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IGH minisatellite suppression of USF-binding-site- and Eμ-mediated transcriptional activation of the adenovirus major late promoter

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ABSTRACT

The 50bp repeat unit of the minisatellite within the D_H-J_H interval of the human immunoglobulin heavy chain locus binds a nuclear factor present in a wide variety of cell types. The binding site contains the myc/HLH motif, CACGTG, and represents a 15 of 17 base match for the USF/MLTF binding site adjacent to the adenovirus major late promoter (MLP). Unlike the USF/MLTF site, the *IGH* minisatellite possesses no enhancer activity. However, it can significantly suppress, in *cis* and in *trans*, USF-site-mediated transcriptional activation of the MLP. In murine myeloma cells, the *IGH* minisatellite can suppress, in *trans*, MLP activation by the murine heavy chain gene enhancer, Eμ. These activities potentially represent a DNA-based form of squelching.

INTRODUCTION

Vertebrate genomes contain dispersed, highly polymorphic elements characterized by the tandem repetition of short (14–100 bp) sequence motifs. These have been designated hypervariable minisatellites (1), variable tandem repeats (VTRs; 2) or VNTRs (3). Although many of these structures are confined to the telomeric regions of chromosomes (4), many of them demonstrate close associations with genes and gene clusters. Examples include *HRAS1* (5), insulin (6), the α-globin cluster (multiple locations; ref. 7), *IGH* (multiple locations; 8, 9), myoglobin (10), apolipoprotein B (11), Type II collagen (12), involucrin (13), and proline-rich proteins (14). The latter two are examples of expressed minisatellite sequences.

Population genetic analysis of one of these VTR loci, VTR_{HRAS1}, has suggested the possibility that minisatellites might influence the behavior of nearby genes. At the VTR_{HRAS1} locus, 1 kb downstream from the *HRAS1* polyadenylation signal, four modal, or common, VTR alleles have given rise through an undefined process of mutation to several dozen non-modal, or rare, alleles (15). These rare alleles appear twice as often in the genomes of cancer patients as in cancer-free controls (2, 16), a result now based on a sample size of nearly 5000 alleles (reviewed in ref. 17, and in preparation). In pursuing an underlying mechanism for this observation, we have recently shown that the VTR_{HRAS1} repeat unit binds four members of the rel/NF-κB family of transcriptional regulatory factors (18). Furthermore, the presence of this minisatellite in reporter gene constructs confers transcriptional activation which is promoter-, cell-type-, and allele-specific (M.Green and T.G.K., in preparation).

The binding of specific transcription factors to VTR_{HRAS1} leads naturally to the speculation that other minisatellites in the vicinity of genes might also interact with particular regulatory proteins. Such interactions might have consequences, either favorable or adverse, for the physiologic function of these binding factors. We chose to examine the minisatellite within the D_H-J_H interval of the human immunoglobulin heavy chain gene, located approximately 1500 bp upstream of the J_H genes and 5 kb upstream of the immunoglobulin heavy chain enhancer (8). The VTR_{IGH} repeat unit is 50 bp long and lacks the NF-κB site of VTR_{HRAS1}. The entire minisatellite is deleted during B cell differentiation at the stage of DJ rearrangement, raising the interesting possibility that any regulatory impact of the VTR would be stage-specific. We now report that VTR_{IGH}, like VTR_{HRAS1}, binds specific, but distinct, nuclear proteins. The apparent effect of this interaction is the sequestration of factor in an inactive form resulting in the suppression of transcriptional activation.

MATERIALS AND METHODS

Cell lines and culture

The following cell lines were used for the preparation of nuclear extracts and/or for the transfection of plasmid DNA: EJ (human bladder carcinoma); HeLa (human cervical carcinoma); Jurkat

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(human CD8+ T-cell leukemia); CEM (human CD4+ T-cell leukemia); BL-1 and BL-31 (Epstein-Barr Virus negative Burkitt lymphoma); RPMI 7951 (human malignant melanoma); PD31 (Abelson-leukemia virus induced murine pre-B cell); human peripheral blood mononuclear cells (PBMC); EBV-immortilized lymphblastoid cell line (LCL), H241 (murine B-cell hybridoma), NIH3T3 (mouse fibroblast), and P3X63-Ag8 (murine plasmacytoma). Cells were maintained either in Dulbecco's MEM plus 10% calf serum or RPMI plus 10% fetal calf serum (FCS).

Oligonucleotides

The oligonucleotides used as targets and competitors in mobility shift assays and as control elements in vectors bearing the reporter gene, chloramphenicol acetyltransferase (CAT) are depicted in Table 1; complementary sequences are not shown. VTR_{IGH} was a 60 bp oligomer representing the 50 bp repeat unit and 10 additional bases from an adjacent repeat. A 25 bp oligonucleotide and complement (MLP1, MLP2), corresponding to position −74 to −49 of the adenovirus major late promoter (MLP; 19, 20) were synthesized for use in mobility shift assays. A 78 bp oligonucleotide and complement (MLP3, MLP4), corresponding to position +1 to −74 of the MLP, were synthesized with XbaI and AccI termini. We also synthesized a 47 bp oligonucleotide and complement (MLP5, MLP6) which represented position +1 to −45 of the MLP (deleting the USF binding site) and containing XbaI and SphI termini. Oligomers corresponding to the 5′ and 3′ ends of the 700 bp XbaI/PstI restriction fragment bearing the immunoglobulin heavy-chain enhancer (21) were synthesized with terminal SphI sites to facilitate subsequent cloning. The VTR_{HRAS1} oligonucleotide represented an entire 28 bp repeat unit, as well as flanking sequences of the neighboring 5′ and 3′ repeat units.

