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# Cell-Type-Specific Control Elements of the Lymphotropic Papovavirus Enhancer

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**Lymphotropic papovavirus (LPV) exhibits a highly restricted host range, in which only cells of primate B-lymphocyte origin are permissive for infection. Its enhancer element contributes to this tropism, since transcriptional potentiation is confined to cells of the hematopoietic lineage. Nuclear extracts from B and T cells, but not from HeLa cells, contain protein factors that interact specifically with the LPV 63-base-pair enhancer repeat, as demonstrated by DNase I footprinting and gel retardation experiments. Within the repeat three sequence motifs were identified: the core motif, the Pu box, and a novel element named T motif. Functional analysis demonstrated that these motifs as well as some sequences upstream of the repeat contribute to the optimal activity of the enhancer. There are clear differences between the patterns of binding of the B and T lymphocyte nuclear proteins to the enhancer which are also reflected in the transcriptional activity of the enhancer in both cell types. Furthermore, the activity of the LPV enhancer and its interaction with nuclear proteins seem to be regulated during B-cell differentiation.**

Enhancers are *cis*-acting regulatory elements that potentiate transcription in a relatively position- and orientation-independent manner (for a review, see reference 17). One of the best studied examples is the simian virus 40 (SV40) enhancer (3, 19, 23, 47). It is located in the nontranscribed regulatory region of the SV40 viral genome between the early and late genes. Its major functional region consists of a 72-base-pair (bp) direct repeat, but approximately 120 bp upstream of the repeat are also required for optimal enhancer activity (47). The whole enhancer region consists of a variety of sequence motifs. Although most single motifs cannot activate transcription to a considerable degree, oligomerization of a motif can create powerful enhancers (11, 26, 33). Interestingly, these artificial enhancers have distinct cell specificities. While an oligomer of the core sequence shows almost the same pattern of activity as the entire enhancer, an oligomer of the "pseudocore" is active only in CV-1 cells and an oligomer of the Sph-I motif is lymphoid cell specific (26, 33).

Enhancers have been characterized in a large number of cellular genes as well (17). Besides being necessary for appropriate levels of expression, in many cases they are also responsible for tissue-specific expression of the corresponding gene. The best-studied example of a tissue-specific cellular enhancer is the immunoglobulin heavy-chain gene enhancer, which is active only in B lymphocytes (2, 15, 25). Like viral enhancers, cellular enhancers consist of different sequence motifs (13). Techniques that can probe DNA-protein interactions (e.g., DNase I footprinting, gel retardation, etc.) have shown that these motifs are the targets of specific nuclear factors (17).

Lymphotropic papovavirus (LPV) belongs to the same family of papovaviruses as do SV40, BK virus, and JC virus (27, 37, 48). LPV infects only mature B lymphocytes from primates (41). Its genome structure is almost identical to that of SV40; the genomes share about 80% homology in their coding regions (28). The major regions of nonhomology are confined to the sequences between the early and late transcriptional start sites which, in LPV, as in SV40, encompass an enhancer. The main feature of the LPV enhancer is a 63-bp direct repeat. In contrast to the SV40 enhancer, the LPV enhancer is only active in cells of the hematopoietic lineage and not in cells of fibroblastoid or epithelioid origin (24). In this respect, the LPV enhancer activity is broader than the infectivity of the virus itself. In accordance with the in vivo results, it was shown that the LPV enhancer activates transcription in vitro when incubated with nuclear extracts of B and T cells but not HeLa cells, a human cell line of epithelial origin (35).

To explain the host range restriction of LPV and the cell type specificity of the LPV enhancer, we found it necessary first to characterize the *cis*-acting regulatory elements contained within the enhancer and the *trans*-acting nuclear proteins that recognize these sequences. Using DNase I footprint analysis, we identified several sequence motifs within the LPV enhancer which interact with nuclear factors present in B and T lymphocytes. Mutational analysis of these motifs demonstrated that they are crucial for enhancer activity. Two of these regulatory elements interact with nuclear factors from both B and T cells (30, 43). A newly identified sequence motif is specifically protected in T cells.

Oligomerization of the area of the LPV enhancer which contains the Pu box and the core sequences creates a powerful cell-type-specific enhancer. Furthermore, both the activity and the interaction of the LPV enhancer with nuclear proteins are regulated during B-cell differentiation.

## MATERIALS AND METHODS

**Cell culture and extract preparation.** HeLa cells were grown in MEM Spinner medium (Flow Laboratories, Inc.) with 1× nonessential amino acids (GIBCO Laboratories); B- and T-cell lines were grown in RPMI 1640 (GIBCO) with 3 g of $NaHCO_3$ per liter. The media were supplemented with 2 mM glutamine, 50 μg of streptomycin per ml, 50 IU of

