The Long Terminal Repeat of VL30 Retrotransposons Contains Sequences That Determine Retinoic Acid-induced Transcription in Cultured Keratinocytes*

(Received for publication, July 13, 1992)

Tahmina Choudhuri Islam, Thomas Henrik Bugge‡, and Staffan Bohm§

From the Center for Biotechnology, NOVUM, Karolinska Institute, S-111 57 Huddinge, Sweden and ‡European Molecular Biology Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

The characterization of retinoic acid (RA)-regulated gene transcription in keratinocytes has important implications as to the function of retinoids in epidermal homeostasis and to the central role retinoids play in the pharmacotherapy of a variety of skin disorders. We show that cultured mouse keratinocytes (Balb/MK) responded to RA with induced expression of VL30 retrotransposons. The induction was rapid, was present at nanomolar concentrations of RA, was independent of new protein synthesis, and occurred both in proliferating and differentiated keratinocytes. The long terminal repeat of a VL30 retrotransposon, expressed in mouse epidermis in vivo, was found to contain two RA-responsive elements (RREs) that independently conferred RA responsiveness on both cultured Balb/MK cells and normal human keratinocytes. Functional characterization and in vitro binding analysis showed that the sequence requirement for binding of retinoid X receptor α (RXRα) and retinoic acid receptor (RAR), either α, β, or γ, together with the consensus sequence requirement for RA-induced transcription in keratinocytes. The VL30 RREs differed functionally from the RA response element present in a VL30 promoter in both cultured Balb/MK cells and normal human keratinocytes. Functional characterization and in vitro binding analysis showed that the sequence requirement for binding of retinoid X receptor α (RXRα) and retinoic acid receptor (RAR), either α, β, or γ, together with the consensus sequence requirement for RA-induced transcription in keratinocytes. The VL30 RREs differed functionally from the RA response element present in the RAR-β2 promoter, in that the VL30 RREs were non-responsive in fibroblasts cultured from human skin. The non-responsive nature has been confirmed by a reduced complex formation between a VL30 RRE and endogenously expressed nuclear factors present in skin fibroblasts. The data suggest that a direct repeat of two half-sites, spaced by two base pairs, with the consensus sequence T(A/G)AACTTTT(T/C)ACC(T/C), bound RAR-RXR heterodimers and mediated, at constitutive receptor levels, a primary RA response on gene transcription specifically in keratinocytes.

Vitamin A (retinol) and its structural analogs (retinoids) are important regulators of a variety of physiological processes, including cell growth and differentiation, tissue development, and cell type-specific gene expression (1-7). These agents have gained significant importance in dermatology for their usefulness in the treatment of many skin diseases, particularly acne, psoriasis, various hyperkeratotic disorders, and certain malignancies (8, 9). The isolation and further characterization of nuclear receptors for all-trans-retinoic acid (RA) and 9-cis-RA have contributed significantly to the understanding of transcriptional regulation by retinoids. At least three different nuclear RA receptors (RAR α, β, and γ) as well as three different 9-cis-RA receptors (RXXR α, β, and γ) have been found (5, 10-13). These receptors exhibit a variable degree of tissue-specific expression and regulate transcription through binding to short cis-acting sequences in their target genes. Several naturally occurring hormone-responsive elements have been shown to contain a direct repeat of a motif with the consensus sequence T(A/C)(A/C)(T/C) (5). The orientation, spacing, and sequence composition of these half-sites dictate the transcriptional regulation mediated by RA, vitamin D, and thyroid hormones (14-16). Recent findings demonstrating that RXXRs can form stable heterodimers not only with RARs but also with vitamin D, thyroid hormone receptors, and the orphan receptor COUP-TF have provided a mechanism for combinatorial diversity in the regulation of RA-responsive genes (11, 17-21). The degree of occupancy of a RA-responsive element in a particular cell type is thus a complex function dependent on the cellular concentrations of RAR, RXR, and other receptors as well as the precise sequence composition of the binding site. The expression of certain proviruses is regulated by hormones. The mouse mammary tumor virus long terminal repeat (LTR) has been extensively studied as a model of a transcriptional unit regulated by steroid hormones (22). A thyroid hormone response element has been identified in the Moloney murine leukemia virus and it was recently reported that the expression of an endogenous retroviral transcript was induced by RA in a human teratocarcinoma-derived cell line (23, 24). We have previously isolated several members of a retrotransposon family (VL30) expressed in mouse epidermis (25). VL30 retrotransposons are like retroviruses flanked by LTRs that contain the sequence information for their transcriptional regulation (26). During the characterization of VL30 expression, we found that topical application of RA caused an increased expression of VL30 RNA in epidermis of mice.*

The abbreviations used are: RA, retinoic acid; RRE, retinoic response element; VLRRRE, VL30 retinoic response element; RXR, retinoic acid receptor; EGF, epidermal growth factor; FCS, fetal calf serum; CAL, chlamydomenoc acid acetyltransferase; bp, base pair(s); TK, thymidine kinase; LTR, long terminal repeat; Luc, luciferase; RSV, Rous sarcoma virus; GRE, glucocorticoid response element.

*This work was supported by the Swedish Work Health Fund (Project 88-0162) and the Swedish Cancer Research Society (Project 0161-D91-01XAD). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M76549.