Plasmids

A plasmid containing the adenovirus major late promoter and USF binding site (pU+MLPCAT) was constructed by inserting the double-stranded oligonucleotide corresponding to positions +1 to −60 of the MLP (MLP3+MLP4) into the pCAT Basic plasmid (Promega) digested with XbaI. A construct lacking the USF binding site (pMLPCAT, pU−MLPCAT) was constructed by ligating MLP5+MLP6 into XbaI/SphI-digested pCAT Basic. The *IGH* minisatellite was inserted into both of these constructs upstream of the MLP (Figure 5) by ligating a 1.5 kb BamHI/BglII fragment containing 400 bp of VTR (8 repeats) and 1100 bp of 5′ flanking sequence in a HindIII site with the appropriate adaptors. These two plasmids were designated pV+U−MLPCAT and pV+U+MLPCAT. A 1.5 kb VTR-containing fragment, cloned from human peripheral blood DNA (T. G. K., unpublished observations), was also ligated into the BamHI site of pBS+ (Stratagene), creating $pVTR_{IGH}$. The immunoglobulin heavy chain enhancer (Eμ) was inserted into pU−MLPCAT and pV+U−MLPCAT to create the plasmids, pEμ+MLPCAT and pV+Eμ+MLPCAT, respectively (Figure 5). The murine IgH enhancer was amplified from the clone pKB6 (22) which contained a rearranged murine heavy chain gene cloned into the plasmid pBR328. Oligonucleotide amplimers (containing SphI sites) corresponded to the 5′ and 3′ ends of the 700 bp XbaI/PstI Eμ fragment. Following PCR amplification, the 700 bp enhancer fragment was digested with SphI and ligated into the SphI site of plasmids indicated above. pRSVCAT (23) and pSV2CAT (Promega) each contain the CAT gene downstream of the RSV 3′ LTR and SV40 virus early promoter and 72 bp enhancer, respectively. In pCMVβgal the β-galactosidase gene is inserted downstream of the cytomegalovirus (CMV) promoter (24).

Gel retardation assays

Crude nuclear extracts were prepared in the manner of Dignam et al. (25). Mobility shift assays were performed as previously described (18). In competition experiments, non-radioactive competitor DNA was added to the reaction mixture prior to the nuclear extracts.

DNA methylation interference

Double-stranded oligonucleotides labelled at either end were methylated according to the procedure of Maxam and Gilbert (26). Mobility shift assays were performed as described above, except that the components and reaction volumes were increased ten-fold. Free and shifted complexes were excised from 6% polyacrylamide gels, bound to DEAE paper (Schleicher and Schuell), and subsequently eluted into high salt buffer (1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris pH 8). Following phenol/chloroform extraction and ethanol precipitation, treatment with 1.0 M piperidine and lyophilization, samples were resuspended in running buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue) and loaded onto 20% polyacrylamide, 7 M urea, sequencing gels.

UV crosslinking assays

Double-stranded oligonucleotides were internally labelled with [α-^{32}P] deoxynucleotides and 5-bromo-deoxyuridine triphosphate by the random priming method (27) and subjected to a large-scale, mobility shift assay as described above. Crosslinking (28) was performed by UV-irradiation of complexes

Table 1. Oligonucleotides

Name	Size	Sequence
VTR_{HRAS1}	46 bp	5′ CACTCGCCCTTCTCTCCAGGGGACGCCACACTCGCCCTTCTCTCCA 3′
VTR_{IGH}	60 bp	5′ TGAGCGGAGCAGGCTCTAAAGGCCGCGTCCTAAACAGTGCGTGGGCCACGTGAGCGGAGC 3′
VTRApoB	45 bp	5′ AAAATATTTAATTATAAATATTTTAATTATAAAATATTTAATTAT 3′
MLP1	25 bp	5′ AGGTGTAGGCCACGTGACCGGGTGT 3′
MLP3	78 bp	5′ ATTATAGGTGTAGGCCACGTGACCGGGTGTTCCTGAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTC-ACT 3′
MLP5	47 bp	5′ ATTGAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTCACT 3′
Eμ1	29 bp	5′ AAAGCATGCTCTAGAGAGGCTTGGTGGAG 3′
Eμ2	34 bp	5′ AAAGCATGCTTCTAAATACATTTTAGAAGTCGAT 3′
5′ VTR_{IGH}	36 bp	5′ AAAAAGCTTAGATCTCCTGCTCAAGGACTCTCTTAC 3′
3′ VTR_{IGH}	30 bp	5′ AAAAAGCTTGCCTGGCTGCCCTGAGCAGGG 3′

in solution at 310 nm for 30 minutes at 4°C. DNA-protein complexes were then digested with 5 units of DNase I (Promega) and 1 unit of micrococcal nuclease (Sigma) for 30 minutes at 37°C prior to electrophoresis in an 8% SDS-polyacrylamide gel. Competition assays were performed by adding non-radioactive DNA from homologous and heterologous sources into the reaction prior to the addition of extract.

DNA transfection

Adherent cells were transfected with plasmid DNA by the calcium phosphate method using a kit obtained from BRL. Cells were plated at 2.25×10^6/100 mm dish 18 hours prior to transfection. A total of 20 μg of DNA, including 2 μg of CMVβgal DNA, was added per dish. Transfected cells were then incubated with 1 ml of 15% glycerol in HEPES-buffered saline for four minutes, followed by a wash with 5 mls of medium and incubation for 48 hours with 10 ml of fresh medium. For competition assays in HeLa cells, 10 μg of pU$^+$MLPCAT and 2 μg of CMVβgal were cotransfected with 0 to 10 μg of pVTR$_{IGH}$. pBS+ DNA was added to keep the total amount of DNA at 20 μg. For competitions with pSV2CAT and pRSVCAT, 2 μg of the test plasmid was cotransfected with 0−16 μg of pVTR$_{IGH}$. P3X63-Ag8 cells were transfected by the electroporation method (29). Exponentially growing cells were counted, washed in RPMI + 20% FCS, and resuspended at 2×10^7 cells/ml. 400 μl of cells were added to a cuvette (BioRad) with 25−50 μg of test plasmid and 10 μg of CMVβgal. The cells and DNA were incubated on ice for 10 minutes, followed by electroporation with a BioRad Genepulser set at 270 volts and 960 μFarads. Pulse lengths varied between 0.4 and 0.5 seconds. The cells were incubated on ice for an additional 10 minutes before being placed in fresh medium at 37°C for 24 hours. Competitions in P3X63-Ag8 cells were performed with 10 μg of either pSV2CAT, pRSVCAT, or pEμ^+MLPCAT and varying amounts (0, 10, 20, 40 μg) of pVTR$_{IGH}$. Ten μg of CMVβgal was included in each reaction, and the total amount of DNA was adjusted to 60 μg with pBS+.

