A novel enhancer in the immunoglobulin λ locus is duplicated and functionally independent of NF κ B

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As a first step toward defining the elements necessary for λ immunoglobulin gene regulation, DNase I hypersensitive sites were mapped in the mouse λ locus. A hypersensitive site found 15.5 kb downstream of C λ 4 was present in all the B-cell but not in the T-cell lines tested. This site coincided with a strong B-cell-specific transcriptional enhancer (E λ 2.4). This novel enhancer is active in myeloma cells, regardless of the status of endogenous λ genes, but is inactive in a T-cell line and in fibroblasts. The enhancer E λ 2.4 functions in the absence of the transcription factor NF κ B, which is necessary for κ enhancer function. No evidence could be found for NF κ B binding by this element. Rearrangement of V λ 2 to J λ 3 or J λ genes deletes E λ 2.4; however, a second strong enhancer was found 35 kb downstream of C λ 1, which cannot be eliminated by λ gene rearrangements. The second λ enhancer (E λ 3.1) is 90% homologous to the E λ 2.4 sequence in the region determined to comprise the active enhancer and likewise lacks the consensus binding site for NF κ B. The data support a model for the independent activation of κ and λ gene expression based on locus-specific regulation at the enhancer level.

[Key Words: Immunoglobulin; enhancer; κ/λ gene regulation; DNase hypersensitivity]

Received November 9, 1989; revised version accepted March 21, 1990.

Immunoglobulin molecules are assembled following the somatic rearrangement and expression of heavy (H)- and light (L)-chain genes in B lymphocytes. The L chains of individual B cells may be of either the κ or λ isotype. In inbred strains of mice, a single locus is found for each isotype, and these show profound differences in their organization (Selsing et al. 1989). Unlike the κ locus, which has clustered variable (V) and joining (J) and only a single constant (C κ) gene segment, the λ locus is an interspersed array of gene segments, arranged as V λ 2-V λ X-J λ 2-J λ 4-V λ 1-J λ 3-J λ 1 (Miller et al. 1988; Carson and Wu 1989; Storb et al. 1989). All λ recombining segments appear to be arranged in the same transcriptional orientation; therefore, rearrangement between any V λ and J λ results in the deletion of intervening DNA (Storb et al. 1989).

Very little is understood concerning the regulation of rearrangement and transcription within the λ locus. Although transcriptional enhancer elements have been localized in the JC introns of immunoglobulin H-chain (Banerji et al. 1983; Gillies et al. 1983; Mercola et al. 1983) and κ -chain (Queen and Baltimore 1983; Bergman et al. 1984; Picard and Schaffner 1983, 1984a) genes, no such regulatory region has been found in the introns of λ genes (Picard and Schaffner 1984a; this study). Transfection studies with functionally rearranged λ genes have shown that high levels of expression in B-lineage cells

are not obtained without the addition of a known enhancer element [such as the SV40 enhancer (Picard and Schaffner 1983, 1984a; Pepe et al. 1986) or H-chain enhancer (Hagman et al. 1989; Neuberger et al. 1989)].

We conducted a systematic search for enhancer elements in murine λ genes by mapping the relative sensitivity to deoxyribonuclease I (DNase I) digestion across the locus. One DNase I hypersensitive site was found to be a strong transcriptional enhancer (E λ 2.4) in B-lineage cells. In contrast to the κ intron enhancer, which is dependent on the transcription factor NF κ B (Sen and Baltimore 1986; Lenardo et al. 1987), no evidence could be found for the utilization of NF κ B by this λ enhancer. A second region (E λ 3.1), cloned by homology with E λ 2.4, was also found to be a strong enhancer in B cells. We suggest that these elements are involved in the regulation of λ rearrangement and transcription.

Results

DNase I hypersensitive sites are located downstream of λ JC clusters

The previously identified immunoglobulin H-chain (Mills et al. 1983) and κ -chain (Parslow and Granner 1982, 1983; Weischet et al. 1982) enhancer elements are contained within regions of DNA that are hypersensi-

tive to nuclease digestion in mouse and human cell lines (for review, see Gross and Garrard 1988). We reasoned that enhancer elements within the murine λ locus should be identifiable by using a similar approach.

A single, very strong hypersensitive site (hsC₄-II) was found 15.5 kb downstream of the Jλ2–Jλ4 cluster in the DNase I-digested nuclei of ABC1 cells, a pre-B-cell line that rearranges λ genes. Another hypersensitive site, hsC₁-I, was found ~8.4 kb downstream of the Jλ3–Jλ1 gene cluster (Fig. 1; Hagman 1989).

We determined whether the observed hypersensitive sites are restricted to λ-producing B cells or are a general characteristic of the λ locus by similarly mapping hypersensitive sites in various cultured cell lines. S194.XXO.Bu.1 (S194) is a κ-producing myeloma whose λ genes are unrearranged (Hagman 1989). Both hsC₁-I and hsC₄-II are present in the chromatin of S194, indicating that λ rearrangement is not a prerequisite for the presence of these sites (Fig. 1; Hagman 1989). In addition, S194 myeloma cells reproducibly demonstrated a unique hypersensitive site, 8.7 kb 3' of Cλ4 (hsC₄-I), which is located at nearly the same position relative to the JC genes as hsC₁-I (Fig. 1; Hagman 1989).

Hypersensitive sites downstream of JC genes were surveyed in B-lineage cells representing the various stages of differentiation to determine whether a developmental hierarchy exists for the appearance of these sites. All B-lineage cells, including lines that represent very early stages of differentiation, exhibit both hsC₁-I and hsC₄-II (not shown; Hagman 1989). Lipopolysaccharide (LPS) treatment is not necessary for the appearance of these sites in pre-B cells, including ABC1. Interestingly, hsC₄-I was only found in the κ-producing S194 myeloma, but other κ myelomas have not been assayed. No evidence was found for the presence of hypersensitive sites between Jλ2 and Jλ4 or between Jλ3 and Jλ1 (not shown; Hagman 1989).

The tissue specificity of λ locus hypersensitive sites was examined next in T-lineage cell lines. As reported by Bier et al. (1985), the immature thymoma BW5147 demonstrates two T-cell-specific hypersensitive sites in the T-cell receptor β JC introns (not shown; Hagman 1989). However, unlike B-lineage cells, no hypersensi-

tive sites are detectable downstream of Cλ4 in BW5147 chromatin (not shown; Hagman 1989). The hypersensitivity at hsC₄-II appears to be B-cell specific. In contrast, nuclease cleavage is observed at ~8.4 kb 3' of Cλ1 (not shown; Hagman 1989), but the hypersensitive site is weaker and often appears as two very close bands, indicating some qualitative difference with the hsC₁-I site seen in B cells. A more mature T-cell line (EL4) also did not possess hsC₄-II, but hsC₁-I was weakly present (not shown; Hagman 1989).

Unexpectedly, two nonlymphoid cell lines exhibited hypersensitive sites in the λ locus. The myeloid–monocytic cell WEHI265.1 has been described as representing an early stage of myeloid development (Kemp et al. 1980), yet both hsC₁-I and hsC₄-II are detectable (data not shown; Hagman 1989). It was reported previously that WEHI265.1 expresses mRNA containing Cμ (probably sterile transcripts), but not κ or λ (Kemp et al. 1980). This cell line may represent a developmental stage at which both myeloid and lymphoid characteristics are expressed. Ltk[−]aprt[−] fibroblastoid cells (L cells) also demonstrate both hsC₁-I and hsC₄-II (not shown; see Discussion).

