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Genetics

An enhancer-blocking element between α and δ gene segments within the human T cell receptor α/δ locus

(boundary element/transcription/VDJ recombination)

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ABSTRACT **T cell receptor (TCR) α and δ gene segments are organized within a single genetic locus but are differentially regulated during T cell development. An enhancer-blocking element (BEAD-1, for blocking element alpha/delta 1) was localized to a 2.0-kb region 3′ of TCR δ gene segments and 5′ of TCR α joining gene segments within this locus. BEAD-1 blocked the ability of the TCR δ enhancer (E_δ) to activate a promoter when located between the two in a chromatin-integrated construct. We propose that BEAD-1 functions as a boundary that separates the TCR α/δ locus into distinct regulatory domains controlled by E_δ and the TCR α enhancer, and that it prevents E_δ from opening the chromatin of the TCR α joining gene segments for VDJ recombination at an early stage of T cell development.**

T lymphocytes express either an αβ or a γδ T cell receptor (TCR) heterodimer that is critical for T cell development and function (1–4). The genes encoding the four TCR proteins consist of multiple variable (V), diversity (D), and joining (J) gene segments that are assembled by the process of VDJ recombination during T cell development in the thymus (5, 6). VDJ recombination of TCR and immunoglobulin gene segments is absolutely dependent on the expression of RAG1 and RAG2 (7, 8), components of the VDJ recombinase (9), in developing T and B cells. However, cis-regulatory elements such as enhancers play a critical role in determining locus-specific developmental control of VDJ recombination by modulating the chromatin accessibility of individual TCR or immunoglobulin gene segments to the recombinase (5, 6, 10).

During T cell ontogeny, the TCR β, γ, and δ genes rearrange early, at the $CD4^-CD8^-$ double negative (DN) stage, whereas the TCR α gene rearranges later, at the $CD4^+CD8^+$ double positive (DP) stage (11–13). Although differentially regulated, the TCR α and δ genes are located in the same genetic locus, with TCR δ gene segments nested between V_α and J_α gene segments (14–16) (Fig. 1). Recent studies indicate that rearrangement at this locus is progressive (18–21). Initial $V_\delta D_\delta J_\delta$ and $V_\gamma J_\gamma$ rearrangement, if productive, can direct the synthesis of a γδ TCR and commit thymocytes to develop along the γδ pathway. However, in thymocytes with nonproductive $V_\delta D_\delta J_\delta$ or $V_\gamma J_\gamma$ rearrangements but a productive $V_\beta D_\beta J_\beta$ rearrangement (22–25), subsequent V_α to J_α rearrangement can delete rearranged TCR δ gene segments, direct the synthesis of an αβ TCR, and commit thymocytes to develop along the αβ pathway.

The TCR α/δ locus contains numerous cis-acting elements that are likely to play important roles in the developmentally regulated rearrangement and expression of gene segments within the locus. These include the promoters upstream of each of the V_α and V_δ gene segments, the TCR δ enhancer (E_δ) within the $J_\delta 3$-C_δ intron (26, 27), the TEA promoter 5′ of J_α gene segments (28, 29), and the TCR α enhancer (E_α) (30, 31), silencer elements (32), and locus control region (33) 3′ of C_α (Fig. 1). To date, several of these elements have been implicated as important developmental regulators of VDJ recombination *in vivo*. For example, in transgenic mice carrying an integrated VDJ recombination substrate, E_δ activates VDJ recombination at the DN stage and in the precursors of αβ and γδ T cells (34, 35), whereas E_α activates VDJ recombination at the DP stage and in the precursors of αβ T cells only (35, 36). Because these results mimic the behavior of $V_\delta D_\delta J_\delta$ and $V_\alpha J_\alpha$ rearrangement, respectively, at the endogenous TCR α/δ locus, E_δ and E_α are implicated as critical developmental regulators of VDJ recombination and lineage commitment at the endogenous locus. The TEA promoter is activated between the DN and DP stages of thymic development, at the immature single positive stage (21). Recent analysis of mice carrying a homozygous deletion of TEA has shown that TEA is critical for the targeting of VDJ recombination events to a discrete window of the TCR α/δ locus that encompasses the most 5′ J_α gene segments (37).

