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Formation of an Active Tissue-Specific Chromatin Domain Initiated by Epigenetic Marking at the Embryonic Stem Cell Stage

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The differentiation potential of stem cells is determined by the ability of these cells to establish and maintain developmentally regulated gene expression programs that are specific to different lineages. Although transcriptionally potentiated epigenetic states of genes have been described for haematopoietic progenitors, the developmental stage at which the formation of lineage-specific gene expression domains is initiated remains unclear. In this study, we show that an intergenic *cis*-acting element in the mouse *λ5-VpreB1* locus is marked by histone H3 acetylation and histone H3 lysine 4 methylation at a discrete site in embryonic stem (ES) cells. The epigenetic modifications spread from this site toward the *VpreB1* and *λ5* genes at later stages of B-cell development, and a large, active chromatin domain is established in pre-B cells when the genes are fully expressed. In early B-cell progenitors, the binding of haematopoietic factor PU.1 coincides with the expansion of the marked region, and the region becomes a center for the recruitment of general transcription factors and RNA polymerase II. In pre-B cells, E2A also binds to the locus, and general transcription factors are distributed across the active domain, including the gene promoters and the intergenic region. These results suggest that localized epigenetic marking is important for establishing the transcriptional competence of the *λ5* and *VpreB1* genes as early as the pluripotent ES cell stage.

Mammalian development is a spatially and temporally regulated process that depends on the differentiation potential of individual cells. This potential is determined by the ability of the cells to establish gene expression programs that are specific to different cell lineages. The patterns of expression of individual genes are regulated by the interaction of transcription factors with a set of *cis*-acting sequences that combine to form a genetic functional unit (reviewed in reference 12). Modification of the amino-terminal regions of the core histones has also been proposed to play an essential role in gene regulation by establishing domains of active or silent chromatin that would mediate the cellular memory of gene expression states (4, 24, 44, 70). The histone modifications identified to date include acetylation, methylation, ubiquitination, and ADP ribosylation (reviewed in references 28 and 80). Of these, histone H3 and H4 acetylation and histone H3 lysine 4 (K4) methylation have been shown to be associated with the transcriptional activation of genes (69). Although patterns of histone modifications have been analyzed for several gene loci, the relationship between the organization of genetic functional units and domains of histone modifications is still not well understood.

Stem cells have the ability to differentiate into multiple cell types as well as to self-renew and maintain their multipotential capacity. In mammals, a specific type of stem cell, the embryonic stem (ES) cell, has been shown to be pluripotent (i.e., able to generate all somatic lineages) (8, 64). ES cells are found in the inner cell mass at the blastocyst stage of development and retain their differentiation potential when grown in culture under appropriate conditions. The pluripotency of ES cells is of immense biological and clinical interest, but little is known about the basis for this phenomenon. Multipotent stem cells are partially committed cells with a relatively broad differentiation capacity, but they can only give rise to a subset of differentiated cell types. An example is the haematopoietic stem cell, which can differentiate into all of the cells of the myeloid and lymphoid lineages but does not normally develop into other cell types (for a review, see reference 16).

Low-level transcription of a number of lineage-specific genes has been observed in haematopoietic progenitors, leading to the suggestion that genes that are destined for activation are already in a transcriptionally permissive configuration (26, 50, 85). Prior to high-level transcription of β-globin genes in erythroid cells, histone H3 acetylation and histone H3 K4 methylation are present at the gene promoters in multipotent haematopoietic progenitors (6). It has also been shown that transcription factor complexes are already partially assembled on regulatory elements of macrophage- and neuron-specific genes in progenitor cells (39, 42, 62, 76, 77). Transcription from *cis*-acting regulatory elements (intergenic transcription) has also been proposed to be important for the generation of potentiated epigenetic states in developmentally regulated chromatin domains (19, 48, 63, 65). The presence of epigenetic modifications in multipotent progenitor cells that specify the activation of genes at later stages suggests that the existence of these marks could be fundamental to the ability of stem cells to activate multiple lineage-specific gene expression programs. If this suggestion is correct, then it would also raise the possibility

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that gene-specific epigenetic marks play a similar role in pluripotent ES cells.

As a model system for investigating the epigenetic regulation of gene expression during B-cell commitment, we are studying the mouse λ*5-VpreB1* locus. Transcription of the *VpreB1* and λ*5* genes is activated during the pro-B-cell stage prior to heavy-chain rearrangement (43, 49). Their protein products associate to form the surrogate light chain which chaperones newly synthesized μ chains to the cell surface to form the pre-B-cell receptor. This receptor is thought to mediate signaling which leads to the proliferation of pre-B cells that have a productive heavy-chain rearrangement. The expression of the *VpreB1* and λ*5* genes is downregulated at the time of immunoglobulin (Ig) light-chain rearrangement, and the genes are silent in mature B cells. The functional unit of the *VpreB1* and λ*5* genes has been localized within a 19-kb fragment containing both genes and 12 DNase I-hypersensitive sites (HS) (47, 49a, 66). The HS are spread over the 19-kb region, where they form a multicomponent locus control region. The expression of the *VpreB1* and λ*5* genes is regulated by interactions between *trans*-acting factors and the locus control region, but the initial events that allow the activators to gain access to regulatory elements at early stages of differentiation have not been defined.

Here we show that the λ*5-VpreB1* domain is already marked by histone H3 acetylation and histone H3 K4 methylation at a discrete site in ES cells and that these modifications are not present in the rest of the locus. The marked region expands in early B-cell progenitors and becomes a localized center for transcription factor and RNA polymerase II (Pol II) recruitment. An extended, transcriptionally active chromatin domain is established and maintained in pre-B cells when the λ*5* and *VpreB1* genes are fully active. Our results provide evidence that epigenetic marking of *cis*-acting elements of tissue-specific genes that are expressed at later stages can already be set up as early as the undifferentiated ES cell stage.

MATERIALS AND METHODS

Sequence of the λ*5-VpreB1* locus. The sequence of the entire 19-kb λ*5-VpreB1* locus has been determined elsewhere (accession number AJ852426 [49a]). The positions of restriction sites were calculated relative to that of an EcoRI site (see Fig. 4D) 2.8 kb upstream from the initiation site for *VpreB1*.

Cells and tissues. E14 and CJ7 ES cells were cultured in Dulbecco minimal essential medium (Sigma) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acid solution, 50 μM 2-mercaptoethanol, and 2,400 U of leukemia inhibitory factor (LIF)/ml in 0.1% gelatin-coated tissue culture flasks. ES cell cultures were always started on mitomycin C-treated SNL feeder layers until the first passage; no more feeder cells were added afterward when the cultures were expanded. To verify the undifferentiated state, the expression of Oct-4 was monitored by fluorescence-activated cell sorting analysis. Ba/F3 cells were grown in RPMI medium (Sigma) supplemented with 15% fetal FCS, 2 mM L-glutamine, 1% nonessential amino acid solution, 50 μM 2-mercaptoethanol, and 10% conditioned medium from WEHI 3 cells as a source of interleukin 3 (IL-3). Primary cultures of pre-B cells from 16.5-day-old fetal livers were established as described previously (66). To generate primary cultures of mature B cells, spleens were disaggregated, and the single-cell suspension was subjected to centrifugation on Ficoll to remove erythrocytes. Cells from the interface were collected and activated in RPMI medium supplemented with 15% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg of gentamicin/ml, and 2.5 μg of lipopolysaccharide/ml for 3 days. Primary mouse embyonic fibroblasts were obtained from whole 16.5-day-old embryos by trypsin treatment and subsequent culturing in Dulbecco minimal essential medium supplemented with 15% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% sodium pyruvate, and 50 μg of gentamicin/ml.

In vitro differentiation of ES cells. E14 ES cells were plated at high densities in uncoated tissue culture flasks by using the medium described above, except that it contained 10% FCS and no LIF. After 3 days of culturing, floating ES cell aggregates were transferred to fresh medium. After another 5 days of culturing, embryoid bodies were disaggregated by trypsin treatment, plated at low densities, and allowed to grow for a further 5 days. At this point, the cell cultures were confluent, with differentiated, flattened fibroblast-like cells, and only a small population of ES cell colonies (<15%) remained.

Reverse transcription (RT). Total RNA was extracted from various cells and tissues by using an RNeasy minikit (Qiagen). Total RNA (1 μg) was treated with 10 U of DNase I (Roche) in a 20-μl volume containing 10 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, and 1 mM dithiothreitol for 1 h at 37°C. DNase I-treated RNA (100 ng) was reverse transcribed in a 20-μl volume with 20 U of Moloney murine leukemia virus reverse transcriptase (Gibco) and 100 ng of random primers according to the manufacturer's instructions.

DNase I hypersensitivity assay. Whole livers were disaggregated in ice-cold phosphate-buffered saline by 30 strokes of a Dounce homogenizer with a "loose" pestle. The cell suspension was washed with phosphate-buffered saline, homogenized, and washed two more times. Preparation of nuclei from all cell types and mapping of DNase I HS were performed as described previously (66) (see Fig. 4D for the HS mapping strategy). Southern blot probe I was generated by PCR with primers PI5′ and PI3′ (Table 1). Probe II was a SacI-SphI restriction fragment (66).

Immunoprecipitation of unfixed chromatin. Preparation of unfixed chromatin and chromatin immunoprecipitation (ChIP) were carried out as described previously (56). At least two independent immunoprecipitations were performed for each cell type and antibody, and 150 μg of unfixed chromatin (measured as DNA at an optical density at 260 nm) was used in each immunoprecipitation. The volume of the samples was adjusted to 1 ml with ChIP buffer 1 (50 mM NaCl, 10 mM Tris-HCl [pH 7.4], 5 mM sodium butyrate, 1 mM EDTA, EDTA-free protease inhibitor cocktail [Roche]), and the samples were precleared for 1 h with 100 μl of protein A-agarose (Sigma) and then centrifuged at 1,400 rpm (Eppendorf Centrifuge 5417R) for 5 min. The supernatants were collected, and 50 to 100 μl was kept as input material. Immunoprecipitation was carried out with rotation at 4°C overnight and the following antibodies: 50 μl of antiserum to tetraacetylated histone H4 (Upstate), 20 μl of antibody to diacetylated histone H3 (Upstate), 50 μl of antiserum to dimethylated histone H3 K4 (Upstate), and 15 μg of nonspecific anti-rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology). Immunocomplexes were collected 3 h after the addition of 100 μl of protein A-agarose at 4°C and then centrifuged for 5 min at 1,400 rpm. Beads were washed once with ChIP buffer 1, once with ChIP buffer 2 (same as ChIP buffer 1 except for 100 mM NaCl), and then twice with ChIP buffer 3 (same as ChIP buffer 1 except for 150 mM NaCl). The bound fraction was eluted twice with 250 μl of ChIP buffer 1 containing 1% sodium dodecyl sulfate. Immunoprecipitated and input samples were extracted twice with phenol-chloroform, ethanol precipitated, and resuspended in 100 μl of Tris-EDTA buffer.