DNase I hypersensitive sites are located upstream of λ variable regions

As expected, the regions of the promoters of Vλ1 and Vλ2 showed hypersensitive sites (hsPr1 and hsPr2; Figs. 1 and 2). Furthermore, an additional hypersensitive site was located ~3 kb upstream of the promoters of both Vλ1 and Vλ2 (hsV₁ and hsV₂; Figs. 1 and 2; Hagman 1989).

Functional assays of hypersensitive sites: hsC₄-II coincides with a transcriptional enhancer

To clone DNA containing the region of the B-cell-specific hypersensitive site (hsC₄-II), a BALB/c liver DNA library was constructed in the cosmid vector pTCF (Grosveld et al. 1982). The library was probed with a 5.8-kb XbaI fragment (isolated from λ2-4.4X as an XbaI–SalI fragment; Hagman et al. 1989), which contains both Cλ2

Figure 1. DNase I hypersensitive sites of the murine λ locus. All segments are drawn approximately to scale. Only regions that were mapped in this study are included. Breaks indicate the boundaries of regions that were mapped in this study. The physical distance between selected points is indicated below (from Storb et al. 1989). (■) Exons. The position of the seven originally identified hypersensitive sites and the two enhancers are indicated. Although not found in the original search, which scanned the region up to 32 kb 3' of Cλ1, the E_{λ3.1} site is also DNase I hypersensitive (hsC₁-II).

Figure 2. Mapping DNase I hypersensitive sites around V λ genes. Map of DNase I hypersensitive sites flanking unrearranged λ variable region segments. (Top) Region flanking V λ 1; (bottom) region flanking V λ 2. Arrows indicate positions of hypersensitive sites. Solid boxes are V region exons. Position of probe used to map hsV sites is indicated below upper map [a]. Segments used in λ promoter test constructions (see Fig. 4) are shown below maps; the short heavy box is 161-bp SstI–SspI V λ 1 promoter fragment. Other segments were added to λ CAT14 as indicated (see text and Materials and methods).

and C λ 4 (indicated as a in Fig. 3). This probe cross hybridizes with both J–C λ region clusters and contains no repetitive DNA. Two independent cosmids (cosA16 and cosC30) were isolated that hybridized both to the original probe and to a unique 158-bp C λ 4 probe (Storb et al. 1989). Both contain identical 16.5-kb *NheI* fragments; in addition, cosA16 contains a 26-kb *KpnI* fragment, indicating that it extends 3' farther (see Fig. 3). CosA16 was selected for further study; a complete map is shown in Figure 3.

The region containing hsC $_4$ -II was assayed for transcriptional enhancer function by linkage to a reporter gene and transient transfection into the λ -producing myeloma line J558L. A minimal V λ 1 promoter (a 161-bp SstI–SspI segment derived from R λ 1; see Materials and methods), containing the known octamer and TATA elements (Falkner and Zachau 1984) necessary for expression in B-lineage cells, was cloned immediately upstream to the chloramphenicol acetyltransferase (CAT) gene derived from pCAT3M (Laimins et al. 1984). The resulting plasmid, λ CAT14 (see Materials and methods), has multicloning sites both up- and downstream of the promoter–CAT gene. The exact site of transcriptional initiation of CAT is unknown but probably occurs within pUC multicloning site sequences downstream of TATA.

Transfection of λ CAT14 into J558L results in an easily measurable increase in CAT expression over the promoterless CAT19-1 (Fig. 4; Table 1). Insertion of a 1-kb *XbaI* fragment (Hagman et al. 1989) containing the H-

chain enhancer ($E_H\lambda$ CAT) increases the conversion of chloramphenicol by extracts of transfected J558L as much as 40-fold. As expected, H-chain enhancer activity is not dependent on orientation (not shown; Hagman 1989).

Estimates from nuclease hypersensitivity mapping placed the hsC $_4$ -II site at 15.5 kb from the C λ 4 exon. The 1.65-kb fragment (*XhoI*–*NheI*) surrounding hsC $_4$ -II (Fig. 3) was inserted in both orientations upstream (A16 λ CAT25 and A16 λ CAT32) and downstream (A16 λ CAT9 and A16 λ CAT31) of λ CAT and transiently transfected into J558L. All four plasmids exhibit very strong enhancement of CAT levels relative to the enhancerless λ CAT14 (Fig. 4; Table 1). A small difference in expression is observed between the two orientations at either site; the fragment has the greatest effect when located downstream in the natural orientation (Table 1). J558L possesses two rearranged λ alleles, one V λ 1–J λ 1 (productive), and one V λ 1–J λ 3 (nonproductively rearranged); therefore, the presence of factors for the $\lambda_{2,4}$ enhancer is not necessarily correlated with rearrangements in the J λ 2–J λ 4 cluster.

All of the A16 plasmids tested above expressed higher CAT levels than those observed with SVCAT (Fig. 4; Table 1), which utilizes the SV40 early region enhancer/promoter (Gorman et al. 1982). Unlike fragments that contain hsC $_4$ -II, plasmids containing either C λ 1 and up to 4.0 kb downstream (a *XhoI*–*EcoRI* fragment isolated from a rearranged λ 1 genomic clone; A1 λ CAT1,3; see Fig. 5), or a 1.4-kb segment containing the entire λ 1J–C

Figure 3. Physical map of cosmid A16 and subclones for assay of enhancer activity. (Top) Map of cosmid A16. Total insert length in cosmid vector TCF (hatched boxes) is 35 kb. (cos) Position of cos sites of vector TCF. Exons are indicated as solid boxes. Positions of hypersensitive sites are indicated as arrows (open arrow for hsC_4 -I, which appears only in S194 cells). Probe a is the 5.8-kb *Xba*I fragment of λ 2-4.4X (Hagman et al. 1989), which was used to isolate this cosmid from a mouse liver library. Probe b is the $C\lambda$ 4 probe used as a secondary screen for confirmation of cosmid identity. (Bottom) Partial restriction map of subclone pA16-N16 and subclones for CAT assay. All segments are shown to scale and were subcloned upstream of the promoter in λ CAT14 or downstream of the CAT gene (indicated in brackets). All segments were tested in both orientations, and the first number of each pair indicates the natural orientation relative to the direction of transcription (-28 has two copies of the segment in the natural orientation). Plus or minus sign indicates enhancement of CAT activity in transient transfection of J558L (Fig. 4). Plasmid designations are indicated to left of bars.

intron (MI λ CAT1) demonstrate no specific enhancer activity in J558L (Fig. 4; Table 1).

The functional enhancer region ($E_{\lambda 2-4}$) was localized further by using overlapping subclones (Fig. 3). Con-

structions containing either a 1.2-kb *Pst*I-*Nhe*I (A16 λ CAT109 and A16 λ CAT110) or a 412-bp *Rsa*I fragment (A16 λ CAT207 and A16 λ CAT208) are active as the 1.65-kb enhancer fragment (Fig. 4;

Figure 4. CAT assay of transient transfections of J558L cells: enhancer activity of hsC_4 -II. J558L cells were transiently transfected by electroporation with plasmids as shown (see Materials and methods). All transfections were performed in duplicate (one set of autoradiographs is shown). A16 λ CAT301 was similarly tested (not shown; see Fig. 3). CAT assays were performed 42 hr post-transfection. Each point represents the extract obtained from $\sim 8 \times 10^6$ cells. Reactions proceeded for 90 min. Plasmids are described in text and in legends to Figs. 2, 3, and 5. *E. coli* is an extract of strain GD32.