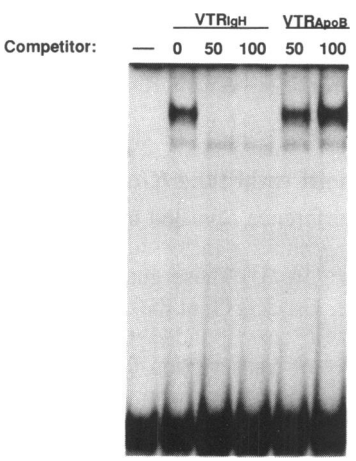

Figure 1. Nuclear factor binding to the *IGH* variable tandem repeat. Two complexes were detected in binding assays employing the 60 bp VTR$_{IGH}$ oligonucleotide target with EJ nuclear extracts (lane 2). Non-radioactive competitors were the VTR$_{IGH}$ oligomer and the 45 bp VTR$_{ApoB}$ oligomer; molar excess is indicated above each lane. The faster migrating complex was not competed by either fragment. The reaction represented in the first lane did not contain extract.

Transient expression assays

CAT assays were performed with 100 μg of heat-inactivated protein as described (23). For time courses, reactions were scaled up four-fold, and aliquots were removed at 0, 3, 6, and 18 hours. Relative CAT activity was obtained by dividing the per cent conversion per mg. protein by the β-galactosidase activity (30).

RESULTS

Ubiquitously-expressed, sequence-specific binding proteins of the *IGH* minisatellite

A 60 bp oligonucleotide corresponding to the consensus of the VTR$_{IGH}$ repeat unit was end-labelled and used as the target in mobility shift assays to determine if any sequence-specific binding of nuclear proteins to VTR$_{IGH}$ occurred. Two shifted complexes were observed with crude nuclear extracts from the EJ cell line (Figure 1). The larger complex was sequence-specific, since it was efficiently competed by 50- to 100-fold molar excess of unlabeled VTR$_{IGH}$ (Figure 1, lanes 3−4), but not with

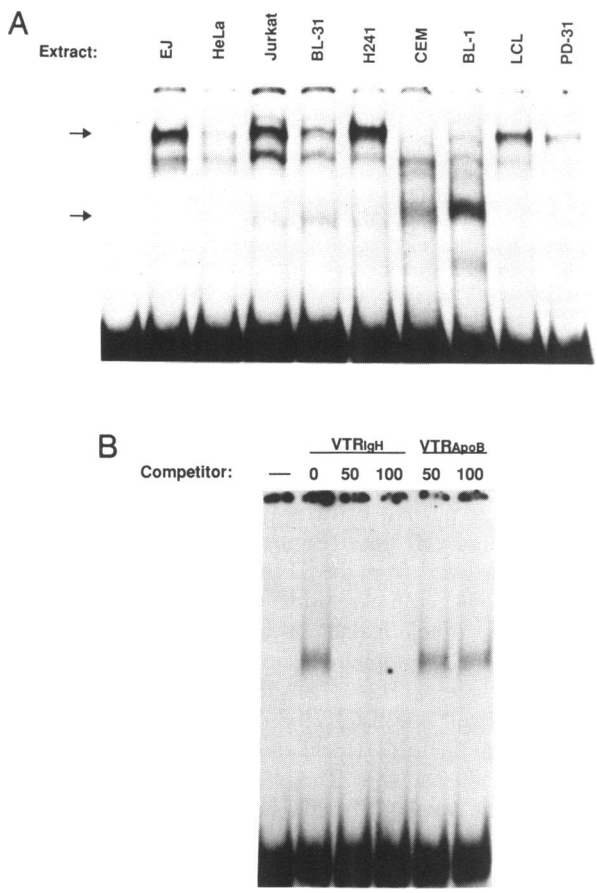

Figure 2. Tissue distribution of the VTR$_{IGH}$-specific complex. (A) Binding of VTR$_{IGH}$ to nuclear extracts from nine different cell lines (EJ, HeLa, Jurkat, BL-31, H241, CEM, BL-1, LCL, and PD31). In addition to the two complexes previously identified with the EJ extract, a complex demonstrating faster migration (lower arrow) was observed with the BL-1 and CEM extracts (lanes 7,8). Small amounts of this complex were observed with Jurkat, BL-31, H241, LCL, and PD31 extracts. (B) The faster complex forming with BL-1 nuclear extracts was examined for sequence specificity. Non-radioactive competitors were VTR$_{IGH}$ and VTR$_{ApoB}$. Molar excess of non-radioactive competitors is indicated above each lane.

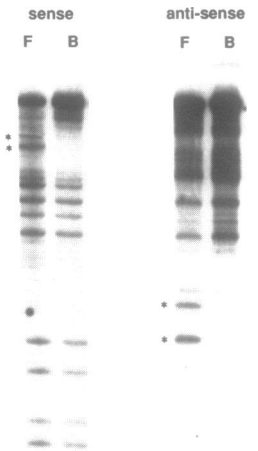

Figure 3. Characterization of the VTR_{IGH} protein binding site by methylation interference. The 60 bp VTR_{IGH} oligomer was methylated on guanines, and shift assays were performed with PD31 nuclear extracts. The free and shifted complexes were excised, cleaved with piperidine, and analyzed on denaturing polyacrylamide gels. Guanines in contact with the binding factor are denoted by asterisks (*) in the G ladder and in the accompanying DNA sequence. The bracket denotes a 17 bp region of VTR_{IGH} which demonstrates a 15-for-17 bp match to the USF/MLTF site in the adenovirus major late promoter.

unlabeled VTR_{ApoB} oligonucleotide (Figure 1, lanes 5–6). The smaller complex, which was not reproducibly observed, was not competed by either DNA.

