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Members of the *rel*/NF-κB family of transcriptional regulatory proteins bind the *HRAS1* minisatellite DNA sequence

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ABSTRACT

The 28 base pair repeat unit of a minisatellite 1000 bp downstream from the human *HRAS1* gene (VTR_{HRAS1}) bound four proteins (p45, p50, p72 and p85) in nuclear extracts from a variety of human cell lines which were indistinguishable from several members of the *rel*/NF-κB family of transcriptional regulatory factors. VTR_{HRAS1} bound the constitutively expressed, but not the inducible, forms of these proteins. Analysis of partially purified binding factors from different cell lines demonstrated qualitative differences in the p50 subunit; phosphocellulose fractionation also revealed considerable heterogeneity in the p72 and p85 subunits. These results suggest the possibility that the *HRAS1* minisatellite, in serving as a tandem array of *rel*/NF-κB binding sites, may function in the transcriptional regulation of HRAS1 and nearby genes.

INTRODUCTION

Variable tandem repeats (VTRs, VNTRs, minisatellites) are highly polymorphic structures characterized by the tandem repetition of short (14–100 bp) sequence motifs (1, 2). Dispersed throughout the genomes of higher vertebrates, VTRs have not been assigned a function. Several observations about tandemly-repetitive elements within viral genomes have led us to speculate that some human minisatellites might serve as regulatory regions for cellular transcription or DNA replication. The Epstein–Barr virus origin of replication, *oriP*, contains a viral 'minisatellite' composed of 20 tandem copies of a 30 bp repeat unit. Each repeat unit binds the viral trans-acting factor, EBNA-1, an interaction which contributes to the lymphocyte-specific transcriptional enhancement by the EBV tandem repeat (3–6). Furthermore, the EBV tandem repeat is required for function of *oriP* in the initiation of DNA replication (3–6). The SV40 virus enhancer can be replaced by a tandem array composed of any one of several short nucleotide sequences present in the larger, native enhancer element (7–9). The enhancer activity of these artificial 'VTRs' is directly proportional to the extent of tandem repetition (8). Given the organizational homology of VTRs with some viral origins/enhancers, as well as the frequent association of VTRs with genes and gene clusters, a comprehensive examination of these regions for functional attributes seems warranted.

We have investigated a VTR located 1 kb downstream from the polyadenylation signal of the human c-Ha-*ras1* gene, *HRAS1*. VTR_{HRAS1} consists of 30 to 100 copies of a 28 bp consensus repeat (10, 11). Four common alleles and more than 25 rare alleles have been described at this locus (12); lineage analysis has revealed that each rare allele is derived from the common allele nearest in size (13). Aggregate results from 11 population studies typing nearly 5000 alleles have demonstrated that rare VTR_{HRAS1} alleles appear in the genotypes of cancer patients more than twice as often as in cancer-free controls (reviewed in ref. 14).

Several laboratories have described moderate enhancer activity for VTR_{HRAS1} which is both position and orientation independent (15, 16). Interestingly, this activity is promoter specific; the VTR enhances transcription from the *HRAS1* (15, 16) and ε-globin (16) promoters in some cell lines, but not from the SV40 early or HSV thymidine kinase promoters (15). To investigate further the potential regulatory role of VTR_{HRAS1}, we have characterized its specific interaction with four proteins present in nuclear extracts from a variety of human and murine cell lines.

MATERIALS AND METHODS

Cell Lines and Nuclear Extracts

Crude nuclear extracts were prepared according to the protocol of Dignam et al. (17) from the following cell lines: 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 (Epstein−Barr Virus negative Burkitt lymphoma); RPMI 7951 (human malignant melanoma); PD31 (Abelson-leukemia virus induced murine pre-B cell); NIH3T3 (murine fibroblast) and human peripheral blood mononuclear cells (PBMC). Cells were maintained in either Dulbecco-modified MEM containing 10% calf serum, or RPMI culture medium containing 10% fetal calf serum. Induced extracts were prepared from Jurkat cells treated for 5 hours with 50 ng/ml of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 2 μg/ml of phyto-hemagglutinin (PHA) (Sigma)(18).

Preparation of Target DNAs

Plasmids containing two distinct *HRAS1* allelic fragments (19), a1 (900 bp) and a2.1 (1700 bp), were digested with BstNI to isolate the 28 bp repeat units. The 35 bp and 37 bp repeat units of $VTR_{4.1}$ were isolated by BglII digestion of the MspI/HaeIII fragment of pBBg3 (20). The 70 bp repeat units of $VTR_{1.1}$ were isolated by digestion of a 2.7 kb PstI fragment containing the VTR with Sau3A (21). All fragments were electrophoresed through 6%-8% polyacrylamide gels and eluted 10−12 hours at 37°C in buffer containing 500 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS. Samples were extracted with phenol/chloroform and precipitated in 2.5 volumes of ethanol. End-labeling was performed with [α-^{32}P]deoxyribonucleotides (NEN) and the large fragment of *Escherichia coli* DNA Polymerase I. All enzymes were purchased from New England Biolabs.