Given the complexity of the TCR α/δ locus and the large number of cis-acting elements that are likely to exert either positive or negative regulatory influences on VDJ recombination and transcription, it will be important to evaluate the mechanisms by which the effects of these elements are restricted to discrete regions of the locus. For example, E_δ is thought to promote accessibility of TCR δ gene segments to the recombinase in DN thymocytes (34, 35). How is E_δ prevented from similarly activating nearby J_α segments at this stage? The activation of J_α segments for recombination to V_α or V_δ segments in DN thymocytes might be expected to prematurely delete TCR δ gene segments and thereby limit the production of γδ lymphocytes.

Boundary elements are thought to separate chromatin into distinct units or domains controlled by different regulatory elements (38–40). Boundary elements such as scs and scs′ in the *Drosophila* 87A7 heat shock locus (41, 42), su(Hw) protein binding sites in the *Drosophila gypsy* transposon (43, 44), 5′HS4 in the chicken β globin locus (45), and Fab-7 in the *Drosophila* bithorax complex (46–49), can block an enhancer from activating a promoter when located between the two in a chromatin-integrated construct, and can insulate a transgene from position effects. The enhancer-blocking activity of boundary elements is clearly distinct from silencing, because it is strictly dependent on boundary element position and occurs without

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Abbreviations: V, variable; D, diversity; J, joining; TCR, T cell receptor; E_δ, TCR δ enhancer; E_α, TCR α enhancer; BEAD-1, blocking element alpha/delta 1; TEA, T-early-α; DN, double negative; DP, double positive; neo, neomycin; hyg, hygromycin; E, 380-bp E_δ fragment; P, $V_\delta 1$ promoter; FBS, fetal bovine serum; KC, *Kpn*I–*Cla*I DNA fragment; BB, *Bgl*II–*Bam*HI fragment; RN, *Eco*RI–*Nsi*I fragment.

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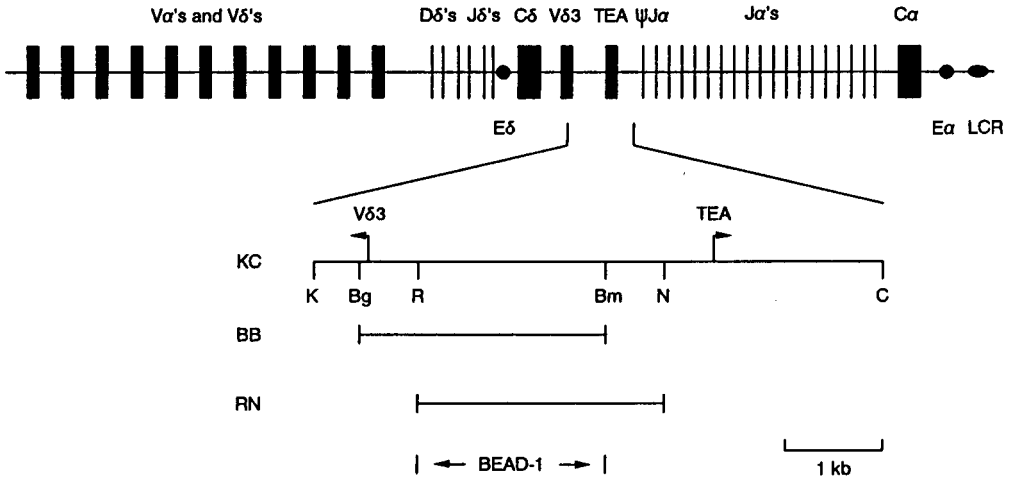

FIG. 1. Schematic map of the human TCR α/δ locus. Filled rectangles represent V_α, V_δ, C_δ and C_α gene segments, as well as the T-early-α (TEA) exon. Vertical lines represent D_δ, J_δ and J_α gene segments. E_δ and E_α are represented by ●, and the TCR α locus control region (LCR) (to date defined only in mouse) is represented as a filled oval. Transcriptional orientation is left to right for all gene segments except $V_\delta 3$ (rearrangement of $V_\delta 3$ occurs by inversion of D_δ, J_δ, and C_δ gene segments). ψJ_α is a nonfunctional J_α thought to serve as an acceptor for initial rearrangement events into the J_α region (17). Restriction enzymes are K, *Kpn*I; Bg, *Bgl*II; R, *Eco*RI; Bm, *Bam*HI; N, *Nsi*I; C, *Cla*I.