We then examined the distribution of the VTR_{IGH} binding proteins in crude nuclear extracts from a variety of lymphoid and non-lymphoid cell lines (Figure 2A). The two complexes observed with extracts from the EJ cell line formed with extracts from the human cervical carcinoma cell line, HeLa; the human T-cell leukemia line, Jurkat; a human Epstein-Barr-virus-negative, Burkitt's lymphoma cell line, BL-31; a mouse hybridoma cell line, H241; an Epstein-Barr-virus-infected, lymphoblastoid cell line, LCL; the Abelson-virus-transformed, mouse pre-B cell line, PD31; and another Burkitt lymphoma cell line, BL-41 (not shown). The same two complexes were observed, albeit weakly, in the human, Epstein-Barr-virus-negative, Burkitt's lymphoma cell line, BL-1 and the human T-cell leukemia cell line, CEM. However, both CEM and BL-1 extracts also demonstrated a very prominent complex which migrated more rapidly than those from other cell lines. Faint bands corresponding to this complex were also present in BL-31 and Jurkat. Competition assays with unlabeled VTR_{IGH} and VTR_{ApoB} oligonucleotides revealed that complex formation in BL-1 was sequence-specific (Figure 2B). Proteolytic digestion introduced during the preparation of BL-1 nuclear extracts seemed an unlikely source of the smaller complex, since the result was obtained with multiple extract preparations in which other nuclear factors (e.g. to VTR_{HRAS1}) were intact (data not shown).

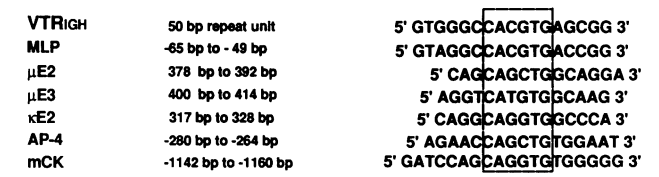

Figure 4. myc/HLH transcription factor binding motifs. myc/HLH binding motifs from the adenovirus major late promoter, the SV40 enhancer, the muscle-specific creatine kinase promoter, and the μE3 and κE2 E box motifs of the immunoglobulin heavy- and light-chain enhancers are compared to VTR_{IGH}. The 6 bp myc/HLH consensus motif is enclosed the box.

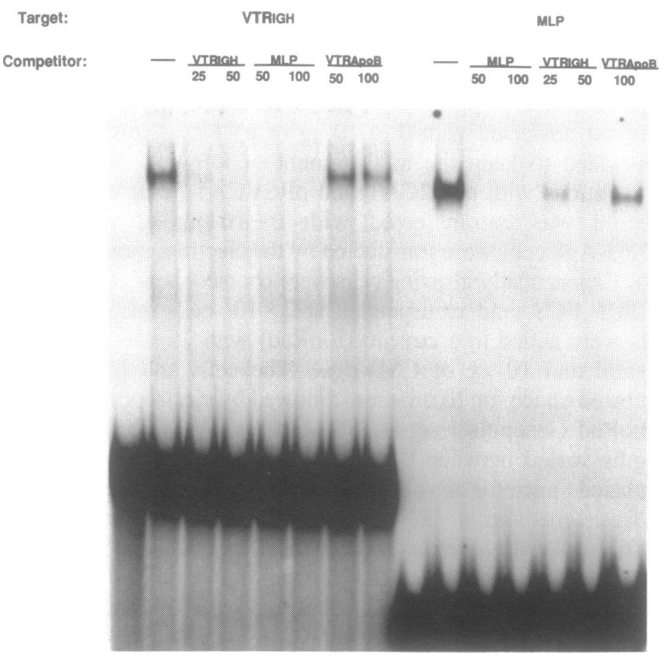

Figure 5. Comparative binding and cross-competition of VTR_{IGH} and adenovirus major late promoter myc/HLH motifs. Crude nuclear extract from the PD31 cell line was incubated with VTR_{IGH} target (lanes 2–8), or adenovirus MLP oligonucleotide (lanes 10–15). Competitors are the non-radioactive VTR_{IGH}, MLP, or VTR_{ApoB} oligonucleotides. Molar excess of competitors is indicated above each lane.

A myc/HLH motif within the *IGH* minisatellite binding site

Methylation interference revealed that two guanines interacted with the VTR_{IGH} binding factors on both the sense and anti-sense strands (Figure 3). These guanines formed the core of a 6 bp palindrome, CACGTG, at the 3′ end of the 60 bp VTR_{IGH} target. This motif corresponded to the binding site of the myc/HLH family of transcription factors (Figure 4) (31–36). Indeed, sequences surrounding the VTR_{IGH} palindrome contained a 15 of 17 bp match for a binding site in the adenovirus major late promoter (MLP; Figure 4) which bound a protein variously designated upstream factor (USF; ref. 35) or major late transcription factor (MLTF; 32). The USF/MLTF binding site was essential for efficient transcription from the MLP (32, 35, 37). To determine if the VTR_{IGH} complex contained proteins related to USF/MLTF, a 25 bp oligonucleotide corresponding to positions −49 to −72 of the MLP (the USF/MLTF binding

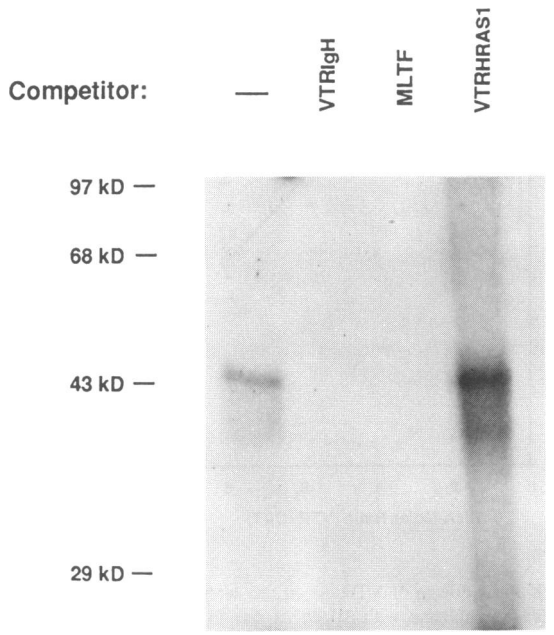

Figure 6. UV crosslinking analysis of the VTR_{IGH}-specific complex. The internally-labelled 60 bp VTR_{IGH} oligonucleotide was UV-crosslinked to proteins in the binding complex formed with BL-41 nuclear extracts, digested with DNase, and analyzed on an SDS-polyacrylamide gel. Competitions were performed with 100-fold molar excess of either VTR_{IGH}, adenovirus MLP (USF/MLTF), or VTR_{HRAS1} non-radioactive oligonucleotides. Relative migrations of molecular weight standards are depicted on the left.