Table 1. Percent chloramphenicol conversion in CAT assays of transient transfections

Plasmid	CAT activity as % chloramphenicol conversion ^b	Plasmid	CAT activity as % chloramphenicol conversion ^b
CAT19-1	0.0 ± 0.0	A16CAT207	41.3 ± 1.4
λCAT14	1.3 ± 0.1	A16CAT208	36.0 ± 8.0
E _μ λCAT	23.3 ± 6.6	A1λCAT1	0.5 ± 0.2
A16λCAT25	26.5 ± 4.0	A1λCAT3	0.4 ± 0.0
A16λCAT32	25.4 ± 3.1	MIλCAT-1	0.4 ± 0.0
A16λCAT28	28.3 ± 5.6	VλCAT2	3.0 ± 1.4
A16λCAT9	50.3 ± 10.2	VλCATXS2	29.1 ± 0.1
A16λCAT31	37.4 ± 2.6	VλCATSX2	40.3 ± 7.6
A16λCAT102	0.2 ± 0.2	VλCATBB2	1.3 ± 0.3
A16λCAT113	0.9 ± 0.5	VλCAT1	2.9 ± 0.3
A16λCAT109	36.7 ± 6.9	SV2CAT	7.1 ± 0.2
A16λCAT110	32.7 ± 1.5		
A16λCAT201	1.7 ± 0.1		
A16λCAT202	1.8 ± 0.1		

^aExperiment shown in Fig. 4 (E_{λ2-4}).^bAll transfections were done in duplicate.

Table 1). Segments flanking this region (A16λCAT102, A16λCAT113, A16λCAT201, A16λCAT202, and A16λCAT301) do not increase expression in this assay; therefore, the minimal critical region for enhancement can be estimated as within the 229-bp *Pst*I–*Rsa*I segment.

hsV₂ has only a minimal effect on CAT expression

The low expression of a functionally rearranged λ2 gene (λ2-4.4X), containing 4.0 kb upstream of Vλ2 in the B cells of transgenic mice (Hagman et al. 1989), indicates

that the presence of *hsV₂* is not sufficient for high-level expression in B cells, but it was possible that it would have an effect in concert with downstream enhancer elements that are not present in λ2-4.4X. This proposition was tested by increasing the length of Vλ upstream sequences in λCAT14 with DNA from rearranged λ genomic clones, transiently transfecting into J558L, and relating CAT expression to the 161-bp promoter (Fig. 4; Table 1). VλCAT1 contains 1.4 kb derived from a rearranged λ1 gene (Rλ1) upstream of CAT and does not contain *hsV₁* (Fig. 2). VλCAT2 has 4.0 kb of Vλ2 upstream region (Hagman et al. 1989) and has *hsV₂* at the natural

Figure 5. Partial physical map of J-CA3-J-CA1 cluster region and subclones for assay of enhancer activity. Exons are shown as solid boxes. The position of *hsC₁-I* is indicated by the solid arrow. Genomic segments were subcloned upstream of the promoter of λCAT14 for CAT assay in one or two orientations, as indicated. All segments are shown to scale. Plasmid designations are indicated to left of bars. Most segments were tested in both orientations, and the first number of each pair indicates the natural orientation relative to the direction of transcription. The other segments were tested in the opposite orientation (the orientation is unknown for S0.7λCAT). Plus or minus sign indicates enhancement of CAT activity in transient transfection of J558L. The raw data (TLC spots) are shown in Fig. 4 (A1λCAT1 and A1λCAT3) only for the fragment used to assay the activity of a putative enhancer described by Bich-Thuy and Queen (1989).

distance from the promoter, whereas the distance between the promoter and hsV_2 is reduced to several hundred base pairs in λ CATBB2 (Fig. 2). λ CAT2 and λ CAT1 had slightly elevated CAT expression in J558L (Fig. 4; Table 1), but this small increase may not be significant.

The 1.65-kb *XhoI*–*NheI* segment comprising hsC_4 -II was subcloned into the downstream multicloning site of λ CAT2. No significant augmentation of CAT activity was observed relative to A16 λ CAT9 and A16 λ CAT31 when hsV_2 was present upstream of the promoter (Fig. 4; Table 1; λ CATXS2 and λ CATXS2). Thus, hsV_2 has no detectable effect on transcription.

hsC₁-I does not function as an enhancer in J558L myeloma cells

None of the plasmids that contain either hsC_1 -I (Fig. 5) or flanking DNA (spanning 3.7 kb) cloned into λ CAT14 exhibited the magnitude of CAT expression seen with the H-chain enhancer in J558L (not shown; Hagman 1989). S1.1 λ CAT1, S1.1 λ CAT3, and B2.5 λ CAT all contain hsC_1 -I; CAT expression by these plasmids was less than or equal to that of the enhancerless λ CAT14. To guard against cloning artifacts, a set of plasmids containing hsC_1 -I was prepared from a second independently isolated phage clone; these also exhibited no enhancer activity. It appears that hsC_1 -I is not functionally similar to hsC_4 -II but may still have a role in transcriptional regulation. The 1.0-kb *SstI* fragment present in S1.0 λ CAT14 does not contain hsC_1 -I and has a very small effect in only one orientation. Further studies

must be conducted to establish the significance of this observation.

E _{λ 2.4} functions in both λ - and κ -producing B cells but not in a T-cell line or in fibroblasts

The enhancer $E_{\lambda 2.4}$ increases the accumulation of CAT in λ -producing cells in the manner of a typical enhancer element, affecting expression independent of orientation and/or position. Because the transcriptional activity in immunoglobulin promoters is specific to B-lineage cells (Queen and Baltimore 1983; Falkner and Zachau 1984; Mason et al. 1985), the relative effects of $E_{\lambda 2.4}$ and other enhancers in other cell types compared with B cells were tested by use of the herpes simplex virus thymidine kinase (HSV-tk) promoter. The enhancerless construction UTKAT can express CAT at low levels in both B and T cells and has been used previously in association with immunoglobulin enhancers (Diamond et al. 1989). Genomic segments containing known enhancer elements were each cloned into the unique *XbaI* site upstream of the HSV-tk promoter in UTKAT. The activity of the $\lambda_{2.4}$ enhancer was compared with the SV40, immunoglobulin H-chain, and κ intron (κ i) and a new transcriptional control region (κ 3' enhancer, or $E_{\kappa 3'}$), recently identified 9 kb downstream of C κ (Meyer and Neuberger 1989).

Transfection of J558L λ -producing myeloma cells with the constructions described above reveals a hierarchy of enhancer activity, measured in terms of relative CAT expression (Fig. 6; Table 2). The HSV-tk promoter alone (UTKAT) functions in J558L and is somewhat more active than the 161-bp $V_{\lambda 1}$ promoter (not shown). Linkage

Figure 6. Activity of enhancers in B- and T-cell lines. All cells were transiently transfected by electroporation with plasmids as shown (Materials and methods). All transfections were performed in duplicate. CAT assays were performed 42 hr post-transfection. Each point represents the extract obtained from ~6 million cells; reaction time was 90 min. Plasmids are described in Materials and methods. *E. coli* is an extract of strain GD32.