Gel Retardation Assays

Mobility shift assays were performed in 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (BME), 4% glycerol, 3 μg poly (dI,dC).poly (dI,dC), 10,000 cpm (0.5−1.0 ng) end-labeled DNA, and 10−15 μg extract as described (18, 22−24). Reactions were incubated for 30 minutes at room temperature and were then subjected to electrophoresis through 6% polyacrylamide gels. Competition experiments were performed as above, except that competitor DNA was added to the reactions prior to the nuclear extracts.

Oligonucleotides

A 46 bp oligonucleotide, AAAAAGCTTCCAGGGGACGCC-ACACTCGCCCTTCTCTGAATTCTTT, and its complement were synthesized which contained the entire VTR_{HRAS1} 28 bp repeat unit, as well as terminal EcoRI and HindIII restriction endonuclease sites. A 22 bp oligonucleotide, GATCTGGGG-ATTCCCCAGGATC (25, 26), corresponding to positions −176 to −160 of the H-2K^b gene promoter, and a 27 bp oligonucleotide, CAACGGCAGGGGAATCTCCCTCTC (27, 28), corresponding to positions −275 to −249 of the IL-2Rα gene promoter, were also synthesized, together with their complements. Both oligomers contained previously characterized NF-κB binding sites (26−27). A 45 bp oligomer, AAATATTTAATTATAAATATTTTAATTATAAAATATT-TAATTAT, representing an entire VTR_{ApoB} repeat unit, was synthesized for use as non-specific competitor. Oligomers were end-labelled using [γ-^{32}P] ATP and polynucleotide kinase.

Methylation Interference

The 46 bp double-stranded oligonucleotide labelled at either end was methylated according to the procedure of Maxam and Gilbert (29). DNA (10^6counts/10 ng) was incubated in 50 mM Na cacodylate (pH 8.0), 10 mM $MgCl_2$, and 1 mM EDTA with 1 μl of dimethyl sulfate for 6 minutes at room temperature. 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, samples were dried and resuspended in 1.0 M piperidine. After heating at 90°C for 30 minutes, DNAs were lyophilized three times from water and resuspended in sample buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). Samples (5000 cpm) were electrophoresed through 20% polyacrylamide, 7 M urea sequencing gels.

UV Crosslinking

The VTR_{HRAS1} 28 bp repeat unit, as well as the H-2K^b and IL-2Rα double-stranded oligonucleotides, were internally labelled for use in UV crosslinking experiments (30) with [α-^{32}P] deoxynucleotides and 5-bromo-deoxyuridine triphosphate by the random priming method (31). Following a large-scale binding reaction as described above, samples in solution were UV-irradiated (310 nm) for 30 minutes at 4°C and then digested prior to electrophoresis with 5 units of DNase I (Promega) and 1 unit of micrococcal nuclease (Sigma) for 30 minutes at 37°C. Samples previously denatured in 1% SDS and 0.15 M BME were then electrophoresed through an 8% SDS-polyacrylamide gel. Competition assays were performed by including non-radioactive DNA in the reaction mixtures.

Fractionation of Minisatellite Binding Proteins

Phosphocellulose (Whatman P11) was washed according to the manufacturer's instructions, and subsequently equilibrated in Buffer A (20 mM Hepes-NaOH pH 7.9, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol) + 0.1 M KCl. Columns were then prepared (32) by washing phosphocellulose (2 ml in a 0.8×4 cm column) with three volumes of Buffer A + 0.1 M KCl + 0.2 mg/ml BSA, followed by three volumes of Buffer A + 0.1 M KCl, and equilibrated with Buffer A + 0.04 M KCl. Crude nuclear extract (3−5 mg) was diluted with Buffer A to a final KCl concentration of 0.04 M and applied to the column bed. The column was washed with three volumes of Buffer A + 0.04

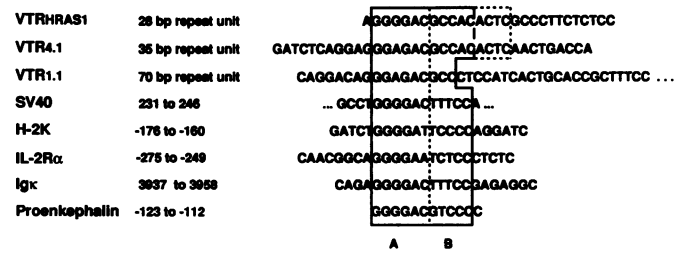

Figure 1: Minisatellite nuclear factor binding sites. Target sequences from the three minisatellites, VTR_{HRAS1}, $VTR_{4.1}$ and $VTR_{1.1}$, the *rel*/NF-κB targets, H-2K^b, and IL-2Rα, and *rel*/NF-κB sites within the SV40 enhancer, kappa light chain enhancer, and proenkephalin promoter are depicted. Homologous regions of the three minisatellites and the *rel*/NF-κB recognition sites in SV40, H-2K^b, IL-2Rα, kappa light chain and proenkephalin are boxed. Half binding sites, as described by Urban and Baeuerle (48), are denoted A and B. Additional homology between VTR_{HRAS1} and $VTR_{4.1}$ is enclosed by the dotted line.