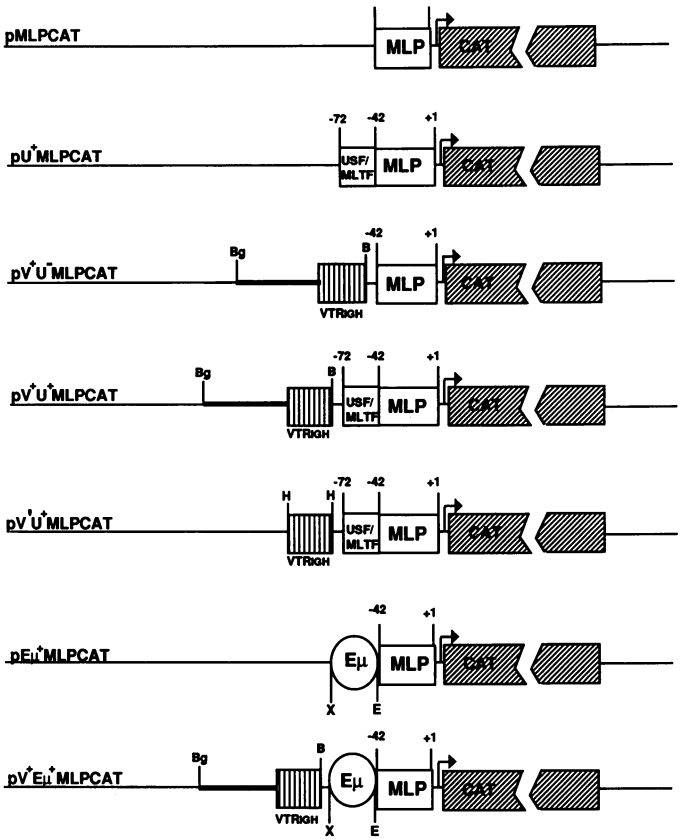

Figure 7. CAT reporter gene constructs. The reporter plasmids used for calcium phosphate transfection and electoporation are depicted. Numbers correspond to positions of the sequence within the adenovirus genome. Arrow indicates the transcriptional start site. Plasmid designations are listed at the left of each construct. Eμ is the murine immunoglobulin heavy chain enhancer. Restriction endonuclease sites: B, BamHI; Bg, BglII ; X, XbaI; E, EcoRI.

site) was constructed and used as the target in a mobility shift assay. Similar complexes were observed to form with the MLP and VTR_{IGH} targets (Figure 5). Slight mobility differences did exist, however, perhaps attributable to the size difference between the two targets (60 bp versus 22 bp; see also Discussion). The MLP complexes were competed by unlabeled MLP and VTR_{IGH} oligomers (Figure 5, lanes 11–14). Conversely, the complexes which formed with VTR_{IGH} could be competed by the MLP oligomer (Figure 5, lanes 5 and 6). Taken together with the methylation interference data, these results suggested that the proteins which bound VTR_{IGH} were highly related to the USF/MLTF transcription factor. The protein binding site for the small complex which was observed with extracts from CEM and BL-1 was identical to that described above. This complex was competed by USF/MLTF oligomer employed as cold competitor and formed with the USF/MLTF target in the presence of BL-1 extracts, as well (data not shown).

Proteins comprising the VTR_{IGH}-specific complex

VTR_{IGH} complexes formed in a DNA-binding reaction with extracts from BL-41 cells were subjected to UV-crosslinking in solution. Analysis of the product on denaturing acrylamide gels indicated two bands: an intense one corresponding to a protein of approximately 43 kD and a weaker one of 40 kD (Figure 6). These proteins were displaced by VTR_{IGH} and MLP cold competitor oligonucleotides, but not by VTR_{HRAS1} oligonucleotide (Figure 6, lanes 2–4). The sizes of these two proteins were consistent with that reported for USF/MLTF (40–45 kD; 33), as well as several μE3 box binding proteins which were also members of the myc/HLH family (34, 38). Since these factors contained a helix-loop-helix region which mediated formation of homo- and heterodimers, the 40 kD and 43 kD proteins may have represented the subunits of a heterodimeric factor binding VTR_{IGH}.

VTR_{IGH} inhibition of transcription by the adenovirus major late promoter

Since VTR_{IGH} bound a factor which closely resembled members of the myc/HLH family, such as USF/MLTF, we investigated potential interactions of the minisatellite with a promoter known to be responsive to these factors, the adenovirus MLP. CAT reporter constructs were prepared (Figure 7) containing the minimal promoter with (pU⁺MLPCAT; bases +1 to −72) or without (pMLPCAT; bases +1 to −42) the intrinsic USF binding site (19, 20). To each of these constructs we appended the 1500 bp BamHI/BglII fragment containing a 400 bp *IGH* minisatellite with 8 repeat units (pV⁺U⁺MLPCAT; pV⁺U⁻MLPCAT). These four plasmids were transfected into HeLa cells; relative CAT activity was determined 48 hours post transfection. The time course of these CAT assays (0, 3, 6, 18 hours) is depicted in Figure 8. For these and all subsequent transient expression assays, the amounts of reporter DNA transfected fell within the linear range of previously determined dose-response curves (DNA transfected vs. relative CAT activity; data not shown).