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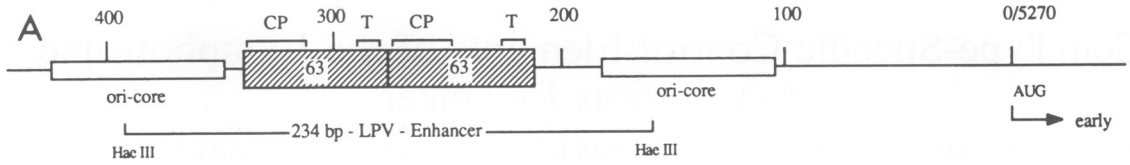
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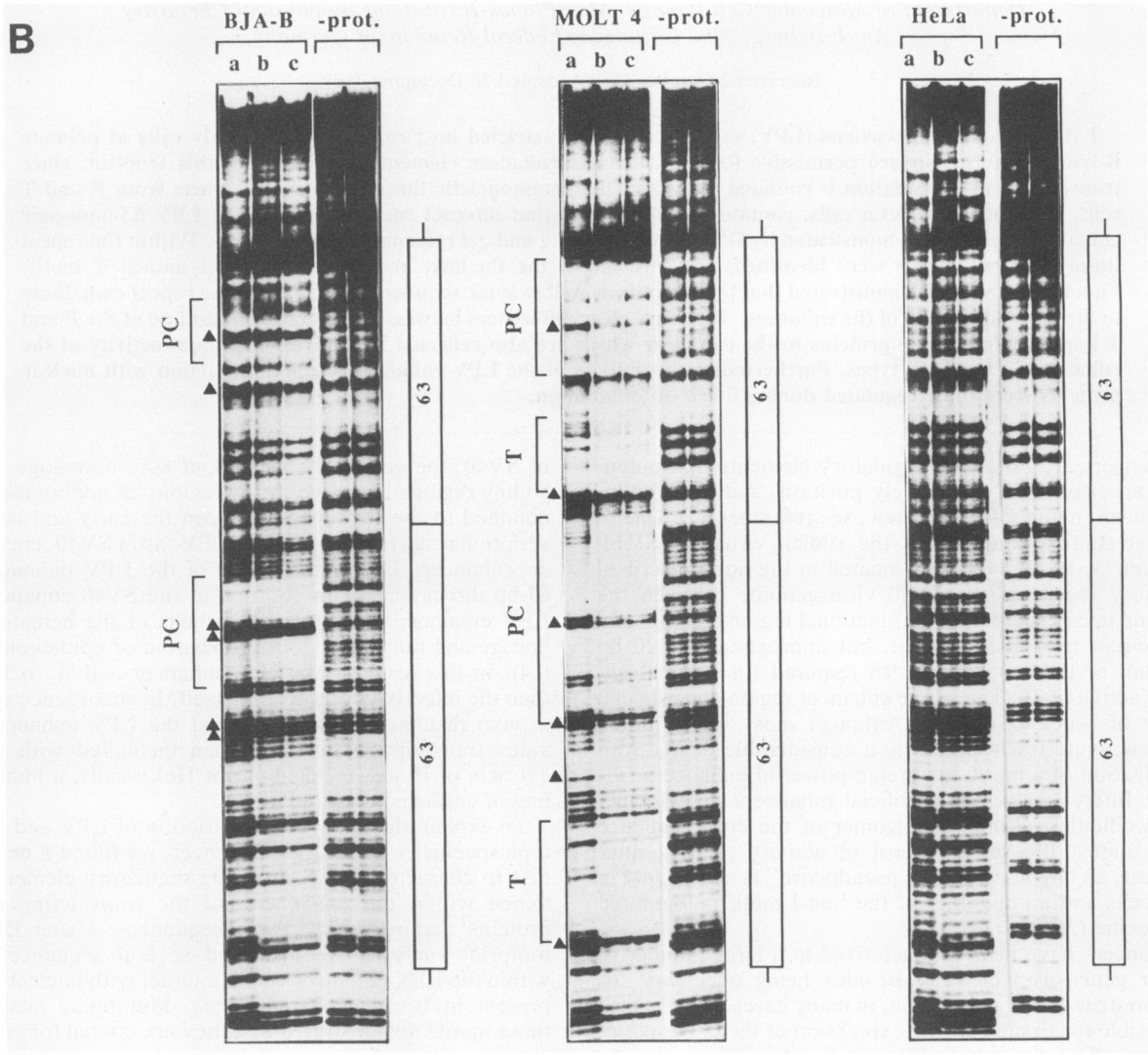
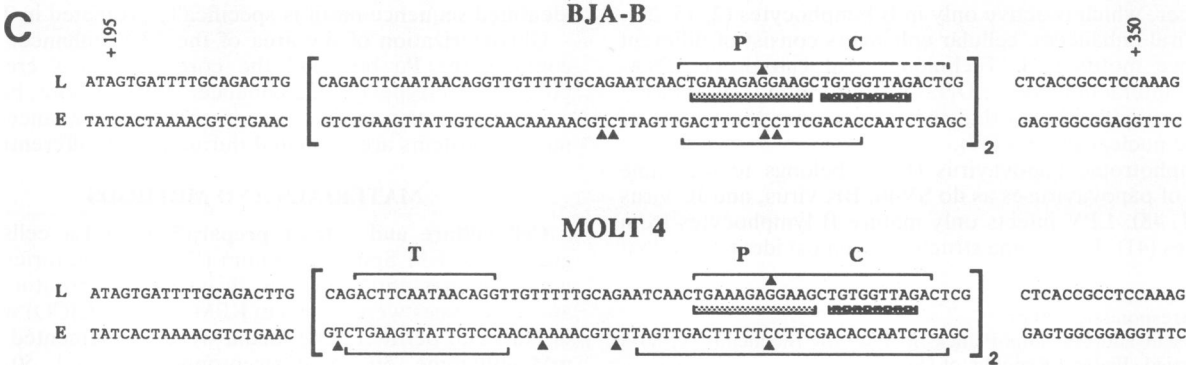
Hae III
Hae III


B
BJA-B -prot.
MOLT 4 -prot.
HeLa -prot.
a b c
PC
T
63


C
BJA-B
+195
+350
P
C
L ATAGTGATTTTGCAGACTTG [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGGAAGCTGTGGTTAGACTCG] CTCACCGCCTCCAAAG
E TATCACTAAAACGTCTGAAC [GTCTGAAGTTATTGTCCAACAAAAACGTCTTAGTTGACTTTCTCCTTCGACACCAATCTGAGC]2 GAGTGGCGGAGGTTTC
MOLT 4
T
P
C
L ATAGTGATTTTGCAGACTTG [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGGAAGCTGTGGTTAGACTCG] CTCACCGCCTCCAAAG
E TATCACTAAAACGTCTGAAC [GTCTGAAGTTATTGTCCAACAAAAACGTCTTAGTTGACTTTCTCCTTCGACACCAATCTGAGC]2 GAGTGGCGGAGGTTTC

penicillin per ml, and 10% fetal calf serum. Nuclear extracts were prepared as described before (6, 46), except that for some extracts the nuclei were prepared by the addition of 0.05% Nonidet P-40 instead of mechanical disruption. No difference in the binding pattern was observed in these cases.

**DNase I footprinting assays.** The 234-bp *Hae*III fragment of LPV (see Fig. 1A) was excised, ligated to *Bgl*II linkers, and cloned into the *Bam*HI site of the SP65 polylinker such that the LPV *Hae*III site at position 158 was located near the *Eco*RI site and the *Hae*III site at position 392 was located near the *Xba*I site of the polylinker. After restriction within the polylinker, the plasmid was dephosphorylated and end labeled, and the LPV enhancer fragment was subsequently excised by a second restriction enzyme digestion. The uniquely end-labeled fragment was used for DNase I footprinting assays and sequencing.

DNase I footprinting reactions were carried out essentially as described previously (34, 45). Briefly, 8 μl of nuclear extract (80 μg) was preincubated for 20 min on ice with 28 ng of pBR322 (restricted with *Hpa*II) in a 10-μl volume containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 20 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol, and 17% glycerol. After preincubation, 2 μl of the labeled template ($10^4$ cpm per reaction; approximately 3 fmol) was added and the samples were incubated for another 10 min on ice. The samples were heated to 20°C, and DNase I (1 mg/ml; freshly diluted in 20% glycerol–1 mM $MgCl_2$–20 mM KCl–1 mM dithiothreitol) was added for 90 s in different amounts (1, 0.5, and 0.25 μg per reaction). Reactions were terminated by the addition of 200 μl of 0.2% sodium dodecyl sulfate–30 μg of tRNA per ml–0.6 M sodium acetate and subsequent extraction with phenol-chloroform. After precipitation with isopropanol, the samples were analyzed on 5% urea–polyacrylamide gels with a G-C sequence ladder run in parallel. The control reactions, which contained no extract, were treated as described for the samples except that the DNase I concentrations used were 20 and 40 ng per reaction. In every case, the procedure was carried out with two to four independently prepared extracts.

**Gel retardation assays.** Oligonucleotides were end labeled with polynucleotide kinase and purified by electrophoresis on 10% polyacrylamide gels. Oligonucleotides (5,000 to 10,000 cpm; approximately 0.15 fmol) were incubated with 5 to 8 μg of nuclear extract in a total volume of 20 μl of 10 mM HEPES (pH 8.0)–5 mM $MgCl_2$–50 mM KCl–1 mM spermidine–0.5 mM dithiothreitol–17% glycerol–1 μg of poly(dI-dC) double-stranded heteropolymer (Boehringer Mannheim Biochemicals) for 15 min at room temperature. DNA-protein complexes were resolved on 5 to 6% polyacrylamide gels (31). The gels were run for 3 to 4 h at 200 V, dried, and exposed for 4 to 12 h to XAR film (Eastman Kodak Co.).

