAi-dependent mechanism become de-repressed (due to mutations in components of the RNAi or chromatin-modifying machinery) unprocessed noncoding transcripts accumulate to levels where they can be detected by RT-PCR. Thus, although both silent and de-repressed states are associated with constitutive bidirectional noncoding transcription, only the latter allows the accumulation of unprocessed transcripts to levels detectable by RT-PCR.

Given the parallels between heterochromatin in fission yeast and Il4 and Il13 transcriptional regulation in mice, we wondered whether intergenic transcription at the Th2 cytokine locus might also engage an RNAi-dependent pathway to maintain Il4 and Il13 transcriptional silence in Th1 cells. A prediction of this hypothesis is that, despite the failure of intergenic transcripts to accumulate to levels detectable by RT-PCR, intergenic transcripts are undergoing constitutive biosynthesis from silent Th2 cytokine loci in Th1 cells. To test this, we performed NRO analysis, hybridizing radiolabeled RNA obtained from resting Th1 and Th2 cells to filter-bound strand-specific RNA probes generated by in vitro transcription of PCR amplicons (see Table II for primer sequences) corresponding to locations A–O. As predicted by the RNAi hypothesis, nascent transcripts mapping to intergenic regions of the Th2 cytokine locus were detected not only from Th2 but also Th1 cells (Fig. 5A). Demonstrating the fidelity of our strand-specific NRO assay, probes for Il4 intronic location H detected strong sense signals in Th2 but not Th1 cells, correlating with the differential transcriptional permissiveness of Il4 in these two lineages (Fig. 5). Interestingly, these sense transcripts were detected in Th2 cells only at intronic location H and not F and G, suggesting the occurrence of a regulated transcriptional pause site in the Il4 second intron between H and G. By contrast, in Th1 cells moderate levels of nascent antisense transcripts occurred at location H, suggesting developmental regulation of the polarity of transcriptional initiation near the Il4 promoter. In contrast to the Th lineage-specific pattern of nascent transcription near the Il4 promoter, high levels of nascent sense and antisense transcripts occurred at the Il13 upstream region (location O) in both Th1 and Th2 cells, suggesting the occurrence of a nearby bidirectional lineage nonspecific promoter. Across the rest of the Th2 cytokine locus, in both Th1 and Th2 cells, low levels of nascent antisense and sense transcripts could be detected. These results demonstrate that at the Th2 cytokine locus nascent intergenic transcripts with the potential to form double-stranded RNA are produced in both Th1 and Th2 cells. Failure in Th1 but not Th2 cells of these transcripts to accumulate to levels detectable by RT-PCR suggests lineage-specific processing/degradation.

Discussion

Quantitative characterization of Th2 cytokine locus-specific intergenic RNA transcripts in relation to developmentally programmed changes in chromatin structure is important for understanding the biological role of intergenic transcription in the regulation of Il4 and Il13 gene expression. Using quantitative RT-PCR, ChIP, pharmacologic transcriptional blockade, and developmental analysis, we have provided strong evidence that histone acetylation and Il4 and Il13 transcriptional permissiveness in Th2 cells are unlikely to depend on the occurrence of intergenic transcription. Using NRO, we find that, despite their failure to accumulate to levels detectable by RT-PCR, intergenic transcripts with the potential to form double-stranded RNA are constitutively synthesized in both Th2 and Th1 cells. Thus, rather than Th2-specific expression, our data support Th1-specific degradation/processing of intergenic transcripts.

Our RT-PCR results are consistent with previous findings demonstrating constitutive biosynthesis of intergenic transcripts from the Th2 cytokine locus of naive CD4 T cells and 48-h Th2- but not Th1-primed CD4 T cells (13, 18, 19). The disappearance of RT-PCR-detectable intergenic transcripts from the Th2 cytokine locus of 48-h Th1-primed cells is thus an early marker of Th1 differentiation. Sensitivity to the adenosine analog DRB indicates that Po-III is responsible for biosynthesis of intergenic transcripts mapping...
to the Th2 cytokine locus. As intergenic transcripts are labile (Fig. 2A and Table I, t$_{1/2}$ = 21–76 min), their constitutive maintenance at a constant level in resting and activated Th2 cells must be achieved dynamically. This is supported by the demonstration that intergenic transcripts accumulate rapidly when released from DRB-mediated transcriptional blockade (Fig. 2B). Dynamic maintenance of constitutive intergenic transcript levels is consistent with a regulatory function. The correlation of H3 acetylation and intergenic transcription at the Th2 cytokine locus in terms of pattern and lineage specificity (compare Figs. 3 and 4 and Ref. 29) together with the demonstration that PolIII possesses histone acetyl transferase activity suggested that intergenic transcription might maintain histone acetylation and Il4 and Il13 transcriptional permissiveness at the Th2 cytokine locus.