M KCl, and fractions were eluted stepwise with 0.35 M KCl (five column volumes), 0.6 M KCl (three column volumes), and 1.0 M KCl + 0.2 mg/ml BSA (two column volumes) in Buffer A. Protein concentrations were determined by the Bradford method. Protein-containing fractions were pooled and dialyzed against Buffer A + 0.1 M KCl. Samples were concentrated by centrifugation through a Centricon 30 microconcentrator (Amicon) prior to binding assays.

RESULTS

Binding of a Nuclear Factor to the *HRAS1* Minisatellite

We used a mobility shift assay to determine if nuclear factors present in various mouse and human cell types bound VTR_{HRAS1}. Since intact VTRs were too large to serve as targets for such assays, individual repeat units were isolated by digestion of VTR_{HRAS1} with BstNI (Figure 1). An end-labelled target DNA representing the 28 bp subunit of VTR_{HRAS1} produced a single complex with extracts prepared from the human bladder carcinoma cell line, EJ (Figure 2A, lane 2). To demonstrate that this complex was sequence-specific, shift assays were performed in the presence of unlabelled competitor DNA (Figure 2A, lanes 3–8). Competitors were the homologous fragment, VTR_{HRAS1}, as well as the repeat units of two related VTRs, $VTR_{4.1}$ (35/37 bp) and $VTR_{1.1}$ (70 bp). $VTR_{4.1}$ was isolated from a genomic library using VTR_{HRAS1} as probe (20), and contained a 15 bp region of homology with VTR_{HRAS1} (Figure 1). $VTR_{1.1}$ was isolated from a genomic library using $VTR_{4.1}$ as probe (21), and contained a shorter region of homology with VTR_{HRAS1} and $VTR_{4.1}$ (Figure 1). When the 28 bp repeat unit of VTR_{HRAS1} was used as non-radioactive competitor, the complex was diminished by a 50-fold excess and almost completely abolished by a 100-fold excess (lanes 3–4). When the 35/37 bp repeat unit of $VTR_{4.1}$ was used as the competitor, the complex was slightly

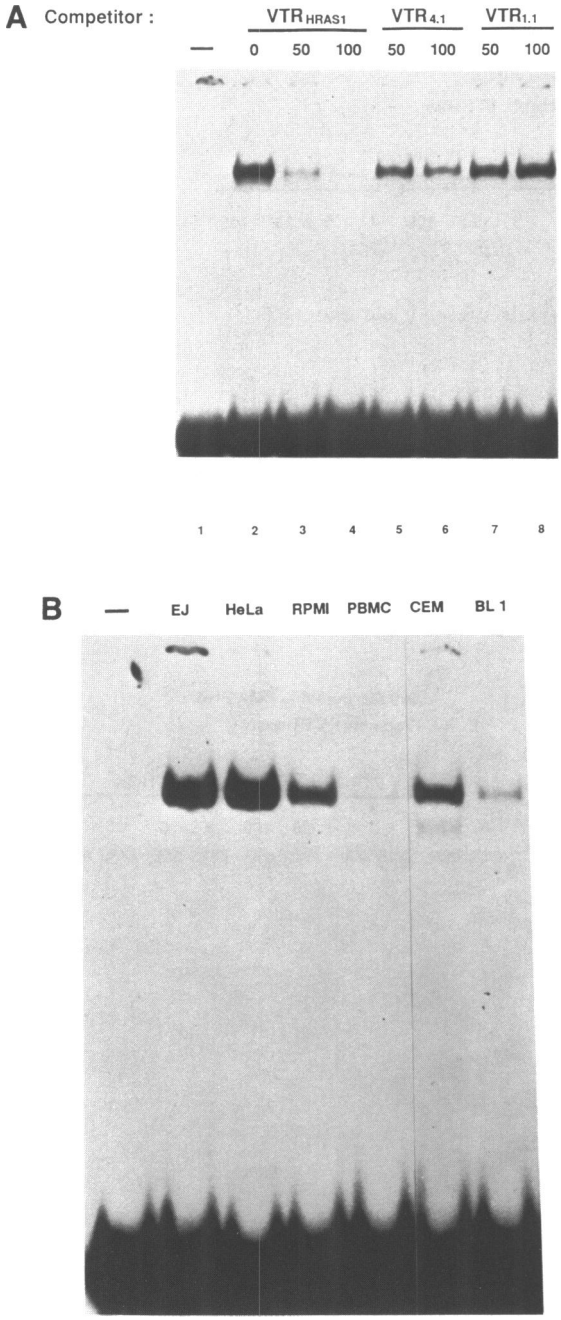

Figure 2: Nuclear factor binding to the *HRAS1* minisatellite. Panel A: One complex is detected in binding assays (see *Methods*) employing the 28 bp VTR_{HRAS1} repeat unit and EJ nuclear extract (lane 2). Non-radioactive competitors are VTR_{HRAS1}, $VTR_{4.1}$ and $VTR_{1.1}$; molar excess is depicted above each lane. Panel B: Binding of VTR_{HRAS1} to nuclear extracts from 6 human cell lines (EJ, HeLa, RPMI 7951, Jurkat, CEM and BL-1) and normal peripheral blood mononuclear cells (PBMC).

