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Inducible and Cell Type-Specific Expression of VL30 U3 Subgroups Correlate with Their Enhancer Design

MATS NILSSON AND STAFFAN BOHM*

Center for Nutrition and Toxicology, Karolinska Institute, NOVUM, S-141 57 Huddinge, Sweden

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The murine VL30 elements constitute one family of retrotransposons represented in 100 to 200 copies that are dispersed among the mouse chromosomes. On the basis of sequence homology, we have subdivided mouse VL30 members into four distinct U3 subgroups. The use of subgroup-specific probes in Northern (RNA) blot analyses shows that individual VL30 U3 subgroups are expressed in a tissue-specific manner. We show by in situ hybridization of mouse skin treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that VL30 expression is induced in epidermal keratinocytes but not in dermal fibroblasts. Transient transfections of reporter gene plasmids together with in vitro binding analysis indicate that TPA-induced VL30 transcription specific for keratinocytes is mediated by two cooperating sequence motifs in juxtaposed position. One sequence motif is shown to constitutively bind CREB- and Jun-related proteins in both keratinocytes and fibroblasts, whereas the other is a target for TPA-induced c-Rel/p65(NF-κB)-binding activity specifically in keratinocytes. These binding sites are found to be conserved within U3 subgroups and individual U3 regions showing induced expression in TPA-treated mouse epidermis. These results together with a sequence comparison between different U3 subgroups indicate that cell type-specific activity of transcription factors known to regulate VL30 transcription and the presence or absence of their cognate binding sites within individual U3 regions determine inducible and cell type-specific VL30 expression. The variable VL30 U3 regions might thus be useful tools to study inducible and cell type-specific transcription in many different cell systems.

The long terminal repeat (LTR) is a distinctive feature that structurally unites proviruses with the broad collection of eukaryotic transposable elements known as retrotransposons (reviewed in references 5, 37, and 38). A particularly potent genetic determinant within the retroviral LTR is the U3 region which contains the transcriptional enhancer and promoter sequences. When the U3 region is viewed as an array of multiple short binding sites for numerous *trans*-acting proteins, it is likely that a difference in the sequence between distinct U3 regions will produce a functional change. Several studies have provided evidence for such a key role for specific U3 sequences in determining viral tissue tropism and transcriptional regulation (12, 13, 16, 40–42, 59, 67, 70).

The murine VL30 elements constitute one family of retrotransposons represented in 100 to 200 copies that are dispersed among the mouse chromosomes (11, 15, 36, 37). The levels of VL30 transcripts are elevated by a wide variety of agents, such as serum, growth factors, calcium, peptide hormones, glucocorticoids, retinoic acid, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), forskolin, and cycloheximide (6–8, 19, 25, 27, 32, 39, 44, 57, 65). Other studies have shown induction of VL30 expression in response to ionizing radiation, anoxia, and transformation caused by simian virus 40 infection or the Ha-*ras* oncogene (3, 19, 50, 52, 68). Because of the heterogeneity in VL30 LTRs, it is possible that only a small fraction of the VL30 population responds to these signals. Three distinct VL30 retrotransposons (NVL3, B10, and RVL3) have been used to map specific binding sites for nuclear proteins involved in mediating transcriptional induction caused by an activated Ha-*ras* oncogene, TPA, forskolin, retinoic acid, epidermal growth factor, and thapsigargin (6, 7, 32, 39, 50).

* Corresponding author. Mailing address: Harvard Medical School, Department of Neurobiology, 220 Longwood Ave., Boston, MA 02115. Phone: (617) 432-4802. Fax: (617) 734-7557.

VL30 transcripts are ubiquitously expressed at relatively high levels in most somatic tissues examined (22, 27, 44, 48). However, analysis of VL30 expression with probes that do not discriminate between different VL30 members might be misleading, in that the predominant size class (5.6 kb) of VL30 RNA probably corresponds to transcripts encoded by different VL30 elements at particular chromosomal locations and/or having unique U3 regions. By the use of S1 nuclease mapping experiments, which distinguish transcripts from distinct VL30 elements, Norton and Hogan have shown both temporal and tissue specific expression of VL30 elements during mouse development (48). It was recently shown by Schiff et al. that VL30 retrotransposons are specifically expressed in steroidogenic cells within all four endocrine tissues engaged in synthesis of steroid hormones in response to their respective pituitary-derived trophic hormone (65). Two related VL30 LTRs which contained hormone-responsive enhancers were cloned from ovaries stimulated with luteinizing hormone. Further support for distinct transcriptional regulation of different VL30 LTRs has come from studies showing that the transcriptional efficiencies mediated by VL30 LTRs differ when analyzed in the same cell type (61). Moreover, induced transcription in response to serum addition is correlated with a specific U3 sequence variant, whereas transformation induces the expression of additional types of VL30 elements (19).

In this study, we have analyzed the relationship between the structural diversity in the U3 region and cell type-specific regulation of VL30 transcription. We have divided sequenced VL30 U3 regions into four U3 subgroups that possess distinct conserved sequence motifs known to be involved in cell type-specific transcriptional regulation. By the use of subgroup-specific probes in Northern (RNA) analysis, it was revealed that U3 subgroups showing high levels of expression in either muscle tissues or spleen specifically contained conserved sequence motifs known to be involved in muscle- and lymphoid-specific transcription, respectively. Furthermore, ke-

ratinocyte-specific c-Rel/NF-κB-binding activity to a distinct TPA response element was found to correlate with keratinocyte-specific transcriptional regulation in cultured cells and the presence of this TPA response element within VL30 U3 regions correlated with TPA-induced expression in keratinocytes in mouse skin in vivo. These results indicated that the specific enhancer design of the individual U3 subgroup determined in which cell type it was expressed or activated in response to extracellular signals.

MATERIALS AND METHODS

Cell culture conditions. All chemicals and media were purchased from Sigma, unless stated otherwise. The mouse fibroblast cell line Swiss/3T3 was cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 10% (vol/vol) fetal calf serum (Hyclone), 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B (Fungizone) per ml. The keratinocyte cell line BALB/MK was cultivated in MCDB 153 medium supplemented with 50 μM $CaCl_2$, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, epidermal growth factor (10 ng/ml), insulin (5 μg/ml), 0.5% Chelex-treated fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml).

Animal treatments. Female SENCAR mice, 6 and 9 weeks old, were purchased from Harlan Sprague-Dawley. The backs of the mice were shaved 48 h prior to topical treatment with 10 nmol of TPA (Pharmacia). TPA was applied in 200 μl of acetone. Control mice were treated with 200 μl of acetone only. Mice were killed by cervical dislocation 3 h after treatment.

In situ hybridization analysis. Formalin-fixed skin tissue was embedded in paraffin, transversally sectioned at 6 to 7 μm, and mounted onto microscope slides pretreated with 3-aminopropyltriethoxysilane (56). Sections were deparaffinized in xylene and hydrated (99-90-70% ethanol). Tissue permeabilization and acetylation were carried out as described previously (54). Sections were prehybridized for 1 to 2 h in a mixture of 50% formamide, 10% dextran sulfate, 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2 M dithiothreitol, bovine (F-V) serum albumin (Pharmacia) (2 mg/ml), yeast tRNA (0.2 mg/ml), and single-stranded calf thymus DNA (1 mg/ml). Hybridization was performed at 52°C for 3 h in a humidified chamber with 1×10^6 cpm of ^{35}S-labeled RNA probes in 40 μl of the buffer described above per cm^2. cRNA was generated from linearized pGEM-3Z containing the cDNA insert from λB10 (full-length 3′ LTR and 309-bp internal VL30 sequences) by using T7 or SP6 RNA polymerase (Promega). ^{35}S-labeled cRNA size was estimated to be 50 to 200 bases by formaldehyde-agarose gel electrophoresis. Sections were washed at 52°C twice for 20 min each time in 50% formamide–2× SSC–10 mM dithiothreitol and then four times for 10 min each time in 2× SSC at room temperature. Nonspecifically bound probe was removed by treatment with RNase A (100 μg/ml) and RNase T1 (1 μg/ml) (Boehringer Mannheim) at 37°C for 30 min and then washed in 50% formamide and 2× SSC at 52°C for 10 min. Sections were dehydrated (70-90-99% ethanol), dipped in photographic emulsion (K-5; Ilford), and left for 1 to 2 weeks at 4°C in the presence of desiccant. The emulsion was developed for 5 min (D19) and fixed (AL4; Kodak), and the sections were counterstained with hematoxylin.

RNA analysis. Total RNA was isolated from SENCAR mouse epidermis by guanidinium thiocyanate-phenol-chloroform extraction as described previously (14). Total RNA (5 μg) was fractionated by electrophoresis through formaldehyde-agarose gels, blotted onto nylon membranes, and fixed by UV irradiation. The mouse multiple tissue Northern blot was obtained from Clontech (Palo Alto, Calif.). The source of RNA on the tissue blot was BALB/c mice. Northern filters were prehybridized, hybridized, and washed according to standard procedures (62) prior to autoradiography.