‡To whom correspondence should be addressed: Karolinska Inst., Center for Biotechnology, NOVUM, Blickgången 6, S-141 57 Huddinge, Sweden. Tel.: 46-8-608-9151; Fax: 46-8-774-5538.
the HRs/J mouse strain. RA influences the cellular homeostasis and the patterns of gene expression in skin (3, 27), and it has recently been shown that the control of keratinocyte differentiation by RA is consistent with the interaction of the retinoid with RA and the regulation of transcription by that ligand-receptor complex (28). Responsive elements that bind RAR/RXR and mediate RA-dependent gene transcription in epidermal keratinocytes will be very useful in the study of how specificity of transcription in response to RA can be maintained in epidermis and is effected by keratinocyte growth and differentiation. Although a few genes expressed in keratinocytes are known to respond to RA (6, 29), no identification of natural RREs mediating a RA response on gene transcription in keratinocytes has previously been reported. We present the characterization of a cell type-specific and primary RA response on gene transcription in keratinocytes that is mediated through two novel RAR/RXR binding sequences present in the long terminal repeat (LTR) of a VL30 retrotransposon expressed in mouse epidermis.

MATERIALS AND METHODS

Cell Culture Conditions. All chemicals, media, and growth factors were purchased from Sigma, unless stated otherwise. Estradiol-17β-stimulated VL30 expression was analyzed in Balb/MK cells grown in Eagle’s minimal essential medium without Ca2+ (GIBCO/BRL), containing 8% (v/v) fetal calf serum (FCS, HyClone), 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 mg/ml fungizone (GIBCO/BRL), and 5 µg/ml dexamethasone (DMSO). Cells were maintained in 125 cm2 non-exchangeable resins (Bio-Rad) to deplete divalent cations, resulting in a final Ca2+ concentration in the medium of 0.02—0.05 mM. Transient transfection studies were done using Balb/MK cells cultivated in MCDB 153 medium, supplemented with 50 µM CaCl2, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 0.5% Chelex-processed FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 mg/ml fungizone. Keratinocytes were isolated and cultivated from adult donors as described previously (30). To allow for maximal growth, the cells were grown in MCDB 153 medium, supplemented with 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.4 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 100 µg/ml bovine pituitary extract (FCS, HyClone), 100 IU/ml streptomycin, 100 IU/ml penicillin (Nordcell), and 0.25 µg/ml fungizone (Nordcell). Fibroblasts were grown from dermal explants in Dulbecco’s modified Eagle’s medium (GIBCO/BRL) supplemented with 10% fetal calf serum (GIBCO/BRL), 100 µg/ml streptomycin, 100 IU/ml penicillin, and 0.25 µg/ml fungizone.

RNA Analysis. Total RNA was isolated from confluent Balb/MK cells by guanidium thiocyanate-phenol-chloroform extraction according to Chomczynski et al. (31). Total RNA (5 µg/lane) was fractionated through the Tris-glycine-agarose gels, blotted onto nylon membranes, and UV cross-linked; filters were subsequently prehybridized, hybridized, and washed according to standard procedures (32) prior to autoradiography.

Reporter Plasmid Constructs. All enzyme reactions were performed according to standard procedures (33). The sequence of the B10 U3 region, the identification of the transcriptional start and the construction of the different B10 CAT reporter plasmids, shown in Fig. 2, have been described elsewhere (33). pT109 Luc contained the −109 to +51 promoter region of the herpes simplex thymidine kinase (TK) gene fused to a luciferase (Luc) reporter gene (34). The VLRE1Luc, VLRE2Luc, M1Luc, M2Luc, M3Luc, M4Luc, and M5Luc constructs were made by cloning the corresponding oligonucleotides into the HincII-Xhol site of plasmid pT109. The 2XVLRELuc was made by cloning the VLRE2 oligonucleotide, in reverse orientation, into the Xhol site of the VLRE1Luc construct. The TK1Luc and TK2Luc contained the LTR sequences −217 to −187 and −200 to −172, respectively. The Xhol-Stul (−301 to −215) and the Rsal-Rsal (−183 to −71) restriction enzyme fragments of the B10 U3 region were cloned into the Smal site of pT109 to create the plasmids named TK-XS and TK-RR, respectively. The plasmids were constructed by sequence analysis. All plasmids were prepared by alkaline lysis, treated with RNase, and purified using Qiagen columns (Diagen, Düsseldorf, Germany).

Transient Transfection Assays. Preconfluent cells grown on 10-cm2 culture dishes were transiently transfected, using cationic liposomes (Lipofectin, Bethesda Research Laboratories), with 2.5 µg of reporter plasmid DNA and 0.5 µg of either pSV2.CAT (35) or pSV2.Luc (36) as internal positive controls. After 18 h the cells were washed free from Lipofectin, starved for EGF for 3 h, and treated for 16 h with 3 µM RA. Chloramphenicol acetyltransferase assays or luciferase assays were performed as described previously (32).