Table 2. Percent chloramphenicol conversion in CAT assays of transient transfections

Plasmid	CAT activity as % chloramphenicol conversion ^b			
	J558L	S194	S107	EL4
UTKAT	2.9 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
SVTKAT1	10.5 ± 0.9	38.8 ± 6.9	0.4 ± 0.1	33.5 ± 1.4
E _H TKAT10	86.8 ± 9.8	12.3 ± 1.8	6.8 ± 0.4	4.6 ± 1.4
E _{κi} TKAT2	11.6 ± 3.8	3.3 ± 0.2	0.5 ± 0.0	1.9 ± 0.2
E _{κ3'} TKAT21	37.9 ± 12.0	5.3 ± 1.6	2.7 ± 0.3	0.5 ± 0.1
E _{λ2-4} TKAT	39.9 ± 1.9	3.1 ± 0.6	3.3 ± 1.8	0.4 ± 0.1

^aExperiment shown in Fig. 6 (E_{λ2-4}).

^bAll transfections were done in duplicate.

of the tk promoter to any of the enhancer elements increases CAT production in J558L. The relative levels of activity may be summarized as E_H > E_{λ2-4}, E_{κ3'} > E_{κi}, SV > UTKAT (Table 2).

The presence of λ enhancer factors is not an exclusive property of cells with λ rearrangements. Transfection of the κ-expressing myeloma S194 demonstrates that E_{λ2-4} functions almost equally well in κ- or λ-expressing cells (Fig. 6; Table 2). The range of CAT expression in S194 is SV > E_H > E_{κ3'} > E_{κi}, E_{λ2-4} > UTKAT (Table 2).

Transfection into the thymoma EL4 confirms the B-cell specificity of the E_{λ2-4} segment (Fig. 6; Table 2). This is not surprising, considering that hscC₄-II cannot be demonstrated in EL4 chromatin (not shown; Hagman 1989). Neither the λ₂₋₄ nor the κ3' segments have any measurable activity in EL4, demonstrating the most restricted tissue specificity observed for any of the immunoglobulin enhancer elements. A small, yet significant increase in CAT is associated with the κ intron enhancer. This may be due to the enhancer fragment employed in this study (900 bp) being longer than in a previous experiment by Queen et al. (1986), who reported no activity in transient assays of other T-cell lines. The relative activity in EL4 may be represented as SV >> E_H > E_{κi} > E_{κ3'}, E_{λ2-4} > UTKAT (Table 2).

Because hscC₄-II was present in L cells, we also determined whether E_{λ2-4} acted as an enhancer in these cells. Whereas the SV40 enhancer strongly increased transcription from the tk promoter in L cells, a low level of enhancement was also seen with E_{λ2-4} (Table 3; see Discussion). However, another fibroblast line, NIH-3T3 cells, showed a decrease in CAT activity with the λ enhancer (Table 3).

Table 3. Percent chloramphenicol conversion in CAT assays of transient transfections

Plasmid	CAT activity as % chloramphenicol conversion ^b	
	L cells	NIH-3T3 cells
UTKAT	3.1 ± 0.1	0.2 ± 0.0
E _{λ2-4} TKAT	4.5 ± 0.4	0.03 ± 0.01
SVTKAT1	14.3 ± 0.1	6.1 ± 0.1

^aCAT assays with E_{λ2-4} in L cells and NIH-3T3 cells.

^bCellular extract quantities were normalized to the activity of a cotransfected β-gal gene; all transfections were done in duplicate.

E_{λ2-4} is regulated independent of the transcription factor NFκB

The myeloma S107 does not express artificially introduced genes that are regulated by the κ intron or SV40 enhancers (Atchison and Perry 1987). This phenomenon is thought to be due to a very low level of the nuclear transcription factor NFκB in these cells.

The same series of UTKAT-based constructions were electroporated into S107 cells to determine whether any of the enhancers can function in the relative absence of NFκB (Fig. 6; Table 2). As reported previously (Atchison and Perry 1987), the H-chain enhancer, which does not compete for NFκB binding (see Fig. 7), has a strong effect on CAT expression in S107. SVTKAT1 and E_{κi}TKAT2 each express only slightly more CAT than the enhancerless construct, in accordance with the known utilization of NFκB by these two enhancer elements (Sen and Baltimore 1986). In contrast, both the λ₂₋₄ and κ3' elements significantly enhance CAT production. This result suggests that different (and possibly new) nuclear factors present in S107 cells are involved in λ transcriptional regulation. The relative production of CAT by these plasmids in S107 may be summarized as E_H > E_{λ2-4}, E_{κ3'} > E_{κi}, SV > UTKAT (Table 2).

E_{λ2-4} does not compete for NFκB binding

In an electrophoretic mobility-shift assay (EMSA), the λ₂₋₄ enhancer did not compete efficiently with a labeled fragment containing a binding site for the transcription factor NFκB (Fig. 7). A 900-bp κ intron segment (κ_i enhancer) competes for factor binding at a molar excess more than sixfold lower than either the λ₂₋₄ or κ3' segment. The inhibition of probe binding observed at a 12-fold molar excess with the competing fragments (other than the κ_i enhancer) is probably nonspecific as a result of the large size of the competing fragments, because no consensus NFκB recognition sequence is present in the E_H segment.

Cloning of a second λ enhancer and comparison with E_{λ2-4}

Because all known rearrangements within the λ locus take place by deletion (Miller et al. 1988; Storb et al. 1989), essential regions that control the expression of rearranged genes cannot be present in DNA that is lost by this process. Although it had been suggested that im-

Figure 7. Competition for nuclear factor binding to NF κ B. EMSAs were performed with 1 ng of 32 P-labeled NF κ B containing oligonucleotide and no, or 2, 4, or 12 M excess of competing DNA fragments; the nuclear extract was from the WEHI231 B-cell line (see Materials and methods). The arrow indicates the major specific retarded band.

munoglobulin enhancer and promoter elements must be linked for only a certain period following rearrangement to both establish and maintain a stable transcription complex (Atchison and Perry 1987), it now appears more likely that stable immunoglobulin H- and κ -chain transcription is perpetuated by the redundancy of enhancer elements. The earlier hypothesis attempted to explain the expression of endogenous immunoglobulin H-chain genes in mature B cells that had deleted functional enhancer elements (Klein et al. 1984; Wabl and Burrows 1984; Eckhardt and Birshtein 1985; Zaller and Eckhardt 1985) and the expression of endogenous κ genes in the absence of functional NF κ B in S107 myeloma cells (Atchison and Perry 1987). It is now suspected that multiple enhancer elements exist for H-chain genes (Grosschedl and Marx 1988; Petterson et al. 1990). Furthermore, the observed expression of κ genes in the absence of NF κ B is almost certainly due to the presence of a second transcriptional enhancer known to be 9 kb downstream of C κ (Meyer and Neuberger 1989).

Rearrangements involving V λ 1 do not delete the λ_{2-4} enhancer, which is located at least 80 kb upstream of V λ 1 (Storb et al. 1989; see Fig. 1). However, rearrangements between V λ 2 (the most 5' gene identified within the λ locus; Storb et al. 1989) and the more distant

J λ 3–J λ 1 cluster are necessarily accompanied by deletion of λ_{2-4} . Such rearrangements do occur at low frequency (Elliot et al. 1982; Reilly et al. 1984), and these events can result in the assembly and expression of functional λ genes. Thus, an additional enhancer element(s) that is maintained following these rearrangements must be postulated.