Sequencing and reporter plasmid constructs. cDNA inserts of λ clones were subcloned into the *Eco*RI site of pGEM-3Z (Promega). Double-stranded DNA was used for dideoxy sequencing (United States Biochemical Corp.). The construction of B10.CAT, which contained the 3′ U3 region of the VL30 cDNA clone B10 fused to the bacterial chloramphenicol acetyltransferase (CAT) gene, has been described elsewhere (6). pBLCAT2 and pT109.Luc contained the −109 to +51 promoter region of the herpes simplex virus thymidine kinase (TK) promoter fused to the CAT or luciferase (Luc) reporter gene, respectively (43, 47). VLTRE.Luc, VLX.Luc, and VLY.Luc were created by subcloning oligonucleotides corresponding to the sequences shown below into the *Hin*dIII and *Xho*I sites of pT109.Luc. The integrity of all plasmid constructs was confirmed by sequence analysis. All plasmids were prepared by alkaline lysis, treated with RNase, and purified using Qiagen columns (Diagen).

Transfections and treatments of cells. Preconfluent fibroblasts were transiently transfected by the calcium phosphate precipitation method (24), while BALB/MK cells were transfected with cationic liposomes (lipofectin; Bethesda Research Laboratories). Cells were grown on 25-cm^2 dishes and transfected with 5 μg of reporter plasmid DNA. The medium was changed 24 h after transfection, and the cells were cultured in the presence or absence of 100 ng of TPA per ml for 16 h. CAT and luciferase activities obtained after transfection were quantified as described previously (62), and the activities of the different reporter plasmids were normalized to the activity of a cotransfected (1 μg per transfection) Rous sarcoma virus LTR-driven luciferase (pRSV.Luc) or CAT (pRSV.CAT) reporter plasmid (47).

Preparation of nuclear extracts and gel shift assay. Nuclear cell extracts were prepared by the method of Struhl et al. (72). Gel shift analysis was performed with 8 μg of extract protein, 0.8 μg of poly(dI-dC), 0.8 μg of poly(dG-dC), 0.6 μg of poly(dA-dT), and 30,000 to 40,000 cpm of ^{32}P-labeled oligonucleotide in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.8)–80 mM KCl–4 mM $MgCl_2$–4% Ficoll. The probe and competitor were added together, and the binding reactions were then incubated for 30 min at room temperature. For some protein-DNA binding experiments (see Fig. 5), binding reactions and nuclear extract preparation were done in the presence of leupeptin (10 μg/ml), 5 μM pepstatin (Boehringer), and aprotinin (Bayer, Inc.) (100 protein kinase K inhibiting units [KIU]/ml). The resulting protein-DNA complexes detected with the VLX probe were resolved on a preelectrophoresed 5% polyacrylamide gel (29:1), with 0.5× TBE (45 mM Tris-borate, 0.5 mM EDTA) as the running buffer. Protein-DNA complexes detected with the VLY and the NF-κB probes were resolved as described above but with 1× TGE (50 mM Tris, 2.7 mM EDTA, 380 mM glycine) as the running buffer.

Oligonucleotides and antibodies used in gel shift analysis. The extracts were preincubated with different antibodies for 30 min at 4°C prior to gel shift analysis. Antibodies recognizing p65(NF-κB) or c-Rel were purchased from Santa Cruz Biotechnology, Inc. These affinity-purified polyclonal antibodies were raised against an amino terminal peptide of human p65 and a carboxy terminal peptide of murine c-Rel, respectively (4, 31). Complementary synthetic oligonucleotides were syn-

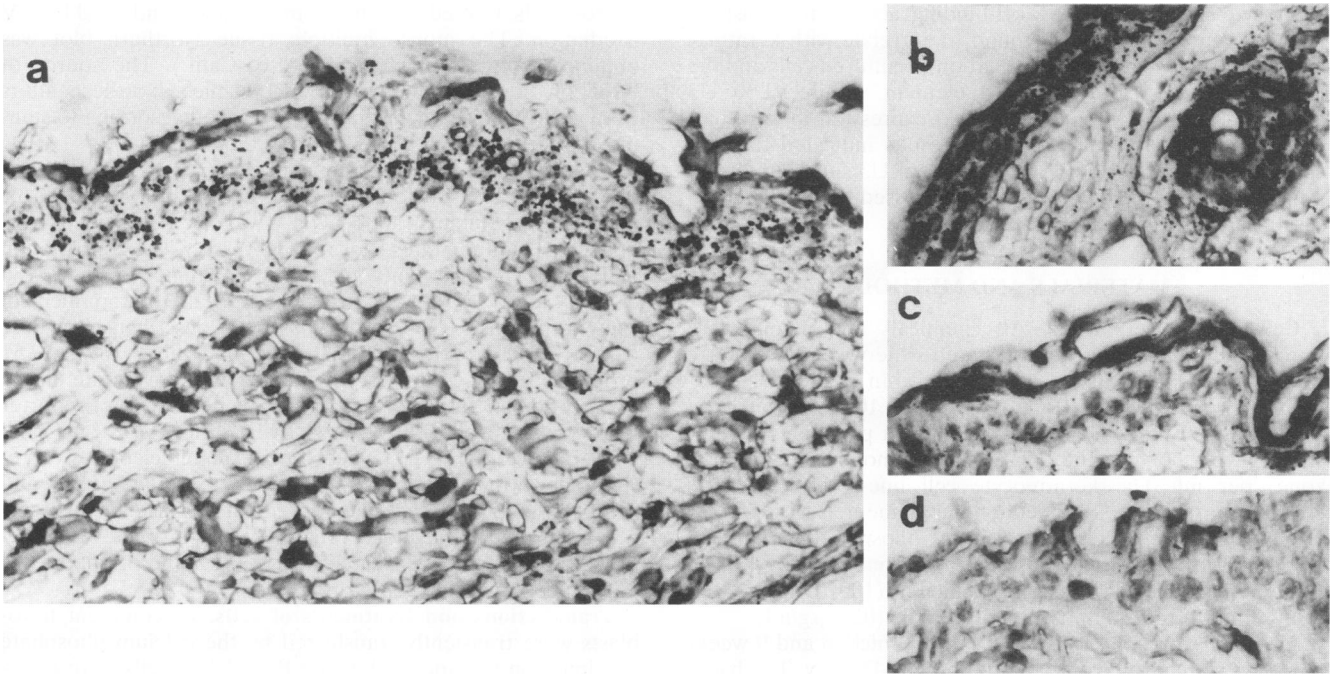

FIG. 1. In situ hybridization showing TPA-induced VL30 expression localized to epidermis and hair follicles. Formalin-fixed sections were hybridized with a ^{35}S-labeled antisense RNA probe derived from the cDNA clone B10 (309-bp element and the 3′ LTR of clone B10). The exposure time was 10 days, and all sections were counterstained with hematoxylin. (a) VL30 mRNA expression 4 h after TPA treatment (100 nmol). (b) VL30 mRNA expression 4 h after TPA treatment in a cross section of a hair follicle. (c) VL30 mRNA expression in a control section (4 h after acetone treatment). (d) The negative control, a section from a TPA-treated mouse hybridized to a ^{35}S-labeled B10 sense RNA probe. Magnification, ×462.

thesized on an Applied Biosystems oligonucleotide synthesizer. The double-stranded oligonucleotides used as gel shift probes were labeled with an appropriate [α-^{32}P]deoxynucleoside triphosphate by Klenow polymerase and purified by electrophoresis through a 12% polyacrylamide gel (29:1). The NF-κB oligonucleotide corresponded to the NF-κB-binding sequence found in the κ light chain enhancer (66). The αCG-CRE oligonucleotide corresponded to the cyclic AMP (cAMP) response element (CRE), including flanking sequences, found in the choriogonadotropin α gene (17). The sense strands of the annealed oligonucleotides are as follows (lowercase letters denote nucleotides present in double-stranded oligonucleotides after a fill-in reaction): VLTRE, agctTCCCTAGTGATGTAAACTTGTACTTTCCCTGCCCAGTtcga; VLX, agctTCCCTAGTGATGTAAACtcga; VLY, agctTAAACTTGTACTTTCCCtcga; NF-κB, agctTGGGGACTTTCCCtcga; αCG-CRE, agctCGAGAAATTGACGTCATGGTAAGtcga.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article have been submitted to the GenBank/EMBL data bank under accession numbers X75481 and X75482.