Preparation of Nuclear Extracts and Gel Shift Assay. Synthetic oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer (Foster City, CA). The double-stranded oligonucleotides used as probes were labeled with [α-32P]dATP or [α-32P]dCTP (Amersham Corp.) by Klenow polymerase and purified by electrophoresis through a 12% polyacrylamide gel (29:1). Whole cell extracts from HeLa cells infected with recombinant vaccinia virus expressing human RARα, β, γ, and RXRα were prepared by lysis using 20 µg on ice in 50 mM Hepes, pH 7.9, 450 mM NaCl, 0.5% Triton X-100, and 1 mM dithiothreitol followed by centrifugation. Nuclear HeLa cell extract was incubated in a binding buffer containing 15% glycerol, 20 mM HEPES, pH 7.9, 5 mM MgCl2, 50 mM KCl, poly(dI-dC) (0.2 mg/ml final concentration), and 0.01% Triton X-100 for 15 min on ice. Nuclear Balb/MK and human fibroblast extracts were prepared according to Struhl et al. (36) and incubated as above in 10% glycerol, 20 mM HEPES, pH 7.9, 5 mM MgCl2, 50 mM KCl, 0.25 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 units/ml aprotinin, 0.2 mg/ml poly(dI-dC), 0.2 mg/ml poly(dA-dT) (Pharmacia). The protein-DNA complexes formed during the bandshift reactions were separated on precooled, preelectrophoresed (1 h, 200 V), 6% polyacrylamide gels using 0.25 X TBE (1 X TBE, 90 mM Tris borate and 1 mM EDTA) as the running buffer at 4°C at 200 V.

RESULTS

RA Induces VL30 Expression in Balb/MK. Initially experiments were performed to determine whether the mouse keratinocyte cell line Balb/MK (37) constitutes a suitable model to study RA-dependent regulation of VL30 gene expression. These cells are non-tumorigenic, dependent on EGF for growth, and differentiate terminally in response to an extracellular Ca2+ concentration greater than 1 mM. Northern blot analyzes were performed using total cellular RNA from ethanol- (vehicle) and RA-treated confluent Balb/MK cells (Fig. 1). VL30 expression was found to be significantly induced by RA, as quantified relative to Northern blot signals generated by a probe recognizing mRNA corresponding to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Fig. 1A shows that the increased VL30 expression detected 5 h after RA treatment was not inhibited by a 1-h pretreatment with the protein synthesis inhibitor cycloheximide. Cycloheximide treatment alone resulted in induced VL30 expression and cycloheximide potentiated the RA induction. This result indicated that VL30 retrotransposons are primary RA response genes in keratinocytes. Fig. 1A also shows that a 30-min pretreatment with the transcriptional inhibitor actinomycin D resulted in abolishment of RA induction, whereas the constitutive VL30 RNA levels were unaffected by the same treatment. This suggested that RA-induced VL30 expression was a consequence of increased transcription. Fig. 1B and C shows the time kinetics and dose-response of RA-induced VL30 expression in Balb/MK cells, respectively. Induced VL30 expression was detected at nanomolar concentrations of RA (Fig. 1C), and the RNA level was increased at 1 h after the administration of RA (Fig. 1B). Additional increases in the VL30 mRNA levels were detected up to the last time point analyzed (16 h). To determine whether the RA response was altered in differentiated keratinocytes, the cells were induced to differentiate by raising the extracellular Ca2+ concentration to 1.5 mM for 48 h before the RA treatment. As shown in Fig. 1B, the differentiated keratinocytes

2 T. Islam and S. Bohm, unpublished observation.
Identification of Two VL30 Retinoic Acid-responsive Elements (VLRREs) That Conferred RA Responsiveness on a Heterologous Promoter—To further pinpoint the sequences of functional significance we cloned different parts of the LTR upstream (~109) to a herpes simplex virus thymidine kinase (TK) promoter driving the luciferase reporter gene (pT109, Ref. 34) and tested the constructs for RA inducibility by transfection into Balb/MK cells. Fig. 3A shows a schematic illustration of these plasmids (named RR.Luc, TK1.Luc, TK2.Luc, XS.Luc, and VLRRER1.Luc) and their relative capacity to direct RA-induced transcription. As expected from the results presented above none of the sequences present 3' to the StuI site mediated any significant RA response on transcription (RR.Luc, TK1.Luc, and TK2.Luc), whereas a 27-bp sequence present 5' to the Xbal site mediated a 13-fold induction (VLRRER1.Luc, Fig. 3). In addition, the Xbal-StuI fragment present in the XS.Luc construct did also mediate RA-induced transcription, although to a somewhat lesser extent (8-fold). These results confirmed the presence of two VL30 Retinoic acid Responsive Elements (VLRREs) within the VL30 LTR. The 5'-VLRRER (VLRRER1) is present within the sequence, TTGTGAAAACATGAACATTACCT, and the 3'-VLRRER (VLRRER2) is present within the 84-bp Xbal-StuI fragment.