**Cell transfections.** Cells were transiently transfected with the Gene Pulser electroporation apparatus (Bio-Rad Laboratories) (5, 32). On the day before transfection, the cells were split to a density of $3 \times 10^5$ cells per ml. On the next day, $2 \times 10^7$ cells were collected by centrifugation, washed once with phosphate-buffered saline, and suspended in 0.8 ml of phosphate-buffered saline. Supercoiled DNA (20 μg) was added, and the cell suspension was pulsed at a voltage setting of 250 to 400 V and a capacitance setting of 960 μF. The average time constant was 12 to 14 ms. The cells were kept for 10 min at room temperature and transferred to 50 ml of medium. Approximately 48 h following transfection, cells were harvested by centrifugation and washed first in 20 ml of 25 mM Tris hydrochloride (pH 7.5)–137 mM NaCl–5 mM KCl–0.7 mM $CaCl_2$–0.5 mM $MgCl_2$–0.6 mM $Na_2HPO_4$ and subsequently in 1 ml of 0.25 M Tris hydrochloride (pH 7.8). The cells were suspended in 0.15 ml of 0.25 M Tris hydrochloride (pH 7.8) and sonicated. Cell debris was removed by centrifugation, and the supernatant (extract) was assayed for chloramphenicol acetyltransferase (CAT) activity (16). Assay mixtures for CAT activity determinations contained 70 μl of extract, 0.2 μCi of [$^{14}$C]chloramphenicol (New England Nuclear Corp.), 20 μl of 4 mM acetyl coenzyme A (Boehringer), and 80 μl of 0.25 M Tris hydrochloride (pH 7.8). Samples were incubated for 90 min at 37°C, extracted with 0.4 ml of ethyl acetate, dried under vacuum, and suspended in 10 μl of ethyl acetate. The acetylated forms of [$^{14}$C]chloramphenicol were separated by thin-layer chromatography.

**Oligonucleotides.** All oligonucleotides were synthesized (BioSearch 8700 DNA synthesizer) with three guanidine or cytidine residues at the ends to allow unidirectional ligation. They were used either for gel retardation experiments or to clone distinct parts of the LPV enhancer. For cloning, the two corresponding single strands were heated together to 95°C and cooled down slowly to room temperature for annealing. The double-stranded forms were separated from single-stranded forms on 10% polyacrylamide gels, cut out, and electroeluted in TE (10 mM Tris hydrochloride [pH 8], 1 mM EDTA [pH 8]) buffer. Subsequently, the oligonucleotides were phosphorylated (polynucleotide kinase; Boehringer), ligated to *Bgl*II linkers, and cloned into the *Bgl*II site of p209A21 (16). For gel retardation experiments, [γ-$^{32}$P]ATP (Amersham Corp.) was used for phosphorylation. The double-stranded forms were isolated after separation from free nucleotides and single-stranded forms on 10 to 15% polyacrylamide gels.

## RESULTS

**Interaction of cell-type-specific factors with the LPV enhancer in vitro.** Transient transfection experiments showed

FIG. 1. (A) Structure and position of the LPV enhancer. The LPV enhancer is situated in the noncoding region (700 bp) which separates the early and late coding regions of the viral genome. Its position and orientation with respect to the early region are indicated. The *Hae*III fragment (positions 158 to 392) containing a 63-bp repeat was shown to act as an enhancer in vivo (24) and in vitro (35). The protected regions in the DNase I footprint analysis are marked C, P, and T. ori, Origin of replication. (B) Comparison of protected regions in the DNase I footprint assay of the LPV enhancer early strand with nuclear extracts from BJA-B, MOLT 4, and HeLa cells. The LPV enhancer fragment was excised from the SP65 vector by restriction with *Eco*RI and *Xba*I. The strand coding for the viral early proteins was radioactively labeled by a fill-in reaction with the Klenow polymerase. After incubation with the extracts, the labeled strand was treated with increasing amounts of DNase I (0.25, 0.5, and 1.0 μg in lanes a, b, and c, respectively). In the control lanes (−prot.), DNA was incubated only with 20 to 40 ng of DNase I. The digestion products were separated on 5% sequencing gels. Protected areas are marked C, P, and T. ▲, DNase I-hypersensitive sites. The positions of the 63-bp repeats are indicated. (C) Summary of the results of the DNase I footprint analysis of the LPV enhancer fragment in B- and T-cell nuclear extracts. The reactions for the late strand were carried out as described for the early strand in panel B. L and E mark the late and early strands, respectively. ▲, DNase I-hypersensitive sites. The Pu box (P) and the core (C) sequences are underscored, the protected regions (P, C, and T) are bracketed, and broken lines indicate weaker protection. The results are shown for one repeat. In the second repeat the corresponding regions were protected (subscript 2). The numbering system is that of Pawlita et al. (28).

that a 234-bp fragment including the LPV enhancer with the 63-bp repeat (Fig. 1A) was active in cells of the hematopoietic lineage but not in epithelial cell lines (24). To identify factors that interact with the LPV enhancer, we prepared nuclear extracts from the human cell lines BJA-B, a Burkitt's lymphoma cell line representing mature B lymphocytes (20), MOLT 4, an acute T-cell lymphoblastic leukemia cell line (22), and HeLa cells of epithelial origin (14) by previously described methods (6, 45) and used them in the DNase I footprint analysis (12). The 234-bp fragment was radioactively end labeled, incubated with the different nuclear extracts, and partially digested with increasing concentrations of DNase I.

Figure 1B shows the protected areas within the 63-bp repeat on the strand coding for the early viral proteins. With the BJA-B extracts, an 18-bp region containing the Pu box (marked P) and the core motif (marked C) was protected in both repeats. This region included a DNase I-hypersensitive site consisting of two cytidine residues (motifs P and C; see below). When the strand coding for the late viral proteins was labeled, a region spanning 22 nucleotides was protected. At the same position as in the strand coding for the early proteins two DNase I-hypersensitive sites were observed (data not shown). Incubation with the MOLT 4 extracts resulted in a footprint in the same area (Fig. 1B), but the protected sequence was more extended, indicating that a modified factor or a different factor was binding in T cells. In addition, a second protected region (marked T) of 16 nucleotides was found (Fig. 1B). In contrast to the results obtained with the B- and T-cell extracts, no protected domains could be seen in footprint experiments with HeLa nuclear extracts (Fig. 1B). The same results were observed with different extract preparations. The data from the DNase I footprint analysis are summarized in Fig. 1C.

The protected region common to B and T cells contains the core motif and the Pu box. The core motif is found in many viral and cellular enhancers (43), and the Pu box, a purine-rich region, is found in many lymphoid cell-specific transcriptional elements, such as the immunoglobulin heavy-chain gene enhancer (1), the 5′ area of the major histocompatibility complex class II gene (7), and the SV40 enhancer (30). In contrast to the core motif and the Pu box, the T motif has not been described before.