RESULTS

Induction of VL30 mRNA expression specifically in mouse epidermis. By differential screening of a mouse epidermal cDNA library, we previously isolated several cDNA clones corresponding to members of the VL30 family that showed induced expression in mouse epidermis 4 h after a single topical application of the skin tumor promoter TPA (8). The localization of mRNA expression before and after treatment with 10 nmol of TPA was studied by the in situ hybridization technique. As a source of a VL30-specific probe, we used a 929-bp-long probe that corresponded to the 3′ LTR and 309 bp of internal sequences of a TPA-responsive VL30 clone designated B10 (6). B10 cRNA probes labeled with ^{35}S in either antisense or sense orientation were synthesized. Figure 1 shows that the site of induced VL30 expression was the keratinocytes in the epidermis, whereas in dermal fibroblasts no significant VL30 expression was detected. VL30 mRNA was induced in both basally and suprabasally located differentiating keratinocytes (Fig. 1A). Enhanced VL30 expression was also seen in keratinocytes within the hair follicles (Fig. 1B). In untreated skin (Fig. 1C), VL30 expression was very low and did not exceed background level. As TPA applied topically penetrates the epidermis (2, 58), this result indicated that the mechanism behind TPA-induced VL30 transcription is cell type dependent.

The function of a TPA response element within a U3 region expressed in the epidermis is cell type dependent. We previously used the U3 region of the VL30 clone B10 to identify sequences mediating TPA-induced VL30 transcription in the keratinocyte cell line BALB/MK (6). The capacity of the B10 U3 region to mediate TPA-induced transcription was analyzed by transient transfections of a fibroblast cell line (Swiss/3T3) and BALB/MK cells. Transfections were done with a plasmid (B10.CAT) which contained the CAT reporter gene under control of the U3 region and the promoter of the VL30 clone B10 (6). Figure 2 shows the CAT activity obtained from this plasmid in untreated and TPA-treated BALB/MK and Swiss/3T3 cells. Basal and TPA-induced CAT activities were detected in keratinocytes but not in fibroblasts, as judged from the activities generated in parallel transfections with an

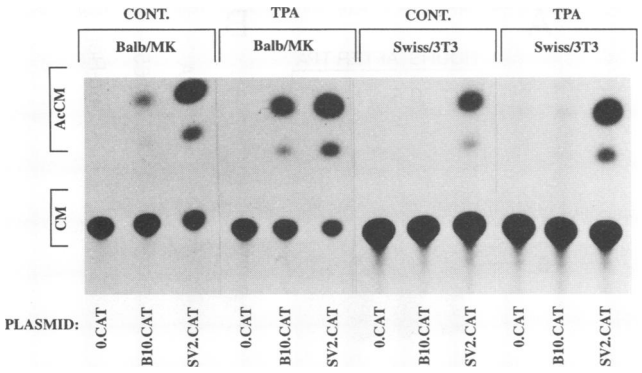

FIG. 2. Cell type-specific transcriptional activation mediated by a VL30 U3 region isolated from TPA-treated epidermis. Shown are representative autoradiographs of thin-layer chromatography analysis of CAT activity in untreated (CONT.) or TPA-treated keratinocytes (BALB/MK) and fibroblast (Swiss/3T3) cells. Preconfluent cells were transiently transfected with a plasmid in which the CAT reporter gene was under the control of the U3 region from a VL30 retrotransposon expressed in TPA-treated epidermis (B10.CAT). Cells were transfected in parallel with the SV2.CAT plasmid, in which CAT expression is driven by the simian virus 40 promoter and enhancer. A promoter- and enhancerless CAT plasmid was used as a negative control (0.CAT). Transfection conditions and TPA treatment are described in Materials and Methods. Aliquots of cell extracts were taken for CAT assay, with the amount adjusted with respect to the protein concentration. CM, chloramphenicol; AcCM, acetylated chloramphenicol.

SV2.CAT plasmid. This plasmid contained the CAT gene under the control of the simian virus 40 promoter and enhancer (43). As a negative control, we used the parental CAT reporter plasmid (0.CAT), which lacked promoter and enhancer sequences. This result indicated that a VL30 U3 region, which was isolated by a method that selected for VL30 elements induced by TPA in keratinocytes in vivo, could mediate transcription in a keratinocyte cell line but not in a fibroblast cell line when analyzed in vitro. A 39-bp VL30 TPA response element (VLTRE), which is functional in keratinocytes, was previously identified within the B10 U3 region (6). To determine whether this response element alone was sufficient to mediate a cell type-specific response, a plasmid containing the herpes simplex virus thymidine kinase promoter (TK) and a luciferase reporter gene under the control of the VLTRE was transiently transfected into keratinocytes and fibroblasts. This construct (VLTRE.Luc, schematically shown in Fig. 3) mediated TPA-induced transcription in keratinocytes, whereas it was inactive as a TPA response element in fibroblasts (Fig. 3). This result indicated that the transcriptional activity of VLTRE was controlled by cell type-dependent *trans* activation in TPA-treated keratinocytes. The TPA-responsive phenotype, with regard to induced transcription mediated by VLTRE, is not restricted to the BALB/MK cell line. (As we have previously shown, VLTRE is functional in primary human keratinocytes [6].) Furthermore, no induced reporter gene activity in response to TPA was obtained when B10.CAT and VLTRE.Luc were analyzed in two additional fibroblast cell lines, BALB/3T3 and NIH 3T3 (9).

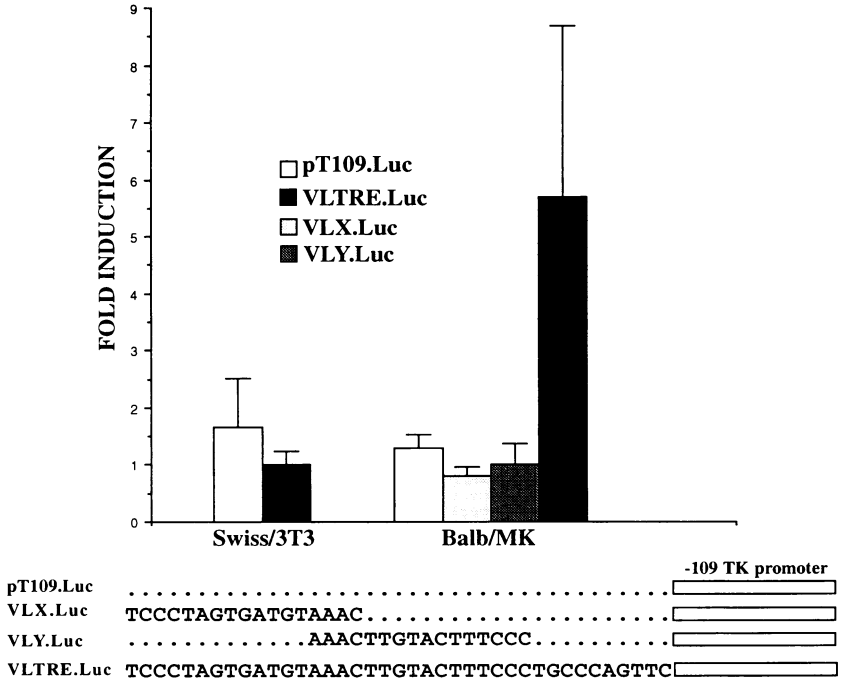

FIG. 3. 39-bp VL30 TPA response element mediated TPA-induced transcription in keratinocytes but not in fibroblasts. Shown are the fold inductions of luciferase activity by TPA (100 μg/ml) in transiently transfected keratinocytes (BALB/MK) and fibroblasts (Swiss/3T3). The cells were transfected in parallel with a thymidine kinase (TK) promoter-driven luciferase plasmid (pT109.Luc) and three derivatives of this plasmid which contained the VL30 TPA response element (VLTRE.Luc) or one of the two protein-binding sequences within VLTRE (VLX.Luc and VLY.Luc), respectively. Transfection conditions and TPA treatment are described in Materials and Methods. Protein aliquots were taken for luciferase assays, with the amounts adjusted with respect to the relative transfection efficiencies, as determined by CAT activity of cotransfected pRSVCAT vector. The bars represent the mean values of the relative fold induction obtained in three independent experiments. The standard deviation of the mean (T bars) is also indicated. Sequences corresponding to VLTRE, VLX, and VLY binding sites are shown.

Previous results have indicated that two nuclear protein-binding sequences within VLTRE collaborate in mediating TPA-induced transcription in keratinocytes (6). To determine whether either of these two sequences alone was able to mediate TPA-induced transcription, two additional plasmid constructs, designated VLX.Luc and VLY.Luc (Fig. 3), were made. These plasmids were found to be nonresponsive to TPA treatment in both Swiss/3T3 and BALB/MK cells (Fig. 3). This result supported the conclusion that the presence of both the VLX- and the VLY-binding sites on the same template was essential for TPA-induced VL30 transcription in keratinocytes.