Mutational Analysis of VLRRER1.Luc Indicated That the Presence of Two Half-sites Were Important for Functionality—Given the functional importance of the 27-bp VL30 sequence present in the VLRRER1.Luc construct, it was searched for DNA elements similar to the consensus half-site TGA(T/C), known to be important for RA-induced transcription of other genes in other cell types. The VLRRER1 sequence contained three putative half-sites: one perfect match, TGAAC(T/C), as well as two imperfect matches, TGAAA and TTACCT, respectively. To further characterize the sequence requirement for the induction response, we synthesized oligonucleotides containing nucleotide substitutions through the sequence comprising VLRRER1 (Fig. 4, M1–M5). These “mutated” variants of VLRRER1 were cloned upstream of the TK promoter, and assayed for their capacity to mediate RA induction. Mutations in the central TGAAC(T/C) motif (M4) or in the 3'-TACCT motif (M5) abolished RA-induced luciferase activity, while mutating sequences immediately 5' to the TGAAC(T/C) motif (M1 and M2) or the 5'-TGAAC(T/C) motif (M3) resulted in no or moderate effects on RA inducibility. This mutational analysis indicated that two half-sites, TGAAC(T/C) and TTACCT, spaced by 2 bp, are required for RA-dependent transactivation of VL30 transcription. The A to G substitution present in mutant M2 was made since several previously isolated VL30 LTRs contain the resulting sequence, AGAACA, juxtaposed to one of the VL30 RRE half-sites. The presence of this glucocorticoid response element half-site did not affect RA-induced transcription in the culture conditions used in this study.

A computer-based search for sequence similarities between the VLRRER1 sequence TGAACCTTTAACC and the entire VL30 LTR sequence revealed the highest sequence similarity to a sequence, TAAACCTTACCC, present within the RA-responsive Xbal-StuI fragment. To test the possibility that this sequence alone mediated RA-induced transcription, an oligonucleotide corresponding to this sequence (VLRRER2, shown in Fig. 3B) was fused to the pT109 luciferase plasmid and assayed for its ability to mediate RA-induced transcription. Fig. 3B shows that this construct, VLRRER2.Luc, was able to mediate a significant RA-induced transcription (4-5-fold). Table I shows a sequence alignment of the identified VL30 RREs to two previously characterized RREs present in
Retinoic Acid-induced VL30 Transcription

FIG. 2. Mapping of RA-responsive regions within the VL30 LTR. Shown is the schematic representation of the different B10 VL30 LTR-driven CAT constructs and their capacity to direct RA-induced transcription in Balb/MK cells. The construction of these plasmids have been described elsewhere (33). The B10.U3 construct contains a full-length (375 bp) U3 region (hatched area) and the first open reading frame in the R region cloned in frame with the CAT gene in a promoterless vector. Transcription start site is indicated by +1 (33). Deletions in the LTR were made by digestion with restriction enzymes indicated as in the figure (Xb, XbaI, −301; St, Stu, −215; Sna, SnaBI, −122). Shown is also a representative autoradiograph of CAT activity in untreated (C) and RA-treated (R) Balb/MK cells. Transient transfection and treatment were done as described under "Materials and Methods." Protein aliquots were assayed for CAT activity with the amounts adjusted with respect to the relative transfection efficiencies, as determined by luciferase activity of cotransfected pRSVLuc vector. The results of four independent experiments showed similar results. Indicated are percent CAT conversion and -fold induction (F. I.).

FIG. 3. Identification of short sequences (VLRRE1 and VLRRE2) mediating RA-induced transcription to a heterologous promoter. Shown is a schematic representation of a number of reporter plasmids containing different parts of the VL30 LTR cloned upstream (−109) to the herpes simplex TK promoter in a luciferase reporter gene plasmid (pT109). The 5′ and 3′ ends of the LTR fragments relative to the transcriptional start (33) are indicated by numbers. The bars in the figure represent mean values of the relative -fold induction calculated from the luciferase activity obtained from each plasmid in untreated and RA-treated transiently transfected Balb/MK cells. A, identification of VLRRE1; B, identification of VLRRE2. Aliquots of cellular extracts from untreated and RA-treated cells were taken for luciferase assays with the amounts adjusted with respect to the relative transfection efficiencies as determined by the CAT activity obtained with a cotransfected pRSV2CAT vector. The different TK promoter-driven plasmids and the parental pT109 plasmid, containing the TK promoter only, were analyzed in parallel. The -fold induction in each experiment was calculated relative to the activity of pT109 (indicated with a horizontal line). The mean values of relative -fold induction and the standard deviations of mean obtained in five independent experiments are shown.

the genes for apolipoprotein A1 and mouse cellular retinol-binding protein type I (7, 38). Also shown are the base pairs deviating from the proposed consensus half-site RRE sequence (5).

The deletion mapping described above (Fig. 2) indicated that the VLRRE1 and the XbaI-StuI fragment together mediate RA-induced transcription in an additive way. One additional plasmid construct was made to test if this also was true for VLRRE1 and VLRRE2 when placed in a heterologous promoter context. This plasmid, designated sXVLRRE.Luc, was made by cloning one copy of VLRRE2 immediately 3′ to the VLRRE1 in the VLRRE1.Luc plasmid. The VLRRE2 was
variants of VLRRE1 calculated from the luciferase activity obtained from each plasmid in lower case letters and arrows indicate nucleotide substitutions and untreated and RA-treated transiently transfected Balb/MK cells. The -fold induction relative to a reporter plasmid containing the TK promoter alone (pT109) was analyzed in three independent experiments and found to be as follows: VLRRE1.Luc, 0.7 ± 0.3; VLRRE2.Luc, 0.9 ± 0.4; 2XVLRRE.Luc, 1.1 ± 0.2. VLRRE1 and VLRRE2 Are Binding Sites for RXR-RAR Heterodimers—Next we wanted to test whether VLRRE1 and VLRRE2 specifically bound nuclear RA receptors. We employed band shift analysis using the VLRRE1 and VLRRE2 as probes in binding reactions containing nuclear extracts from HeLa cells infected with recombinant vaccinia virus expressing either RARα or RXRa. Fig. 5A shows that RARα and RXRa alone did not demonstrate appreciable binding to VLRRE1 and VLRRE2, whereas a strong enhancement of binding to both VLRREs were observed when using mixed extracts, containing both RARα and RXRa. Thus, effective binding of RAR to the VLRREs requires the presence of an auxiliary receptor like RXRa.