Immunoprecipitation of fixed chromatin. ChIP of formaldehyde-cross-linked chromatin was carried out essentially as described previously (72). Fixed chromatin from 3×10^8 to 5×10^8 cells was sonicated by using Soniprep 150 equipment to obtain DNA fragments of between 200 and 500 bp. Chromatin (500 μg) (measured as described above) was immunoprecipitated with mouse monoclonal antibodies overnight in sonication buffer (72) containing 0.1% bovine serum albumin. The antibodies used were as follows: 50 μg of purified anti-PU.1 antibody (BD Pharmingen), 50 μg of purified E2A (E12/E47) antibody (BD Pharmingen), 50 μl of anti-TATA-binding protein (TBP)-associated factor 5 (TAF5) antibody solution, 50 μg of purified anti-TAF10 antibody, 50 μg of purified anti-TBP antibody (84), and 50 μl each of anti-Brg1 antibody solution (54), anti-TRRAP antibody solution (22), and anti-Pol II antibody solution (5). To demonstrate the specific binding of transcription factors above the background, precipitations were also carried out with 50 μl each of anti-Gal4 antibody solution and anti-VP16 antibody solution (83) and without antibody. Eluates were resuspended in 20 to 50 μl of Tris-EDTA buffer.

Real-time PCR and data analysis. The total amount of DNA in each sample was determined by Pico Green fluorimetry (Molecular Probes). cDNA and ChIP material were analyzed by using a DNA Engine Opticon system (MJ Research Inc.) and SYBR green master mix (Applied Biosystems). Reactions were carried out in duplicate in 20- to 25-μl volumes. Primers were designed by using Primer Select or Primer Express software (see Fig. 2A for positions of primers that were used in ChIP); sequences, concentrations, and annealing temperatures are shown in Table 1. RT-PCR primer sequences are available upon request. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, annealing at a primer-dependent temperature for 15 s, and extension at 72°C for 15s. For each experiment, the threshold

TABLE 1. PCR primers and conditions used for ChIP in this study

Primer	Primer sequence (5′-3′)	Position (kb)[a]	Concn (nM)[b]	Annealing temp (°C)	Primer	Primer sequence (5′-3′)	Position (kb)[a]	Concn (nM)[b]	Annealing temp (°C)
1	TTC TTT GCC ACA CCT CAC TAA T	0.204	900	52	30	GGG GGA TAT TAC CTG CTC TTT	7.394	300	52
2	CCT TTT GGG GCT CCT CA	0.31	900	52	31	TGG ATA TCA GTC AGG CAG AG	7.869	900	60
3	TGC CGG TCA CAG GTC AG	0.522	900	57	32	CCC GGT TGT GGT TGG	7.968	900	60
4	GAT GGC TCA GCG CTC TTA CAA	0.623	900	57	33	CTA AGA GGA GGG GGC TGT GC	8.487	900	70
5	TAC CCA AGT TTT CTC CTA ATC TGC	0.856	900	57	34	GGA GGT GGG CTA TGG ATG TGG	8.586	900	70
6	GGC CTG CGG TTT GCG TTC T	0.974	900	57	35	TCA GCA ACC ATT CAT CTA CTA TCA	9.001	900	65
83	AGT CCG AGA ACA GCC TGG GT	0.888	300	68	36	CCT GCC TCT TCC TCC CAA CT	9.119	900	65
84	AGT TGT GCT GCC CAC AGA GG	0.998	300	68	37	AGG CCC TAA CAG CTT CAT CTA CTC	9.777	300	55
708	TTT ATT TTT ATT TGA TAT GCA TTG GT	1.587	900	57	38	GCA TCT GGG CCT CGG TTT A	9.894	900	55
808	ATA TGG CAG CTC ACA ACT ATC CC	1.665	400	57	39	GGC TGC ACC TGG AAA ACC TTA	11.701	900	55
11	TAG AAG GTT GAG ACA GCG AGT TAG	1.831	900	58	40	ATC AAA ATC TTC CCC TCA ATC TGT	11.801	300	55
12	AGG TGC TTT GTT TTG TTT TGT CTT	1.934	900	58	43	AGG AGA CCA GAA GGG GCA GTT	13.382	900	53
13	CAA ACC CAG AGC CAC AAA G	2.759	900	62	44	GGT TCA ATG TTT AAG AGC AAG TTT	13.491	900	53
14	GGG GAG AGG GTC ACT GC	2.875	900	62	45	ACC TTT GCT TTC TTG TTG TT	14.357	900	50
15	GGG CTT CTT TGC TCC TCC TAT GG	3.027	900	60	46	TTG AAA TTT TTA GGG ACT GTG AGA	14.456	900	50
16	GAA GAT GCT AAT GGT GGC TGA TGC	3.137	900	60	47	CAC AGA AGA GCA GAG AAC AGA	15.322	900	60
17	CAA GTG ACA GGT GTG GAG CAA GTT	3.939	900	60	48	CAC CCC ATG AGA CAA CCA G	15.432	900	60
18	GGA GAG CAC ACC CCA GTA GGA TTA	4.046	900	60	49	ACC CAG TAA GCA AGT TTT CA	16.262	900	55
19	GTG ACA GAC CCG TTA CCA A	4.445	900	52	50	ATA AGC TCT CCT CCC TCA AG	16.372	300	55
20	AGA AAG AGA AGG GGA AAA AGA G	4.589	300	52	51	TGG AGT ATG AGG CAG TGA TTG TTA	16.731	300	53
25	TCC CCA TTG CCA GAT AGA GAC ACA	5.434	900	61	52	AGA GTT TTG ACG GCT TCC AGA	16.861	900	53
26	TGG GCC CAA CAG ATT AAC ACA GAG	5.538	900	61	53	TGT AAG CTC CTG TAT TTG GTT TTT	16.977	900	54
27	TGA CTT GCT TGT GCT TGC CTG GAC	5.732	300	58	54	GCC CTA CAG AGC TTT TGG AGA	17.103	900	54
28	ATA ATA ACA AAG TAC TGA GAA AAC	5.841	900	58	55	ACC CCT CCC TCC CCG TAT G	17.927	900	62
57	TGG ATA ACT GCA GGA AGC TGT	6.608	600	68	56	TCC TTC CCT GAC CAC TCC	18.031	900	62
58	GCA GTG CCA GAT CTC CAG AC	6.728	600	68	P15′	TTC TGC CAA GGC TCT GAC ACTA	0.098	400	57
29	GGA TAG ACT TTG CAT GTT TTT GAT	7.285	300	52	P13′	GCA ATG TTG GTA GAC AGA GCT G	0.424	400	57

[a] Positions correspond to the 5′ end of each primer in the 19-kb locus sequence.
[b] Final concentration of each primer in the PCR.