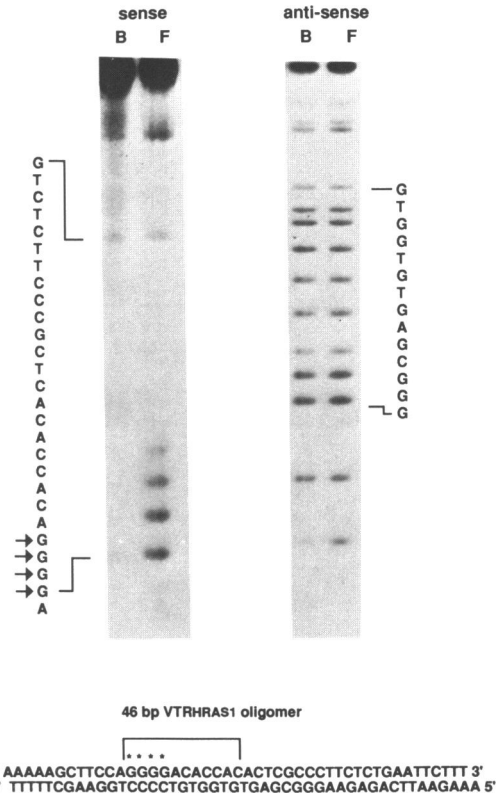

Figure 3: Analysis of the DNA binding site within the *HRAS1* minisatellite repeat unit. Methylation interference was performed with each strand of the 46 bp oligonucleotide described in *Methods*. The products were analyzed on polyacrylamide gels. Guanines involved in contacts with the binding factor are denoted by asterisks (*) in the G ladder and accompanying DNA sequence. Brackets contain the 11 bp region of the *HRAS1* repeat unit homologous to NF-κB binding site.

diminished at 50- and 100-fold excess (lanes 5–6). The 70 bp repeat unit of $VTR_{1.1}$ showed no significant competition (Figure 2A, lanes 7–8). The entire target sample of 28 bp repeat units could be shifted into the complex if additional extract were added (data not shown), demonstrating that all repeat units were capable of binding the factor. Identical results were obtained with subunits of two different alleles of VTR_{HRAS1} (19): a1, the most common allelic form; and a2.1, a unique VTR cloned from lymphocyte DNA of a familial melanoma patient (data not shown).

Cell-Type Distribution of the *HRAS1* Minisatellite Binding Factor

The distribution of VTR binding activity was examined in crude nuclear extracts from various human and murine cell lines (Figure 2B). The EJ complex was observed with extracts from