repressing either the enhancer or the promoter (50, 51). The locations of *Drosophila* scs and scs′, which flank a pair of divergently transcribed heat shock genes, and 5′HS4, which lies at one end of the chicken β globin locus, suggest that these elements function to prevent crossregulation between these and adjacent loci. Fab-7 lies between the iab-6 and iab-7 domains of the bithorax complex, and is required for the independent regulation of these domains (46, 47). Due to the close apposition of differentially regulated gene segments within the TCR α/δ locus, we wondered whether a boundary element with enhancer-blocking activity might be located between TCR δ and J_α gene segments, such that it would prevent E_δ from opening J_α segments for VDJ recombination during the early stage of T cell development.

MATERIALS AND METHODS

DNA Constructs. Constructs were generated as follows: A 2.3-kb neomycin (neo) gene fragment was excised from the plasmid pSRαNeo (52) by digestion with *Bam*HI, treatment with the Klenow fragment of DNA polymerase I, and subsequent digestion with *Hin*dIII. The plasmid pV_δ1-CAT (26), which carries a 1.6-kb V_δ1 promoter fragment, was digested with *Kpn*I, treated with T4 polymerase, and digested with *Hin*dIII to remove the chloramphenicol acetyltransferase gene, and the neo gene was inserted in its place. A 380-bp E_δ (E) fragment (53) was then cloned upstream of the V_δ1 promoter (P) to generate E-P-Neo. All other fragments were introduced by blunt-end ligation into the *Xba*I site upstream of E_δ, the *Eco*RV or *Sal*I sites between E_δ and the V_δ1 promoter, or the *Xho*I or *Kpn*I sites downstream of Neo. To generate the plasmid pTK-hyg, a 2.2-kb fragment carrying the thymidine kinase promoter and hygromycin B (hyg) gene was excised from the plasmid pMEP4 (Invitrogen) by digestion with Pflm I, treatment with T4 polymerase, and digestion with *Not*I, and the resulting fragment was then ligated into *Eco*RV and *Not*I digested pBluescript KS^+ (Stratagene). All plasmids were purified by two CsCl density gradient centrifugation steps and were linearized by *Not*I digestion. Following three phenol and two chloroform extractions, linearized plasmids were ethanol precipitated and resuspended in 10 mM Tris·HCl, pH 8.0, and 1 mM EDTA.

Soft Agar Colony-Forming Assay. The human T cell leukemia Jurkat was cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA). The culture was split 24 hr before harvesting for transfection. Transfection with each construct was performed in triplicate. Jurkat cells were adjusted to 1.0×10^7 cells/ml in cold RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), and for each transfection, 1.1 pmol (5.0–10.0 μg) of linearized plasmid was introduced into 0.4 ml of cell suspension in a 2.0 mm-gap cuvette. Cells were electroporated using a BTX (San Diego) Electrocell Manipulator ECM 600 at 250 V, 600 μF, 129 Ω in low voltage mode. After electroporation, 1.0 ml cold RPMI 1640 medium supplemented with 20% FBS was added to the cuvette, and cells were placed on ice for 20–30 min. The cell suspension was then transferred into 10 ml of RPMI 1640 medium supplemented with 10% FBS, and cells were cultured at 37°C for 48 hr. Following culture, cells were pelleted, resuspended in 1.0 ml RPMI 1640 medium supplemented with 10% FBS, and were plated following addition of 30 ml of soft agar plating medium [1 vol 0.66% agar (Sigma)/0.64 vol 2× RPMI 1640 medium/0.16 vol FBS/0.2 vol Jurkat conditioned medium/0.02 vol 10× PBS] containing 1,000 μg/ml active G418 (Life Technologies, Gaithersburg, MD). G418-resistant colonies were counted 3–4 weeks after plating and selection.

Cotransfection and Cloning by Limiting Dilution. Jurkat cells were cotransfected with linearized test construct and linearized pTK-hyg at a molar ratio of 6:1. At 24 hr posttransfection, cells were plated into 96-well plates at 100 cells per well in 200 μl of selection medium [RPMI 1640 medium supplemented with 10% FBS/10% Jurkat conditioned medium/300 units per ml hyg (Calbiochem)]. Following expansion of hyg resistant clones, test construct integration and copy number was determined by slot blot analysis (Schleicher & Schuell) of duplicate 5 μg samples of genomic DNA using a ^{32}P-labeled neo probe. Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics).