**Mutational analysis of the LPV enhancer in vitro.** To analyze the sequence requirements for the binding of the nuclear factors in more detail, we synthesized and tested several 39-bp oligonucleotides spanning the area of the Pu box and the core motif in gel retardation assays with nuclear extracts from BJA-B, MOLT 4, and HeLa cells (Fig. 2). The oligonucleotide termed L 30 represents the wild-type sequence (Pu box and core), while L 31, L 32, L 33, and L 38 contain different introduced mutations. L 37 contains only the Pu box wild-type sequence.

The wild-type oligonucleotide (L 30) formed a specific complex with a nuclear protein present in BJA-B cells (lower arrowhead in Fig. 2). The same complex was found with nuclear extracts from the B-cell line Namalwa, a human Burkitt's lymphoma cell line (data not shown). This factor was present only in very small amounts in MOLT 4 and HeLa extracts. On the other hand, the MOLT 4 extract contained a different factor that formed a weak protein-DNA complex and that was not seen with the other extracts (upper arrowhead in Fig. 2). No specific complex could be detected with HeLa nuclear extracts. The specificity of binding was tested in competition experiments in which only the wild-type oligonucleotide (L 30) competed effectively for binding (data not shown). One band was detected with all oligonucleotides (asterisk in Fig. 2) and was most likely due to binding of a protein to three protruding guanosine or cytidine residues which were introduced into all oligonucleotides to facilitate unidirectional cloning (36). The amount of this protein varied according to the conditions of cell growth and nuclear extract preparation (see, for example, the two BJA-B nuclear extracts in Fig. 2).

Two independent mutations in the Pu box region (L 31 and L 38; Fig. 2) led to a dramatic decrease in the binding of the B- and T-cell-specific factors, confirming the specificity of the factors binding to the Pu box. Surprisingly, a mutation in the core motif (L 32) had no effect on the binding of the cell-specific factors. The altered G residue was previously shown to be critical for the function of the core motif in the SV40 enhancer (43). Simultaneous mutations in both the Pu box and the core motif (L 33) resulted in a pattern similar to the one observed with oligonucleotides L 31 and L 38, in which only the Pu box was changed. These results demonstrate that the Pu box sequences were sufficient for the binding of the cell-type-specific factors seen in the gel retardation assays. To confirm these results, we synthesized a shorter oligonucleotide (L 37) containing the wild-type Pu box but not the core motif. This oligonucleotide was sufficient to form the B-cell-specific complex with BJA-B nuclear extracts (Fig. 2).

In addition to the described complexes, faint protein-DNA interactions were found with the L 30 wild-type oligonucleotide but were not further investigated. The introduction of mutations into this oligonucleotide (L 31, L 32, L 33, and L 38) gave rise in some cases to new, faint complexes which had mobilities different from those of the complexes eliminated by the mutations. These complexes might have been due to weak interactions with proteins that took place when the specific factors did not bind or, alternatively, to the creation of new binding sites.

An oligonucleotide spanning the T motif was also tested in gel retardation assays. No specific complexes were formed with any of the extracts, indicating that this sequence by itself was not sufficient for binding (data not shown).

**Characterization of the LPV enhancer in vivo.** To test whether the binding sites identified in vitro contributed to the activity of the LPV enhancer in vivo, we introduced deletions and specific mutations and cloned the corresponding fragments in front of the SV40 promoter of plasmid p209A21 driving the *cat* gene (21). The constructs were introduced by electroporation into BJA-B, MOLT 4, and HeLa cells. In all transfection experiments, the parental vector p209A21 was used as a negative control and pSV2CAT (16), which contains the SV40 enhancer, was used as a positive control. Plasmid pLPV $CAT_s$ (24), which contains the entire LPV enhancer, was also used. In B and T cells, all constructs were compared with pLPV $CAT_s$, the activity of which was set arbitrarily at 100%. The different constructs are shown in Fig. 3A.

As previously described, pLPV $CAT_s$ is active in BJA-B and MOLT 4 cells but not in HeLa cells (24). In HeLa cells, activity was not detectable with any of the LPV enhancer constructs, whereas the SV40 enhancer stimulated transcription considerably (data not shown). The two 63-bp repeats alone without further 5′ or 3′ sequences (pLPV 1) were almost as active as the wild-type enhancer in MOLT 4 cells (Fig. 3A). In BJA-B cells, the activity was reduced to 80%. A single 63-bp repeat without any surrounding sequences (pLPV 4) was not able to stimulate transcription in BJA-B cells, and only 10% of the activity was maintained in MOLT