The VLY sequence is a target for TPA-induced c-Rel/NF-κB-binding activity in BALB/MK cells but not in Swiss/3T3 cells. Given the functional importance of VLTRE, we employed gel shift analysis in order to identify the proteins binding to this sequence. The VLY-binding site contained a sequence, GTACTTTCCC, which was identical to a previously identified DNA element involved in TPA-induced transcription of the human urokinase gene (26). Hansen et al. designated this DNA sequence the Rel related binding element (RRBE) since it was found to bind both NF-κB(p50/p65) and a novel complex between the p65 subunit of NF-κB and c-Rel (26). Figure 4A shows that an oligonucleotide corresponding to the VLY sequence was the target for a transient TPA-induced binding activity in BALB/MK cells, which reached a maximum 1 h after the treatment. Two closely migrating complexes, designated A, and a faster-migrating complex, designated B, were detected with the VLY probe. Figure 4B shows that formation of both A and B complexes was inhibited by antibodies specific for the p65 subunit of NF-κB, while complex formation of A but not B was inhibited by antibodies specific for c-Rel. Interestingly, with nuclear extracts prepared from untreated and TPA-treated Swiss/3T3 cells, no complexes comigrating with A and B were detected (Fig. 5A). When nuclear extracts were prepared from BALB/MK and Swiss/3T3 cells in the presence different protease inhibitors (see Materials and Methods), a fast-migrating complex was formed. This complex formation (indicated with an asterisk in Fig. 5A) was not affected by the TPA treatment; nor was it supershifted or abolished when antibodies specific for p65 and c-Rel were included in the binding reaction. We next compared the complex formation observed with the VLY sequence with that generated by the classical NF-κB-binding site present in the enhancer of the κ light chain gene (66). Figure 5B shows that this type of NF-κB-binding site formed a TPA-induced complex in both BALB/MK and Swiss/3T3 nuclear extracts. This complex, designated B′, comigrated with complex B and was recognized by antibodies specific for p65, while no supershift or abolished complex formation was obtained in the presence of the c-Rel-specific antibodies (Fig. 5B). These results suggested that the VLY sequence was a target for TPA-induced binding activity of a c-Rel/p65 heterodimer (complex A) as well as a p65-containing complex (B) in keratinocytes but not in fibroblasts. We did not detect any c-Rel/p65 heterodimer-containing complex when the κB sequence was used as probe. However, the NF-κB-binding site formed a p65-containing complex (B′) which lacked the c-Rel component in both TPA-stimulated keratinocytes and fibroblasts. This result was intriguing since it implied that the B or the B′ complex contained a protein which together with p65 could distinguish between VLY and the NF-κB-binding site present in the enhancer of the κ light chain gene. The use of antibodies specific for the p50 subunit may resolve whether B and/or B′ contained a factor different from a p65/p50 heterodimer.

The VLX sequence corresponds to a nonconsensus CRE-binding sequence (CREB). The other sequence motif present within VLTRE is the VLX-binding sequence (shown in Fig. 5). We previously showed that this sequence formed a large number of complexes containing proteins related to members of the CREB and Jun families of transcription factors (7). Figure 5C shows gel shift analysis using an oligonucleotide corresponding to the VLX-binding site as the probe. The individual nature of these complexes has been studied in gel shift analysis using CREB- c-Jun-, JunB-, and JunD-specific antibodies and bacterially expressed CREB (7). Use of an excess of unlabeled oligonucleotides corresponding to VLX- and VLY-binding sites and a consensus CRE present in the choriogonadotropin α gene (αCG-CRE) (17) as competitors confirmed that the proteins binding to VLX were related to CREB proteins (Fig. 5C). Most of the proteins binding to VLX in BALB/MK and Swiss/3T3 cells were also found to be immunochemically related to CREB and c-Jun (data not

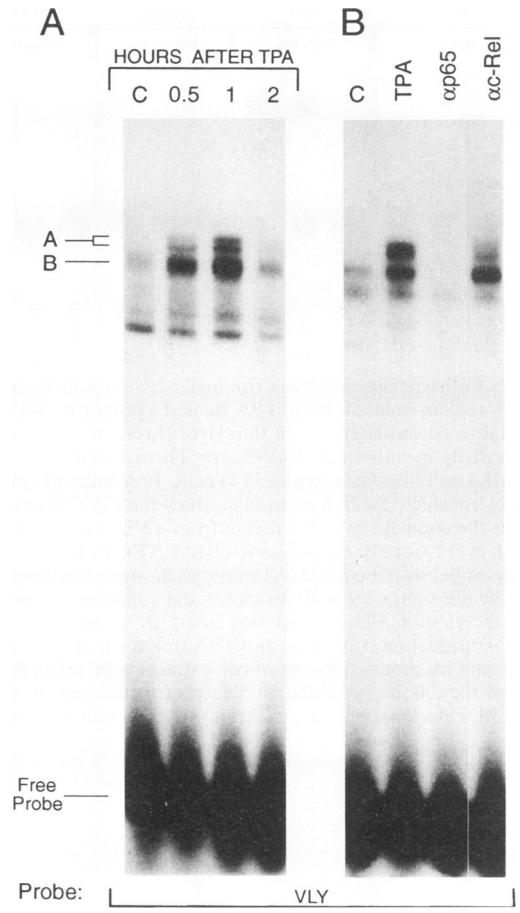

FIG. 4. TPA-induced complex formation in nuclear keratinocyte extracts between VLY and a c-Rel/p65 heterodimer and a factor containing p65 but not c-Rel. (A) Gel shift analysis using nuclear extracts (8 μg per incubation) prepared from BALB/MK cells. Protein-DNA complexes (indicated as A and B) were resolved by 5% nondenaturating polyacrylamide gel electrophoresis and autoradiography of the fixed and dried gels. Cells were treated for the time indicated with TPA prior to the preparation of nuclear extracts. C, control extracts from untreated cells. Equal amounts of protein from the different extracts were incubated with a ^{32}P-labeled probe corresponding to the VLY sequence (see Materials and Methods or Fig. 5 for the sequence). (B) The extracts were preincubated for 30 min at 4°C with antibodies recognizing the p65(NF-κB) subunit (αp65) and c-Rel (αc-Rel), respectively.

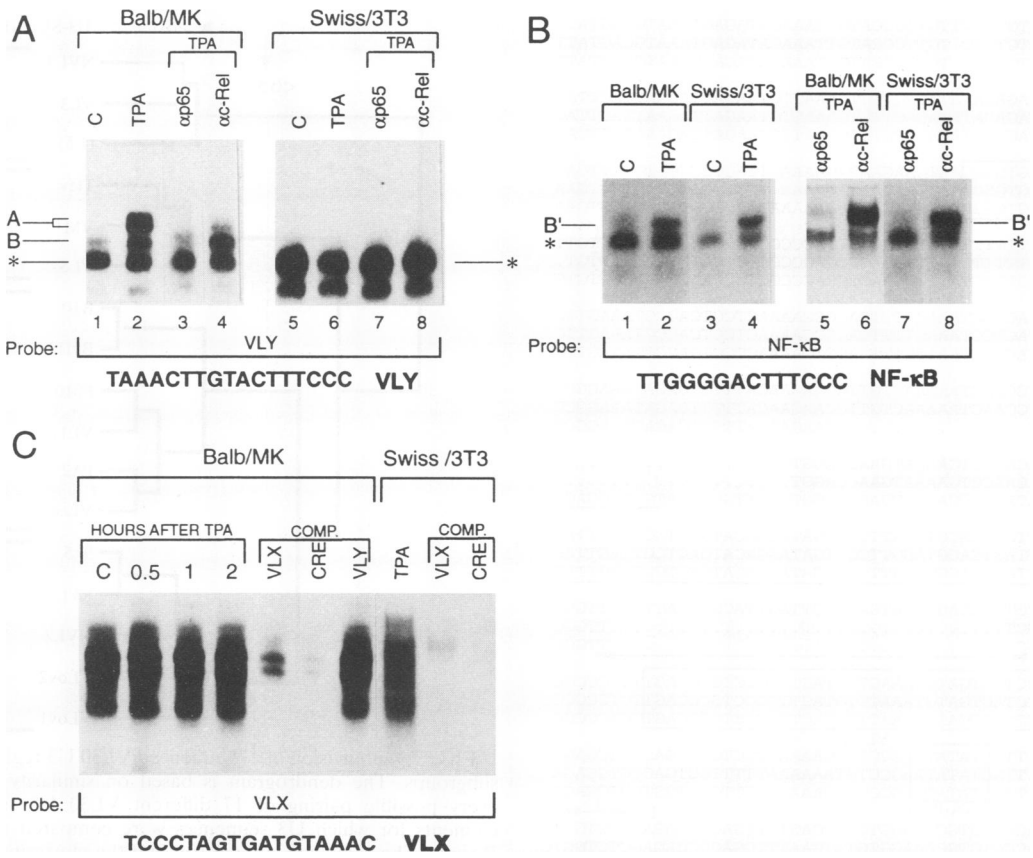

FIG. 5. The complex formation with the VLY sequence was cell type specific and differed from that of the NF-κB-binding site in the κ light chain enhancer. Equal amounts of protein from BALB/MK and Swiss/3T3 nuclear extracts (8 μg per incubation) were incubated with ^{32}P-labeled oligonucleotides corresponding to the VLY sequence (A), the NF-κB-binding site in the κ light chain enhancer (B), or the VLX sequence (C). Protein-DNA complexes (labeled in left and right margins) were resolved by 5% nondenaturating polyacrylamide gel electrophoresis and autoradiography of the fixed and dried gels. The complexes indicated by asterisks were detected when a number of protease inhibitors were included in nuclear extract preparation and binding analysis (see Materials and Methods). Extracts were prepared from untreated cells (C) or 1 h after treatment with TPA, and the immunological analysis was performed as described in the legend to Fig. 4. αp65, antibody to p(65)NF-κB subunit; αc-Rel, antibody to c-Rel. (C) Competition experiments (Comp.) used a 100-fold molar excess of unlabeled oligonucleotides corresponding to VLX, VLY, and a consensus CRE, respectively (see Materials and Methods for the corresponding sequences).