Subsequently we tested if VLRRE1 bound RARα and RXRa in a fashion that correlated with functionality. The different mutated VLRRE1 variants (Fig. 4A, M1–M5) were used as probes in binding reactions containing RARα and RXRa. Fig. 5B shows that the sequences mediating RA-induced transcription, VLRRE1, M1, M2, and M3, also formed DNA-heterodimer complexes, whereas the non-functional sequences, M4 and M5, were unable to form such complexes. This showed that the sequence requirement for in vitro binding correlated with those required for RA-induced transcriptional activation in vivo and thus imply a functional role for RAR-RXR heterodimers in RA-dependent VLR30 transcription in keratinocytes.

Since RARγ is specifically expressed in skin (10, 29) we wanted to test the possibility if VLRRE1 bound RARγ homodimers and/or RARγ-RXRa heterodimers with different efficiency compared to RARα, RARβ homodimers, and/or RARα, RARβ receptors in heterodimers with RXRa. Fig. 5C shows gel shift analyses using nuclear extracts from HeLa cells infected with recombinant vaccinia virus expressing either RARα, RARβ, RARγ, or RXRa. No apparent differences were found in the relative amount of complexes formed between VLRRE1 and the different RAR homodimers and RAR-RXRa heterodimers. A similar pattern of complex formation was observed when RREβ was used as probe (Fig. 5C). Based on previous reports we interpreted the appearance of the faint shifts in RAR extracts to be a consequence of heterodimerization of RAR with endogenous RXR present in the HeLa cells (11, 17). All RAR/RXR complexes detected with the VLR30 RREs and RREβ probes were formed in a sequence-specific manner as judged by the ability of a 30-fold molar excess of unlabeled RREs to compete for binding, whereas an unrelated nucleotide sequence used in the same molar excess did not affect the complexes formed (data not shown).

Efficiency of RA to Induce Transcription via VLRREs and RREβ Differ in Normal Human Skin Fibroblasts and Keratinocytes—To examine the possibility that the RA response mediated by VLRRE was cell type-dependent, we transfected

![Fig. 4. Mutational analysis of the VLRRE1. Shown are the sequences of oligonucleotides corresponding to VLRRE1, mutated variants of VLRRE1 (M1–M5) and the RRE present in the human RARE promoter (44) cloned upstream of pT109) to the herpes simplex TK promoter in a luciferase reporter gene plasmid (pT109). The bars in the figure represent mean values of the relative -fold induction calculated from the luciferase activity obtained from each plasmid in untreated and RA-treated transiently transfected Balb/MK cells. Lowercase letters and arrows indicate nucleotide substitutions and putative RRE half-sites, respectively. A putative GRE half-site is indicated with a dotted arrow. Aliquots of cellular extracts from untreated and RA-treated cells were taken for luciferase assays with the parental pT109 plasmid, containing the TK promoter alone (pT109) were used as controls. The -fold induction in each experiment were calculated relative to the activity of pT109 (indicated with a horizontal line). The mean values of relative -fold induction and the standard deviations of mean obtained in five independent experiments are shown.](image-url)
Human skin fibroblasts

Human epidermal keratinocytes

FIG. 5. The VL30 RREs form specific complexes with RAR/RXR. Gel shift analysis were performed using nuclear extracts prepared from HeLa cells infected with wild type (WT) or recombinant vaccinia virus expressing either different RAR isotypes or RXRα. The arrows denote the specific complex and the sequences of the oligonucleotides used as probes are shown in Figs. 3 and 4. A, autoradiographs of a gel shift experiment using VLRE1 and VLRE2 as probes in vaccinia virus extracts containing RARα and/or RXRα, for sequences see Fig. 3, A and B). B, autoradiographs of a gel shift experiment using the mutated VLRE1 variants as probes in vaccinia virus extracts containing RARα and/or RXRα (M1 and M2; sequences are shown in Fig. 4). C, autoradiographs of a gel shift experiment using VLRE1 or RREβ as probes in vaccinia virus extracts containing different RAR isotypes (RARα, RARβ, or RARγ) and/or RXRα (for sequences, see Fig. 4).

FIG. 6. VL30 RREs mediate RA-induced transcription in human keratinocytes but not in human skin fibroblasts. Cells were transfected with VLRE1.Luc, VLRE2.Luc, RRE6.Luc (for sequences see Figs. 3 and 4), and the parental plasmid, pT109.Luc, containing the TK promoter only. Transfections and RA treatment were as described under “Materials and Methods.” Aliquots of cellular extracts from untreated and RA-treated cells were taken for luciferase assays with the amounts normalized according to the protein concentration of each cell extract. The bars in the figure represent mean value of relative -fold induction obtained in three independent experiments. The standard deviation from the mean is also indicated.

normal human foreskin fibroblasts with the plasmids, VLRE1.Luc, VLRE2.Luc, and RRE6.Luc. Fig. 6 shows that the latter construct, RRE6.Luc, did mediate approximately an 10-fold induction of luciferase activity, whereas both VLRE1.Luc and VLRE2.Luc were found to be inactive as RREs in human skin fibroblasts. In parallel experiments normal human keratinocytes were used as recipient cells in transfection studies. In these cells, RA responsiveness mediated through VL30 RREs was cell type-dependent and occurred in normal epidermal keratinocytes but not in normal dermal fibroblasts. However, VL30 RREs were found to be RA-responsive in NIH3T3 fibroblasts (data not shown), indicating that RA-induced transactivation mediated through the identified RREs could occur in embryonically derived cells of a non-epithelial origin.