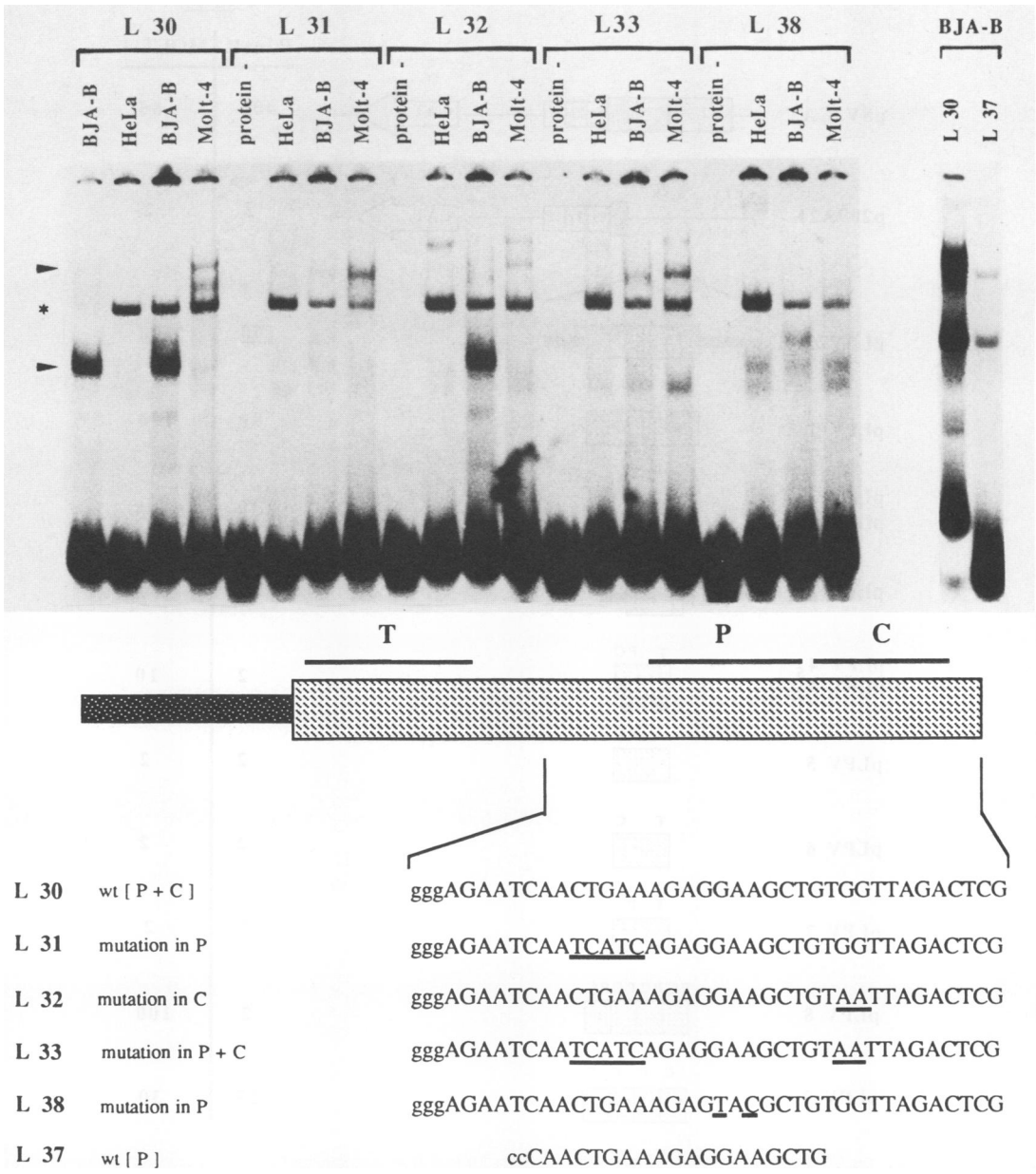
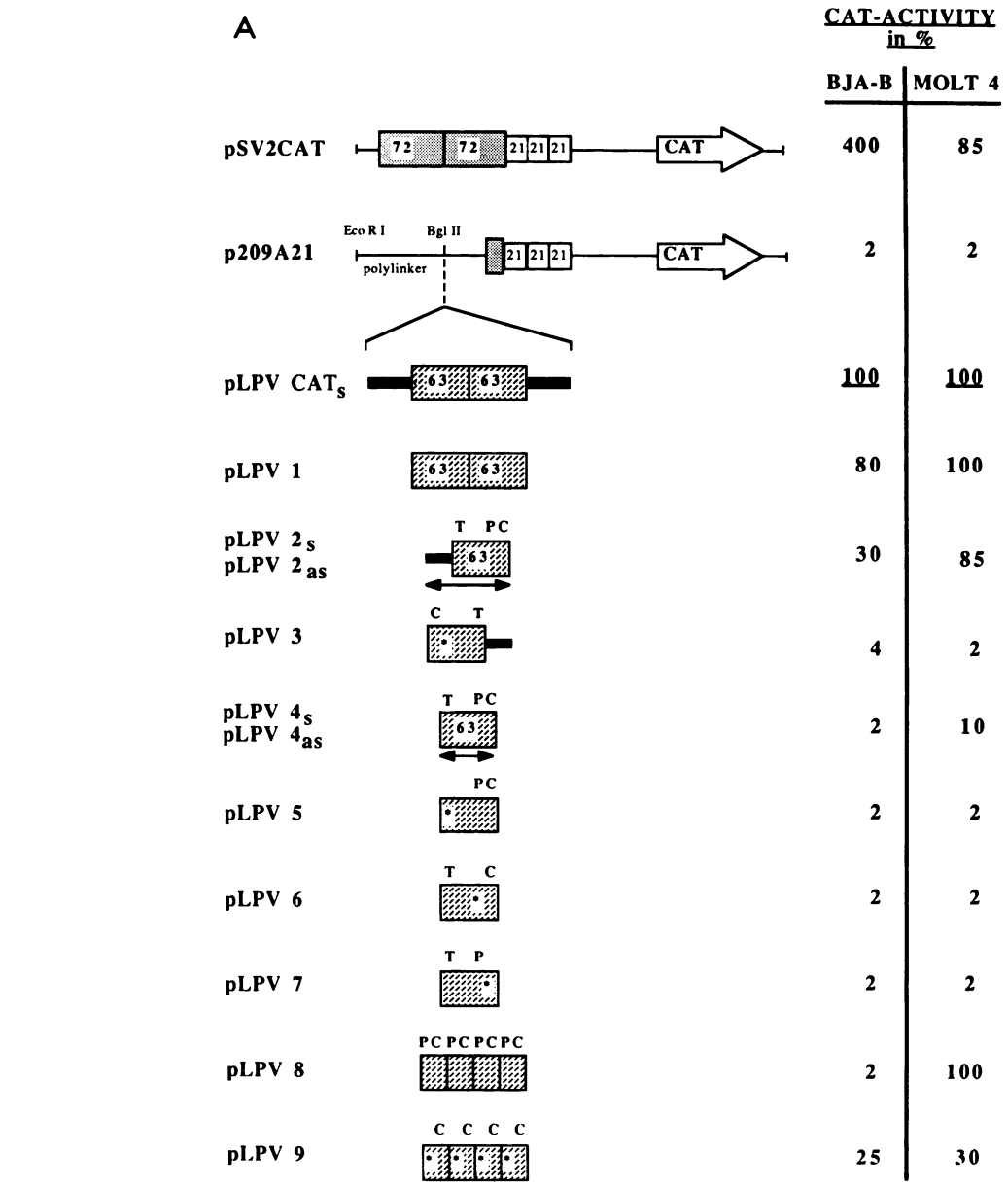



FIG. 2. Comparison of the patterns of binding of nuclear extracts from BJA-B, MOLT 4, and HeLa cells to the Pu box-core region of the LPV enhancer in gel retardation assays. The gel retardation assays were carried out as described in Materials and Methods. The sequences of the synthetic double-stranded oligonucleotides spanning the Pu box and core sequences are shown. Mutated nucleotides in oligonucleotides L 31, L 32, L 33, and L 38 are underlined. L 37 contains only Pu box sequences and was used to determine whether the Pu box itself is sufficient to bind the B-cell-specific factor (rightmost two lanes). For the wild-type (wt) oligonucleotide L 30, the control (−protein) is shown in Fig. 4, and the result for two independently prepared BJA-B extracts is shown here instead. The upper arrowhead indicates the T-cell-specific protein-DNA complex, and the lower arrowhead indicates the B-cell-specific complex. The common band (asterisk) in all lanes is due to nonspecific binding of a factor to the protruding ggg or ccc (corresponding strand) ends of the oligonucleotides. L 37 contains only two c's.

4 cells. In contrast, one repeat plus 32 bp of upstream sequences (pLPV 2) was active in BJA-B cells (30%) and MOLT 4 cells (85%). Thus, the presence of two copies of the repeat is necessary for full activity of the enhancer, but upstream sequences contribute to the transcriptional stimulation as well. The activity of the upstream sequences was most pronounced in B cells, whereas in T cells the effect was observed only when one of the repeats was present. The same results were obtained independent of the orientation of the inserted fragment.

Mutations in the Pu box (pLPV 3 and pLPV 6; same mutations as in the L 38 oligonucleotide; see above) resulted in a complete loss of activity in both BJA-B and MOLT 4 cells, confirming the importance of the Pu box for enhancer activity in vivo. Mutations were introduced into the core and T motifs in pLPV 4 (constructs pLPV 7 and pLPV 5, respectively). Both constructs were inactive in MOLT 4 cells, demonstrating that both the T and the core motifs contribute to enhancer activity in T cells.