shown). No increased VLX complex formation was observed in nuclear extracts prepared from TPA-treated BALB/MK and Swiss/3T3 cells (Fig. 5C and data not shown).

Nucleotide sequence analysis of three TPA-induced VL30 clones. An interesting question was whether TPA induced the expression of a single, as opposed to multiple, distinct VL30 elements in mouse epidermis. To answer this, we sequenced the 3′ LTRs of three cDNA clones: B10, PA2, and PB10. These VL30 clones were individually isolated by differential screening, and we have previously published the U3 sequence of clone B10 (6). Figure 6 shows that aligned 3′ LTR sequences of these clones displayed a low rate of divergence, although differences included nucleotide substitutions, deletions, and short insertions (ranging between 5 and 23 bp). These types of sequence differences were typical of the heterogeneity found between distinct VL30 elements and were not indicative of the sequence variation normally associated with alternative processing pathways of a single RNA precursor species. Thus, this result led us to conclude that at least three VL30 sequences that were TPA inducible in epidermis were encoded by distinct, independent retrotransposon elements in the genome. The VLTRE sequence (boxed in Fig. 6) was found to be conserved within the isolated VL30 U3 regions.

Division of VL30 genes into four U3 subgroups. To relate the VL30 sequences induced by TPA in mouse epidermis to other members of the VL30 family, we calculated the percent sequence similarity in the U3 and R regions between the isolated LTRs and all VL30 LTR sequences previously published. Shown in Fig. 7 is a dendrogram based on similarity scores between every possible pair of published VL30 U3 sequences. The computer program and parameters used for this calculation are given in the legend to Fig. 7. The R regions of all members were found to be highly homologous, showing 87 to 99% sequence similarity (data not shown). With respect to the internal VL30 sequences of B10 (230 bp), PA2 (410 bp), and PB10 (130 bp) characterized so far, all had a very high percent similarity (96 to 98%) to the recently sequenced VL30 clones NVL3 (1) and BVL1 (28) (data not shown). One interesting observation was that four distinct VL30 U3 subgroups could be distinguished (Fig. 7). The percent sequence similarity within a particular U3 subgroup was in general >80% of their nucleic acid sequences, whereas between groups sequence similarity was in general <65%. Three different VL30 elements, NVL3, VL3, and 1.5, constituted U3 subgroup I (U3I). VL3 was isolated from a genomic library, while NVL3 was isolated from type C retroviral particles

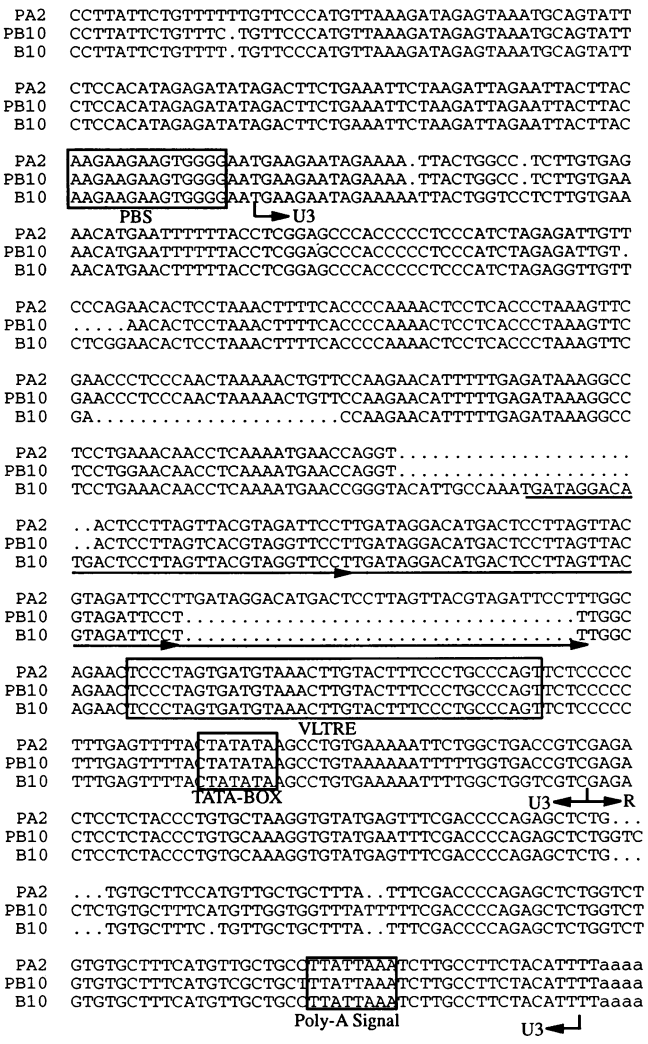

FIG. 6. Nucleotide sequences of isolated VL30 LTRs of B10, PA2, and PB10. Nucleotide sequences of VL30 LTRs (3′) with partial 5′ VL30 element flanks corresponding to three VL30 cDNA clones isolated by a differential screening strategy which selected for genes induced by TPA in mouse epidermis. The VL30 element-U3 and U3-R borders are indicated. The plus primer binding site (PBS), TATA box, polyadenylation signal (poly-A signal), and the VL30 TPA responsive element (VLTRE) are boxed. The internal 35-bp direct repeat region is underlined with arrows.

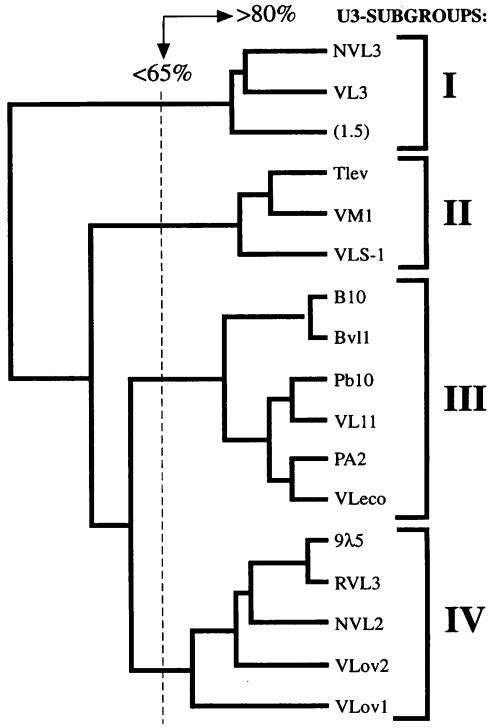

FIG. 7. Subdivision of 17 sequenced VL30 U3 regions into four U3 subgroups. The dendrogram is based on similarity scores between every possible pairing of 17 different VL30 U3 sequences. VL30 elements for which U3 sequences were compared are described in Results. The sequence comparison and the clustering strategy represented by the dendrogram were done according to the PILEUP computer program of the sequence analysis software package from the Genetics Computer Group, Inc. (GCG package) (23). The gap creation penalty and gap extension penalty used were 5.0 and 0.3, respectively. The PILEUP program uses the unweighted pair-group method using arithmetic averages (69) for clustering strategy and the method of Needleman and Wunsch (46) to calculate pairwise alignment. U3 regions sharing >80% of their nucleic acid sequences were considered to constitute a subgroup (I, II, III, and IV). Between U3 subgroups, sequence similarity was usually <65%. Brackets indicate that the calculations performed with the sequence of the 1.5 VL30 U3 region were done by using a partial sequence which represented 57% in length of the corresponding U3 regions contained in the other members of U3I. The sequence analysis was performed with U3 sequences derived from 3′ LTRs, except for VL11, RVL3, and the solo LTRs (VLeco and VLS1).