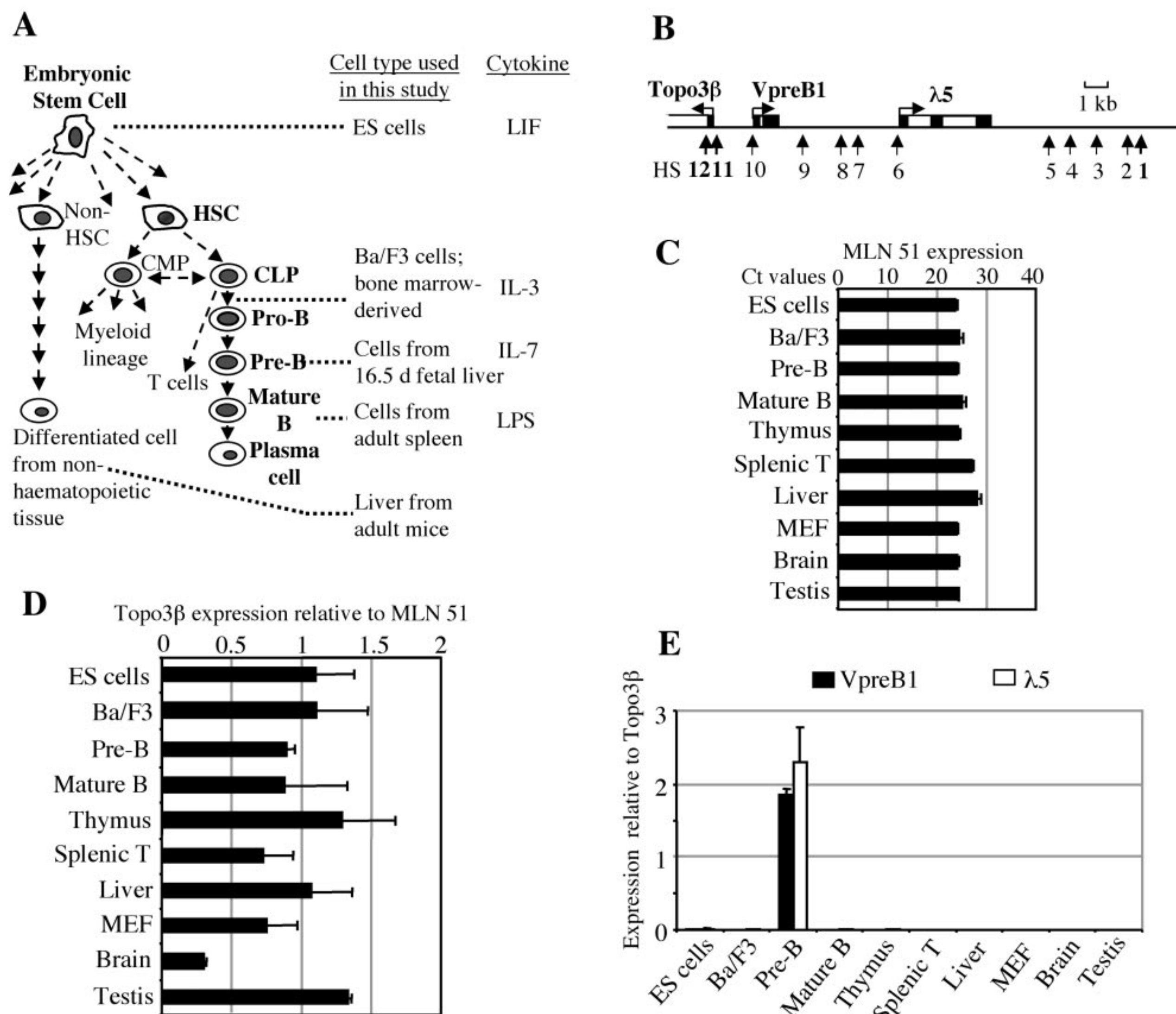
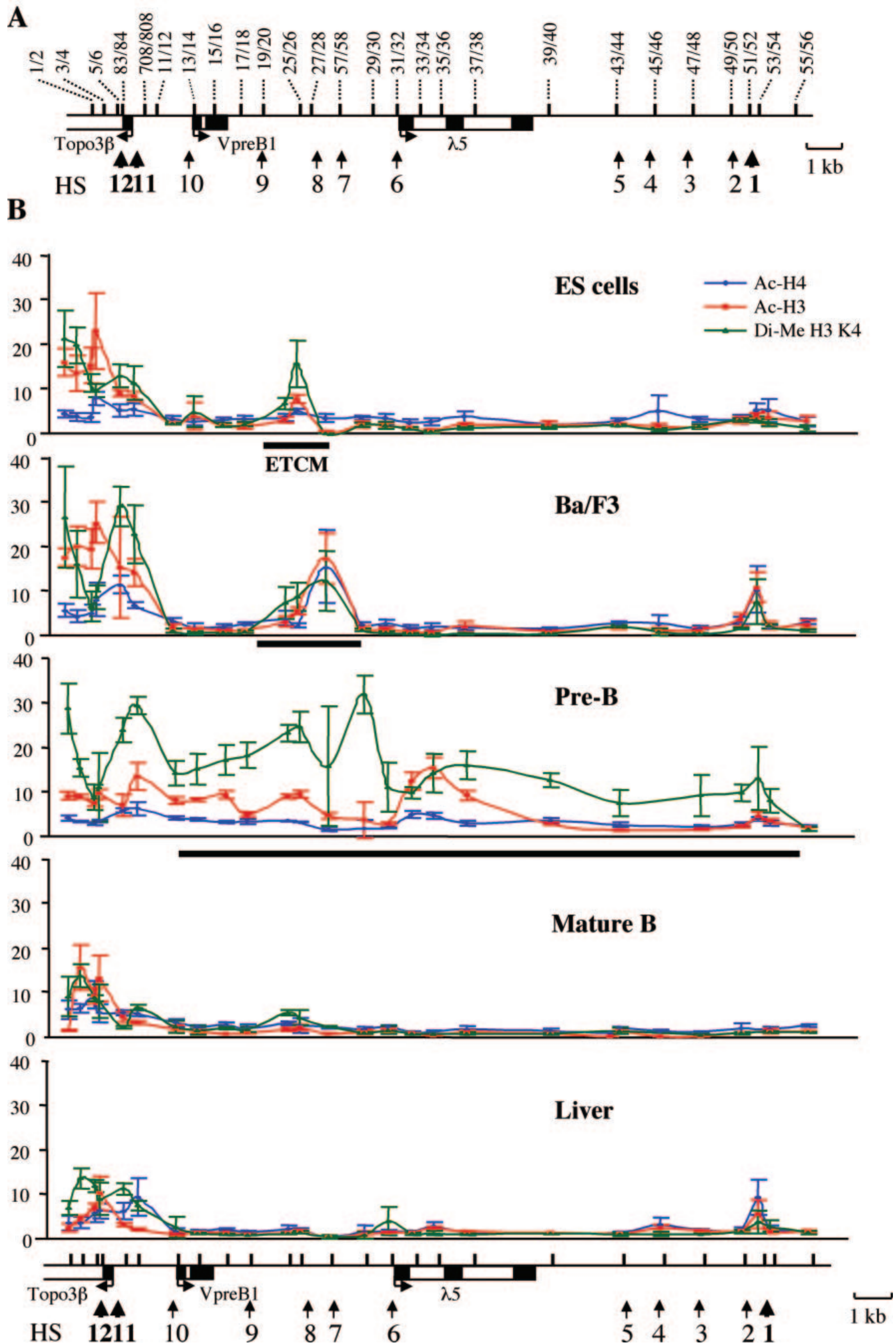


FIG. 1. Experimental system and gene expression analysis. (A) Scheme showing haematopoietic cell differentiation and cell types that were used to represent differentiation stages. ES cells give rise to more specialized, multipotent stem cells, such as haematopoietic stem cells (HSC). HSC develop into common myeloid progenitors (CMP), which generate the myeloid lineage. Common lymphoid progenitors (CLP) give rise to the lymphoid lineage. The relationship between CMP and CLP is controversial (reviewed in reference 32). The B-cell lineage differentiates through progenitor B cells (pro-B cells) and precursor B cells (pre-B cells) and finally generates antibody-producing plasma cells. Ba/F3 cells are IL-3-dependent early pro-B cells. d, day; LPS, lipopolysaccharide. (B) Organization of the *λ5-VpreB1* locus. *VpreB1* and *λ5* are transcribed on the same DNA strand (sense direction), whereas *Topo3*β is transcribed on the opposite strand (antisense direction). Black and white boxes symbolize exons and introns, respectively. Vertical arrows show the positions of the 12 previously mapped DNase I HS (49a, 66). Constitutive HS1, HS11, and HS12 are shown in bold type; the other HS (HS2 to HS10) are pre-B-cell specific. (C) Pattern of expression of the *MLN51* gene. Ct values represent the cycle numbers in real-time PCR at which signals start to be detectable above the threshold. Accordingly, the higher the Ct value, the lower the expression level. MEF, mouse embryonic fibroblasts. (D) Expression of the *Topo3*β gene. Real-time RT-PCR signals for *Topo3*β were normalized to those for *MLN51*. (E) Expression of *VpreB* and *λ5*. Signals were normalized to those for *Topo3*β. Error bars in panels D and E indicate standard deviations.

was set to cross a point at which real-time PCR amplification was linear (0.02 to 0.05 for the majority of the experiments). To test the efficiency of different primer pairs in real-time amplification, we carried out reactions with various known concentrations of a plasmid containing the appropriate region. Calibration curves were generated by plotting the cycle number at which the PCR signal rose above the background (Ct value) against the logarithm of the number of template molecules. The slope of the standard curve was used to determine the efficiency of the PCR (75), which was above 70% with all primer pairs.

To quantify mRNA, a 2.5-μl sample from the RT reaction was added to each PCR. Immunoprecipitated material from ChIP assays was quantified by using a previously described method (45). From the samples (input or immunoprecipitated), equal amounts (0.5 to 2 ng) of DNA were analyzed in every PCR. The enrichment of a given target sequence precipitated by an antibody was determined as the fold difference between the amount of target sequence in the immunoprecipitated fraction and the amount of target sequence in the input DNA (fold enrichment). Average fold enrichment was calculated from at least two independent immunoprecipitations and PCR duplicates for each immunoprecipitation. Normalized values reflecting the binding of transcription factors were expressed as a percentage of input material (fold enrichment multiplied by 100). Error bars in the plots represent the standard deviations of the whole set of values obtained for each PCR amplicon position. The background immunoprecipitation of unfixed chromatin with nonspecific rabbit Ig or no antibody was



A
1/2
3/4
5/6
83/84
708/808
11/12
13/14
15/16
17/18
19/20
25/26
27/28
57/58
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45/46
47/48
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53/54
55/56
Topo3β
VpreB1
λ5
1 kb
HS 12 11 10 9 8 7 6 5 4 3 2 1
B
ES cells
Ac-H4
Ac-H3
Di-Me H3 K4
ETCM
Ba/F3
Pre-B
Mature B
Liver
0
10
20
30
40

determined for each cell type (data not shown). Quantification of transcription factor binding above the background was achieved by subtracting an average of normalized values obtained from three negative controls (treatment of fixed chromatin with anti-Gal4 and anti-VP16 antibodies and without antibody). Thus, enrichment (percentage of input material) reflects only the specific binding of transcription factors and not the affinity of antibodies for different sites.

RACE. Rapid amplification of cDNA ends (RACE) was carried out by using a SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions (see Fig. 7A for positions of primers that were used in RT and PCR). Primer sequences are available upon request. For mapping of initiation sites for intergenic transcripts, nested PCR products were analyzed by blotting and hybridization with a restriction fragment (SphI-BamHI; 5.4 to 6.8 kb) prior to cloning. Nested PCR products were directly cloned into the pGEM-T Easy vector system (Promega) and bacterial strain DH5α. Colonies containing a fragment from the intergenic regions were identified by colony hybridization with the SphI-BamHI probe. At least 10 independent clones were sequenced with each strategy.

RESULTS

Expression status of the λ*5-VpreB1* locus at successive stages of B-cell differentiation. The different stages of B-cell development have been extensively characterized on the basis of the expression of cell surface markers and the rearrangement status of the Ig loci. We investigated gene expression and epigenetic characteristics of the entire λ*5-VpreB1* locus from undifferentiated ES cells through successive stages of B-cell differentiation. Differentiation stages were represented by primary cells from fetal and adult mice and by nontransformed cell lines that provided enough material for the analysis of chromatin structure. The system is shown in Fig. 1A. When cultured under the appropriate conditions (see Materials and Methods), ES cells remain undifferentiated and retain their capacity to give rise to every cell type in the animal (64). Ba/F3 cells are nontransformed, IL-3-dependent bone marrow-derived early B-cell precursors (14, 23, 57) that have been used as models to represent the early pro-B-cell stage of B-cell development (38, 68, 82). They have been found to be positive for very early B-cell markers, such as Mb-1, B29, and E47 monomers and germ line μ transcripts, but do not express markers of later stages of B-cell development, such as CD19, B220, Pax5, EBF, TdT, Rag1, IL-7R, VpreB, and λ5 (71). To study chromatin structure in pre-B cells, primary cultures were established from 16.5-day-old fetal livers and grown on ST-2 feeder cell layers in the presence of IL-7. Primary mature B cells were obtained by lipopolysaccharide activation of cells from adult spleens.