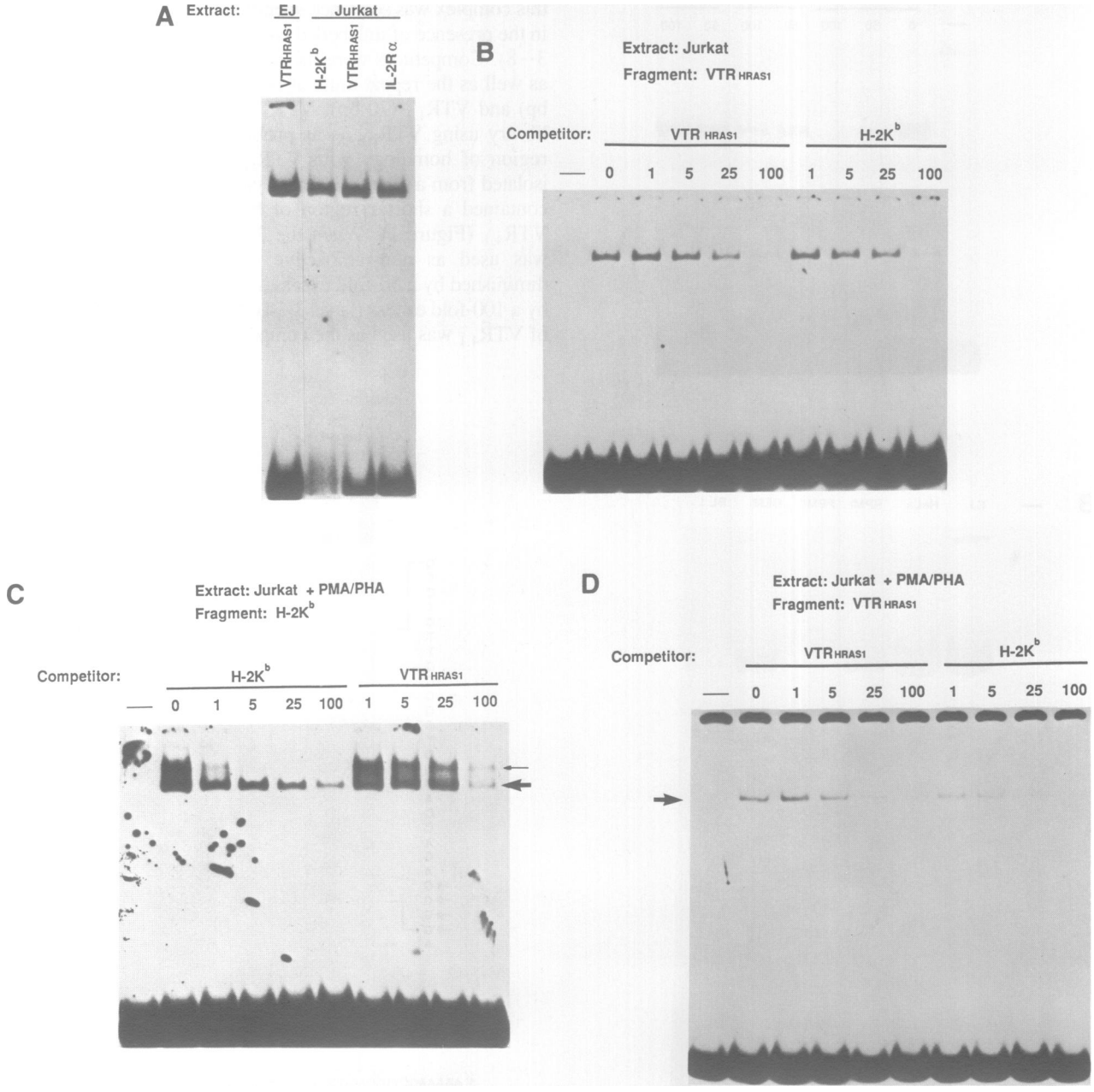

Figure 4: Comparative binding of VTR_{HRAS1}, $H\text{-}2K^b$ and IL-2Rα targets. Panel A: Crude nuclear extracts from the indicated cell lines were incubated with the two *rel*/NF-*x*B targets, $H\text{-}2K^b$ and IL-2Rα (Figure 1), as well as VTR_{HRAS1}. The major complexes in each extract-target combination comigrate. Panel B: Labelled VTR_{HRAS1} was incubated with extracts from uninduced Jurkat cells. Factor binding was also performed in the presence of non-radioactive VTR_{HRAS1} or $H\text{-}2K^b$ competitor. Panel C: Labelled $H\text{-}2K^b$ was incubated with extracts from Jurkat cells treated for 5 hours with TPA (50 ng/ml) and PHA (2 μg/ml) prior to extract preparation. Factor binding was performed in the presence of non-radioactive VTR_{HRAS1} or $H\text{-}2K^b$ competitor. Large arrow: constitutive complex. Small arrow: inducible complex. Panel D: Labelled VTR_{HRAS1} was incubated with the identical extract as in (C) above. Again, factor binding was performed in the presence of non-radioactive VTR_{HRAS1} or $H\text{-}2K^b$ competitor. Large arrow: constitutive complex.

the human cervical carcinoma cell line, HeLa, the human melanoma cell line, RPMI 7951, actively proliferating human peripheral blood mononuclear cells (PBMCs), two human T leukemia cell lines, Jurkat (see Figure 4A) and CEM, and the EBV-negative human Burkitt lymphoma cell line, BL-1. Extracts from a murine Abelson-virus-transformed pre-B cell line, PD 31, and a murine fibroblast cell line, NIH3T3, yielded one complex slightly retarded in migration relative to the human ones (data not shown). A faint complex which migrated faster than the principal species was observed in CEM, RPMI 7951, and BL-1 cell lines, as well as PBMCs. At present, we attribute no significance to differences in the amount of VTR_{HRAS1} binding factor present in each human cell type, since parallel differences were observed for another, unrelated, binding factor (USF/MLTF) in these same extracts (W.L.T. and T.G.K., manuscript in preparation).