produced in NIH 3T3 fibroblasts (10). The 1.5 element corresponded to a partial VL30 U3 sequence identified by differential screening of a cDNA library, among genes induced in T lymphocytes in response to glucocorticoid treatment and increased cAMP levels (27). The U3 regions present in three distinct genomic VL30 clones, Tlev1 (51), VM-1 (33), and VLS-1 (60), constituted U3 subgroup II (U3II). The TPA-inducible VL30 elements which were characterized in epidermis together with some previously characterized VL30 elements designated BVL-1, VL11, and Vleco constituted the third U3 subgroup (U3III). BVL-1, VL11, and VLeco were isolated from genomic libraries (28, 29, 34). Vleco was a solo LTR that was found to be integrated within a murine leukemia provirus (29). Five different VL30 U3 regions constituted U3 subgroup IV (U3IV). These were the U3 regions present in 9λ5, which was isolated by differential screening of a cDNA library prepared from simian virus 40-transformed fibroblasts (68); RVL3, which was recently used to define sequence motifs mediating induced transcription in the Rat-1 cell line to treatment with thapsigargin in combination with epidermal growth factor or TPA (39); NVL1, which was identified in type C retrovirus particles produced in NIH 3T3 fibroblasts (10); and VLov1 and VLov2, which were cloned from a cDNA library made from luteinizing hormone-stimulated ovaries (65). The VLov1 U3 region was placed in U3IV, since it showed the highest sequence homology with members within this group, although its sequence homology to these members was lower than 80%.

From this sequence comparison, it was evident that those VL30 members that were known to be expressed could be found in three different U3 subgroups (I, III, and IV). Thus,

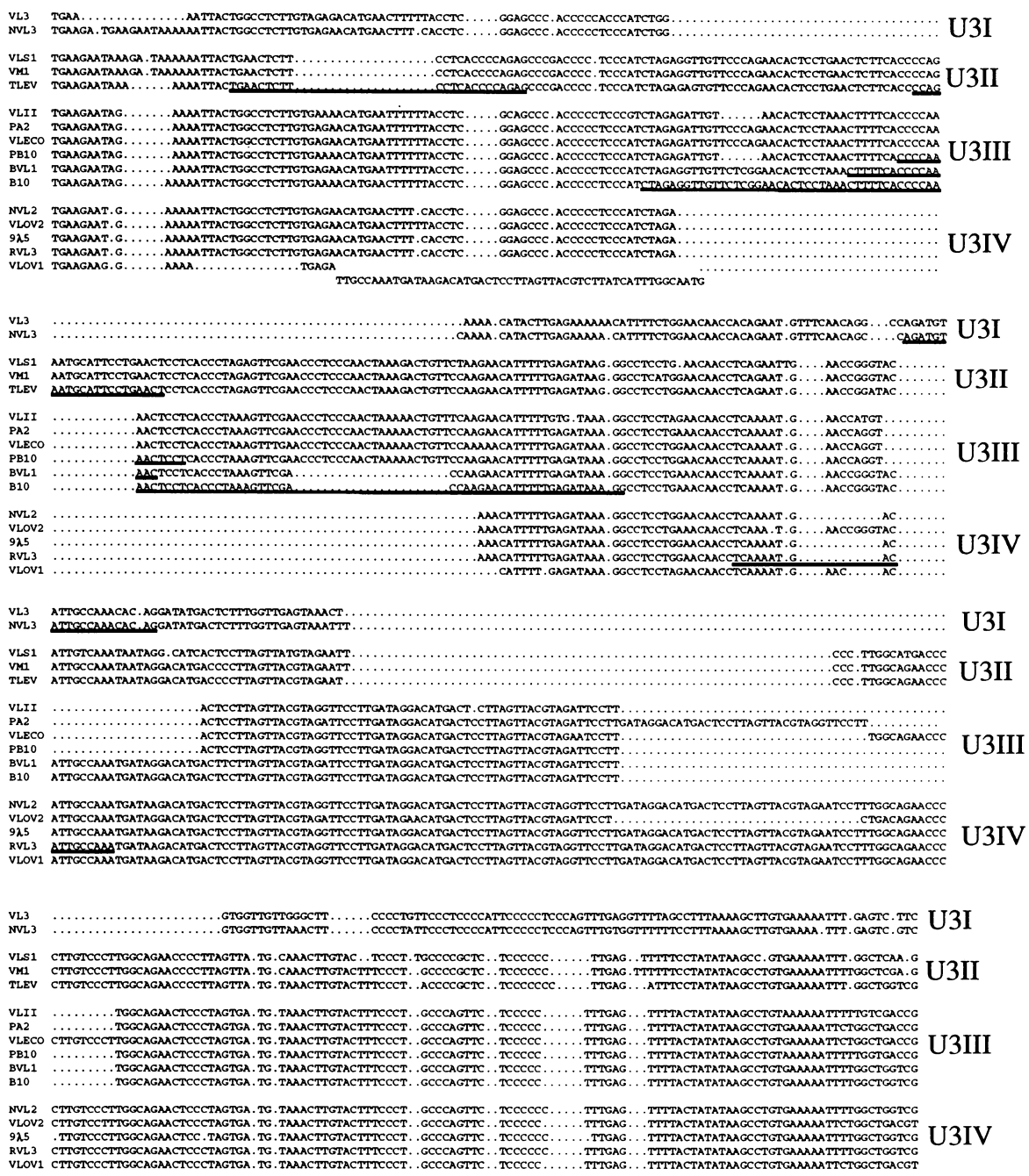

FIG. 8. Sequence alignment of VL30 U3 regions comprising four distinct U3 subgroups designated U3I, U3II, U3II, and U3IV. Underlined sequences correspond to U3-specific oligonucleotide probes used for the Northern blot analysis shown in Fig. 9.

expressed VL30 members that have been isolated from different sources show a high percent sequence variation in their U3 regions. We showed in this study that three expressed VL30 elements (B10, PA2, and PB10) isolated from the same source, i.e., TPA-treated epidermis, had high percentages of sequence similarity in their U3 regions. This result indicated that the specific sequence composition of a U3 region correlates to expression in a cell type- and/or stimulus-specific manner.

Tissue-specific expression of U3 subgroups. A sequence alignment of the different U3 subgroups is shown in Fig. 8. The U3 regions within each U3 subgroup displayed blocks of conserved sequences, whereas between groups, numerous point mutations, sequence duplications, deletions, and/or insertions could be observed. To test the possibility that different U3 subgroups were expressed in a tissue-specific manner, we employed subgroup-specific oligonucleotides as probes in Northern blot hybridization experiments with poly(A)$^+$ RNA isolated from eight different types of tissue of adult mice. Initially, this tissue blot was hybridized to a general VL30 probe corresponding to 600 bp of the 3′ end of the B10 clone

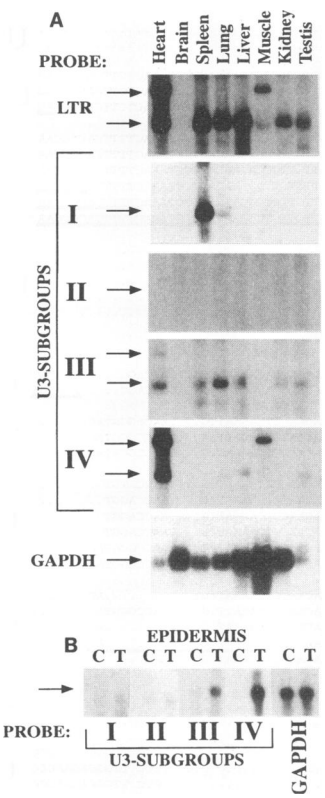

FIG. 9. Tissue-specific expression of distinct U3 subgroups. Autoradiographs of Northern blots analysis using RNA were prepared from different mouse tissues (the RNA sources are given in Materials and Methods). Hybridizations were performed with oligonucleotide probes (for sequences, see Fig. 8) specific for each of the defined U3 subgroups or a U3-nonspecific probe (LTR) corresponding to the full-length 3′ LTR and 309-bp internal sequences of the VL30 clone B10. The upper and lower arrows indicate VL30 transcripts corresponding to 10 and 5.6 kb, respectively. For comparison, hybridization to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe is included. (A) VL30-specific signals on Northern blots generated after repeated hybridizations and rehybridizations of a single tissue blot with group-specific probes. (B) Northern blot analysis performed (as for panel A) with U3-specific probes and RNA preparations from untreated mouse epidermis (C) and 4 h after a single topical treatment with 10 nmol of TPA (T).