VpreB1 and λ*5* have been shown to be expressed at the pro- and pre-B-cell stages of B-cell development (49) (Fig. 1E). The two genes are located within a 10-kb genomic fragment on mouse chromosome 16 (Fig. 1B). Upstream from the transcription start site for *VpreB1*, sequence analysis identified another gene, *Topoisomerase 3*β (*Topo3*β), which is transcribed on the strand opposite that of *VpreB1* and λ*5*. The promoter of the *Topo3*β gene resembles that of a housekeeping gene and overlaps with a CpG island. To determine the expression pattern for *Topo3*β, we performed quantitative real-time RT-PCR. PCR signals were normalized to those obtained for the metastatic lymph node 51 (*MLN51*) gene (20), which was chosen as a reference because its expression is constant in every cell type examined (Fig. 1C). The results of RT-PCR showed that *Topo3*β was expressed at every differentiation stage and in every tissue that we analyzed (Fig. 1D). Expression varied by less than twofold, with the exception of expression in the brain, which was present at a lower level. These results show that *VpreB1* and *Topo3*β have entirely different expression patterns (Fig. 1E), despite the fact that their transcription start sites are separated by only 1.5 kb.

Histone modifications mark a discrete intergenic site in the λ*5-VpreB1* locus in ES cells. Current evidence indicates that histone modifications mark differentially expressed chromatin domains and also shows that these epigenetic modifications are present in multipotent haematopoietic stem cells before transcription is fully activated (3, 6). We set out to investigate at which differentiation stage these epigenetic marks are established. The small size of the λ*5-VpreB1* domain allowed us to carry out a high-resolution search for histone modifications across the entire locus by ChIP analysis.

The pattern of histone modifications across the λ*5-VpreB1* locus was determined at five differentiation stages (Fig. 2). Unfixed chromatin was isolated from cells and tissues and digested with micrococcal nuclease to produce fragments containing one to three nucleosomes. Digested chromatin was subjected to immunoprecipitation with antibodies recognizing diacetylated histone H3, tetraacetylated histone H4, and dimethylated histone H3 K4. PCR primers were designed to cover the entire 19-kb region at intervals of 0.2 to 1 kb (Fig. 2A and Table 1). The specificity of ChIP analysis was monitored by using either nonspecific rabbit IgG antibody or no antibody. The results of ChIP analysis are shown in Fig. 2B.

Previous studies with immunostaining suggested that the ES cell genome is subject to global histone acetylation. Our ChIP analysis revealed no evidence of generalized acetylation or histone H3 K4 methylation across the λ*5-VpreB1* locus. Instead, we detected a tightly localized peak of histone H3 acetylation and histone H3 K4 dimethylation between the *VpreB1* and λ*5* genes in ES cells (Fig. 2B). In Ba/F3 early pro-B cells, the histone modification mark expands in both directions from

FIG. 2. Patterns of histone modifications in stem cells and differentiated cells. (A) Positions of primers that were used in this study. Thick vertical lines in the locus map indicate PCR amplicon positions; numbers above the locus map correspond to primer pairs. Primer sequences and PCR conditions are shown in Table 1. (B) Histone modification state of the λ*5-VpreB1* locus at different stages of B-cell development. ChIP analysis was performed with unfixed chromatin and antibodies against acetylated (Ac) histones H4 and H3 and dimethylated (Di-Me) histone H3 K4 as described in Materials and Methods. A similar analysis with nonspecific IgG antibody or no antibody confirmed that histone modification signals were detected above the background (data not shown). Fold enrichment of target sequences in the immunoprecipitated material relative to the input material is shown on the *y* axis of each plot. *x* axes represent positions across the λ*5-VpreB1* domain. Error bars indicate standard deviations. A locus map is shown below the plots; amplicon positions are indicated by black vertical lines. A thick black horizontal line below a plot indicates the region in the λ*5-VpreB1* domain that is marked by acetylation and K4 dimethylation of histone H3. The tightly localized mark in ES cells was termed the ETCM.

FIG. 3. Loss of ETCM during in vitro differentiation of ES cells. (A) Morphology of ES cells after in vitro differentiation. (B) Expression of ES cell factors during in vitro differentiation of ES cells. The cells were tested for Oct-4 and Nanog expression by RT-PCR. The measurements for each stage were obtained from three sequential fivefold dilutions. Two independent ES cell lines, E14 and CJ7, expressed these ES cell factors, and their expression was downregulated in embryoid bodies (EB) and lost in differentiated E14 ES cells. *MLN51* served as a loading control. (C) In vitro differentiated (Diff.) ES cells do not express B-cell-specific genes. Real-time RT-PCR signals were normalized to those for *MLN51*. MEF, mouse embryonic fibroblasts. Error bars indicate standard deviations. (D) ChIP analysis of the *λ5-VpreB1* domain in in vitro differentiated ES cells. Explanations of the plots are given in the legend to Fig. 2B. Note the reduction in histone modifications at the ETCM in differentiated ES cells. The low residual signal could be attributed to a small number of undifferentiated ES cells that remained in the differentiated culture.

this discrete site toward the *VpreB1* and λ*5* genes. In pre-B cells, in which the *VpreB1* and λ*5* genes are fully active, H3 K4 dimethylation extends across the rest of the 19-kb region. Acetylation of H3 in pre-B cells shows a more localized pattern of peaks and troughs that extend only as far as the 3′ end of the λ*5* gene. A low and uniform level of histone H4 acetylation is observed across the locus, in keeping with previously published results describing relatively constant levels of H4 acetylation across the human growth hormone and human β-globin loci (36, 70) but in contrast to the nonuniform state of H4 acetylation across the murine β-globin locus (17, 34). Our results suggest that H4 acetylation is not involved in the establishment of the active λ*5-VpreB1* domain during B-cell development. In mature B cells and adult liver, histone modifications are largely absent from the locus. Peaks of histone acetylation and H3 K4 dimethylation are present around the initiation site for the *Topo3*β gene in all cell types examined. This finding is consistent with the observation that the *Topo*3β gene is ubiquitously expressed. We observed a sharp transition between the histone modification states of the *Topo3*β promoter and the silenced *VpreB1* promoter in cells that do not express the *VpreB1* and λ*5* genes. The transition occurs in the region between the transcription initiation site for *VpreB1* and a point approximately 600 bp upstream of the *VpreB1* start site.

To further investigate the relationship between the localized intergenic histone modification mark and B-cell lineage-specific expression of the λ*5-VpreB1* locus, ES cells were allowed to differentiate in vitro into nonlymphoid cells (see Materials and Methods) (Fig. 3). After 2 weeks of differentiation, the cell cultures were confluent, with flattened fibroblast-like cells, and only a small population of ES cell colonies remained (Fig. 3A). Differentiation was confirmed by showing that the expression of the known ES cell-specific factors Oct-4 (55) and Nanog (9) was silenced in in vitro differentiated ES cells (Fig. 3B). The facts that these cells were adherent in culture and that B-cell-specific transcripts of the *Mb-1* (67), *B29* (27), and *VpreB1* and λ*5* genes were absent (Fig. 3C) showed that the ES cells did not differentiate into the B-cell lineage. ChIP analysis of the λ*5-VpreB1* locus indicated that the intergenic histone H3 acetylation and histone H3 K4 dimethylation marks largely disappeared in differentiated ES cells (Fig. 3D). The low residual level of modification that was still detectable was likely due to a small number of undifferentiated ES cells remaining in the cultures.

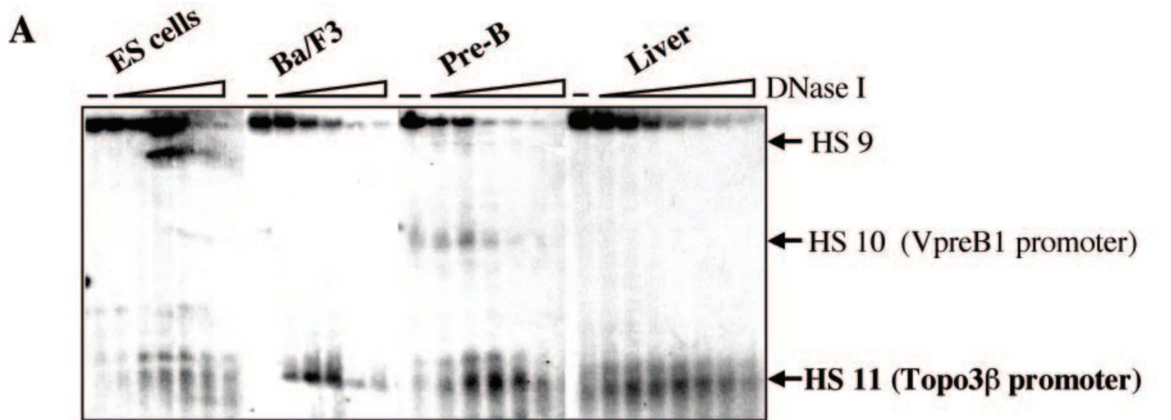
These results show that a localized epigenetic mark is present in the λ*5-VpreB1* domain in pluripotent ES cells. The mark expands during commitment to the B-cell lineage and disappears during differentiation into nonlymphoid cells. Functional analysis has shown that the region containing the mark is able to enhance the transcription of a transgene driven by the *VpreB1* promoter in pre-B cells (49a). Colocalization of the mark with a transcriptional enhancer suggests that this region is involved in initiating the formation of a transcriptionally active chromatin structure in the λ*5-VpreB1* domain. Therefore, we propose the name early transcription competence mark (ETCM) to describe this type of modification.