Northern Blot Analysis. Total RNA was isolated from individual E-2.7-P-Neo-scs′ or E-RN-P-Neo-scs′ positive, hyg resistant Jurkat cell clones as described (54). RNA samples (5.0 μg) were denatured and electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane (Micron Separations, Westboro, MA). Neo transcripts were detected using a ^{32}P-labeled neo probe, and RNA loading was assessed using a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase probe. Hybridization signals were quantified using a PhosphorImager.

RESULTS

***Drosophila* scs and scs′ Function as Enhancer-Blocking Elements in Human Cells.** We measured enhancer activity and enhancer-blocking in a soft-agar colony forming assay (45) adapted to the human T cell leukemia Jurkat. This assay uses a transfected bacterial neomycin resistance gene (neo) reporter construct and measures the number of G418 resistant colonies following transfection and selection in soft agar as a readout to reflect neo expression. This allows a direct measure of reporter gene expression that is free of the potential bias introduced by a two step process in which stable transfectants are initially selected on the basis of drug resistance, and the expression of a linked or cotransfected reporter is subsequently determined.

We initially tested the ability of E_δ (E) and the $V_\delta 1$ promoter (P) to drive neo gene expression. In the experiment shown, inclusion of E_δ in the construct (E-P-Neo) increased the number of G418-resistant colonies by 16-fold as compared with a construct driven by the $V_\delta 1$ promoter alone (P-Neo) (Fig. 2*A*). In nine independent experiments using different DNA preparations, the mean ± SD fold-increase in colony number attributable to E_δ was 14 ± 5 (Figs. 2 and 3, and data not shown). Thus, the colony assay provides a sensitive and reproducible measure of enhancer activity. We then tested the utility of E-P-Neo for measurement of enhancer blocking activity, by introducing the *Drosophila* scs and scs′ boundary elements (1.8 and 0.5 kb, respectively) to generate E-scs-P-Neo-scs′ and scs-E-P-Neo-scs′. In these constructs scs′ should insulate P-Neo from copies of the enhancer located downstream in tandemly arrayed multicopy integrants. With scs inserted between the enhancer and promoter, the colony number decreased to the basal level observed with the promoter alone (Fig. 2*A*). However, with the scs inserted upstream of the enhancer the colony number remained high. This position dependence indicates that scs blocks E_δ from activating the promoter but has no intrinsic silencing activity directed toward either E_δ or the promoter. The decreased colony number with E-scs-P-Neo-scs′ was not due to a distance effect since replacement of scs with a 1.35kb ϕx DNA fragment did not provide any enhancer-blocking activity (Fig. 2*A*). A 1.3-kb DNA fragment that spans the human $D_\delta 3$ and $J_\delta 1$ gene segments, inserted both upstream and downstream of P-Neo, failed to provide any enhancer-blocking activity as well (Fig. 2*B*). Taken together, these results both validated the assay system and showed that the *Drosophila* scs and scs′ boundary elements function well as enhancer-blocking elements in human Jurkat cells. To our knowledge, this is the first data indicating that scs and scs′ function as enhancer-blocking elements in vertebrates. The mechanism of scs and scs′ action must be highly conserved.