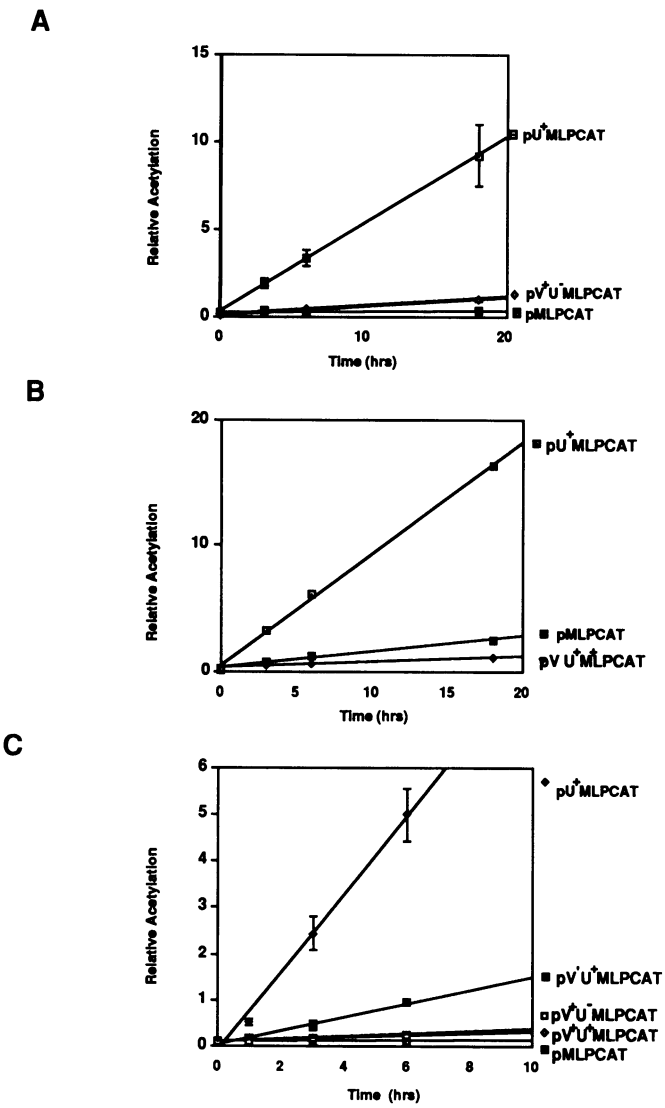

Figure 8. *cis* effects of VTR_{IGH} on the adenovirus major late promoter. (A) HeLa cells were transfected in duplicate with CAT constructs containing the adenovirus major late promoter without the USF/MLTF binding site, to which VTR_{IGH} had (pV^+U^-MLPCAT) or had not (pMLPCAT) been appended. The MLP construct which contained the USF/MLTF binding site (pU^+MLPCAT) was used as a positive control. 18 μg of test DNA and 2 μg of CMVβgal DNA were transfected per reaction. CAT assays were performed for the indicated time periods; additional acetyl CoA was added at eight hours. Data were plotted by Cricket Graph after normalizing per cent acetylation for protein content and β-galactosidase activity (relative acetylation). In this and all subsequent Figures, two or three experiments were performed with comparable results. Data from a representative experiment are presented. (B) Same as above, except that CAT constructs consisted of the adenovirus MLP with the USF/MLTF binding site, to which VTR_{IGH} had (pV^+U^+MLPCAT) or had not (pU^+MLPCAT) been appended. (C) Same as (B), except that the clone bearing flanking deletions of VTR_{IGH} ($pV'U^+$MLPCAT) was employed in place of pV^+U^+MLPCAT.

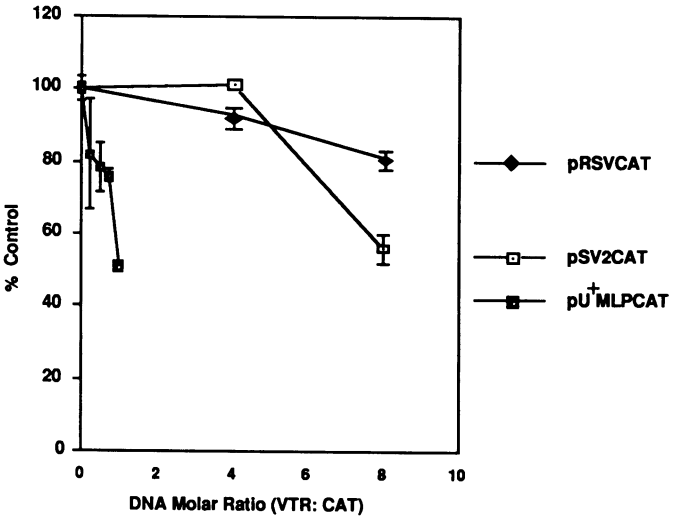

Figure 9. *trans* effects of VTR_{IGH} on the adenovirus MLP. HeLa cells were transfected in duplicate with 10 μg of the adenovirus MLP CAT construct containing the USF/MLTF binding site (pU^+MLPCAT) and increasing amounts (0, 2.5, 5.0, 10.0 μg) of $pVTR_{IGH}$. 2 μg of pRSVCAT or pSV2CAT were transfected with increasing amounts (0, 8.0, 16.0 μg) of $pVTR_{IGH}$. 2 μg of CMVβgal DNA was included in each reaction. The total DNA transfected per reaction was adjusted to 20 μg with pBS+. The relative acetylation when no $pVTR_{IGH}$ was co-transfected equaled 100%. The molar ratios are computed directly from the amount of DNA transfected because of the approximate equivalence of plasmid sizes.

The presence of the USF/MLTF site (pU^+MLPCAT) resulted in a ten-fold enhancement of CAT gene transcription (Figure 8A). This activity level in vivo correlated well with the enhancement obtained from in vitro transcription of an MLP template linked to a USF site (32, 35, 37). The 1.5 kbfragment containing the minisatellite could not substitute for an intact USF/MLTF binding site, since the pV^+U^-MLPCAT plasmid did not demonstrate any significant increase of CAT activity over background (Figure 8A). The addition of VTR_{IGH} to the MLP construct containing the USF/MLTF site (pV^+U^+MLPCAT) produced quite a different result: USF-site-mediated enhancement was inhibited by 90% (Figure 8B). We confirmed that this inhibition resided within the minisatellite by replacing the 1.5 kb fragment with a deleted segment containing only VTR_{IGH} and the 20 bp on each flank required for subcloning by PCR amplification. Once again, strong inhibition could be demonstrated; pV^+U^+MLPCAT possessed only 20% of the activity of pU^+MLPCAT (Figure 8C).