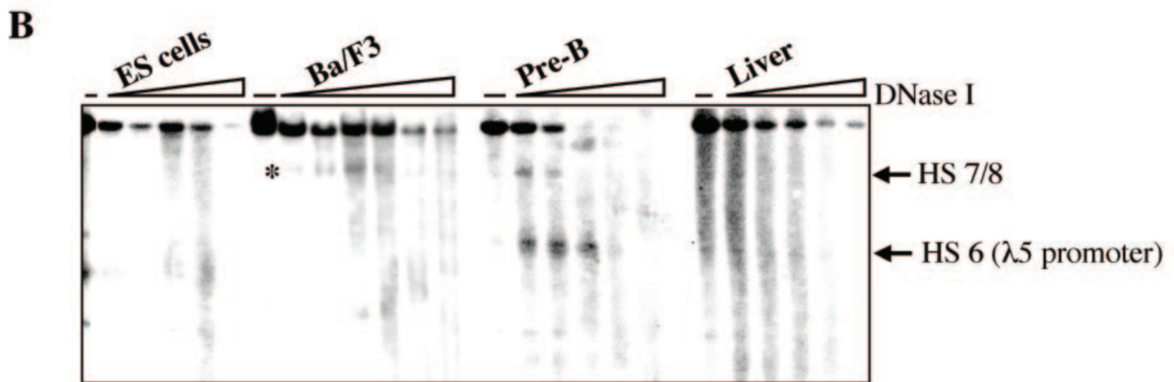
The ETCM becomes hypersensitive to DNase I digestion prior to the activation of *VpreB1* and λ5 transcription. It was of particular interest that the marked site is a *cis*-acting regulatory element that is likely to be involved in the assembly of transcription factor complexes during B-cell differentiation. It has been reported that DNase I HS formation precedes the activation of the β-globin gene during erythroid cell commitment in multilineage progenitors (29). Two DNase I HS (HS7 and HS8) have been mapped to the ETCM region in pre-B cells (49a, 66). In ES cells, the mark coincides with the region in which HS8 is located; it then spreads to encompass HS7 and HS8 in early pro-B cells. To investigate the timing of HS formation during differentiation, the DNase I hypersensitivity of the entire locus was analyzed in ES cells, Ba/F3 early pro-B cells, and pre-B cells (Fig. 4). In Ba/F3 early pro-B cells, in which *VpreB1* and λ*5* are not yet expressed, an HS appeared between the genes near the locations of HS7 and HS8 in pre-B cells (Fig. 4B and D). The mapping strategy used in this study did not discriminate between HS7 and HS8 due to their close proximity; they are separated by less than 100 bp. In pre-B cells, multiple HS, including HS7 and HS8 and the promoters of *VpreB1* (HS10) and λ*5* (HS6), were observed. A previously characterized 3′ HS (HS1) (66) and an HS at the ubiquitously expressed *Topo3*β promoter (HS11) were detected in all cell types examined. The appearance of the otherwise pre-B-cell-specific HS7 and HS8 in early pro-B cells shows that marking of the intergenic *cis*-acting regulatory element by histone modifications is followed by further structural changes that precede transcriptional activation.

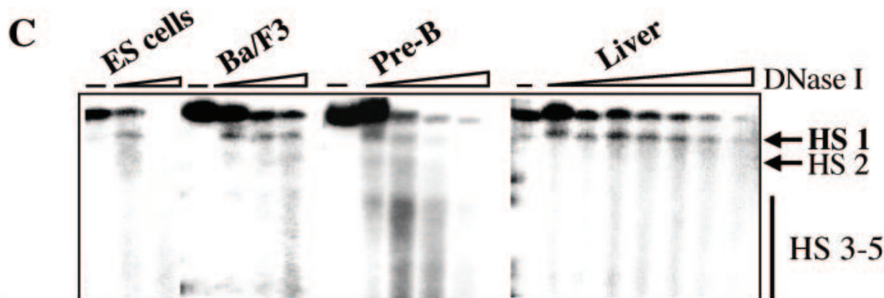
The ETCM is a center for general transcription factor recruitment during B-cell development. There is evidence that general transcription factors and RNA Pol II can bind to *cis*-acting regulatory elements, such as enhancers (30, 35, 48, 62, 73). Therefore, we investigated the recruitment of the general transcription machinery (including factors that are involved in transcriptional initiation and others that are parts of chromatin-modifying complexes) to the ETCM by ChIP analysis. TBP, TAF5, and TAF10 are components of the transcription factor IID (TFIID) complex, which plays a role in the formation of the preinitiation complex (52). TAF5 and TAF10 are subunits of the TBP-free TAF-containing (TFTC) complex, which contains GCN5 histone acetyltransferase and plays a role similar to that of the yeast SAGA complex in gene regulation (7, 84). Brg1 is a subunit of the SWI/SNF ATP-dependent chromatin-remodeling complex (51). Transformation-transactivation domain-associated protein (TRRAP) is a coactivator in histone acetyltransferase complexes, such as STAGA, PCAF, TFTC, and TIP60 (7, 18, 46).

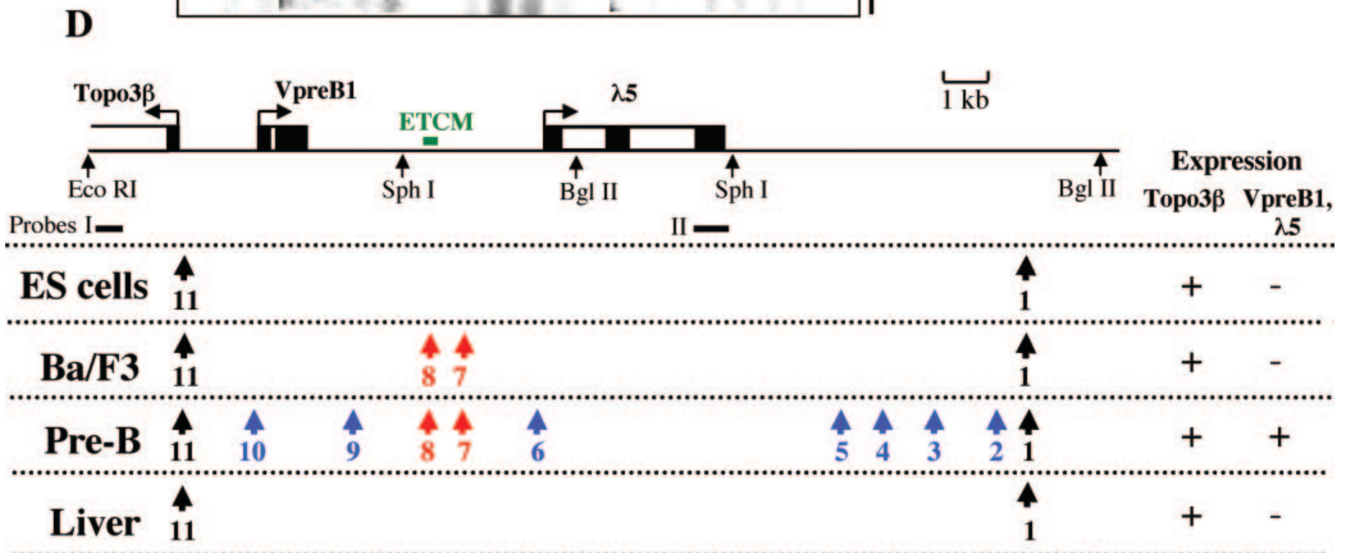
The results of ChIP analysis are shown in Fig. 5. They reveal a complex relationship between binding of these factors and changes in epigenetic marking of the locus during B-cell differentiation. In undifferentiated ES cells, we detected TAF10, TBP, and Pol II but not Brg1 in the ETCM region (Fig. 5A). Surprisingly, at this stage, Pol II binding was observed only in the ETCM region and not at the promoters of the *VpreB1* and λ*5* genes. The binding of TAFs, TBP, and TRRAP was also observed at the promoters of the genes and in a region immediately downstream from the *VpreB1* gene. It is notable that the neighboring HS7 region, which is not part of the ETCM, showed no evidence of factor binding in ES cells.

In Ba/F3 early pro-B cells, in which λ*5* and *VpreB1* are not yet expressed, the presence of the transcription factors investigated was less detectable at many sites (including the gene promoters) than it was in ES cells (Fig. 5A). In contrast, in the


A
ES cells
Ba/F3
Pre-B
Liver
DNase I
HS 9
HS 10 (VpreB1 promoter)
HS 11 (Topo3β promoter)


B
ES cells
Ba/F3
Pre-B
Liver
DNase I
*
HS 7/8
HS 6 (λ5 promoter)


C
ES cells
Ba/F3
Pre-B
Liver
DNase I
HS 1
HS 2
HS 3-5


D
Topo3β
VpreB1
ETCM
λ5
1 kb
Eco RI
Sph I
Bgl II
Sph I
Bgl II
Probes I
II
Expression
Topo3β
VpreB1, λ5
ES cells
11
1
+
-
Ba/F3
11
8
7
1
+
-
Pre-B
11
10
9
8
7
6
5
4
3
2
1
+
+
Liver
11
1
+
-

ETCM region and at HS7, increased levels of TBP and TAFs were observed. Interestingly, the recruitment of transcription factors to HS7 coincided with the expansion of the region of histone modifications (Fig. 5A). At this stage, the chromatin-modifying factors Brg1 and TRRAP became localized to the ETCM region, and DNase I HS7 and HS8 were detected for the first time. Another important observation is that Pol II still bound only to the ETCM region and was absent from the rest of the locus. These results suggest that the HS7-HS8 region is a center for transcription factor recruitment and that the increasing complexity of factor binding correlates with localized changes in the chromatin structure of the region immediately prior to the activation of the locus in early pro-B cells.

In pre-B cells, in which the locus is fully active, the binding of general transcription factors and Pol II was detected across the λ*5-VpreB1* domain (Fig. 5B). A dramatic increase was observed in the abundance of factors such as TBP, Brg1 and, most interestingly, Pol II, in the ETCM region and at the active gene promoters (compare scales in Fig. 5A and B). In contrast to the findings at the earlier differentiation stages, in pre-B cells, TAF5 and TAF10 were preferentially detected at HS8. The finding that this substantial increase in factor binding to the locus was observed at HS8 and in the ETCM region at the pre-B-cell stage indicates that the region continues to be a major center for the recruitment of general transcription factors when the λ*5-VpreB1* domain is active.