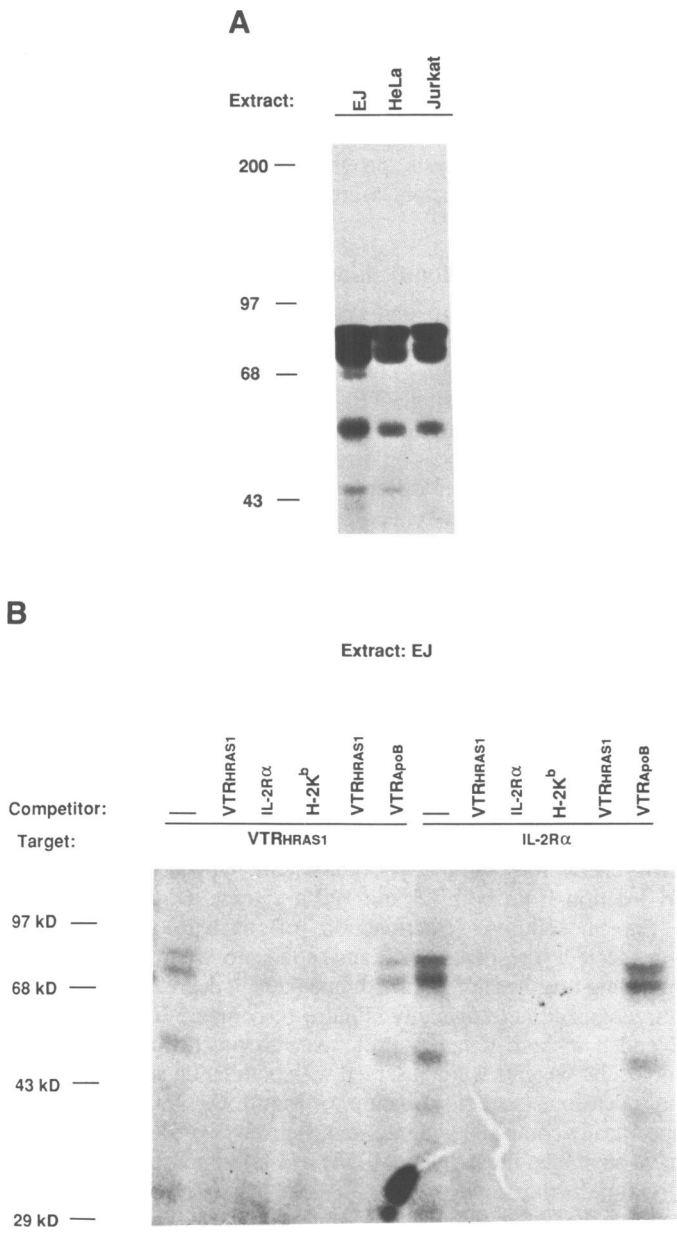

Figure 5: Characterization of the *HRAS1* minisatellite binding factor by UV crosslinking. Panel A: An internally-labelled VTR_{HRAS1} 46 bp oligonucleotide was incubated with the indicated extracts and subjected to UV crosslinking and SDS-polyacrylamide gel electrophoresis as described in Methods. Protein molecular weight markers are depicted to the left. Panel B: An internally-labelled VTR_{HRAS1} 46 bp oligomer or 27 bp IL-2Rα oligomer was incubated with EJ crude nuclear extract, then subjected to UV crosslinking, DNase digestion and polyacrylamide gel electrophoresis. Competitions were performed with 100 fold molar excess of the indicated unlabeled oligonucleotides.

Characterization of the Minisatellite Binding Site

The position of the binding site within the 28 bp repeat unit of VTR_{HRAS1} could be inferred from comparative studies with the related repeat units of $VTR_{4.1}$ and $VTR_{1.1}$. As shown in Figure 2A, $VTR_{4.1}$ competed for the factor which bound VTR_{HRAS1}, but $VTR_{1.1}$ did not. We confirmed by direct binding studies with each of these targets that $VTR_{4.1}$ possessed a lower affinity for the VTR_{HRAS1} binding factor, while no specific binding to $VTR_{1.1}$ was detected in any extract tested (data not shown). Comparison of regions of homology (Figure 1) revealed that VTR_{HRAS1} and $VTR_{4.1}$ shared a 15 bp region with only one mismatch, while the comparable region in $VTR_{1.1}$ bore a 6 bp deletion. Thus, parallel studies with different VTR subunits comprised a form of mutation analysis which strongly suggested the binding site. Methylation interference (Figure 3) confirmed that four adjacent guanines at the 5′ end of the 15 bp region were important for binding of the complex. (Note the equal reappearance of submolar bands immediately below the four protected G's in both 'free' and 'bound' lanes representing the sense strand.) We did not detect any guanines interacting with protein on the opposite strand (Figure 3), a result also obtained by others for some target sites binding factors we eventually identified as *rel*-related proteins (see below).

Binding of the *HRAS1* Minisatellite to the *rel*/NF-κB Family of Regulatory Proteins

The 15 bp region of VTR_{HRAS1} defined by comparative studies with other VTR subunits and by methylation interference contained an 11 bp sequence similar to a site found in a number of viral and cellular promoter and enhancer regions (Figure 1) which bound the transcriptional regulator, NF-κB, and other members of the *rel* family of DNA binding proteins (33−35). Both constitutively expressed (35, 36) and inducible forms (11, 37−39) of these factors have been characterized. It has been suggested that the constitutive form of NF-κB contributes to the basal level of transcription of some inducible genes (40). To test the possibility that members of the NF-κB family were binding to VTR_{HRAS1}, we constructed oligomers representing two distinct NF-κB targets (Figure 1). The first was a site within the murine H-$2K^b$ gene promoter which was used to purify the constitutively expressed form, KBF-1/p50 (25, 41). The second, from the human IL-2 receptor α chain gene (IL-2Rα), was the target used to identify both constitutively expressed and inducible members of the NF-κB/*rel* family (11, 28; see Figure 1). Both NF-κB targets produced one principal complex with nuclear extracts from a variety of cell lines (Figure 4A), confirming the results of others (11, 26, 28, 42, 43). In all extracts we examined, this complex comigrated with the one produced by a VTR_{HRAS1} target (Figure 4A). Furthermore, complexes produced with VTR_{HRAS1} could be effectively competed with H-$2K^b$ (Figure 4B and 4D) or IL-2Rα (data not shown), and *vice versa* (Figure 4C, bottom complex). Although a relatively large molar excess of non-radioactive VTR_{HRAS1} was required for specific

competition of the constitutively expressed factors from the homologous target in assays performed with our crude extracts (Figure 2A), the stoichiometry was not informative since we did not establish that the competition occurred in the linear range of the binding reaction. Of greater moment was our observation that this result could be reproduced exactly when both the target and competitor were H-2K^b (Figure 4C, bottom complex). Therefore, VTR$_{HRAS1}$ served as an array of binding sites either for the constitutive form of NF-$\varkappa$B or for a very closely related nuclear factor. (See also UV crosslinking studies, below).