It was previously shown that enhancers with a distinct




B

| | | T | P | C |
|---|---|---|---|---|
| pLPV 2 | GCTAGGGTTGCCATAGTGATTTTGCAGACTTG | [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGGAAGCTGTGGTTAGACTCG] | | |
| pLPV 3 | GCTAGGGTTGCCATAGTGATTTTGCAGACTTG | [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGtAcGCTGTGGTTAGACTCG] | | |
| pLPV 4 | | [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGGAAGCTGTGGTTAGACTCG] | | |
| pLPV 5 | | [CAGACTTCAcTcACAGGTTGTTTTTGCAGAATCAACTGAAAGAGGAAGCTGTGGTTAGACTCG] | | |
| pLPV 6 | | [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGtAcGCTGTGGTTAGACTCG] | | |
| pLPV 7 | | [CAGACTTCAATAACAGGTTGTTTTTGCAGAATCAACTGAAAGAGGAAGCgGgGGTTAGACTCG] | | |

tissue specificity could be obtained by oligomerization of certain sequence motifs (13, 26, 33). We were interested in determining whether oligomerization of the area containing the Pu box and core motif could result in an enhancer element. The wild-type oligonucleotide L 30 (Pu box and core) and the mutant oligonucleotide L 31 (mutated in the Pu box) were oligomerized four times, inserted in p209A21 (constructs pLPV 8 and pLPV 9, respectively), and transfected into BJA-B, MOLT 4, and HeLa cells. Both constructs were inactive in HeLa cells (data not shown). The wild-type tetramer was a very strong enhancer in T cells but was inactive in BJA-B cells. The mutated tetramer was only 30% as active as the wild-type tetramer in T cells. Surprisingly, the mutated tetramer showed comparable activity in B and T cells (Fig. 3A).

**LPV enhancer activity during B-lymphocyte differentiation.** B lymphocytes follow a precise program of differentiation. In pre-B cells, only immunoglobulin μ heavy-chain genes are expressed. In later stages, transcription of the κ or λ light chains follows and immunoglobulin M molecules appear on the surface of virgin $B_\mu$ cells. These cells give rise first to mature $B_{\mu+\delta}$ cells and, following antigen stimulation, to blast and plasma cells (4).

The transcriptional factor NF-κB, involved in the expression of the immunoglobulin genes, is only present in mature B cells, whereas the factor OTF-2 is also present in earlier stages (34, 38–41, 44). Since it has been demonstrated that sequences homologous to the Pu box are also present in the immunoglobulin heavy-chain enhancer and are required for activity (30), we were interested in determining whether the LPV enhancer is regulated during B-cell differentiation and whether the activity correlates with the presence of the corresponding nuclear factors. To answer the first question, we transfected several cell lines representing different stages of B-lymphocyte differentiation with plasmids pLPV $CAT_s$ and pSV2CAT (as a control). The activity in all cell lines was compared with the activity of pSV2CAT in BJA-B cells, which was set at 100. The SV40 enhancer strongly stimulated transcription in all cell lines, whereas the LPV enhancer was only active in mature B cells (BJA-B and Daudi) as well as in the plasma cell line R-LICR (Table 1). In these three cell lines, the activity of the LPV enhancer was significantly lower compared with that of the SV40 enhancer, but the ratio of SV40 to LPV enhancer activity was constant. In pre-B cells (cell lines 207 and 697) or in cells in an early stage of differentiation (LBW 4) (8, 10, 18), the LPV enhancer was not active.

Subsequently, nuclear extracts from BJA-B cells (representing mature B cells) and 207 cells (representing pre-B cells) were compared in gel retardation assays. The B-cell-specific factor binding to the Pu box (arrowhead in Fig. 4) was much more abundant in the mature B-cell line BJA-B. The pre-B cell line 207 contained only low levels of the factor. As a control, an oligonucleotide containing the XP-1-binding site of the immunoglobulin μ heavy-chain gene enhancer was used in these experiments (9). This oligonucleotide detected equal amounts of the corresponding factors in both cell lines, indicating that the difference observed was specific for the Pu box-binding protein.

TABLE 1. LPV enhancer activity during B-lymphocyte differentiation[a]

| Cell type | CAT activity with: | | |
|---|---|---|---|
| | p209A21 | pSV2CAT | pLPV $CAT_s$ |
| 207 | 1 | 50 | 1 |
| 697 | 1 | 57 | 1 |
| LBW 4 | 1 | 67 | 1 |
| BJA-B | 2 | 100 | 51 |
| Daudi | 1 | 60 | 30 |
| R-LICR | 1 | 43 | 20 |

[a] Cell lines in different stages of B-lymphocyte differentiation were transfected with plasmid pLPV $CAT_s$. Cell lines 207 and 697 are pre-B cells. LBW 4 is a cell line in an early stage of differentiation, while Daudi and BJA-B represent mature B cells. R-LICR is a plasma cell line. p209A21 and pSV2CAT wre transfected in parallel as controls. The activity of pSV2CAT in BJA-B cells was set at 100, corresponding to 85% CAT conversion (for details, see Materials and Methods).

## DISCUSSION

LPV represents an ideal system for studying cell-type-specific gene expression. Its enhancer, a 234-bp fragment containing a direct repeat of 63 bp, is active only in cells of the hematopoietic lineage of primates (24). The aim of this study was to identify and characterize the *cis*-acting elements and their corresponding *trans*-acting factors that are responsible for this tissue specificity and to compare the requirements for enhancer activity in B and T lymphocytes. The results presented here demonstrate that three sequence elements, namely, the core, the Pu box, and the T motif, bind nuclear proteins present only in lymphoid cells. Mutational analysis showed that these motifs all contribute to enhancer activity. Besides the two 63-bp repeats which contain these three transcriptional elements, sequences outside the repeats also contribute to optimal enhancer activity. Thus, the LPV enhancer, like most transcriptional elements, consists of different sequence motifs. Since it is composed of only a small number of motifs which are active only in lymphoid cells, it is not surprising that the activity of the whole enhancer is also restricted to these cells. In contrast to this very specific enhancer, the SV40 enhancer is highly active in a variety of cell types. In this case, many motifs, such as the core, the pseudocore, the Sph-I motif, the octamer, the Pu box, and the P motif, contribute in various extents to enhancer activity (19, 26).

The three motifs protected in the footprint analysis of the LPV enhancer are the Pu box, the core, and the T motif. The

FIG. 3. (A) Functional analysis of the LPV enhancer by the CAT assay. For all transfection experiments, 20 μg of supercoiled DNA was used. The DNA was introduced into the cells by electroporation, and 48 h after transfection the CAT extracts were prepared (for details, see Materials and Methods). The CAT activity of the LPV enhancer (pLPV $CAT_s$) was set in all cell lines at 100%. pSV2 CAT and p209A21 were used as positive and negative controls, respectively. On the left side of the panel, the LPV constructs used for transfection are shown. The three motifs, T motif (T), Pu box (P), and core motif (C), are indicated. Asterisks indicate mutated sequences. On the right side of the panel, the CAT activities of the different constructs (as percentages) are compared with that of pLPV $CAT_s$. pLPV $CAT_s$ represents the wild-type enhancer, pLPV 1 contains the two 63-bp wild-type repeats without any surrounding sequences, pLPV 8 is a tetramer of oligonucleotide L 30, and pLPV 9 is a tetramer of oligonucleotide L 31 (Fig. 2). The subscripts "s" and "as" refer to sense and antisense, respectively. (B) Sequences of the LPV CAT constructs used for transfection in BJA-B, MOLT 4, and HeLa cells. pLPV 2 and pLPV 4 contain only wild-type sequences. In the other constructs, the introduced mutations are underlined. All constructs were made with synthetic oligonucleotides. The brackets indicate the protected areas in the DNase I footprint analysis (Fig. 1B and C).