Expansion of the region of histone modifications correlates with the binding of transcription factors PU.1 and E2A. The expansion of the marked region and the formation of the general transcription factor recruitment center in early pro-B cells suggest that chromatin-modifying complexes and basal factors are recruited to the λ*5-VpreB1* locus by lineage-specific factors that can recognize a specific sequence in the intergenic region. E2A is a ubiquitously expressed factor which is upregulated in pro- and pre-B cells (33) and has been shown to be directly involved in activating λ5 and VpreB1 expression (71). PU.1 is expressed in haematopoietic stem cells and in lymphoid and myeloid progenitors, in which it plays an important role in specifying the choice between lymphoid and myeloid lineages (10). Its expression is maintained in all differentiating haematopoietic cells except for T cells (1, 2, 25). Multiple consensus recognition sequences for E2A and PU.1 are found across the locus, and the HS7-HS8 region also contains conserved binding sites for E2A and PU.1, with a cluster of PU.1 binding sites at HS7 (49a).

ChIP analysis was used to assess the binding of these factors to the intergenic region and the gene promoters at different stages of development (Fig. 6). Neither factor bound to the locus in ES cells, despite the fact that E2A is expressed at this stage. In Ba/F3 early pro-B cells, the binding of PU.1 coincided with the expansion of the region of histone modifications. Interestingly, the highest level of PU.1 binding at HS7 correlated with the expansion of the histone modification mark and the recruitment of general transcription factors at the early pro-B-cell stage. In pre-B cells, in which the λ*5* and *VpreB1* genes are active, continued binding of PU.1 was accompanied by a striking increase in the binding of E2A in the intergenic region. These results suggest that lineage-specific factors, such as PU.1 and E2A, contribute to the establishment of the active λ*5-VpreB1* domain by directing the recruitment of chromatin-modifying and general transcription factor complexes during B-cell differentiation.

The intergenic region contains active promoters. The observation that general transcription factors and Pol II bind to the ETCM region raised the possibility that factor recruitment is due to the presence of intergenic promoters. Sense and antisense intergenic transcription was described previously for several tissue-specific domains in mammals (19, 48, 60, 63, 65, 78, 79) and *Drosophila* (13, 61). Quantitative RT-PCR analysis detected significant levels of intergenic transcripts in the λ*5-VpreB1* locus in pre-B cells (data not shown). To test whether sites of recruitment of general transcription factors coincided with intergenic promoters, transcription initiation sites were mapped by RACE (Fig. 7A). RACE-PCR products were cloned, and bacterial colonies were screened for the presence of locus sequences by colony hybridization. Positive clones were sequenced to determine initiation sites. Significant changes were observed in the patterns of transcription initiation sites during differentiation from ES cells to pre-B cells (Fig. 7B and C).

Multiple initiation sites were detected in ES cells, indicating that these cells are transcriptionally permissive. Correct initiation from the λ*5* promoter was not observed at this stage. In Ba/F3 early pro-B cells, only a single intergenic initiation site was detected; it was located in the ETCM region and was transcribed in the antisense direction relative to the *VpreB1* and λ*5* genes. Two main clusters of start sites were observed in pre-B cells. One group of transcripts (antisense relative to the genes) initiated at HS8, where the ETCM is located. The other major cluster of start sites, transcribing in the same direction as the *VpreB1* and λ*5* genes (sense direction), was located near HS9. This analysis showed that intergenic promoters are located at HS8 and HS9. However, the fact that levels of Pol II at HS8 in ES cells were 20-fold higher than at HS9 suggests that high-level Pol II recruitment is a specific feature of the ETCM and is not due solely to the presence of an intergenic promoter. Taken together, our results suggest that the ETCM is a localized center for the recruitment of transcription factors, including RNA Pol II, from undifferentiated ES cells to (and through) the pre-B-cell stage.

FIG. 4. Changes in patterns of DNase I HS during differentiation. The 12 HS in pre-B cells were mapped elsewhere (49a). (A) Pattern of HS at the 5′ region of the λ*5-VpreB1* domain. DNA was digested with EcoRI and SphI, Southern blotted, and hybridized with probe I (see the map in panel D). (B) Pattern of HS in the central part of the λ*5-VpreB1* domain. DNA was digested with SphI, Southern blotted, and hybridized with probe II (see the map in panel D). The appearance of HS7 and HS8 in Ba/F3 early pro-B cells is indicated by an asterisk. (C) Mapping of HS downstream of the λ*5* gene. DNA was digested with BglII, Southern blotted, and hybridized with probe II. (D) Summary of HS patterns at successive differentiation stages. Restriction sites and locations of probes used for mapping are shown below the locus map. Color key: black, constitutive HS; blue, pre-B-cell-specific HS; red, HS found in early pro-B and pre-B cells.

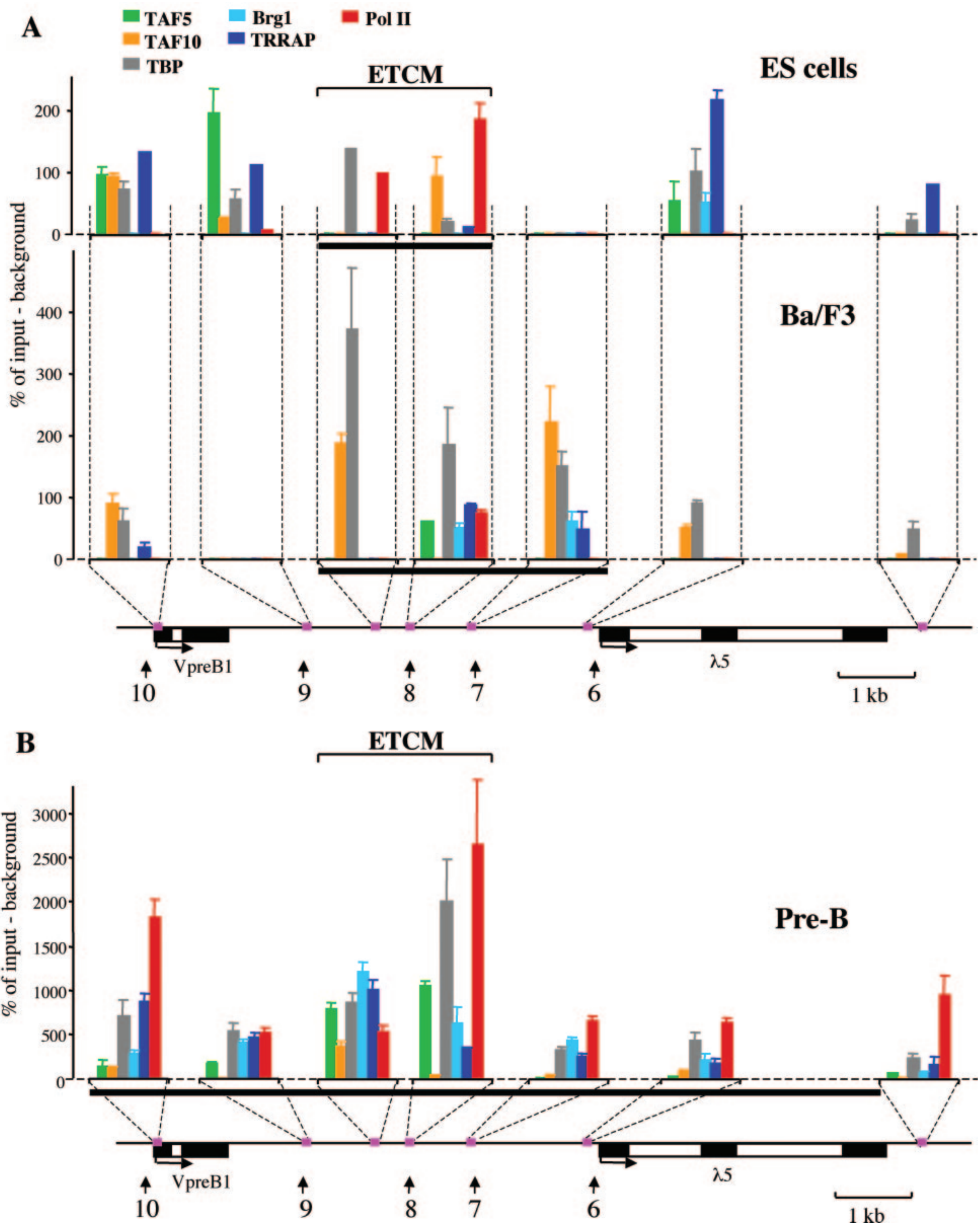


FIG. 5. The ETCM is a center for general transcription factor recruitment during B-cell development. (A) Binding profiles for general transcription factors at early stages of B-cell differentiation. ChIP analysis was performed with fixed chromatin as described in Materials and Methods. The percentage of target sequences in the immunoprecipitated material relative to the input material is shown on the *y* axis of each plot. Background immunoprecipitation (an average normalized value obtained by treatment of chromatin with two nonspecific antibodies and with no antibody) was subtracted from normalized specific ChIP signals (obtained by immunoprecipitation with antibodies to general transcription factors) at each position. *x* axes represent positions across the λ*5-VpreB1* domain. A locus map is shown below the plots; amplicon positions are indicated by pink boxes. A thick black horizontal line below each plot indicates the region in the λ*5-VpreB1* domain that is marked by acetylation and K4 dimethylation of histone H3. (B) Binding profiles for general transcription factors at the pre-B-cell stage. Quantification, symbols, and color codes are explained in panel A. Because of the higher level of transcription factor binding in pre-B cells, the scale on the *y* axis is different from that in panel A. Error bars in panels A and B indicate standard deviations.