By screening a phage library with the 412-bp *Rsa*I fragment comprising the active region of λ_{2-4} , we found and cloned a second λ enhancer, λ_{3-1} (Fig. 8). By pulsed-field gel electrophoresis and conventional Southern blotting of genomic DNA analyzed with a variety of restriction enzymes and hybridization probes, λ_{3-1} maps 35 kb 3' of C λ 1 (not shown).

Figure 9 shows the comparison of the DNA sequences of λ_{2-4} and λ_{3-1} . The overall homology between the two enhancers is 87.8%, with the highest match (>90%) in the active enhancer region. The overall relationship presumably can be ascribed to the relatively recent event that duplicated the mouse λ genes (Selsing et al. 1982). The pseudogene C λ 4 has diverged from its homolog C λ 1 by 15.2% (Selsing et al. 1982). The slightly higher conservation of the λ enhancers may reflect on their functional significance.

A comparison of both sequences with the GenBank data base, using the FASTA program (Pearson and Lipman 1988), revealed no significant homologies to the region delineating the active enhancer. The sequence revealed only two motifs with significant identity with other known immunoglobulin enhancer and promoter elements. A perfect 9-bp match with the known μ E2 sequence located in the H-chain enhancer (Sen and Baltimore 1986) was present in λ_{2-4} . It appears that all known immunoglobulin enhancer elements contain at least one E motif (Sen and Baltimore 1986; Meyer and Neuberger 1989; Petterson et al. 1990; this paper). The μ E2 sequence is located at the extreme 5' end of the *Pst*I–*Rsa*I fragment estimated to contain the essential enhancing activity (Fig. 3). This μ E2 sequence is conserved at seven or eight positions in λ_{3-1} but may still be functional. The λ_{2-4} enhancer segment also contains a 7-bp sequence (7-mer) that has recently been shown to be a second binding site (Landolfi et al. 1986; Kemler et al. 1989; Poellinger and Roeder 1989) for the octamer-binding transcription factors (transcription factors NF-A1 and NF-A2; nomenclature of Staudt et al. 1986). However the 7-mer was bound with much lower affinity without an adjacent consensus octamer (LeBowitz et al. 1989; Poellinger et al. 1989) and is unlikely to be functional in the λ enhancers, because it is not perfectly conserved in λ_{3-1} . No match of >6 of 11 with the NF κ B-binding site of the κ JC intron or 6 of 8 with the immunoglobulin octamer was present. Binding motifs for factors AP-1 and Sp-1 were identified, but both are outside of what is estimated to be the minimal enhancer segment.

Enhancer activity of λ_{3-1}

Regions of the λ 1–3 enhancer were subcloned into a λ

Figure 8. Physical map of $E_{\lambda 3-1}$ enhancer phage clone and subclones for assay of enhancer activity. (Top) Map of phage $\phi 14.2$. Total insert length in phage λ DASH2 vector (hatched boxes) is 13.4 kb. (■) Region of 90% sequence homology with $E_{\lambda 2-4}$. (Bottom) Subcloned fragments are shown to scale. They were introduced upstream of the promoter in OP_{λ} CAT1 and OP_{λ} CAT2 or downstream of the CAT gene (indicated in brackets). $E_{\lambda}P_{\lambda}$ CAT101– $E_{\lambda}P_{\lambda}$ CAT104 were tested in both orientations; the first number of each pair indicates the natural orientation relative to the direction of transcription. $E_{\lambda}P_{\lambda}$ CAT105, $E_{\lambda}P_{\lambda}$ CAT106, and $E_{\lambda}P_{\lambda}$ CAT107 are inserted downstream and in inverted orientation. Plus or minus sign indicates enhancement of CAT activity in transient transfection of J558L.

promoter–CAT expression vector, upstream and downstream of the reporter gene and in both orientations (Fig. 8). The CAT constructs were cotransfected with a β -gal-expressing control plasmid into J558L myeloma cells. Strong enhancer activity was seen with a 535-bp fragment 3' of the *Sst*I site at position 267 (Table 4). Strong enhancer activity is found with the enhancer in either orientation and either position but appears to be highest when placed downstream and in the natural orientation ($E_{\lambda}P_{\lambda}$ CAT103). This arrangement was also most active for $E_{\lambda 2-4}$ (Table 1).

Discussion

Two strong transcriptional enhancers have been identified in the λ locus

The λ enhancer $E_{\lambda 2-4}$ appears to be highly B-cell-restricted. B-cell restriction has not yet been assessed for $E_{\lambda 3-1}$ but is expected because of the high degree of homology between the two enhancers. $E_{\lambda 2-4}$ is a strong enhancer in myeloma cells, but inactive in T cells, in contrast to the H-chain and κ intron enhancers. L cells seem to be a special case. They show *hsC₄-II* and a small enhancement of CAT activity with $E_{\lambda 2-4}$. We suspect that lineage-specific gene expression in L cells may be less restricted than is desired. Unusual B-cell-like properties of these cells include the ability to express transfected major histocompatibility complex (MHC) class II and Ii-chain genes (J. Miller, pers. comm.). NIH-3T3 cells probably represent more typical fibroblasts; $E_{\lambda 2-4}$ is not an enhancer in these cells.

The DNase I hypersensitive site defined as *hsC₄-II* is present in an unusually broad distribution of phenotypes within the B lineage, as it was observed in pro-B, pre-B, and mature B-cell lines. In contrast, the hypersensitive site associated with the activated κ intron enhancer is not present in the pre-B cell line 38B9 (J. Hagman, not shown) and appears to be correlated with stage-dependent transcription (Parslow and Granner 1983) in 70Z/3 cells. The *hsC₄-II* observed in early B-cell lines may be

qualitatively different than that observed in myeloma cells. It will be interesting to test whether the $E_{\lambda 2-4}$ element is an enhancer in early B cells. The relative roles of the other κ hypersensitive sites cannot be determined at this time but may be analogous to other gene systems (such as the human β -globin locus; Grosfeld et al. 1987; Ryan et al. 1989) in which only a subset of identified hypersensitive sites have demonstrable enhancer activity.

The orientation and arrangement of λ genes necessitates the use of multiple downstream transcription control regions

The presence of transcriptional enhancer elements downstream of the $J\lambda 2$ – $J\lambda 4$ gene cluster and the $J\lambda 3$ – $J\lambda 1$ cluster confirms the prediction that λ genes have partitioned sequences that regulate transcription between gene segments that are separate in germ line DNA (see Fig. 1). Like other immunoglobulin genes, rearrangement of the germ line sequence by the immunoglobulin-specific recombinase juxtaposes a V region promoter with a downstream enhancer element to assemble a complete transcription unit. It is expected that the λ enhancers, like other immunoglobulin enhancers, may regulate both the rearrangement and transcription of gene segments to which they are linked.

Unrearranged λ gene segments and the enhancers identified in this study may be ordered along chromosome 16 as $V2$ – V_X – $J\lambda 2$ – $J\lambda 4$ – $E_{\lambda 2-4}$ – $V1$ – $J\lambda 3$ – $J\lambda 1$ – $E_{\lambda 3-1}$. Thus, each cluster of two JC genes has its own downstream enhancer. The fact that $V1$ – $J\lambda 3$ – $J\lambda 1$ is surrounded by two enhancers may explain why rearrangement of $V1$ – $J\lambda 3$ and $V1$ – $J\lambda 1$ occurs at a much higher frequency than rearrangement of $V2$ – $J\lambda 2$ (Persiani et al. 1987). Finally, the expression of $V2$ to $J\lambda 3$ or $J\lambda 1$ rearranged genes is driven by $E_{\lambda 3-1}$, because $E_{\lambda 2-4}$ is deleted by this rearrangement (Storb et al. 1989).