Given the number of potential myc/HLH binding sites within VTR_{IGH}, the ability of the 1.5 kb fragment to inhibit USF-mediated MLP transcription may have represented the sequestration of myc/HLH binding factor by the minisatellite. Since such inhibition should also be observed in *trans*, this hypothesis was tested by cotransfection of pU^+MLPCAT and a plasmid subclone of the 1.5 kb BamHI/BglII fragment bearing VTR_{IGH}. We employed a constant amount of pU^+MLPCAT and the requisite amount of VTR_{IGH} to achieve 0.25:1, 0.5:1, 0.75:1, and 1:1 molar ratios (VTR:U^+MLP; a 1:1 molar ratio represented the maximum which could technically be achieved by $CaPO_4$ transfection of HeLa cells.) A 1:1 molar ratio resulted in 50% inhibition of CAT gene activity. The same molar ratio did not significantly inhibit CAT gene activity expressed from either the SV40 or RSV viral promoter/enhancer (Figure 9). Larger molar ratios of VTR_{IGH} (8:1 VTR_{IGH}:pSV2CAT or RSVCAT; higher molar ratios could be obtained by using less CAT DNA for constructs with viral enhancers) resulted in 40% and 20% inhibition of transcription, respectively. The SV40 72 bp enhancer contained a 6 bp consensus sequence for a USF/MLTF binding site (39, 40) which may have contributed to such inhibition at high molar ratios.

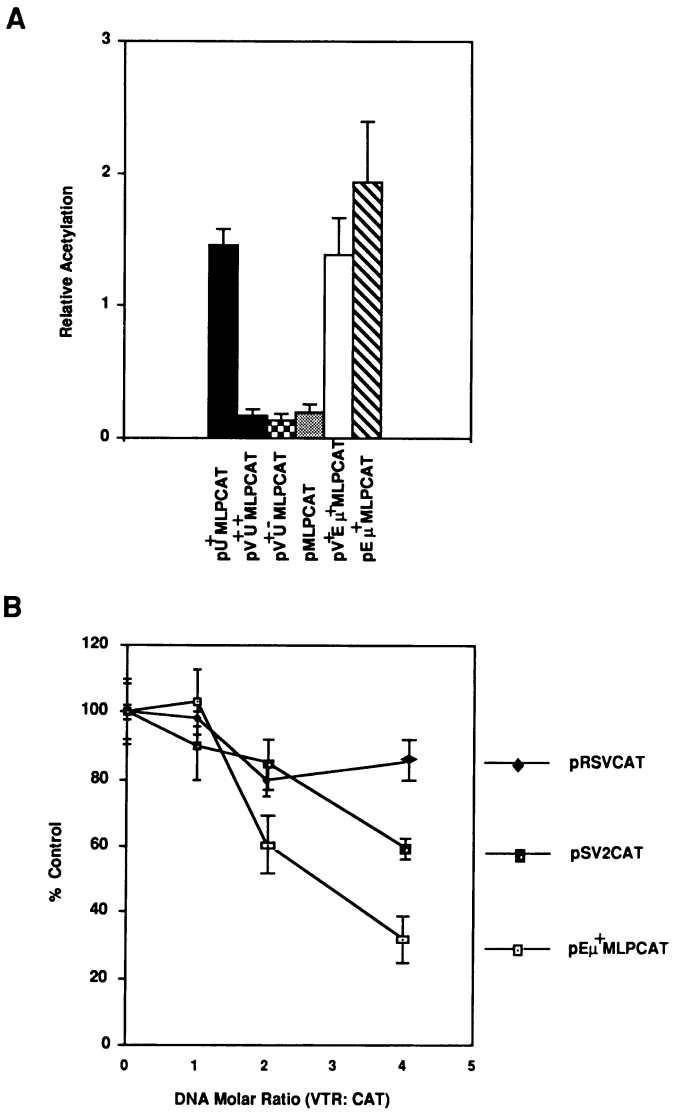

Figure 10. *cis* and *trans* effects of VTR_{IGH} on the *IgH* enhancer. (A) Murine B cells (P3X63-Ag8) were electroporated in duplicate with 25 μg of the indicated constructs and maintained for 24 hr. Results from CAT assays employing a single 18 hour time point are presented. (B) 10 μg of pRSVCAT, pSV2CAT, and pEμMLPCAT were electroporated into p3X63-Ag8 cells with increasing amounts (0, 10.0, 20.0, 40.0 μg) of $pVTR_{IGH}$. 10 μg of CMVβgal DNA were also added to each reaction, and the total amount of DNA was adjusted to 60 μg with pBS+. CAT assays were performed as in (A).

Inhibition of Eμ-mediated MLP transcription by a molar excess of VTR_{IGH}

VTR_{IGH} is located approximately 5 kb upstream of the immunoglobulin heavy chain enhancer, Eμ. Several motifs within Eμ, designated E boxes (31, 34, 38, 41) contain a 6 bp consensus sequence (CANNTG) which is related to the USF/MLTF binding site. In fact, USF/MLTF binds the μE3 box; and a μE3 binding protein, TFE3, binds the USF/MLTF site (31). Given these correspondences, VTR_{IGH} inhibition of USF-mediated activation of MLPCAT raised the possibility that interaction of the minisatellite with Eμ might also occur. The 700 bp PstI/XbaI fragment containing the murine IgH enhancer was PCR-amplified and cloned into the SphI site of pMLPCAT ($pE\mu^+MLPCAT$; Figure 7). An additional plasmid bearing VTR_{IGH} immediately upstream of Eμ ($pV^+E\mu^+MLPCAT$; Figure 7) was also prepared. These plasmids, together with pMLPCAT, $pU^+MLPCAT$, $pV^+U^-MLPCAT$ and $pV^+U^+MLPCAT$, were electroporated into the murine myeloma cell line, P3X63-Ag8. A representative assay is shown in Figure 10A.