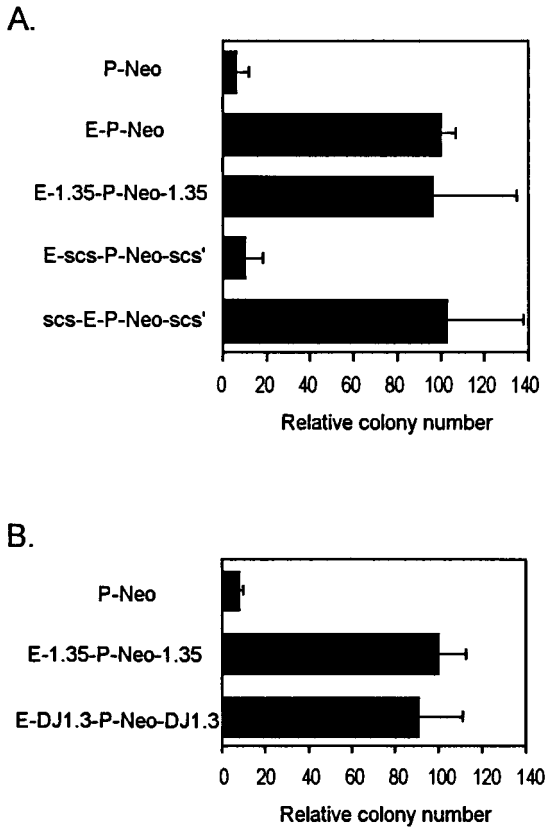

FIG. 2. Enhancer-blocking by *Drosophila* scs and scs′ in human cells as measured by colony formation. Constructs were transfected in triplicate into Jurkat cells and colony number was determined following growth in soft-agar containing G418. Results are presented as mean ± SD, with the colony number for E-P-Neo or E-P-Neo-scs′ normalized to 100. E is E_δ, P is the $V_\delta 1$ promoter, 1.35 is a control ϕx *Hae*III DNA fragment, and DJ1.3 is a fragment spanning $D_\delta 3$-$J_\delta 1$ from the human TCR α/δ locus. (*A*) Enhancer blocking by *Drosophila* scs and scs′. The absolute number of colonies for E-P-Neo was 64. (*B*) Absence of enhancer blocking by a 1.3-kb DNA fragment spanning the $D_\delta 3$ and $J_\delta 1$. The absolute number of colonies for E-1.3-P-Neo-1.3 was 269.

Identification of Enhancer-Blocking Activity Between the $V_\delta 3$ and TEA Promoters by a Colony Assay. Since scs′ appeared to function in Jurkat cells, we used E-P-Neo-scs′ as the base construct for further experiments, and cloned test fragments from the human TCR α/δ locus between the enhancer and promoter to identify those with enhancer-blocking activity. A 5.8-kb *Kpn*I–*Cla*I DNA fragment (KC, Fig. 1), which spans from $V_\delta 3$ to 5′ of ψJ_α, was used to generate E-KC-P-Neo-scs′ and was found to completely block the ability of E_δ to activate the $V_\delta 1$ promoter (Fig. 3*A*). However, due to the large size of the KC fragment, apparent enhancer blocking could result from the increased distance between E_δ and the $V_\delta 1$ promoter, which might inhibit enhancer-promoter communication, or from the increased size of the test plasmid, which might inhibit transfection or integration efficiency. Furthermore, two divergently transcribed promoters, the $V_\delta 3$ promoter (26) and the TEA promoter (28, 29), are located within the KC fragment. Apparent enhancer blocking activity could therefore result from promoter competition, as E_δ is closer to the $V_\delta 3$ and TEA promoters than to the $V_\delta 1$ promoter in this construct.

To eliminate these possibilities, a 2.5-kb *Bgl*II–*Bam*HI fragment (BB) which lacks the TEA promoter, and a 2.5-kb *Eco*RI–*Nsi*I fragment (RN) which lacks both promoters, were cloned into E-P-Neo-scs′ to generate E-BB-P-Neo-scs′ and E-RN-P-Neo-scs′, respectively. Insertion of either BB or RN between the enhancer and promoter decreased the colony number to basal level (Fig. 3*B*), arguing that the $V_\delta 3$ and TEA promoters are dispensable for enhancer-blocking activity. Because insertion of a 2.7-kb control fragment (two copies of the 1.35-kb ϕx fragment) did not affect colony number, inhibitory effects attributable to the distance between the enhancer and the promoter and to overall plasmid size could be eliminated as well (Fig. 3*C*). Importantly, although enhancer-blocking was independent of the orientation of BB or RN (Fig. 3*B*) it was strictly dependent on the position of these fragments within the construct (Fig. 3*D*). That BB fails to influence colony number when positioned upstream of E_δ rules out inhibition by a mechanism that involves enhancer or promoter silencing, and rules out the possibility of an inhibitory effect of the BB fragment on integration efficiency. Furthermore, because the results for each construct were confirmed using a minimum of two, and more typically three or more different DNA preparations in independent experiments, spurious sample to sample variation in transfection