No enhancer activity was found in the 4.0 kb downstream of $C\lambda 1$ (Fig. 5), contrary to a recent report

Figure 9. Nucleotide sequence of E_{K2-4} (*top*) and E_{K3-1} (*bottom*). The sequence is shown in the same orientation as the direction of transcription of the λ genes. Nucleotides are numbered above and below. Gaps have been introduced for maximum alignment of the two sequences. Selected restriction enzyme cleavage sites are shown. The two putative regulatory motifs described in the text are boxed and identified above. The sequences have been submitted to EMBL/GenBank Data Libraries.

Although a number of short sequences related to the

Evidence for the regulation of E₁₂₋₄ by transcription factors other than NFκB

Regulation of κ/λ gene expression

The identification of these novel enhancers with unique properties in the λ locus is the first step toward understanding the regulatory events controlling the utilization of κ and λ genes. Our data may be supportive of a regulatory scheme involving the sequential activation of

Table 4. Percent chloramphenicol conversion in CAT assays of transient transfections

Plasmid	CAT activity as % chloramphenicol conversion ^b
OP _κ CAT1 ^c	2.1 ± 0.8
OP _κ CAT2 ^c	2.8 ± 0.2
E _κ P _κ CAT101	23 ± 5
E _κ P _κ CAT102	22 ± 8
E _κ P _κ CAT103	75 ± 20
E _κ P _κ CAT104	55 ± 4
E _κ P _κ CAT105	9.3 ± 0.1
E _κ P _κ CAT106	1.5 ± 0.5
E _κ P _κ CAT107	0.7 ± 0.2

^aCAT assays with E_{κ3-1} in J558L cells.

^bCellular extract quantities were normalized to the activity of a cotransfected β-gal gene; all transfections were done in duplicate.

^cPromoters 1 and 2 are in two different orientations within the Bluescript CAT vector. The enhancer plasmids 101 and 102 used promoter 2; the rest used promoter 1.

κ, followed by λ, genes through the action of unique factors on their respective enhancer elements. The generation of productive H-chain rearrangement (Alt et al. 1986), followed by the interaction of the κ_i enhancer with NFκB, may be prerequisites for the recombination of V_κ and J_κ gene segments (Kelley et al. 1988). At a later stage, and independent of productive κ expression, unique factors may bind to activate the λ enhancers. The presence of nonproductive rearrangements on both κ alleles would prolong the presence of recombinase until λ rearrangement recognition sequences are accessible, resulting in rearrangement at λ alleles. In light of our evidence for separate L-chain *trans*-activating factors, a stochastic mechanism (unregulated) for L-chain rearrangement (Coleclough et al. 1981; Coleclough 1983) seems less likely.

It has been proposed that SJL strain mice have a mutation in an enhancer region regulating the production of λ chains (Sanchez et al. 1985). A regulatory gene (rλ1) has been identified genetically that segregates with the SJL phenotype (low λ1 expression), and the inheritance of this phenotype is tightly linked to the Cλ gene (Arp et al. 1982; Epstein et al. 1983). The overall map of the λ locus, obtained by pulsed-field gel electrophoresis, is unchanged in SJL mice (U. Storb, unpubl.), but it will be interesting to determine whether E_{κ3-1} is mutated in SJL mice. Differential enhancer function has also been suggested (Onodera et al. 1983) as an explanation for the predominance of λ1 L-chain expression over the other isotypes (~80% λ1) in normal mouse sera (10% λ2, 10% λ3). We may now begin to assess the roles of rearrangement, transcriptional control, and antigen-driven selection of B cells in deriving these ratios.

Materials and methods

Cell culture and LPS induction

All cell lines listed below were grown in media supplemented

with sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, penicillin, and streptomycin. J558L (provided by S. Morrison), Ltk⁻aprt⁻ (provided by R. Palmiter), NIH-3T3 cells (a gift of J. Miller), and S-194/5.XXO.Bu.1 (S194; American Type Culture Collection) were grown in Dulbecco's modified essential medium (DMEM) with 10% bovine calf serum (HyClone). Cells (70Z/3.12) (ATCC) were grown in RPMI 1640 and the same supplements as described above. The myeloma S107 (the kind gift of M. Atchison), pre-B cell line ABC1.28.13.8 (kindly provided by E. Selsing), the EL4 thymoma (the gift of J. Bluestone), and immature B-cell lines BASC6 C2, HAFTL 1, and BAMC1 (all kindly provided by M. Lieber) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). The thymoma BW5147 (provided by J. Miller) and the myeloid cell line WEHI265.1 (ATCC) were grown in DMEM containing 10% FBS. Line 54hi 17.2.3.1.2 is a B-cell hybridoma (Gollahon et al. 1988).

For induction with LPS, cell cultures were established at 10^5 /ml and incubated for 24 hr with 10 μg/ml *Escherichia coli* LPS (Sigma; Serotype 0111:B4).

Isolation of nuclei and limited DNase I digestion

Nuclei were isolated as reported previously (Storb et al. 1981) with the following modifications. Cells were harvested by centrifugation and resuspended in ice-cold 50 mM Tris-HCl (pH 7.5), 50 mM KCl, and 15 mM MgCl₂ ($1 \times$ TKM). After centrifugation, cells were resuspended in $\frac{1}{2} \times$ TKM, pelleted again, and resuspended at 5×10^7 /ml in $\frac{1}{2} \times$ TKM. Ten percent Triton X-100 in $\frac{1}{2} \times$ TKM was added to a final concentration of 0.5 or 1.5% (Ltk⁻aprt⁻) and left on ice for 5 min. The lysate was then overlaid with 1 ml 60% sucrose and $\frac{1}{2} \times$ TKM and centrifuged at 1000 rpm in a refrigerated Sorvall RT6000B. Nuclei were harvested from the interface and resuspended in 10 ml 250 mM sucrose, 10 mM Tris-HCl (pH 7.8), and 10 mM MgCl₂ (STM) plus 0.5% Triton X-100 and centrifuged again onto a sucrose cushion. Nuclei were again recovered and resuspended in 10 ml STM and counted, pelleted, and resuspended at 1.4×10^8 /ml in STM for nuclease digestion.

For nuclease digestion, 0.25 ml of nuclei were warmed for 1 min at 37°C, followed by an additional 2 min after addition of DNase I (Cooper Biomedical; DPRF) in STM. DNase I digestion was terminated by the addition of nuclei to 0.75 ml lysis buffer to make 100 mM EDTA, 1% SDS, and 100 μg/ml proteinase K and left overnight at 37°C. Lysates were then extracted twice with phenol and twice with chloroform, and the DNA was recovered by combination with 5 ml 80% ethanol, spooling the DNA onto a glass rod, and redissolving in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA for restriction enzyme digestion.

Southern analysis of DNA

DNA samples (15 μg) were digested overnight in potassium glutamate buffer (Hanish and McClelland 1988) with 1.5 U/μg restriction enzyme and electrophoresed and blotted as described previously (Hagman et al. 1989). Molecular size markers (Supermarker) were purchased from Discount DNA (Charleston, SC).