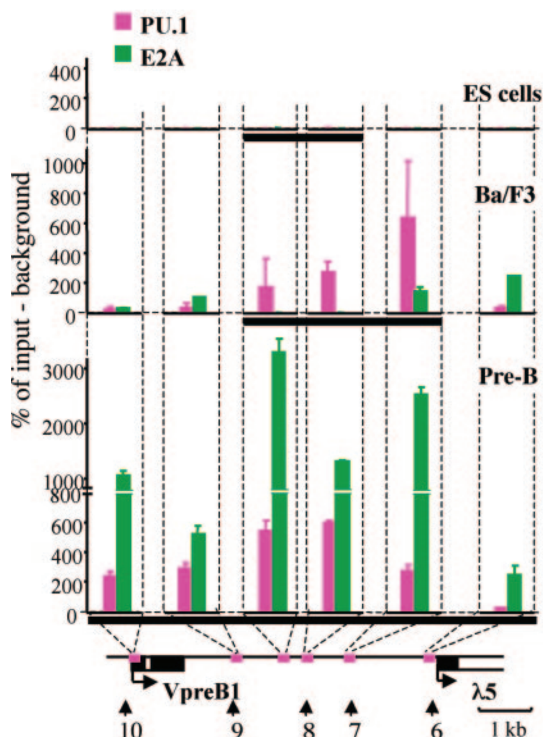


FIG. 6. ChIP analysis of binding of lineage-specific transcription factors E2A and PU.1. ChIP was performed with fixed chromatin as described in Material and Methods. Explanations are given in the legend to Fig. 5. A thick black horizontal line below each plot indicates the region in the *λ5-VpreB1* domain that is marked by acetylation and K4 dimethylation of histone H3.

DISCUSSION

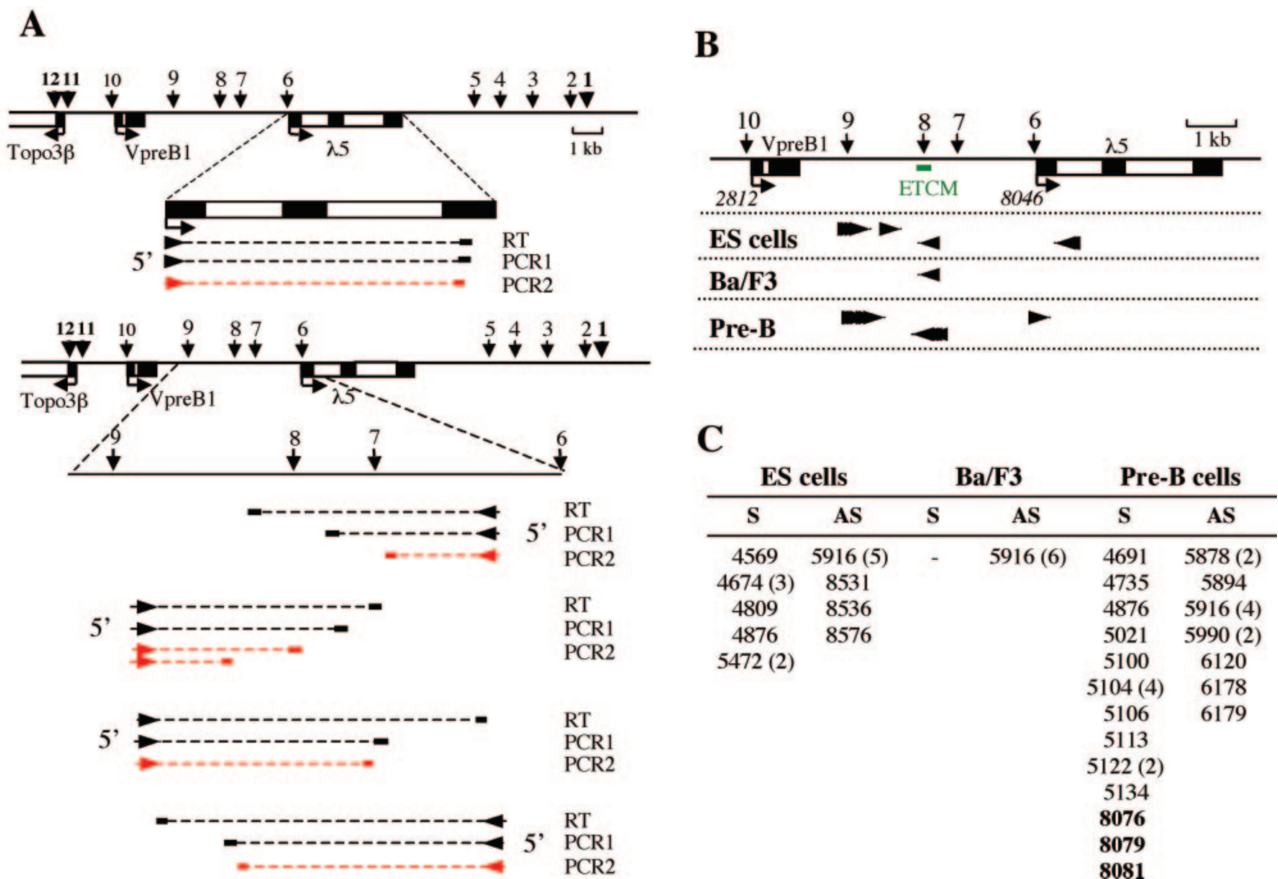
Localized epigenetic marking of the *λ5-VpreB1* domain in ES cells. A defining characteristic of stem cells is their ability to activate multiple gene expression programs that give rise to different specialized cell types. Stem cells also have the important property of being able to divide and self-renew while maintaining their multipotential capacity. Differentiation potential can be described in terms of the number of genes that can be activated as the cell (and its daughter cells) progress through a particular lineage. A fundamental question in stem cell biology concerns the nature of the epigenetic mechanisms that regulate this potential. Analysis of global histone modification patterns in ES cells has suggested that the ES cell genome is subject to generalized histone acetylation and histone H3 K4 methylation (37, 41). Our high-resolution analysis of the histone modification pattern across a specific gene expression domain raises doubts about this conclusion, as most of the *λ5-VpreB1* domain shows no evidence of general histone H3 and H4 acetylation and histone H3 K4 methylation at the ES cell stage. A similar absence of general acetylation and H3 K4 methylation was observed at the α-globin locus in ES cells (3).

Instead of global histone modifications, our results demonstrate the existence of a tightly localized mark of H3 acetylation and histone H3 K4 methylation (the ETCM) which is already established in the *λ5-VpreB1* locus in ES cells. Several lines of evidence suggest that the ETCM is likely to contribute to the establishment of the active epigenetic state of the *λ5-VpreB1* domain. A key observation is the fact that the region of histone modifications expands as the cells progress toward the B-cell lineage and disappears when they differentiate into nonlymphoid cells. In addition, the ETCM region is a center for the recruitment of transcription factors and RNA Pol II at all stages from ES cells to pre-B cells. The progressive recruitment of lineage-specific and general transcription factors during B-cell differentiation correlates with the formation of the first B-cell lineage-specific DNase I HS in the locus. In pre-B cells, in which the *VpreB1* and *λ5* genes are expressed, the HS7-HS8 region shows the highest level of transcription factor and Pol II binding and also has enhancer activity. A schematic representation of the successive changes in the epigenetic state of the *λ5-VpreB1* locus during B-cell differentiation is shown in Fig. 8.

Establishment of the active *λ5-VpreB1* domain during B-cell development. The establishment of a localized epigenetic mark at early stages of development implies that sequence-specific factors bind to the region and initiate the recruitment of histone-modifying enzymes and general transcription factors. The factors that establish the ETCM in ES cells have not been identified, but we do have evidence that the expansion of the mark in early pro-B cells involves the action of transcription factor PU.1, which is known to play a key role in determining the choice between lymphoid and myeloid lineages. High levels of PU.1 binding are observed at HS7 at the time when histone acetylation and K4 methylation and recruitment of general transcription factors expand into this region. It is interesting that Brg1 and TRRAP, factors that are involved in modifying chromatin structure, became detectable at HS7 and HS8 at this stage. PU.1 has been shown to cooperate with various transcription factors and chromatin-remodeling factors to activate or repress transcription (15, 40, 53, 59, 74).

As the cells progress to the pre-B-cell stage, expansion of the region of histone modifications across the *λ5-VpreB1* domain is accompanied by high-level binding of E2A to the locus (Fig. 8). The highest level of E2A binding is observed at HS7 and HS8, and PU.1 also continues to bind to the same region. Interestingly, E2A has been shown to act in synergy with PU.1 to activate transcription in B lymphocytes (81). These data, together with the fact that the ETCM region is a center for general transcription factor binding, suggest that the factor recruitment center at HS8 is likely to be formed by cooperation between lineage-specific activators, chromatin modifications, and the general transcription machinery. The presence of a factor recruitment center at HS7-HS8 could facilitate the binding of transcription factors to other regions of the locus both by creating a locally accessible chromatin structure and by direct contacts with these regions through loop formation (21, 31, 58).

The fact that active intergenic promoters have been detected in the locus raises the question as to whether intergenic transcription has a role in generating the active, open *λ5-VpreB1* domain during B-cell development (19, 48, 60, 65). Theoretically, the factor recruitment that we observed at HS7-HS8 might be due partly to the presence of intergenic promoters. However, two lines of evidence suggest that intergenic transcription on its own may not be sufficient for the establishment of the active epigenetic structure of the *λ5-VpreB1* domain.



ES cells		Ba/F3		Pre-B cells	
S	AS	S	AS	S	AS
4569	5916 (5)	-	5916 (6)	4691	5878 (2)
4674 (3)	8531			4735	5894
4809	8536			4876	5916 (4)
4876	8576			5021	5990 (2)
5472 (2)				5100	6120
				5104 (4)	6178
				5106	6179
				5113	
				5122 (2)	
				5134	
				8076	
				8079	
				8081	

FIG. 7. Mapping of transcription initiation sites by RACE. (A) Mapping of λ*5* transcription start sites (upper panel) and intergenic initiation sites (lower panel). RT and PCR approaches are shown for each mapping strategy. 3′ primers (RT, PCR1, and PCR2) that were used for each region are indicated as short thick horizontal lines extended by dotted lines that represent the part of the transcript that was converted into cDNA. RT was performed with locus-specific 3′ primers to detect RNAs transcribed from either the leading or the lagging DNA strand. The orientation of transcription corresponding to each strategy is indicated by arrowheads. Nested PCR products (PCR2; red lines) were cloned and sequenced. (B) Pattern of intergenic transcription initiation sites. Numbers in italics below the locus map indicate positions of transcription initiation sites for the three genes relative to the EcoRI site 2.8 kb upstream from *VpreB1*. Arrowheads indicate the transcription initiation sites that were mapped in various cell types. The direction of the transcripts is also represented by these arrowheads. (C) Positions of transcription initiation sites. Numbers correspond to positions in the 19-kb locus sequence (as in panel B). Correct initiation sites for the λ*5* gene are shown in bold type. Numbers in parentheses after position numbers indicate how many sequenced clones contained that particular initiation site when there was more than one. S, sense; AS, antisense (relative to the directions of *VpreB1* and λ*5* transcription).