Plasmids previously tested in HeLa behaved similarly in B cells. The USF site conferred the same degree of transcriptional activation on the adenovirus MLP. VTR_{IGH} was incapable of supplanting the USF site; it was no more successful as an enhancer in B cells than in HeLa cells. However, the minisatellite again inhibited USF-mediated transcriptional activation nearly completely. The presence of the IgH enhancer in $pE\mu^+MLPCAT$ resulted in 10- to 15-fold enhancement of transcription from the adenovirus MLP in several experiments, a result comparable to that previously observed by others in these cells (42). The addition of VTR_{IGH} to the Eμ reporter ($pV^+E\mu^+MLPCAT$) showed only a small, statistically insignificant, degree of inhibition.

The IgH enhancer, possessing multiple enhancer motifs, obviously represented a more complex activation element than the single USF site present in $pV^+U^+MLPCAT$. Such complexity may have masked any inhibitory effect of the minisatellite, since other contributing activation sites would presumably have remained unaffected. Alternatively, theminisatellite binding site may have discriminated between USF and TFE3, effectively sequestering only the former. To pursue potential VTR_{IGH}/Eμ interactions further, we cotransfected a constant amount of $pE\mu^+MLPCAT$ with increasing amounts of plasmid ($pVTR_{IGH}$) containing only the 1.5 kb fragment bearing VTR_{IGH}; total DNA transfected was held constant by the addition of appropriate amounts of pBS+. In *trans*, progressive inhibition of Eμ-mediated enhancement of the MLP by VTR_{IGH} was observed until, at a 4:1 molar ratio of VTR to Eμ, a 68% reduction in transcriptional activation was achieved (Figure 10B). Under the same conditions, VTR_{IGH} inhibited transcription of the RSV promoter/enhancer by only 18%. Once again, however, the SV40 promoter/enhancer proved susceptible to inhibition by the minisatellite. In these experiments, pSV2CAT activity was reduced by 40% (Figure 10B).

DISCUSSION

Analysis of repeat units from a minisatellite within the D_H-J_H interval of the human immunoglobulin heavy chain gene, designated VTR_{IGH}, demonstrated the binding of two members (43 and 40 kD) of the myc/helix-loop-helix (HLH) family of transcription factors. This identification was based on a similar recognition sequence (CACGTG), tissue distribution, and molecular weight for the minisatellite binding factor as for previously identified members of this family (USF/MLTF; 31, 34, 35, 38). In addition, a recognition site from the adenovirus major late promoter for the myc/HLH factor, USF/MLTF, competed protein binding by VTR_{IGH}, and vice versa. Finally, DNA sequence surrrounding the VTR_{IGH} binding site was a 15/17 match for the MLP binding site of USF/MLTF. These results, in conjunction with the functional correlations discussed below and our previous finding that the *HRAS1* minisatellite bound members of the *rel*/NF-κB family of transcription factors (18), suggest that a subset of minisatellites may interact with important components of the transcriptional regulatory apparatus.

Despite its similarity to the USF site, VTR_{IGH} did not enhance transcription from the basal MLP in either HeLa or

P3X63-Ag8 murine myeloma cells. It strongly suppressed activation of the MLP by a native USF binding site both in *cis* and in trans; *trans* suppression of Eμ was also observed in B cells. While our studies did not address the mechanism for this paradoxical behavior, three possibilities are consistent with our findings. The first USF binding site in the *IGH* minisatellite in V'U$^-$MLPCAT is 97 bp further upstream than the USF site in U$^+$MLPCAT; conceivably, the absence of transactivation reflects the increased distance (or altered phasing) of this and more distal minisatellite binding sites relative to the MLP. If VTR_{IGH} binds *exactly* the same factor as the USF site, it is difficult to envision its sequestration in an inactive form on the VTR without the participation of another, inhibitory protein which binds to a distinct site within the VTR. In the most general formulation of this mechanism, the inhibition could arise by interaction of the factor with itself on the tandem array of VTR_{IGH} binding sites. Alternatively, VTR_{IGH} may bind a heterodimer composed of only one of the subunits of USF/MLTF; the other, VTR_{IGH}-specific subunit, by analogy to the combinatorial variation of activity in both the myc/HLH (33, 34, 38, 39, 43–45) and NF-κB (46–48) families of transcription factors, would confer inhibitory activity. This latter possibility, however, is inconsistent with recent observations in vitro demonstrating the propensity of both TFE3 and USF for homodimerization (49) and also requires that the common subunit be in dynamic equilibrium with the USF-site-specific and VTR-specific factors. The presence of the VTR would, therefore, alter the equilibrium state in favor of VTR binding and inhibition. Both of these possibilities for VTR_{IGH} interaction with the USF site assume limiting quantities of USF dimer (first mechanism) or common subunit (second mechanism), a qualifying circumstance which must be invoked for VTR_{IGH} interactions with Eμ binding factors, as well (see below). Regardless of the mode of inhibition, it should also be noted that all of the mechanisms represent novel, DNA-based forms of 'squelching' (50). The binding site of the minisatellite, rather than an overexpressed protein ligand (50), competitively sequesters factor from cognate promoters.

Our *trans* experiments suggested that the IgH minisatellite was capable of binding factors which governed the activity of the immunoglobulin heavy chain enhancer, while parallel co-transfections with the SV40 promoter/enhancer and the RSV 3' LTR ruled out the interpretation that VTR_{IGH} was merely competing for non-specific elements of the transcription apparatus. The requirement for a 4-fold molar excess of VTR binding sites to produce a 70% inhibition of Eμ could not be taken as evidence that the affinity of VTR_{IGH} for immunoglobulin enhancer binding proteins, such as TFE3 (31), was necessarily lower than that of native sites within the enhancer. Mercola et al. (51) demonstrated that an 8:1 molar excess of Eμ was required in *trans* for a comparable degree of inhibition of Eμ-mediated activation. Thus, the minisatellite was a slightly better inhibitor-in-*trans* of the immunoglobulin enhancer than the precisely homologous structure; our result probably reflected a relatively large excess of binding factors over targets in murine myeloma cells. We concluded that VTR_{IGH} could influence the function of Eμ, but that such interactions, like those with the MLP USF site, would likely demonstrate a critical dependence upon ambient factor-to-target ratios. Future studies must establish whether the IgH minisatellite contributes to the coordinate regulation of IgH gene transcription and/or rearrangement by titrating myc/HLH proteins away from the IgH enhancer.

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