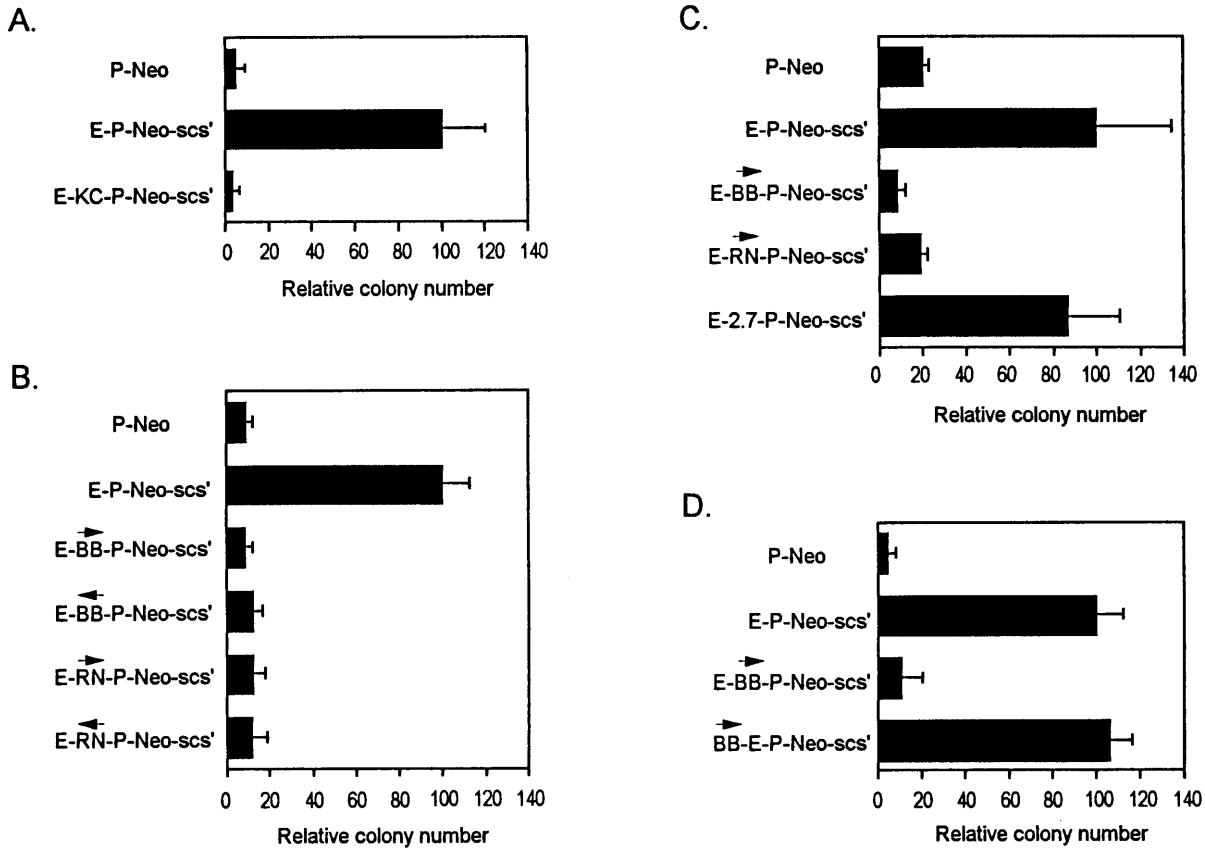

FIG. 3. Enhancer-blocking by the putative boundary element as measured by colony formation. Experiments were conducted and presented as described in the legend to Fig. 2. KC, BB, and RN are test fragments from the human TCR α/δ locus, and 2.7 is two copies of the 1.35-kb ϕx *Hae*III fragments in tandem. (*A*) Enhancer-blocking by the KC fragment. The absolute number of colonies for E-P-Neo-scs′ was 51. (*B*) Enhancer-blocking by BB and RN is independent of their orientation. The absolute number of colonies for E-P-Neo-scs′ was 93. (*C*) Enhancer-blocking by BB and RN is not a distance effect. The absolute number of colonies for E-P-Neo-scs′ was 208. (*D*) Enhancer-blocking by BB is distinguishable from silencing. The absolute number of colonies for E-P-Neo-scs′ was 68.

efficiency can be ruled out as well. We conclude, rather, that the RN and BB fragments display bona fide enhancer-blocking activity similar to that described for previously characterized boundary elements. On this basis, we hereafter refer to the enhancer-blocking element defined by these fragments as BEAD-1, for *B*locking *E*lement *A*lpha/*D*elta *1*.