containing the full-length 3′ LTR and a 309-bp internal sequence. Figure 9A shows that this probe recognized VL30 transcripts from all tissues tested except the brain. In addition to the 5.6-kb transcript, we observed a highly expressed RNA transcript of approximately 10 kb in heart and skeletal muscle. This hybridization pattern is in agreement with that obtained by Harrigan et al. (27). The tissue blot was then rehybridized with probes specific for each U3 subgroup. A U3I-specific probe hybridized predominantly to a 5.6-kb VL30 RNA in spleen. A relatively weak signal corresponding to this U3 subgroup was also obtained using lung poly(A)$^+$ RNA. Two different U3II-specific probes did not give rise to significant signals in any tissue analyzed. Two U3III-specific probes hybridized weakly to transcripts in all tissues examined except brain and muscle. A more intense signal was detected in lung poly(A)$^+$ RNA with these probes. A U3IV-specific probe predominantly recognized the 5.6-kb transcript in poly(A)$^+$ RNA from heart and the 10-kb transcripts in poly(A)$^+$ RNA from heart and muscle. Relatively weak signals were also generated by this probe in lung, liver, and testis samples. As a reference, the filter was rehybridized with a probe that recognized glyceraldehyde-3-phosphate dehydrogenase mRNA (21). These results showed that members of the VL30 family were expressed in a tissue-specific manner and that a rough assignment of expressed VL30 elements to different U3 subgroups could be done. Assuming that the nonspecific LTR probe used recognized all expressed VL30 U3 regions, a comparison of the relative signal intensities (data not shown) indicated that additional VL30 members not included in the four subgroups were expressed, for example, in tissues such as kidney.

TPA inducibility of VL30 in epidermis correlates with the presence of VLTRE. To test the correlation between regulated VL30 expression and the presence of a characterized response element, we prepared RNA from untreated and TPA-treated mouse epidermis. Figure 9B shows that U3III- and U3IV- but not U3I- and U3II-specific probes recognized TPA-induced VL30 expression in epidermis. This expression pattern correlated with the presence of a conserved VLTRE in U3III and U3IV, while in U3I and U3II the corresponding region contained conserved core and OCT motifs, respectively (see the discussion below).

Enhancer design of U3 subgroups. Figure 10 illustrates that the sequence variation between the U3 subgroups correlated with variations in the number and types of sequence motifs known to be involved in VL30 transcriptional regulation as well as to a large number of putative binding sites for other known transcription factors. VL30 U3 regions corresponding to each U3 subgroup were marked as follows. Retinoic acid response elements (boxed within the B10 U3 region), which have been shown to bind heterodimers of retinoic acid receptor (RAR) and the retinoid X receptor (RXR), mediated retinoic acid-induced B10 transcription in normal human keratinocytes but not in normal skin fibroblasts (32). The corresponding sequences (also boxed) in the other U3 subgroups differ from those in the B10 U3 region in the nucleotide spacing between the direct repeats of these putative retinoic acid elements. Moreover, CRE- and Jun-binding sequences, one of which is identical to the VLX site within the VLTRE, are located within the B10 U3 region. These sites cooperated in mediating adrenocorticotropin- and forskolin-induced transcription in the steroidogenic adrenal cell line Y1 but not in the BALB/MK, Swiss/3T3, or Hepa1 cell line (7). During the characterization of TPA-, cAMP-, and retinoic acid-induced transcription, we encountered other binding activities in nuclear extracts recognizing sequence motifs within the B10 U3 region (9). These DNA motifs were similar to the CArG box and AP1- and NF1-binding sequences (Fig. 10). Formation of the complex with oligonucleotides corresponding to these sequence motifs as probes was inhibited by an excess of unlabeled oligonucleotides corresponding to the serum response element in the c-*fos* promoter, the AP1-binding site in the collagenase promoter, and a consensus NF1-binding site, respectively (9). The activities of the CREB and Jun-binding sites, the CArG box, and the putative AP1-binding motif within the RVL3 U3 region (U3IV) have also been studied in Rat-1 fibroblasts by Lenormand et al. (39). Owen et al. identified two juxtaposed binding sites for nuclear factors which mediated induced NVL3 (U3I) transcription in response to an activated Ha-*ras* gene in NIH 3T3 fibroblasts (50). These two sites resembled the Ets- and AP1-binding sites, respectively (55). The site resembling an Ets-binding sequence has been shown to be recognized by a 120-kDa protein designated *ras*-responsive factor-1 (55). Three putative binding sites for additional transcription factors are the leukemia virus b sequence (LVb),

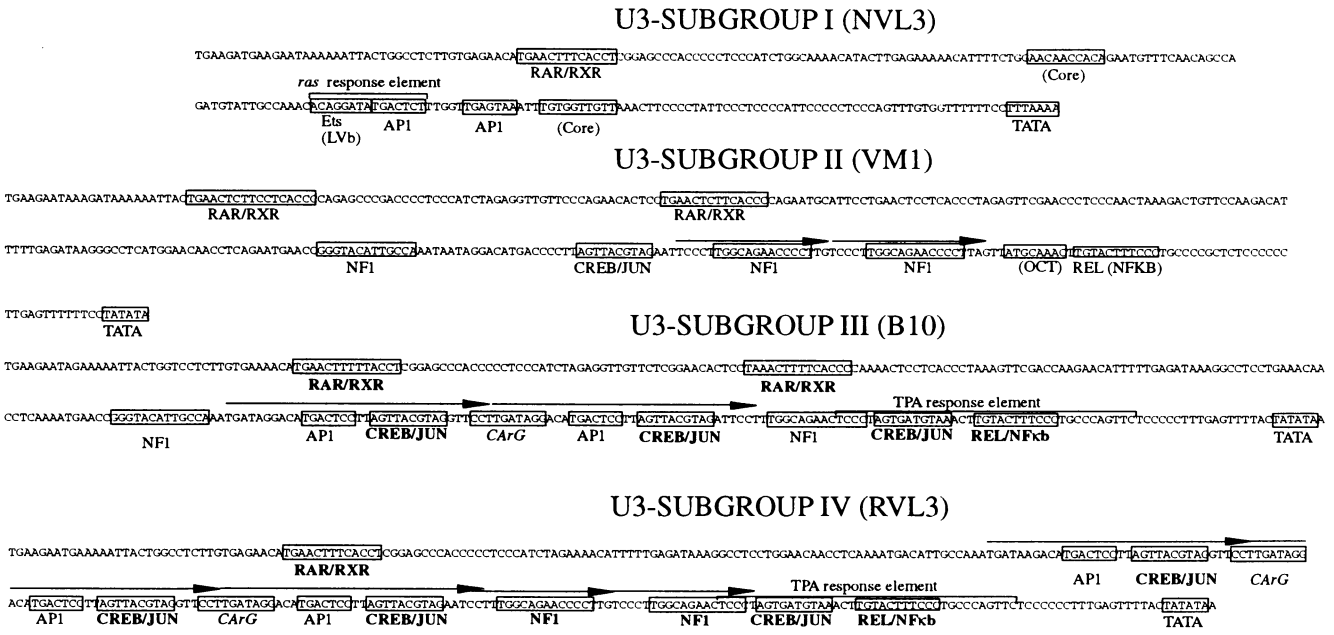

FIG. 10. Enhancer design of the distinct U3 subgroups showing representative U3 regions corresponding to each U3 subgroup. Binding sites for nuclear proteins, which are conserved within each U3 subgroup, are enclosed (boxes). The origins of the binding sites chosen are given in Results. The direct repeat regions in U3III and U3IV are indicated with arrows. Known binding activities such as RAR/RXR, CREB/Jun, and c-Rel/p65(NF-κB) are indicated in boldface letters. Identified response elements such as the CArG box and the Ha-*ras* oncogene response element are indicated in italics. Binding activities to known binding sequences identified by competition experiments are indicated, and putative binding sites which are conserved in a U3-specific manner are indicated in parentheses.

the core sequence, and a consensus OCT sequence (Fig. 10) (20, 35, 70, 71).