In a separate series of experiments, we also analyzed the fold induction mediated by 2XVLRE2.Luc, which contained one copy of each VL30 RRE. No significant induction was mediated by this construct in human skin fibroblasts when analyzed in four independent experiments (fold induction, 1.2 ± 0.3).

Identification of Endogenous Nuclear Proteins Binding to VLREs—We employed gel shift analyses using nuclear extracts prepared from Balb/MK cells to identify specific interactions of VLRE with endogenous proteins. Two main complexes were formed with radioactively labeled VLRE1 containing RARα and/or RXRα (indicated by A and B in Fig. 7A). Both complexes formed were specific as judged by the ability of unlabeled VLRE1 to compete for binding, whereas an unrelated (GRE) oligonucleotide used in the same molar excess (100-fold) did not compete for binding. The formation of complex A was abolished by an excess of unlabeled oligonucleotides corresponding to either VLRE2 or RREβ. Competition with mutated VLRE1 variants M3 and M4 (see above) indicated that the sequence requirement for complex A formation correlate with the sequence requirement for RA-dependent transcriptional activation. This result suggested that complex A contained a nuclear protein involved in RA-induced transcription.
Arrows A, complexes with VL30 RREs and RREP,. Nuclear extracts (2.5 pg) were incubated with ^32P-labeled VLRRE1 and RREP, as probes. Balb/MK nuclear extract. A 100-fold molar excess of unlabeled oligonucleotide (a, b, and c) represent the specific DNA-protein complexes that are discussed in the text and the minus sign (−) indicates that no competitor was added in that incubation. A, shown is an autoradiograph of a gel shift analysis using the VLRRE1 as probe in Balb/MK nuclear extract. A 100-fold molar excess of unlabeled VLRRE1 and RREP, were found to be qualitatively similar in the two extracts. The RREP, probe formed a complex that migrated equivalent to the VLRRE1-complex A (discussed above), whereas both probes generated one additional complex in fibroblast extracts (designated C in Fig. 7B). The relative ratio of the signal intensities of the complexes formed in the different extracts was found to be dependent on the type of probe used. In fibroblast extracts, the amounts of complex A and C formed with VLRRE1 were lower than A and C formed with RREP, whereas in a parallel incubation using Balb/MK extracts, the same probes generated equivalent amounts of complex A. This result indicated that the non-responsiveness of VL30 RREs in fibroblasts correlated with relatively reduced complex formation between VLRRE and endogenously expressed nuclear factors present in skin fibroblasts.

Complex A formed with RREP, in Balb/MK extract was not affected by unlabeled VLRRE1, whereas unlabeled RREP, in the same molar excess completely abolished the formation of this complex. In the reverse experiment using labeled VLRRE1 as probe in Balb/MK extract, both unlabeled VLRRE1 and RREP, abolished the formation of complex A. A similar pattern of competition was observed for complex A and C formation in fibroblast extracts, except for some inhibition by unlabeled VLRRE1 of complex A formation when RREP, was used as probe. These cross-competition experiments indicated that VLRRE1 and RREP, have different affinity and/or specificity to endogenous factors present in Balb/MK and fibroblast extracts.

**DISCUSSION**

Present in this article is the identification and characterization of VL30 retrotransposon sequences mediating a primary cell type-specific RA response on gene transcription. Induced expression of endogenous VL30 RNA was detected 1 h after administration of RA, and the induction was found to correspond to a cycloheximide-resistant primary transcriptional event. The induction was detected at a nanomolar concentration, well within the range of RA concentrations required for trans-activation by RAR (0.5–50 nM) (39). These results indicate that RA-induced VL30 expression reflects an early and direct consequence of retinoid action rather than a secondary response due to changes in cellular proliferation and/or differentiation. These results also indicate that RA-induced VL30 expression occurs without the requirement of RA-induced RAR and RXR expression. Consistent with these findings, previous studies have shown that RA does not possess any mitogenic effect for quiescent Balb/MK cells (40) and that the expression of RAR (α, β, γ) and RXRα is not induced by RA in human epidermis (29, 41). Furthermore, we report similar time kinetics and magnitude of VL30 induction in both proliferating and differentiated keratinocytes. It has been shown that RA inhibits the transcription of differentiation-specific keratin genes in keratinocytes (3, 6). Since the time kinetics of this inhibition and the VL30 induction are similar, a comparative study of the RA-dependent transcriptional regulation of these genes might generate information about how RA can exert opposite effects on gene transcription in keratinocytes.