Confirmation of Enhancer-Blocking Activity by a Cotransfection Assay. As the colony-forming assay actually measures neo gene expression at the protein level, we sought to confirm the identification of BEAD-1 using a different assay that more directly measures its effect on the ability of E_δ to activate neo gene transcription. To do so, E-2.7-P-Neo-scs′ and E-RN-P-Neo-scs′ were each cotransfected with pTK-hyg into Jurkat cells at a molar ratio of 6:1, and individual hyg resistant clones were generated by limiting dilution and selection in suspension culture. Seven E-2.7-P-Neo-scs′ and eight E-RN-P-Neo-scs′ hyg-resistant clones were generated, and the level of neo gene transcripts in these clones was tested by Northern blotting (Fig. 4). All seven E-2.7-P-Neo-scs′ clones expressed readily detectable neo gene transcripts. However, transcripts were undetectable in six out of the eight E-RN-P-Neo-scs′ clones. The nonexpressing E-RN-P-Neo-scs′ clones included several with reporter gene copy numbers that were similar to those of the E-2.7-P-Neo-scs′ clones, arguing that this result is not attributable to differences in copy number (Fig. 4). These data therefore offer strong confirmation of the enhancer-blocking activity of both BEAD-1 and scs′ in this system. Of note, expression was clearly detectable in two E-RN-P-Neo-scs′ clones, and the level of neo gene expression per copy in these clones was in the same range as those of the E-2.7-P-Neo-scs′ clones. Such expressing clones might arise due to construct integrations in which BEAD-1 or scs′ has been interrupted. Alternatively, a fraction of integration sites might be intrinsically permissive for promoter activity, or might overcome the enhancer-blocking effect of either BEAD-1 or scs′.

DISCUSSION

Considering the data obtained in both the colony and cotransfection assays, we conclude that an enhancer-blocking ele-

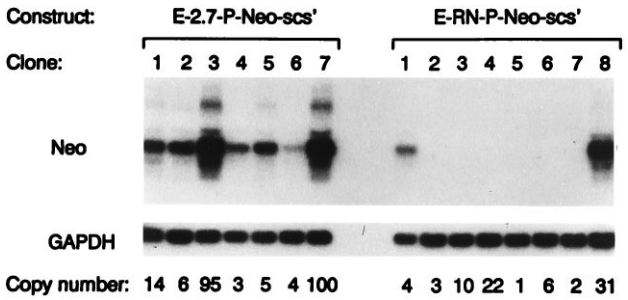

FIG. 4. Enhancer blocking by BEAD-1 as measured by Northern blot analysis of neo gene expression in stably transfected cell clones. Constructs were cotransfected into Jurkat cells along with pTK-hyg. Total RNA was isolated from individual hyg resistant Jurkat clones and was analyzed on a Northern blot that was serially hybridized with ^{32}P-labeled neo and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Neo gene copy number in each clone was determined by slot blot analysis of genomic DNA using the same probes. Northern blot and slot blot hybridization signals were quantified using a PhosphorImager.

ment, denoted BEAD-1, is located within a 2.0-kb region between the $V_\delta 3$ and TEA promoters. BEAD-1 displays potent enhancer-blocking activity as measured by its ability to prevent E_δ from activating transcription from a nearby promoter when interposed between the two in a chromosomally integrated substrate. We propose that BEAD-1 plays a similar role within the endogenous TCR α/δ locus, and that it functions as a boundary that separates the locus into two distinct regulatory domains. Because E_δ is located 5′ of BEAD-1 along with the $V_\delta 3$ promoter and any additional upstream (i.e., V_α and V_δ) promoters, BEAD-1 should not interfere with the activation of these promoters by E_δ. However, since BEAD-1 separates E_δ from the TEA promoter and any additional downstream promoters, BEAD-1 should effectively block the activation of these promoters by E_δ. Because E_δ is activated prior to the TEA promoter in developing thymocytes (21, 35), BEAD-1 may be crucial to maintain independent and developmentally appropriate regulation of TEA transcription.