DISCUSSION

One characteristic feature of VL30 retrotransposons is the remarkable inducibility of their expression by a wide range of agents and physiological stimuli. One possibility is that this feature is determined by the structural diversity of VL30 U3 sequences. In this work, we show that a homology study of 17 sequenced VL30 LTRs reveals one interesting observation: it is possible to organize VL30 retrotransposons into four distinct U3 subgroups (U3I, U3II, U3II, and U3IV). A sequence alignment of all groups reveals that each U3 subgroup contains different variants and/or a combination of response elements known to regulate VL30 transcription. We have previously identified response elements that determine cell type-specific VL30 transcription in response to retinoic acid in keratinocytes as well as cAMP-induced transcription in steroidogenic adrenal cells (7, 32). On the basis of these findings, we have addressed the question of whether the presence or absence of specific transcription factor-binding sites within distinct U3 subgroups correlates with their expression in different mouse tissues. In situ hybridization of mouse skin reveals that keratinocytes in epidermis, but not dermal fibroblasts, show an increase in VL30 expression in response to TPA treatment. Previously, we have identified a 39-bp sequence, designated VLTRE, which mediates TPA-induced VL30 transcription in keratinocytes. We report here that VLTRE is conserved within the U3 regions of three different VL30 LTRs identified among cDNA clones corresponding to genes induced in mouse epidermis by TPA (8). The fact that all sequences regulating transcription of retrotransposons are contained in the transcript provides the opportunity to use short oligonucleotides specific for each U3 subgroup as probes to study the regulation of expression of individual U3 subgroups by Northern blot analysis. By this strategy, we have found that VL30 expression corresponding to U3III and U3IV is TPA inducible in mouse epidermis, whereas both U3I and U3II are silent. Interestingly, both U3III and U3IV contain a conserved VLTRE, whereas the corresponding positions in nonresponsive subgroups U3I and U3II have a core motif and an OCT motif, respectively, which also is a specific feature of their U3 subgroup. By transient transfection analysis, VLTRE was shown to mediate induced transcription in response to TPA in cultured keratinocytes, whereas it is inactive as a TPA-responsive element in a fibroblast cell line. By in vitro binding analyses, we have identified two nuclear factors in keratinocytes that show TPA-induced complex formation with a sequence (VLY) present within the VLTRE. One of these DNA-protein complexes is recognized by both c-Rel- and p65(NF-κB)-specific antibodies, while the other complex is recognized only by the p65(NF-κB)-specific antibody. Correlating with the nonresponsive phenotype of fibroblasts, TPA-induced binding to VLY is not detected in nuclear extracts made from this cell type. In fact, we have not detected any proteins related to c-Rel and p65 binding to VLY in fibroblast extracts. TPA-induced binding activity of a p65-containing complex can, however, be detected in extracts from both cell types by using the NF-κB-binding site present in the κ light chain enhancer as a probe. Most notably, the c-Rel/p65-containing complex is not formed with the κB sequence as probe. Furthermore, the differential appearance of two p65-containing complexes (B and B′), produced by the use of keratinocyte and fibroblast extracts, indicates that VLY and the classical NF-κB-binding site are specifically recognized by two different p65-containing factors lacking c-Rel. The

functional studies indicate that the VLY sequence alone is insufficient to mediate a full response, as is a juxtaposed sequence, VLX, which is a target for constitutive binding of proteins belonging to the CREB and Jun families of transcription factors. It is thus possible that TPA-induced transcription specific for keratinocytes includes cell type-specific activation and/or expression of factors within the c-Rel/NF-κB family of transcription factors as well as cell type-specific constraints imposed by attractive or repulsive interactions with CREB- and Jun-related proteins binding to the juxtaposed VLX site.

Since it is generally believed that the VL30 expression level is high in all tissues, we wanted to test the possibility that VL30 expression appears ubiquitous when expression is analyzed with a probe recognizing all VL30 members but cell type specific when U3-specific probes are used. Indeed, this pattern of VL30 expression is evident in Northern blot analyses of RNA isolated from eight different mouse tissues.

U3I is found to be specifically expressed in spleen. One VL30 element within U3I that is known to be expressed (element 1.5) has been isolated among genes specifically induced by cAMP and glucocorticoids in T cells (27). Specific to this U3 subgroup are conserved LVb and core motifs which have been shown to mediate T cell-specific expression of the Moloney murine leukemia virus (70, 71). These results indicate that U3I might be expressed in the T-cell population of spleen. Another VL30 member within U3I is NVL3, which is expressed in NIH 3T3 fibroblasts and has been used to study induced transcription in response to an activated Ha-*ras* oncogene and TPA in these cells (50). The correlation between Ha-Ras/TPA-induced transcription in fibroblasts and the high level of constitutive expression in spleen tissue remains to be determined. However, it is interesting that both the LVb and the core motifs in the Moloney murine leukemia virus LTR are essential for TPA-induced transcription in Jurkat T cells (71).

Members within U3II have not been reported to be expressed, and accordingly no expression of these U3 regions is found in the tissues examined in this study. The fact that all tissues (except epidermis) have been taken from the BALB strain of mice and that the members of U3 subgroup II are cloned from genomic libraries made from the same strain argues against the possibility that a mouse strain-specific difference in the existence of genomic U3II members can explain the lack of expression. From the structural analysis of U3 regions within this subgroup, it is evident that many transcriptional regulatory sequence motifs are present, such as a TATA box, RAR/RXR-, CREB/Jun-, NF1-, and c-*rel*/NF-κB-binding sites. We therefore favor the hypothesis that members of this U3 subgroup are transcriptionally competent, but it remains to be determined in which cell type and/or by which intracellular signal pathway their expression is regulated.

Schiff et al. have used the method of in situ hybridization to analyze the distribution of VL30 expression in mice from late embryonic stages (65). In their study, it was shown that the level of VL30 expression is high in steroidogenic tissues and detectable in the epithelial tissue layers of most internal organs. The only U3 regions within U3III that have been shown to be expressed are the ones described in this study. The fact that these U3 regions correspond to VL30 elements expressed in epithelial tissue (epidermis) suggests that the relatively low level of constitutive expression of U3III detectable in most organs may arise from expression in the epithelial layers of these organs. However, the highest relative level of expression of U3III is detected in lung and expression of both U3III and U3IV is detected in the testis, which is a steroidogenic organ. Members within U3IV have been isolated from steroidogenic cells, and we have used B10 (belonging to U3III) to identify sequence motifs that mediate steroidogenic cell-specific transcription. U3III expression is found to be relatively weak in testes. However, it should be noted that high levels of VL30 expression in Leydig cells of testes can be detected after administration of human chorionic gonadotropin, a treatment mimicking a luteinizing hormone stimulation (65).

Constitutive high levels of U3IV expression are detected specifically in cardiac and skeletal muscles. The LTRs corresponding to this U3 subgroup may therefore be useful in the elucidation of transcriptional regulatory mechanisms specific for muscle genes in both skeletal and cardiac muscle cells. In this respect, it is intriguing that U3IV specifically contains two copies of the CArG element, which has been shown to be one element involved in muscle-specific gene regulation (45, 53, 64). The finding that U3 subgroup IV shows a higher relative level of expression in heart than in skeletal muscle and the identification of a heart-specific 5.6-kb VL30 transcript are observations that can be explored in studies concerning differences in the transcriptional regulation between skeletal muscle and cardiac muscle cells. The U3IV subgroup has also been shown to respond to cAMP, TPA, epidermal growth factor, and Ca^{2+} agonist-induced transcription in cell types other than muscle cells (39, 65). One possibility is that cardiac cells have constitutive activation of a particular signal pathway which is utilized for inducible gene transcription in other cell types.

The 100 to 200 VL30 members are dispersed in the mouse genome (11, 36, 37). It is therefore likely that mechanisms other than *trans* repression and/or *trans* activation contribute to the individual patterns of constitutive expression in different mouse tissues. The lack of detectable VL30 expression in brain might be an indication of such a *cis*-acting regulatory mechanism. However, it is possible that the fraction of VL30 elements which is present in a transcriptionally accessible chromatin configuration in a given situation is only transcribed when it contains the appropriate transcription factor binding sites which are utilized in that particular cell type.

The high level of VL30 expression in kidney that can be detected with a probe recognizing all VL30 members and the relatively low level of VL30 expression in this organ detectable with the U3-specific probes argue for the existence of additional U3 subgroups. Another indication of this possibility is the finding that the U3 region of VLov1 shows the highest sequence similarity to members of U3IV but to a lesser degree (<80%) than the other members within this U3 subgroup.

Retroviral recombination in general is thought to require prior transcription of the virus and packaging of the respective RNA molecules (30, 73). Thus, transcriptional activation and subsequent packaging are prerequisites and the driving force for recombinogenic activity. Of the known retrotransposon families, VL30s are the only ones which so far have been shown to be packageable and hence rescuable as C-type pseudoviruses (37). The relatively high number of VL30 family members having variable U3 regions is therefore most likely a consequence of a high rate of recombination, errors during replication, and transposition into the germ line (37). One established theory is that a DNA entity which is successful in replicating itself could stay in the genome as a "selfish" DNA (18, 49, 63). VL30 members may therefore prosper in the mouse genome by retaining and/or acquiring specific binding sites for transcription factors, or combinations thereof. We present results here that strongly support the conclusion that VL30 members are transcribed independently in a stimulus- and cell type-specific manner and that the regulation of VL30 transcription is dependent on both the presence of specific *cis* elements within the U3 region and cell type-specific differences in the activity of transacting factors. Thus, the close association

between transcription and recombination, the existence of U3 with variations in transcription factor binding sites, and the expression of these in a cell type- and stimulus-specific manner implies a novel approach to the study of cell type-specific transcription by comparing expressed U3 regions with other U3 regions that are not affected by the particular transcriptional regulatory mechanism of interest.

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