Absence of *HRAS1* Minisatellite Binding to the Inducible Form of NF-$\varkappa$B

We tested VTR$_{HRAS1}$ binding to the inducible form of NF-$\varkappa$B, as well. Shift assays were performed with nuclear extracts from Jurkat cells pretreated with TPA and PHA, which are potent inducers of one form of NF-$\varkappa$B (18). Extracts from induced cells (Figure 4C) yielded an additional complex with the NF-$\varkappa$B target, H-2K^b (and IL-2Rα; not shown), which migrated more slowly than the complex from uninduced cell extracts (Fig. 4C; small arrow). Furthermore, the new complex was observed in all induced cell lines tested (HeLa, EJ, Jurkat, PD31; results with Jurkat are presented in Figure 4). It has been proposed that this slower migrating complex represents a p50/p65 heterodimer form of NF-$\varkappa$B, while the faster migrating form (Fig. 4C and 4D; large arrow) consists of the p50 homodimer and perhaps other heterodimeric forms (36). The inducible complex was never observed to bind to a VTR$_{HRAS1}$ target incubated with TPA/PHA-treated extracts (Figure 4D). In addition, VTR$_{HRAS1}$ was much less efficient as a non-radioactive competitor for H-2K^b binding of NF-$\varkappa$B than the homologous competitor. As shown in Figure 4C (upper band), a 100-fold molar excess of non-radioactive VTR$_{HRAS1}$ produced the same result as the smallest (1X) amount of non-radioactive H-2K^b. However, competition did eventually occur, perhaps reflecting a sequestration of p50 by unlabeled VTR$_{HRAS1}$.

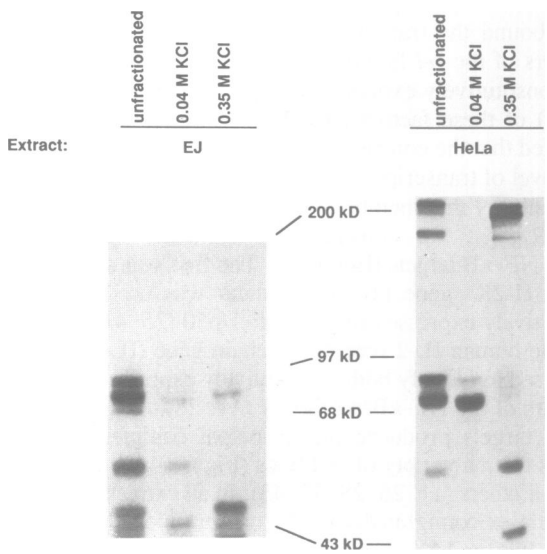

Figure 6: UV Crosslinking analysis of EJ and HeLa phosphocellulose fractions. The internally labelled 46 bp VTR$_{HRAS1}$ oligonucleotide was UV-crosslinked to either unfractionated or phosphocellulose column fractions from EJ and HeLa cells. Results from two separate SDS-polyacrylamide gels are presented.

Further Characterization of *HRAS1* Minisatellite Binding Proteins

UV crosslinking studies were performed to determine the number and molecular weights of proteins comprising the VTR$_{HRAS1}$ shifted complex. Four proteins of 85, 72, 50, and 45 kD which bound to VTR$_{HRAS1}$ were observed in extracts from a EJ, HeLa and Jurkat (Figure 5A). Since four proteins of this description have been described in complexes with NF-$\varkappa$B binding sites (28, 33, 44), we performed a direct comparison. As shown in Figure 5B (compare lanes 6 and 7), proteins of identical size and number bound the IL-2Rα target (originally used to define the *rel*-related family of transcription factors) and VTR$_{HRAS1}$. The binding of these proteins was sequence-specific, since competition occurred with unlabeled VTR$_{HRAS1}$ (lanes 2,8), as well as the NF-$\varkappa$B targets, H-2K^b (lanes 3,9) and IL-2Rα (lanes 4,10), but not with a non-specific competitor, VTR$_{ApoB}$ (lanes 5,11). Thus, UV-crosslinking analysis provided additional evidence that VTR$_{HRAS1}$ binding factors were members of the NF-$\varkappa$B/*rel* family.