In order to identify sequences mediating the RA response, we used a LTR of a VL30 retrotransposon (B10) expressed in epidermis (25, 33). The U3 region, TATA box, and cap site from B10 could confer RA responsiveness on a reporter gene (CAT) in transiently transfected cells. Deletion analysis of
the B10 U3 region made it possible to limit the target sequence for RA action to two retinoic acid response elements, VLRLRE1 and VLRLRE2, which independently conferred RA responsiveness on a heterologous promoter (TK) in both the mouse keratinocyte cell line and in normal human keratinocytes. Transfection experiments using TK promoter constructs that contained each VL30 RRE alone or both together indicated that these sequences mediate RA-induced VL30 transcription in an additive rather than a synergistic manner. Functional characterization of mutated VLRLRE1 variants and sequence similarities between VLRLRE1 and VLRLRE2 indicates that RA can mediate induced transcription in keratinocytes through the sequences TGAACCTTTTACCT (VLRLRE1) and TAAACTTTACCC (VLRLRE2). These sequences consist of two directly repeated hexamer motifs (underlined) separated by 2 bp. Both VLRLREs were found to specifically bind recombinant vaccinia virus expressed RAR-RXR heterodimers, and the analyses of VLRLRE1 mutants provide a strong correlation between RA inducibility in vivo and efficiency of RAR-RXR binding in vitro. These analyses also indicate that the integrity of both half-sites is important for RAR-RXR binding and RA-induced transcription.

It has been shown that half-site sequences, spaced by 3, 4, and 5 bp, mediate a relatively selective positive regulation by vitamin D, thyroid hormone, and RA, respectively (14–16). Furthermore, RXR heterodimerizes not only with RARs but also with vitamin D and thyroid hormone receptors, which increases both DNA binding and transcriptional function of these receptors (11, 17–20). The identified VL30 RREs were found to be inactive as vitamin D-responsive elements, indicating that these sequences mediate a specific response to RA. Half-sites spaced by 1 bp have been shown to be regulated by RXR and the orphan receptor COUP-TF (42). In this regard it is interesting that the half-sites in the VLRLREs are spaced by the remaining option of 2 bp instead of the proposed optimum of 5 bp. However, this is not unique for VLRLRE, since both the mouse cellular retinol binding protein 1 (mCRBP1) and apolipoprotein AI genes contain RREs with an identical organization of the half-sites (assuming hexameric half-sites; Refs. 7 and 38). In addition, these RREs and the VLRLREs exhibit a significant sequence similarity, suggesting that these regulate transcription by similar mechanisms. In this respect it is interesting to note that the RRE present in the apolipoprotein AI gene has been shown to bind heterodimers between RXRa and a novel member of the steroid hormone receptor superfamily, ARP-1, and that the intracellular levels of this orphan receptor controls the responsiveness to RA (43).

The TGAACCT half-site of VLRLRE1 matches the proposed consensus half-site sequence, TGA(C/A)(C/T), and identical sequences are present in other RREs such as those identified in the promoters of RARδ, alcohol dehydrogenase, and complement factor H (15, 44–46). Although several RRE half-sites have been identified that deviates from the consensus sequence, no sequence identical to the VLRLRE half-sites TTACCT, TAAACT, and TCACCC is present in previously characterized RREs (5). It is interesting to note that the second nucleotide in these VLRLRE half-sites is not conserved and deviates from the proposed consensus sequence. It has been suggested previously that half-sites deviating from the idealized TGAAC or TGACC sequences represent degenerated motifs with regard to RAR binding and RA responsiveness. Our results are not fully compatible with this model, since VLRLRE2 (with two degenerated motifs) formed complexes with RAR-RXR heterodimers with approximately the same efficiency as VLRLRE1 (one degenerated motif) and RREδ2 (two idealized half-sites). However, the magnitude of -fold induction was in the order RREδ2 > VLRLRE1 > VLRLRE2 in Balb/MK cells and human keratinocytes. The finding that RREδ2 but not the VLRLREs function in human skin fibroblasts shows that the VLRLREs mediate a more specific RA response than RREδ2. It is therefore likely that the potency of a half-site in mediating RA-induced transcription is dependent both on the binding specificity of the RARE and cell type-specific differences in the levels of distinct nuclear receptors. Since retroviruses are dependent on transcription for their propagation there, is a substantial evolutionary pressure to acquire powerful enhancer motifs within their LTRs. It is therefore tempting to speculate that a VL30 LTR, regulating transcription in keratinocytes, has evolved RREs that are functionally optimal in this cellular environment. In this regard it is interesting to note that we found the RREδ2 coupled to a heterologous promoter, functional in transiently transfected human keratinocytes, whereas it has been reported that the expression of the endogenous RARE gene is not induced by RA in these cells (41). This suggests that the RREδ2 is repressed in keratinocytes when present in its normal sequence context. This result is in agreement with a recent report by Vollberg et al. (28).