Probes

The Cλ1, Cλ2, mousetubulin β5 (Hagman et al. 1989), 3'C1 (Storb et al. 1989), Cκ (Storb et al. 1986), Cλ4 (Gollahon et al. 1988), and Vλ1 (Miller et al. 1988) probes have been described previously. The T-cell receptor β J-C intron probe is a 0.9-kb *SacII*-*XbaI* fragment of plasmid 2B4-X, the gift of M. Davis.

Phage and cosmid cloning

High-molecular-weight DNA was prepared from BALB/c liver according to a modification of the method of Blin and Stafford (1976). Insert DNA was prepared by partial *Mbo*I digestion of high-molecular-weight DNA, followed by size-fractionation and ligation into the *Bam*HI site of the cosmid vector pTCF (Grosveld et al. 1982). Transduction and screening of replica filters was performed as described previously (Hanahan and Meselson 1980; Chaplin et al. 1983). Hybridizing colonies were picked from master filters, as described by Grosveld et al. (1981), colony purified, and stored frozen at -70°C in 25% glycerol/LB broth.

The isolation of phage clones KH5 (containing JCA3, JCA1, and 12-kb 3') and KZ24 (containing 17 kb 3' of CA1) has been described previously (Miller et al. 1981; Miller 1983).

A probe derived from $E_{\lambda 2.4}$ cross-hybridized to a 14-kb *Bam*HI fragment. Thus, for the cloning of phage $\phi 14.2$, containing $E_{\lambda 3.1}$, C57Bl/6 kidney DNA was digested to completion with *Bam*HI, and size-fractionated by gel electrophoresis, and the 13- to 15-kb fragments were isolated by electroelution into hydroxylapatite (Tabak and Flavell 1978). The isolated fragments were cloned into the vector λ DASH2 (Stratagene), packaged with Gigapack Gold packaging extract (Stratagene), and screened on P2PLK17 and PLK17 (Stratagene), according to the manufacturer's instructions.

Construction of plasmids for transient CAT expression and sequencing

The promoterless CAT expression plasmid pCAT3M (Laimins et al. 1984) was provided by D. Gius. The CAT gene (*Bgl*II–*Bam*HI) was ligated into the *Bam*HI site of pUC19 to make pCAT19-1. To make λ CAT14, the λ 1 promoter was ligated upstream of CAT by digesting CAT19-1 with *Xba*I, filling in the overhanging ends with Klenow fragment, digesting with *Hind*III, and ligating to a 203-bp *Hind*III–*Ssp*I fragment (containing –31 to –191 of the λ 1 promoter) derived from a murine λ 1 clone (R λ 1, the gift of N. Hozumi).

CAT expression plasmids containing DNA from the hsC_1 -I region (see Fig. 5) was constructed as follows. The 4.9-kb *Eco*RI fragment of phage clone KH5 was subcloned to make KH5-E4.9. The 0.7-kb *Sst*I fragment of KH5-E4.9 was ligated into the upstream polylinker of λ CAT14 to make S0.7 λ CAT. B2.5 λ CAT and HC1.5 λ CAT, the 2.5-kb *Bam*HI or 1.5-kb *Hind*III–*Cl*AI fragments of KH5-E4.9 were ligated upstream of the promoter of λ CAT14. *Sst*I fragments (1.0- or 1.1-kb) were derived from a second phage clone (KZ24) and subcloned into the upstream polylinker of λ CAT14 to make S1.1 λ CAT3 and 4 and S1.0 λ CAT14 and 16.

CAT plasmids containing DNA from the hsC_4 -II region (see Fig. 3) were prepared from cosA16 (see Results). pA16-N16 contains the 16.5-kb *Nhe*I fragment of cosA16 subcloned into the *Xba*I site of pBSC (P. Engler). The 1.65-kb *Xho*I–*Sal*I fragment of pA16-N16 was subcloned into the *Sal*I site of λ CAT14 to make A16 λ CAT25, A16 λ CAT32, and A16 λ CAT28 (A16 λ CAT28 has two copies of the fragment in the same orientation as A16 λ CAT25) and into the *Sal*I site of SKC (P. Engler) to make SKA16-9 and 10. A16 λ CAT9 and A16 λ CAT31 were made by insertion of the blunt-ended *Xho*I–*Sal*I fragment into the downstream *Sma*I site of λ CAT14. The 1.2- and 0.9-kb *Pst*I fragments of pA16-N16 were ligated into the *Pst*I site of λ CAT14 in both orientations to make A16 λ CAT109 and A16 λ CAT110, and A16 λ CAT102 and A16 λ CAT113, respectively. The same *Pst*I fragments were subcloned into the *Pst*I site of SKC to make SKA16P0.8-2 and SKA16P1.2-5. Fragments of 412 and 280 bp were isolated from a *Rsa*I complete digest of

SKA16-9 and ligated in both orientations to λ CAT14, which had been digested previously with *Sal*I and blunt-ended with the Klenow fragment, producing A16 λ CAT207 and A16 λ CAT208, and A16 λ CAT201 and A16 λ CAT202, respectively. A16 λ CAT301 was produced by religation of A16 λ CAT25, following digestion with *Bgl*II and *Hind*III and treatment with the Klenow fragment to create blunt ends. A 1.4-kb *Sst*I fragment containing the λ 1 major intron (from R λ 1) was subcloned upstream of the promoter of λ CAT14 to yield M1 λ CAT1. The immunoglobulin H-chain enhancer was isolated as a 1.0-kb *Kpn*I–*Sal*I fragment of pHen18-3 (Hagman et al. 1989) and ligated similarly into λ CAT14.

Plasmid λ 2A2 has been described previously (Hagman et al. 1989). λ CAT2 was made by insertion of the *Sst*I–*Bam*HI promoter–CAT fragment of λ CAT14 into *Sst*I–*Bam*HI-digested λ 2A2. The same procedure was used to construct λ CAT1, except that λ 1A1 (which was constructed by subcloning the 7.8-kb *Eco*RI insert of R λ 1 into the *Eco*RI site of pUC19) received the promoter–CAT fragment. λ CAT.BB2 was derived from λ 2A2 by digestion with *Bgl*II and *Bam*HI, followed by ligation to the 1.8-kb *Bam*HI fragment of λ CAT14. λ CAT.SX and λ CAT.XS were made by inserting the 1.65-kb *Xho*I–*Sal*I fragment of pA16-N16 into the downstream polylinker of λ CAT2. Plasmids A1 λ CAT1 and A1 λ CAT3 containing the 4.0-kb *Xho*I–*Sal*I fragment of λ 1A1 subcloned into the unique *Sal*I site of λ CAT14 in opposite orientations.