Although in this report we have measured the ability of BEAD-1 to block effects of E_δ on gene expression, we hypothesize that BEAD-1 will also function within the endogenous locus to block effects of E_δ on VDJ recombination. This proposal rests on previous data indicating that enhancers can function to increase local chromatin accessibility (55–57), that a boundary element can block the formation of accessible chromatin by an enhancer (45, 58), and that chromatin accessibility is a critical regulator of VDJ recombination (59). Our previous data argues that during the DN stage of T cell development, E_δ induces local chromatin accessibility, and hence VDJ recombination, of TCR δ gene segments (34, 35). We propose that BEAD-1 functions within the endogenous TCR α/δ locus to prevent E_δ from providing accessibility to J_α gene segments, either by blocking a global increase in accessibility that is propagated from E_δ into the J_α region, or by specifically preventing E_δ from interacting with the TEA promoter, which has itself been implicated in 5′ J_α segment accessibility (37). By blocking E_δ-induced accessibility of J_α gene segments, BEAD-1 would prevent V_δ-J_α or V_α-J_α recombination in DN thymocytes, and thereby prevent premature deletion of TCR δ gene segments that might inhibit the production of γδ T cells. These predictions are currently being tested *in vivo* by genetic manipulation of the endogenous TCR α/δ locus.

Based upon the recent evidence supporting a progressive model for VDJ recombination at the TCR α/δ locus (18–21), DN thymocytes with nonproductive TCR γ or TCR δ rearrangements but a productive TCR β rearrangement (22–25) can differentiate via the immature single positive stage to the DP stage. During this transition, activation of the TEA promoter (21, 37) and E_α (35, 36) provide access to J_α chromatin, thereby facilitating the rearrangement of V_α gene segments to J_α gene segments. Of note, the presence of BEAD-1 in the middle of the TCR α gene (between V_α and J_α segments) suggests that BEAD-1 is unlikely to block the process of VDJ recombination *per se*. In other words, V_α to J_α rearrangement is likely to be permitted so long as V_α segments and J_α segments are both accessible to the recombinase due to enhancer or promoter activity in each region. It remains possible, however, that BEAD-1 can block the process of VDJ recombination, but that V_α to J_α rearrangement is permitted because BEAD-1 is not active at the DP stage. Interestingly, the first V_α to J_α rearrangement will delete not only the TCR δ gene, but BEAD-1 as well. The deletion of BEAD-1 may be critical to allow E_α to activate transcription from the promoter of the rearranged V_α gene segment.

Only a limited number of boundary elements have been identified to date. Although several models have been proposed to explain the enhancer-blocking and insulating activities of boundary elements (45, 50, 51, 60), the mechanism remains unknown. The binding of su(Hw) protein to specific sites within the *gypsy* transposon is necessary for *gypsy* boundary function (43, 44). However, su(Hw) does not influence the activity of scs and scs′. The protein BEAF32 binds to scs′ and localizes to interbands and puff boundaries on polytene chromosomes (61) suggesting that it may be a fairly general component of chromatin boundaries. Nevertheless, it does not bind to scs. Clearly, a single protein cannot account for all examples of boundary activity in *Drosophila*.

Matrix-attachment regions physically separate chromatin into looped domains by attaching the chromatin fiber to nuclear matrices (62). In some instances, matrix-attachment regions and boundary elements appear to colocalize (63–65). We have therefore asked whether BEAD-1 functions through association to nuclear matrices and physically separates the TCR α/δ locus into looped chromatin domains. No matrix-attachment regions were detected in the BEAD-1 region (unpublished observations) using both the *in vitro* and *in vivo* matrix-attachment region assays (66, 67).

Because the *Drosophila* scs and scs′ elements display enhancer blocking activity in human Jurkat cells (this report) and chicken 5′HS4 has weak insulating activity in transgenic *Drosophila* (45), at least some examples of boundary function are mediated by mechanisms that have been highly conserved through evolution. With this in mind, we have asked whether BEAD-1 is functional in *Drosophila*. BEAD-1 failed to block the *white* enhancer from activating mini-*white* gene expression (unpublished observations), suggesting that BEAD-1 function requires factors that are distinct from and less conserved than those that mediate scs, scs′, and 5′HS4 activity. Additional insights into the mechanism of BEAD-1 action will require, as a first step, a more precise definition of the minimal functional enhancer-blocking element. Experiments directed toward this goal are currently in progress.

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