Cell-Type-Specific Modification of NF-$\varkappa$B p50 subunit

Published reports of cell-type specificity in VTR$_{HRAS1}$ transcriptional regulation (15), as well as our own results demonstrating VTR$_{HRAS1}$ enhancer activity in EJ, but not in other cell lines expressing VTR$_{HRAS1}$ binding factors (M. Green and T.G.K., in preparation), suggested the possibility that we might observe differences on further fractionation of minisatellite binding proteins from different cell sources. Therefore, we partially purified *rel*-related proteins on phosphocellulose columns. Examination of eluted fractions by gel shift assays showed no appreciable differences between the representative extracts from EJ and HeLa cells with either VTR$_{HRAS1}$ or H-2K^b targets (data not shown). However, when these same fractions were analyzed by UV-crosslinking (as described in Methods, binding reactions were irradiated directly in solution), we repeatedly observed a marked difference in fractionation of the p50 subunit. In the EJ cell line, p50 eluted in the 0.04 M fraction (Figure 6, left panel, lane 2), while in the HeLa cell line, p50 eluted in the 0.35 M KCl fraction (Figure 6, right panel, lane 3). Other proteins did not demonstrate cell-type-specific differences; for example, p45 consistently appeared in the 0.35 M fraction from both EJ and HeLa extracts (Figure 6).

Phosphocellulose fractionation of nuclear extracts also unexpectedly resolved p72 and p85 into a multiple bands, revealing that these proteins exhibited a high degree of previously unrecognized heterogeneity (Figure 6, compare lane 1 to lanes 2 and 3 in both panels). Proteins with much larger apparent molecular weights were observed with both HeLa and EJ extracts on occasion (see, for example, Figure 6); these could be specifically competed with non-radioactive NF-$\varkappa$B targets. Others have attributed these species to the cross-linking of two or more proteins to the same probe (33, 44). However, we could not exclude the possibility that these represented other proteins which recognized the NF-$\varkappa$B site, such as MBP-1/PRDII-BF1 (290 kD; ref.45), H2TF-1 (110 kD; ref.45), or other rel-associated proteins (p115, p124; ref.46).

DISCUSSION

We have demonstrated that the *HRAS1* minisatellite, in side-by-side comparisons with two distinct and previously well-characterized *rel*/NF-$\varkappa$B target sequences, bound factors with similar recognition sequences, tissue distribution, gel shift patterns

and apparent molecular weights as members of the *rel*/NF-κB family. Like the binding of purified NF-κB to certain targets (HIV-1 enhancer; ref. 47), *rel*/NF-κB factors interacting with VTR_{HRAS1} target demonstrated G contacts on only one strand. The wide variation of homo- and heterodimer formation displayed by *rel*/NF-κB family members on traditional NF-κB target sequences, with one important exception described below, were recapitulated with the VTR_{HRAS1} repeat unit. We also observed differences in p50 fractionation, as well as size heterogeneity of other factors (Figure 6), which have not yet been elucidated. In other contexts, the diversity of these protein interactions was considered responsible for multiple effects on gene transcription (33, 44).

VTR_{HRAS1} bound exclusively the constitutively expressed forms of NF-κB, namely the homo- and heterodimers of p50, p72 and p85 (36, 40). The inability of VTR_{HRAS1} to bind the inducible form of NF-κB likely resulted from its inability to bind p65. The recognition site for NF-κB consists of two half-sites, designated A and B, with p50 binding the A site with high affinity and p65 interacting with the B site (48,49). The VTR_{HRAS1} A site was identical to that of IL-2Rα and other targets for the inducible form of NF-κB (Figure 1). However, the VTR_{HRAS1} B site differed considerably from these other recognition sequences. Interestingly, the proenkephalin NF-κB binding site, with a B half-site similar to that of VTR_{HRAS1}, did not bind p50p65, either (40).

Given the complex array of proteins we observed by the UV crosslinking studies, we propose the hypothesis that VTR_{HRAS1} can integrate potentially competing regulatory signals to the enhancers and promoters of *HRAS1* and other nearby genes. Our own studies of transcriptional regulation by VTR_{HRAS1} (M. Green and T. G. K., in preparation) and those of others (15,16) support this notion. One prediction of this hypothesis would be that other minisatellites might also bind discrete proteins and influence gene expression. We expect that these proteins would be distinct from the recently described 'minisatellite-specific' factor, Msbp-1 (50), which apparently recognizes the core homology (1) in a minisatellite subfamily. Many VTRs associated with genes, such as VTR_{HRAS1}, lack this core sequence and, in general, display a widely divergent set of repeat units. Pursuing the hypothesis that VTRs of this description would be good candidates for transcriptional control elements, we have demonstrated that the minisatellite within the D-J intron of the human immunoglobulin heavy chain gene (51) binds several members of the c-*myc* family (52) of helix-loop-helix proteins. VTR_{IgH} strongly represses transcription associated with two regulatory structures which contain highly related *myc*HLH binding sites, namely, the adenovirus major late promoter and the murine immunoglobulin heavy chain gene enhancer (W. L. T. and T. G. K., manuscript in preparation). We have most recently identified, but not yet characterized, a factor specific for the AT-rich minisatellite just downstream from the human apolipoprotein B gene (P. DiCroce and T. G. K., unpublished). Further studies with these three VTRs should help elucidate the potential regulatory role of minisatellites linked to genes and gene clusters.

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