CAT plasmids containing the $E_{\lambda 3.1}$ fragments were constructed as follows. OP λ CAT1 and OP λ CAT2 were made by inserting the *Kpn*I fragment of λ CAT14 containing the λ 1 promoter and CAT gene in either orientation into the *Kpn*I site of Bluescript SK[–] (Stratagene). The 535-bp *Sst*I–*Xba*I fragment of phage $\phi 14.2$ was blunt-ended and inserted into the *Sma*I site of Bluescript SK[–] to generate pSXO.5 and pXS0.5 (differing only in orientation). The *Hind*III–*Spe*I fragments of pSXO.5 or pXS0.5 were cloned into the *Hind*III/*Spe*I-cut backbone of OP λ CAT2 to generate E_{λ} P λ CAT101 and E_{λ} P λ CAT102, respectively, and into OP λ CAT1 to generate E_{λ} P λ CAT103 and E_{λ} P λ CAT104, respectively. The 5.9-kb *Hind*III–*Nhe*I and 2.3-kb *Hind*III–*Xba*I fragments of phage $\phi 14.2$ were inserted into the *Hind*III/*Spe*I-cut backbone of OP λ CAT1 to generate E_{λ} P λ CAT105 and E_{λ} P λ CAT106, respectively. The 3.1-kb *Sst*I–*Nhe*I fragment of phage $\phi 14.2$ (blunt-ended by mung bean nuclease on the *Sst*I site) was inserted into the *Eco*RV/*Spe*I-cut backbone of OP λ CAT1 to generate E_{λ} P λ CAT107.

Plasmid UTKAT was the kind gift of E. Reinherz (Probst and Moore 1986). DNA segments containing putative and known enhancer elements were blunt-ended with Klenow fragment and ligated to UTKAT, which had been blunt-ended at the single *Xba*I site upstream of the HSV-tk promoter. κ Intron and downstream enhancers were subcloned, respectively, as a *Hind*III fragment containing 9.0 bp of the major intron (Engler et al. 1990) and as a 1-kb *Xho*I–*Xba*I fragment derived from pB₁14 (Brinster et al. 1983). A segment containing the SV40 early region 72- and 21-bp repeats was transferred as a 250-bp *Hind*III (*Pvu*II in wild-type SV40)–*Nco*I segment from plasmid IL2R2S (a gift of P. Engler). The 1.0-kb H-chain enhancer and 1.65-kb *Xho*I–*Sal*I fragment of pA16-N16 were described above.

Transient transfection of lymphoid cells

Supercoiled plasmid DNAs were ethanol-precipitated to sterilize and then dissolved in 10 mM Tris-HCl (pH 7.2) and 0.1 mM EDTA for transfection. Cells (15×10^6 to 20×10^6) were transfected with supercoiled plasmid DNA by electrophoresis with an ISCO 494 power supply (set at 2000 V, but discharge is actually 160 V) according to the method of McDougall et al. (1988),

with the following modifications. All transfections were performed at least in duplicate and were carried out at room temperature. Cells were incubated with plasmid (10 μ g of λ CAT14 or equimolar concentrations of the other constructs) and carrier DNA (sonicated salmon testes DNA, 500 μ g/ml) for 2 min, pulsed once, allowed to sit undisturbed for 10 min, resuspended gently, and plated immediately in 40 ml medium. Electroporation with a 25-fold excess (by weight) of carrier DNA was found to increase the CAT level produced by λ CAT14 at least fivefold and to normalize the DNA concentration-dependent cell death characteristic of electroporation. At 44–48 hr post-transfection, cells were counted and lysed in 0.25 M Tris-HCl (pH 7.8) at 170×10^6 cells/ml.

In the experiment shown in Table 4, transfection efficiency was normalized by cotransfection of 2 μ g of plasmid p1924 (Nielsen et al. 1983) containing the *E. coli* β -galactosidase gene under the control of the SV40 enhancer and β -globin promoter. β -Galactosidase activity was measured essentially as described (Nielsen et al. 1983).

Transient transfection of fibroblastoid lines

L cells and NIH-3T3 cells were transfected by calcium phosphate-mediated transfection essentially as described by Kingston (1989). Cells were plated at a density of 1×10^6 /100-mm tissue culture plate 24 hr prior to the addition of the DNA suspension. A molar equivalent of 2.5 μ g of UTKAT of each plasmid was used per plate, along with 22.5 μ g of sheared salmon testes DNA as a carrier. The cells were incubated with the DNA for 15 hr. Excess DNA was removed with two washes of PBS, and the cells were allowed to incubate for an additional 48 hr at 37°C in 5% CO₂.

Assay of CAT expression

Cell lysis and CAT assays were performed essentially according to Gorman et al. (1982), except that cell extracts were heated at 60°C for 10 min, chilled on ice for 5 min, and centrifuged at 15,000g for 10 min prior to analysis. For quantitation, duplicate spots from thin layer chromatography were counted by liquid scintillation spectrophotometry.

DNA sequencing

Double-stranded DNA sequencing was performed by use of the Sequenase II (U.S. Biochemical Corporation) kit with the Reverse Sequencing Primer (no. 1201; New England Biolabs) and the KS and SK primers (Stratagene). Each strand was sequenced by use of independent clones and with complete agreement between complementary strands. One strand of the E_{κ2.4} inserts subcloned from pA16-N16 to make plasmids A16 λ CAT201, A16 λ CAT202, A16 λ CAT207, and A16 λ CAT208 (see Fig. 3) was sequenced completely. In addition, much of the sequence was confirmed by partial sequencing of two additional plasmids, SKA16P0.8-2 and SKA16P1.2-5 (see Construction of plasmids for transient CAT expression and sequencing). The E_{κ3.1} sequence derives from single-stranded sequencing of the inserts of plasmids E_κP_κCAT107, pSXO.5, and pXSXO.5.

EMSA

EMSAs were performed as described (Singh et al. 1986). The WEHI231 (B-cell line) nuclear extract (Dignam et al. 1983) and the DNA fragment containing the NF κ B-binding site were the gifts of H. Singh. The NF κ B-binding site (pUC κ B1) had the sequence

CTCTCGGAAAGTCCCCTCTGTTGGTAC
CATGGAGAGCCTTTTCAGGGAGACAAC

(the NF κ B site is underlined); it was cloned into the *Kpn*I site of pUC18. The fragment was excised with *Eco*RI and *Bam*HI and labeled with [α -³²P]CTP by using the Klenow fragment of DNA polymerase I. For the competition assay the following gel-purified fragments were used: E_{κ1} = 901-bp *Hind*III fragment (the 3' *Hind*III site is artificial; Engler et al., unpubl.); E_{κ3} = 800-bp *Xho*I–*Xba*I fragment of pB1-14 (Brinster et al. 1983); E_{κ2.4} = 1.1-kb *Pst*I fragment of SKA16 X S10 (the 3' *Pst*I site is naturally an *Nhe*I site); E_H = 1-kb *Xba*I fragment from pHen18-3 (Hagman et al. 1989).

Acknowledgments

J.H. and C.M.R. contributed equally to this work. We thank G.S. Provost, G. Bozek, and P. Morrison for excellent technical assistance and J. Snoddy and H. Singh for assistance with computer analysis of the enhancer sequence. We also thank P. Engler, B. Rogerson, and H. Singh for useful comments concerning this manuscript, H. Singh for help with the EMSA, and M. Glymour for her assistance in preparing this manuscript. We are very grateful to S. Morrison, R. Palmiter, M. Atchison, E. Selsing, J. Bluestone, M. Lieber, J. Miller, L. Laimins, N. Hozumi, P. Engler, E. Reinherz, and H. Singh for gifts of cell lines, DNA, probes, and nuclear extract. This work was supported by National Institutes of Health grants AI-24780 and GM-38649 to U.S. C.M.R. was supported by NIH training grant GM-07281 and is the recipient of a Medical Student Award from the Arthritis Foundation.

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Genes Dev. 1990, **4**:

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