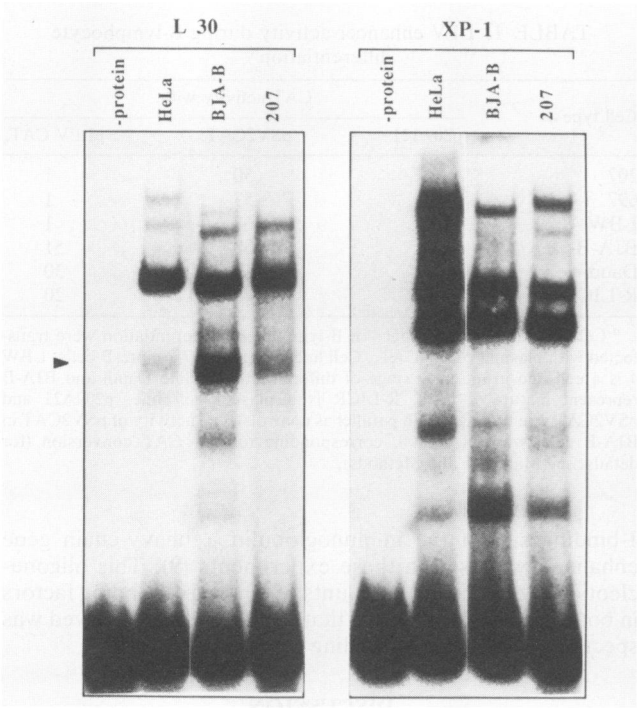



FIG. 4. Binding of nuclear factors to the Pu box-core region of the LPV enhancer in the pre-B cell line 207 and the mature B-cell line BJA-B. The sequence of oligonucleotide L 30 is given in Fig. 2. In the leftmost lanes of each panel the oligonucleotide was incubated without extract. 207 is a pre-B cell line, and BJA-B is a mature B-cell line. The arrowhead indicates the B-cell-specific complex formed with Pu box sequences. The XP-1-binding site of the immunoglobulin μ heavy-chain gene enhancer was incubated with the same extracts as a control.

Pu box is found in a large number of lymphoid cell-specific transcriptional elements, such as the major histocompatibility complex class II genes and the immunoglobulin enhancer (1, 7). It was first identified in the SV40 enhancer, in which it is found approximately 120 bp upstream of the 72-bp repeats. Duplication of a DNA segment containing the Pu box creates a lymphoid cell-specific enhancer (30). In the case of LPV, oligomerization of the Pu box-core sequence also creates a cell-type-specific enhancer but, in contrast to that in SV40, it is active only in T cells. As the DNase I footprinting and gel retardation experiments showed, different factors are bound in B and T lymphocytes. It has been shown for the octamer motif that the same sequence binds a plethora of factors which can, in turn, mediate different tissue specificities (36). Recently, a 100-kilodalton protein from mouse lymphocytes that interacted with sequences similar to the Pu box and present in the immunoglobulin Cμ enhancer was purified and shown to mediate immunoglobulin enhancer activity (1); however, it is unclear whether this factor binds the LPV Pu box. As the in vivo experiments demonstrated, the Pu box is essential for B- and T-cell activity, although the oligomer works only in T cells. Mutations in the Pu box lead to a dramatic decrease in activity in both cell types. So, although the Pu box is required for activity in B cells, it probably requires other sequences for its activity. If these sequences are not present, the binding of the factor might be inhibitory.

The Pu box factor seems to be regulated during B-cell differentiation. Strong binding activity is detected in mature B cells but not in pre-B cells. In this respect, the Pu box-binding protein resembles nuclear factor NF-κB, which is induced in mature B cells (38, 42), and not OTF-2, which is also present in pre-B cells. It is not yet clear whether the Pu box-binding factor can be induced in other cell types, like NF-κB can (38). The LPV enhancer is only active in B cells, such as BJA-B, that show strong binding of the factor, providing additional evidence that the Pu box is a crucial element for transcriptional activity. It will be interesting to see if the Pu box interacts with proteins of other cells of the hematopoietic lineage. Moreover, it will be interesting to see if oligomerized L 30 is active in these cells or if its activity is restricted to T lymphocytes.

The core motif is found in many transcriptional elements, and oligomers of the core motif are active in a wide variety of cells (26). However, the core motif found in the LPV enhancer binds lymphoid cell-specific proteins since, in contrast to the results with nuclear extracts of lymphoid cells, it is not protected in the DNase I footprint analysis with HeLa extracts. In addition, an oligomer of the mutated oligonucleotide L 31, which contains a tetramer of the wild-type core sequence, is active in B and T cells but not in HeLa cells. Interestingly, the LPV core is identical to the core in the immunoglobulin enhancer, and mutational analysis showed that the core contributes to immunoglobulin enhancer activity (29). It is noteworthy that in the immunoglobulin Cμ enhancer the Pu box and the core motif are also found in close proximity and the core represents the strongest homology between these two lymphoid cell-specific elements (34).

Although the core motif is clearly protected in the footprinting experiments, an oligonucleotide consisting only of the core sequence does not bind proteins in the gel retardation experiments. It is probable that the core-binding protein needs additional sequences not present in the oligonucleotide and/or that the binding of proteins outside the core sequence is required. This might also be true for the T motif: the same sequence which is protected in the DNase I footprinting experiments with T-cell nuclear extracts does not form a complex with nuclear proteins in the gel retardation assays. Mutational analysis proved that this sequence, like the Pu box and the core motif, contributes to enhancer activity in T lymphocytes. However, computer analysis shows that no sequence homologous to the T motif has been described to date in any regulatory element.

Thus, all three motifs identified in the 63-bp repeats of the LPV enhancer by in vitro experiments are involved in mediating transcriptional activity in a cell-type-specific manner. In addition, sequences upstream of the repeats contribute to the overall activity. The results presented here describe the activity of the LPV enhancer in B and T cells. Since the LPV enhancer is also active in other cell types of the hematopoietic lineage, such as erythrocytes, macrophages, and granulocytes (24), further insights into its activity should come from characterizing *cis*-acting elements and the corresponding *trans*-acting factors present in these cells and comparing them with the ones analyzed in B and T lymphocytes. Moreover, purification and cloning of the proteins interacting with the LPV enhancer and regulating its activity will help to better understand its cell type specificity and regulation of transcription in cells of the hematopoietic lineage.

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The first two authors contributed equally to this work.