When using nuclear keratinocyte extract in gel shift analysis, we detected a VLRLRE1-protein complex (complex A) that was formed in a manner that correlated with functionality. Furthermore, the formation of this complex was specifically abolished by an excess of VLRLRE2 and RREδ2. A corresponding complex was formed in keratinocyte extracts when RREδ2 was used as the probe. Interestingly, the intensity of the VLRLRE1-specific complex was found to be significantly lower in the fibroblast extracts, whereas the intensity of the RREδ2-specific complex was equal in keratinocyte and fibroblast extracts. This result is intriguing because of the lack of RA responsiveness of VLRLRE1 in fibroblasts and suggests that the affinity of a specific nuclear factor or factors to these sequences differs in the two cell types. However, these results do not exclude the presence of a putative repressor in fibroblasts that inhibits RA responsiveness mediated through VLRLRE but not RREδ2. The evolutionary conservation of RAR and RXR isotypes as well as their tissue-specific expression suggest that the possibility to form distinct RXR-RAR heterodimers may determine a tissue-specific response. Since the major receptor is represented by the RXRa-RXRα heterodimer (12, 41). Interestingly, we found by using the vaccinia system that VLRLRE did not discriminate between RARα-, RARγ-, and RARγ-RXRα heterodimers. A similar result was obtained when RARδ was used as probe. Assuming that the binding specificities of overexpressed RARs and RXR reflect the accurate in vivo situation, these results indicate that the observed differences between VLRLREs and RREδ2, in both binding to endogenous proteins and functionality, probably reflect an involvement of additional proteins such as other RXR isotypes, RAR isoforms, or coregulators not tested in this study. It is difficult to speculate which factor(s) may determine the observed differences between the RREs analyzed in this study, since no exhaustive characterization of the differential expression of RAR isoforms and RXR isotypes in keratinocytes and skin fibroblasts presently is available. However, the differences between VL30 RREs and RREδ2 in binding to endogenous proteins and functionality may facilitate the identification of the specific factors determining the response through VL30 RREs in vitro. Regarding RAR isoforms, it is interesting to note that transcripts cor-

**Retinoic Acid-induced VL30 Transcription**
responding to RARγ1 and RARγ2 are expressed in skin (41), and that RARγ1 can inhibit the activity of RARγ2, RARδ, and RARε receptors in CV-1 cells when using the RREs as a target (48). It is also interesting that RA-induced transcription mediated through a recently identified RRE, present in the promoter of the RARγ2 gene, is cell type-specific and is not observed in all cell lines that contain receptors activating the RRE (49). However, it should be noted that it has also been shown that RARγ2 expression is not induced by RA in human skin (41).

In conclusion, we have demonstrated that RA induces the transcription of the VL30 retrotransposon through two novel RREs that are targets for binding of RAR-RXR heterodimers in vitro. The induction is a primary RA response that occurs at constitutive receptor levels specifically in epidermal keratinocytes. Our study thus establishes the VL30 LTR-dependent transcription unit as a useful model to understand retinoid-dependent signal transduction in the cutaneous environment.

REFERENCES
1. Summerbell, D., and Maden, M. (1990) Trends Neurosci. 13, 142–147
2. Eichele, G. (1989) Trends Genet. 5, 246–251
3. Kogan, R., Trasler, G., and Fuchs, R. (1987) J. Cell Biol. 105, 427–440
4. Glass, C. K., Devary, O. V., and Rosenfeld, M. G. (1990) Cell 63, 729–738
5. Glass, C. K., DiRenzo, J., Kurokawa, R., and Han, Z. (1991) DNA Cell Biol. 10, 625–636
6. Stellman, V., Lessk, A., and Fuchs, E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4582–4586
7. Rottman, J. N., Widom, R. L., Nadal-Ginard, B., Mahdavi, V., and Kartenhans, S. K. (1991) Mol. Cell. Biol. 11, 3814–3820
8. Otvos, L., Jr. (1984) Pharmacol. Rev. 36, 83–97
9. Hong, W. K., Lipman, S. M., M., N., Karp, D. D., Lee, J. S., Byers, R. M., Schantz, S. P., Kramer, A. M., Lotan, R., Peters, L. J., Dimery, J. W., Brown, B. W., and Geopfert, H. (1990) New Engl. J. Med. 323, 796–801
10. Zelemt, A. (1989) Nature 343, 714–717
11. Leff, M., Kastner, P., Lyons, R., Nadejda, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Garnier, J. M., Mader, S., and Chambon, P. (1992) Cell 68, 373–385
12. Mangelsdorf, D. J., Rongmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A., and Evans, R. M. (1992) Genes & Dev. 6, 329–344
13. Brand, N., Petkov, M., Krut, A., Chambon, P., de T. H., Marchio, A., Tiollais, P., and Dejean, A. (1988) Nature 332, 850–853
14. Naar, A. M., M., K., L., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) Cell 65, 1267–1279
15. Vivanco Ruiz, M. D., et al. (1990) EMBO J. 9, 2629–2638
16. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Cell 65, 1253–1260
17. Bugay, T. H., Poli, L., Lonnomy, O., and Stukenberg, H. G. (1992) EMBO J. 11, 1409–1418
18. Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M., and Ozato, K. (1992) EMBO J. 11, 1419–1435
19. Zhang, X. K., Hoffmann, B., Tian, P. B., Graupner, G., and Pfahl, M. (1992) Nature 355, 441–446
20. Klesewer, S. A., Umesono, K., Mangelsdorf, D. J., and Evans, R. M. (1992) Cell 65, 454–464
21. Yu, V. C., Deisert, C., Andersen, B., Holloway, J. M., Devay, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) Cell 70, 621–631
22. Beato, M. (1980) Cell 66, 335–344
23. Kastner, P., Baert, R., Prat, D. R., and Taissey, M. A. (1989) Nature 340, 242–244