First, the fact that histone modifications and high-level Pol II binding were detected only at the ETCM region and not at the other intergenic initiation sites in ES cells suggests that the presence of an intergenic promoter is not in itself sufficient to generate the mark and establish a factor recruitment center during B-cell development. Second, expansion of the region of histone modifications does not coincide with spreading of the transcribed region in early pro-B cells. Nevertheless, the possibility remains that intergenic transcription facilitates the distribution of transcriptionally active complexes across the active λ*5*-*VpreB1* locus and plays a role in the maintenance of the active state of the domain in pre-B cells.

Assembly of general transcription factor complexes on an epigenetically marked intergenic regulatory element. The binding of TFIID subunits, such as TBP and TAFs, to the intergenic region was surprising, as the recruitment of these factors was believed to be specific to gene promoters. As discussed above, our results suggest that high-level general transcription factor binding is a specific characteristic of the ETCM region and is not due solely to the presence of an active intergenic promoter. There are several possible explanations for the quantitative and qualitative differences in general transcription factor binding at different sites in the locus and at different stages of development (Fig. 5 and 8). The process of assembly and disassembly of general transcription factor complexes is very dynamic, and the rates of turnover of different complexes can vary between different sites (e.g., at HS7-HS8 versus gene promoters). Moreover, TBP occupancy and TRRAP occupancy at the same site can indicate the presence of both TFIID and TFTC-like complexes, as TRRAP has been shown to be a subunit of both TFIID and TFTC but TBP is found only in TFIID (see Results for a more detailed description of these factors and complexes). The fact that we detected components of different complexes at the same site could be due to the sequential recruitment of complexes during the transcriptional initiation and elongation cycle (reviewed in reference 11) or to

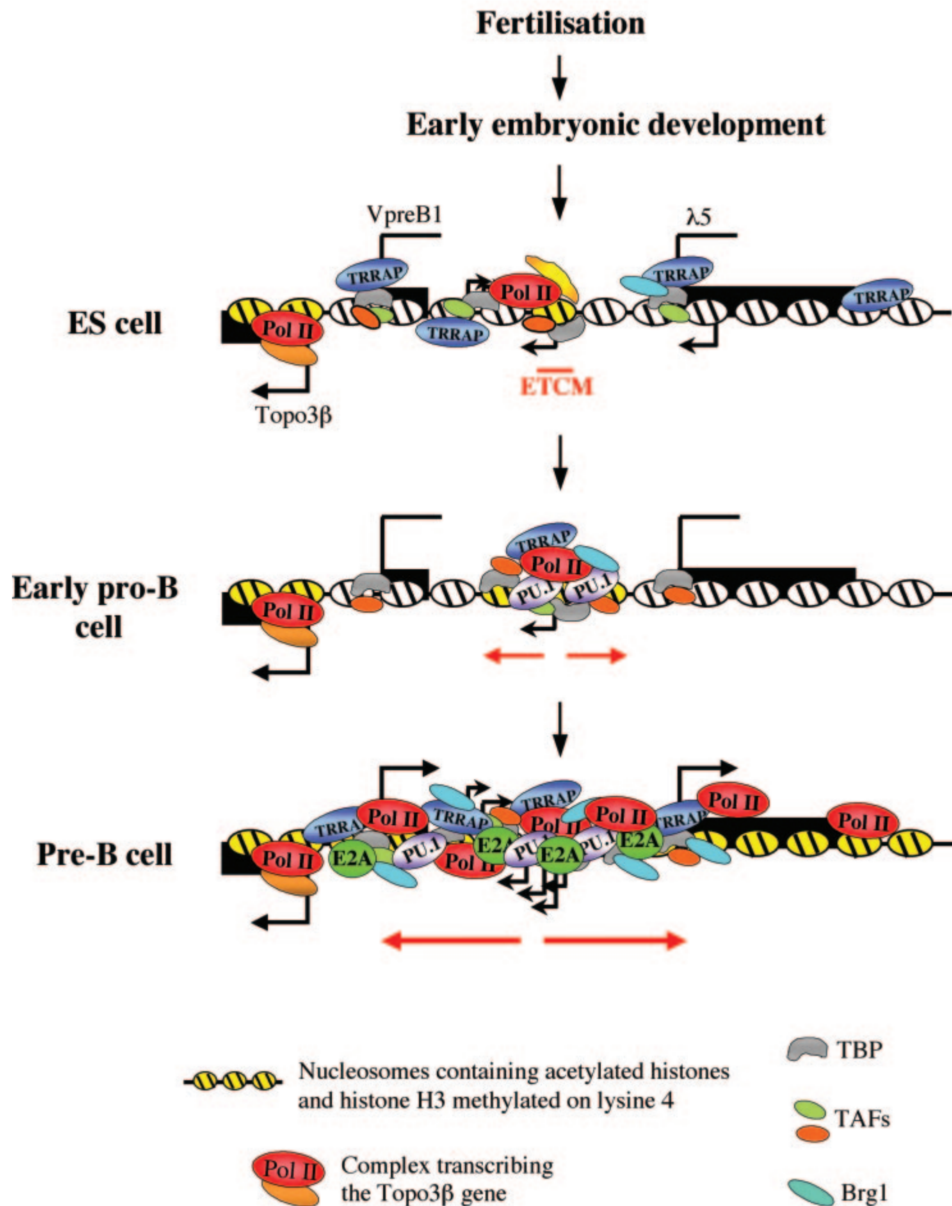


FIG. 8. Schematic illustration of the formation of the active *λ5-VpreB1* domain. The *Topo3*β gene is expressed throughout the entire differentiation program. *VpreB1* and *λ5* are in a transcriptionally potentiated but inactive state in ES cells and in early pro-B cells. Black arrows represent intergenic transcription initiation sites. The ETCM is a localized histone modification mark in ES cells that is likely to be established by sequence-specific transcription factors (yellow shapes). In addition to factor recruitment to the ETCM region, the *λ5-VpreB1* domain is permissive for the binding of general transcription factors at the ES cell stage. Note that Pol II binds only to the ETCM. Expansion of the mark in early pro-B cells (red arrows) would facilitate the recruitment of more transcription factors, the binding of which is reflected in the appearance of DNase I HS in the ETCM region. In early pro-B cells, before the activation of *VpreB1* and *λ5* transcription, the region forms a center for the recruitment of PU.1 and general transcription factors, and the rest of the locus becomes less permissive for factor binding. At the pre-B-cell stage, the epigenetically modified region expands, and additional transcription factor complexes and Pol II are recruited to the entire *λ5-VpreB1* domain. The binding of E2A is likely to be important for the generation of the active epigenetic state of the locus. This process leads to the activation of the *VpreB1* and *λ5* promoters.

cells within the population being at different stages of the cell cycle.

In summary, our data provide evidence for the formation of a dynamically regulated transcription factor recruitment center in the ETCM region from the early stages of B-cell differentiation. These data are consistent with a model proposing that an important function of the epigenetically marked intergenic enhancer is to act as a nucleation center for the assembly of preinitiation complexes prior to their recruitment (or transfer) to the promoters of the genes.

Differential regulation of the closely linked *λ5-VpreB1* and *Topo3*β domains. In pre-B cells, the *λ5-VpreB1* locus is part of a continuous domain of histone H3 K4 methylation that extends across the neighboring *Topo3*β promoter. A particularly

interesting feature of the λ5-*VpreB1* locus is the very short distance between the promoters of the ubiquitously expressed *Topo3*β gene and the developmentally regulated pre-B-cell-specific *VpreB1* gene. In mature B cells and liver, the *VpreB1* promoter remains silent and unaffected by the high levels of histone modifications on the nearby *Topo3*β promoter, despite the fact that the transcription start sites of the two genes are only 1.5 kb apart. The sharp transition of histone modifications suggests that the border of the *Topo3*β and *VpreB1* domains falls within a very short region of approximately 600 bp and that the active *Topo3*β domain does not spread further from this point. These results suggest a tightly controlled epigenetic determination of differentially expressed domains over a very short genomic distance. Since this short region does not include any obvious elements, such as DNase I HS or intergenic promoters, that can have an insulator function, it is more likely that the separate histone modification profiles of the *Topo3*β and *VpreB1* genes are generated by the differential assembly of transcription factor complexes on their *cis*-acting regulatory elements.

Localized epigenetic marking and regulation of differentiation potential. It is widely accepted that the establishment of tissue-specific gene expression domains occurs through a series of sequential events that involve the potentiation of a domain before the genes located within it are fully activated. There is evidence that a specific subset of genes that are expressed late in haematopoietic differentiation are already in a permissive state in haematopoietic stem cells (26). Histone modifications and partial assembly of transcription factor complexes have also been observed on promoters in the α- and β-globin loci and at the c-*fms* and lysozyme genes in haematopoietic progenitors (3, 6, 39, 76, 77). These results have established a paradigm that links the epigenetic potentiation of genes to the differentiation capacity of multipotent stem cells. The basis for the pluripotency of ES cells is much less clear. Current thinking on this issue has tended to focus on the idea of nonspecific global accessibility of the ES cell genome. This accessibility would become restricted as the cells differentiate and acquire specific epigenetic modifications.

Our results suggest an alternative scenario in which there is no fundamental difference between the mechanisms that underlie the multipotency of haematopoietic stem cells and the totipotency of ES cells. According to this model, the transcriptional competence of individual gene loci would be determined by localized chromatin modifications in stem cells, and the differentiation potential of these cells would correlate with the number of marks that are present in the cells. The number and the distribution of the marks would determine the ability of the cells to differentiate along different lineages. The model predicts that more gene loci would be marked in pluripotent ES cells than in multipotent stem cells. As cells differentiated toward a specific lineage, they would lose marks on genes that belong to alternative lineages. The tightly localized nature of the ETCM suggests that such marks would be detected only by high-resolution mapping of the type described in this study. Analysis of the epigenome at this level of resolution is likely to provide further information on the distributions of the marks and their relationship to the differentiation capacities of totipotent and multipotent cells.

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