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## LITERATURE CITED

1. **Araki, K., H. Maeda, J. Wang, D. Kitamura, and T. Watanabe.** 1988. Purification of nuclear trans-acting factor involved in the regulated transcription of a human immunoglobulin heavy chain gene. Cell **53**:723–730.
2. **Banerji, J., L. Olson, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–749.
3. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV 40 DNA sequences. Cell **27**:299–308.
4. **Blackwell, T. K., and F. W. Alt.** 1988. Immunoglobulin genes, p. 1–60. *In* B. D. Hames and D. M. Glover (ed.), Frontiers in molecular biology. Molecular immunology. IRL Press Ltd., Washington, D.C.
5. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**:1311–1326.
6. **Dignam, J. D., R. M. Lebovitz, and G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
7. **Dorn, A., H. J. Fehling, W. Koch, M. Le Meur, P. Gerlinger, C. Benoist, and D. Mathis.** 1988. B-cell control region at the 5′ end of a major histocompatibility complex class II gene: sequences and factors. Mol. Cell. Biol. **8**:3975–3987.
8. **Edwards, P. A. W.** 1981. Some properties and applications of monoclonal antibodies. Biochem. J. **200**:1–10.
9. **Ephrussi, A., G. M. Church, S. Tonegawa, and W. Gilbert.** 1985. B lineage-specific interaction of an immunoglobulin enhancer with cellular factors *in vivo*. Science **227**:134–140.
10. **Findley, H. W., Jr., M. D. Cooper, T. H. Kim, C. Alvarado, and A. H. Ragab.** 1982. Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. Blood **60**:1305–1309.
11. **Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon.** 1988. Cooperativity and hierarchical levels of functional organization in the SV 40 enhancer. Cell **54**:943–953.
12. **Galas, D., and A. Schmitz.** 1978. DNase I footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. **5**:3157–3170.
13. **Gerster, T., P. Matthias, M. Thali, J. Jiricny, and W. Schaffner.** 1987. Cell type-specificity elements of the immunoglobulin heavy chain gene enhancer. EMBO J. **6**:1323–1329.
14. **Gey, G. O., W. D. Coffman, and M. K. Kubicek.** 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. **12**:264–265.
15. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
16. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
17. **Hatzopoulos, A. K., U. Schlokat, and P. Gruss.** 1988. Enhancers and other *cis*-acting regulatory elements, p. 43–96. *In* B. D. Hames and D. M. Glover (ed.), Frontiers in molecular biology. Transcription and splicing. IRL Press Ltd., Washington, D.C.
18. **Hendershot, I., and D. Levitt.** 1982. Differential regulation of membrane and secretory μ chain synthesis in human B cell lines. J. Exp. Med. **156**:1622–1634.
19. **Herr, W., and J. Clarke.** 1986. The SV 40 enhancer is composed of multiple functional elements that can compensate for one another. Cell **45**:461–470.
20. **Klein, G., B. Giovanella, A. Westman, J. S. Stehlin, and D. Munford.** 1975. An EBV-genome-negative cell line established from an American Burkitt lymphoma; receptor characteristics, EBV infectibility and permanent conversion into EBV-positive sublines by *in vitro* infection. Intervirology **5**:319–334.
21. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
22. **Minowada, J., T. Ohnura, and G. E. Moore.** 1972. Rosette-forming human lymphoid-cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. J. Natl. Cancer Inst. **49**:891–895.
23. **Moreau, P., R. Hen, B. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon.** 1981. The SV 40 72 bp repeat has a striking effect on gene expression both in SV 40 and other chimeric recombinants. Nucleic Acids Res. **9**:6047–6068.
24. **Mosthaf, L., M. Pawlita, and P. Gruss.** 1985. A viral enhancer element specifically active in human haematopoietic cells. Nature (London) **315**:597–600.
25. **Neuberger, M. S.** 1983. Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. EMBO J. **2**:1373–1378.
26. **Ondek, B., A. Shepard, and W. Herr.** 1987. Discrete elements within the SV 40 enhancer region display different cell-specific activities. EMBO J. **6**:1017–1025.
27. **Padgett, B. L., C. M. Rogers, and D. L. Walker.** 1977. JC virus, a human polyomavirus associated with progressive multifocal leukoencephalopathy: additional biological characteristics and antigenic relationships. Infect. Immun. **15**:656–662.
28. **Pawlita, M., A. Clad, and H. zur Hausen.** 1985. Complete DNA sequence of lymphotropic papovavirus: prototype of a new species of the polyomavirus genus. Virology **143**:196–211.
29. **Perez-Mutul, J., M. Macchi, and B. Wasylyk.** 1988. Mutational analysis of the contribution of sequence motifs within the Ig enhancer to tissue specific transcriptional activation. Nucleic Acids Res. **16**:6085–6096.
30. **Petterson, M., and W. Schaffner.** 1987. A purine-rich DNA sequence motif present in SV 40 and lymphotropic papovavirus binds a lymphoid-specific factor and contributes to enhancer activity in lymphoid cells. Genes Dev. **1**:962–972.
31. **Piette, J., M. H. Krysze, and M. Yaniv.** 1985. Specific interaction of cellular factors with the B enhancer of polyomavirus. EMBO J. **4**:2675–2685.
32. **Potter, H., L. Weir, and P. Leder.** 1984. Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA **81**:7161–7165.
33. **Schirm, S., J. Jiricny, and W. Schaffner.** 1987. The SV 40 enhancer can be dissected into multiple segments, each with a different cell specificity. Genes Dev. **1**:65–74.
34. **Schlokat, U., D. Bohmann, H. Schöler, and P. Gruss.** 1986. Nuclear factors binding specific sequences within the immunoglobulin enhancer interact differentially with other enhancer elements. EMBO J. **5**:3251–3256.
35. **Schöler, H. R., and P. Gruss.** 1985. Cell type-specific transcriptional enhancement *in vitro* requires the presence of *trans*-acting factors. EMBO J. **4**:3005–3013.
36. **Schöler, H. R., A. K. Hatzopoulos, R. Balling, N. Suzuki, and P. Gruss.** 1989. A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. EMBO J. **8**:2543–2550.
37. **Seif, I., G. Khoury, and R. Dhar.** 1979. The genome of human papovavirus BKV. Cell **18**:963–977.
38. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
39. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319**:154–157.
40. **Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Sharp, and D. Baltimore.** 1986. A lymphoid specific protein binding to the octamer motif of immunoglobulin genes. Nature (London) **323**: 640–643.

41. **Takemoto, K. K., A. Furuno, K. Kato, and K. Yoshiike.** 1982. Biological and biochemical studies of African green monkey lymphotropic papovavirus. J. Virol. **42**:502–509.
42. **Wall, R., M. Briskin, C. Carter, H. Goven, A. Taylor, and P. Kincade.** 1986. A labile inhibitor blocks immunoglobulin κ light chain gene transcription in pre-B leukemic cell line. Proc. Natl. Acad. Sci. USA **83**:295–298.
43. **Weiher, H., M. König, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**: 626–631.
44. **Weinberger, J., D. Baltimore, and P. A. Sharp.** 1986. Distinct factors bind to apparently homologous sequences in the immunoglobulin heavy chain enhancer. Nature (London) **322**:846–848.
45. **Wildeman, A., P. Sassone-Corsi, T. Grundström, M. Zenke, and P. Chambon.** 1984. Stimulation of *in vitro* transcription from the SV 40 early promoter by the enhancer involves a specific *trans*-acting factor. EMBO J. **13**:3129–3133.
46. **Wildeman, A. G., M. Zenke, C. Schatz, M. Wintzerith, T. Grundström, H. Matthes, K. Takahashi, and P. Chambon.** 1986. Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. **6**:2098–2105.
47. **Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV 40 enhancer function. EMBO J. **5**:387–397.
48. **zur Hausen, H., and L. Gissmann.** 1979. Lymphotropic papovaviruses isolated from African green monkey and human cells. Med. Microbiol. Immunol. **167**:137–153.