However, a 3-h time course of DRB-mediated inhibition of PolIII-dependent transcription had only a slight inhibitory effect on H3 acetylation. Small (2-fold) decreases in acetylation levels were detected at several locations after 2 h of DRB treatment. However, as a 3rd consecutive hour of inhibition provided no further decrease, it would appear that the majority of histone acetylation is independent of ongoing intergenic transcription. The inhibition time course was more than two orders of magnitude greater than the steady-state half-life of acetylated histone H3 (~5 min) (39, 40–43) and thus is likely to have been sufficient to reveal any effects resulting from the loss of an important acetylation maintenance mechanism. These results suggest that neither the process nor—given that intergenic transcripts themselves are labile—the product of intergenic transcription is required for the maintenance of histone acetylation in Th2 cells. Nonetheless, we cannot exclude the possibility of a long-lived intergenic transcription-dependent factor required for histone acetylation.

Arguing against this latter possibility are our developmental studies that also fail to support a role for intergenic transcription in the maintenance of histone acetylation and Il4 and Il13 transcriptional permissiveness. First, in naive CD4 T cells, histone acetylation is absent from the majority of the Th2 cytokine locus, despite the occurrence of intergenic transcripts in a pattern and at levels only slightly reduced from those detected in Th2-primed CD4 T cells (12, 14, 29). Second, intergenic transcripts rapidly disappear following 48-h of Th1 priming, despite the continued capacity to express Il4 and Il13 at levels undiminished from those of naive CD4 T cells. Taken together, these findings argue strongly that intergenic transcription is not required at the Th2 cytokine locus of Th2 cells to maintain histone acetylation or Il4 and Il13 transcriptional permissiveness.

Although the structure of intergenic transcripts was not addressed directly in the current study, certain features were ascertained indirectly. First, the minimum length of intergenic transcripts in Th2 cells, corresponding to the size of the PCR amplicons used for their detection, is between 196 and 496 nucleotides. Second, complete insensitivity to induction by PMA/ionomycin—a stimulus that greatly increased the level of Il4 and Il13 mRNA—argues against intergenic transcripts being uncoupled cytoplasmic mRNAs (as their abundance would be expected to increase in tandem with cytokine mRNAs). Second, dissimilar turnover rates of the intergenic transcripts flanking Il4 vs those spanning the Il4 second intron suggests the existence of at least three distinct RNA species in the vicinity of the Il4 gene: Il4-3′-intergenic, Il4-5′-intergenic and Il4-intronic (likely Il4 precursor). Supporting the classification of Il4 intron-spanning transcripts as pre-mRNAs is their extremely rapid turnover (t$_{1/2}$ = 8–9 min), significantly faster than the labile RNA class represented by myc (t$_{1/2}$ = 51 min). Thus, rather than spanning the Il4 transcription unit, intergenic transcripts appear to occur separately on either flank. It is likely that the intergenic transcripts detected upstream of Il13 represent yet a third distinct group. Consistent with the possibility that intergenic transcript promoters occur near locations B, E’, I, J, and N is the corresponding elevation in the level of nascent transcripts near these locations in Th1 cells (Fig. 5, locations C, I, K, L, M, and O).

In fission yeast, noncoding transcripts mapping to centromeric repeats embedded in transcriptionally silent heterochromatin (telomeres, centromeres, and the silent mating type locus) show striking similarities to the intergenic transcripts that map to the Th2 cytokine locus in CD4 T lymphocytes. In both cases, RT-PCR suffices for the detection of noncoding transcripts from de-repressed loci (seen with Th2 priming of CD4 T cells and mutation of the RNAi and chromatin-modifying machinery in yeast cells). By contrast, RT-PCR no longer suffices for the detection of noncoding transcripts mapping to repressed loci (seen with Th1-priming of CD4 T cells and unmanipulated WT yeast cells). Despite this failure of RT-PCR to detect steady-state levels of noncoding transcripts in WT yeast, NRO assays reveal constitutive biosynthesis of nascent transcripts (12, 14, 29). Recent studies have shown that heterochromatin in yeast is maintained by the constitutive biosynthesis of noncoding double-stranded RNAs that target the RNAi machinery as it in turn recruits repressive chromatin-modifying machinery (12, 14, 29). A byproduct of the targeting role of double-stranded RNAs is their rapid processing and hence diminished steady-state levels.

In this light, it is interesting to note that NRO analysis reveals that intergenic transcripts are synthesized from the Th2 cytokine loci of both Th2- and Th1-primed cells (Fig. 5), revealing yet another similarity to the yeast system. Furthermore, at the majority of locations transcription appears to be bidirectional, raising the possibility that double-stranded RNA species capable of recruiting the RNAi machinery could operate, as they do for heterochromatin in yeast, to silence Th2 cytokine genes in T lymphocytes.

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Disclosures

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References


