MOLECULAR AND CELLULAR BIOLOGY, June 1987, p. 2296–2298
0270-7306/87/062296-03$02.00/0

Vol. 7, No. 6

Individual Mouse VL30 Elements Transferred to Rat Cells by Viral Pseudotypes Retain Their Responsiveness to Activators of Protein Kinase C

KARIN D. RODLAND,[1] ANTHONY M. C. BROWN,[2] AND BRUCE E. MAGUN[1]*

Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, Oregon 97201,[1] and Department of Microbiology and Immunology, Medical School, University of California at San Francisco, San Francisco, California 94143[2]

Received 1 December 1986/Accepted 11 March 1987

By exploiting the retroviral characteristics of mouse VL30 elements, we transferred individual copies of VL30 to Rat-1 cells by infection. Analysis of clonal isolates containing single VL30 elements integrated into the Rat-1 genome indicates that responsiveness to activators of protein kinase C is an inherent property of at least some of the VL30 sequences.

Mouse VL30 elements are a family of retroviruslike DNA sequences which are present in 100 to 200 copies per genome (6). They have a mean size of 5.2 to 5.6 kilobases and are typically flanked by long terminal repeats (LTRs) which show many similarities to the LTRs of murine retroviruses (5). The LTRs include a 36-base-pair sequence related to the enhancer element of murine sarcoma virus, as well as terminal inverted repeats homologous to those of murine leukemia virus (MLV; 5). VL30 elements appear to be flanked by 4-base-pair direct repeats of host cellular DNA sequences (5), which is characteristic of integration by a specific retroviral mechanism. The elements encode a 30S polyadenylated RNA species which can be packaged into MLV viral particles (10, 11).

VL30 RNA is expressed at high levels in transformed cells and cells undergoing rapid proliferation but is present at relatively low levels in quiescent cells (3, 6). The abundance of this RNA is rapidly increased when quiescent cells are stimulated with epidermal growth factor (EGF) or phorbol ester tumor promoters (4, 9). We have previously shown that VL30 RNA levels can be induced by phorbol esters and diacylglycerols even in the absence of EGF receptors, suggesting that induction of VL30 RNA is a consequence of activation of protein kinase C (9).

Further studies of the mechanism by which this induction occurs are complicated by the presence of multiple copies of VL30 in the mouse genome and by the considerable heterogeneity among individual elements (6). These factors raise the possibility that individual VL30 elements respond to different regulatory pathways and that only a subset of the elements is induced by EGF and phorbol esters, potentially via the influence of adjacent host cellular DNA sequences rather than via sequences within the VL30 elements themselves.

To determine whether the response to the activation of protein kinase C is an autonomous property of VL30 elements and, if so, to facilitate the identification of the relevant regulatory sequences, we studied the expression of individual VL30 elements in isolation by transferring them to rat cells which contained no sequences closely homologous to VL30. To transfer intact VL30 elements in the absence of flanking mouse genomic DNA, we took advantage of the retroviruslike properties of the elements. Since VL30 RNA can be packaged at low frequency into MLV viral particles (10), we expected that the MLV-packaging cell line Psi-2, commonly used to produce helper-free stocks of recombinant retroviral vectors (8), might package VL30 RNA into pseudovirions. If so, these could be used to infect Rat-1 cells.

Psi-2 cells were treated with 100 ng of 12-0-tetradecanoyl-13-phorbol acetate (TPA) per ml to maximize the expression of VL30 RNA, and the production of VL30 pseudovirions by Psi-2 cells was verified by RNA dot-blot analysis of viral particles concentrated from the culture medium (data not shown). Medium from TPA-stimulated cells was then used repeatedly to infect Rat-1 cells in the presence of Polybrene. No effort was made to remove TPA from this medium before infecting the Rat-1 cells. After three sequential rounds of infection, the recipient Rat-1 cells were cloned by serial dilution in microtiter wells, and 70 independent clonal lines were obtained.

To screen for rat cells which had received a functional mouse VL30 element, total cellular RNA was prepared from each clonal isolate after treatment with TPA for 4 h; the RNA was size fractionated by electrophoresis in agarose-formaldehyde gels, immobilized on nitrocellulose, and hybridized with the BVL-1 plasmid described by Hodgson et al. (5). This plasmid contains one LTR and the entire intervening sequence of a single mouse VL30 element. Of the 70 clonal lines examined, 3 (designated RVL-1, -2, and -3) expressed high levels of VL30 RNA, while the remaining lines were negative. The results obtained from two of these positive lines (RVL-1 and RVL-2; Fig. 1, lanes 4 and 9, respectively), together with the results from 8 other clones which expressed no VL30 RNA, are shown in Fig. 1. The expression of normal-sized VL30 RNA in rat cells from the infected population indicated that the RNA was indeed packaged into infectious particles by Psi-2 cells; our data suggested that the titer of such particles was approximately 10^2 infectious units per ml.

We next wished to confirm that the transferred VL30 elements were integrated within the Rat-1 cellular DNA, and we therefore analyzed restriction digests of high-molecular-weight DNA from clonal lines of infected cells. Genomic

* Corresponding author.

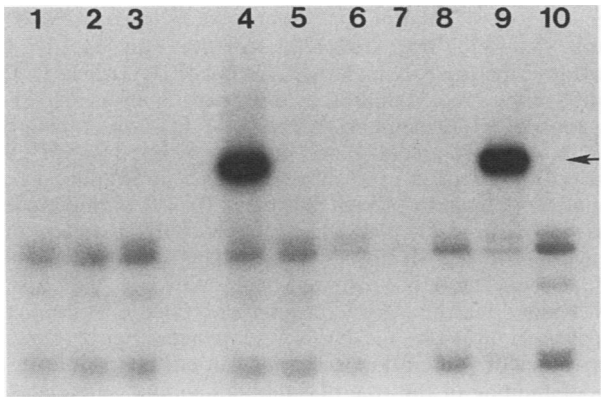

FIG. 1. Expression of exogenous VL30 elements in infected Rat-1 cells. Confluent 10-cm plates representing nine clonal isolates of VL30-infected Rat-1 cells and the uninfected Rat-1 cell line were serum deprived for 24 h and then exposed to 10 ng of TPA per ml for 4 h. Total cellular RNA was then extracted and purified (1), size fractionated by electrophoresis, transferred to nitrocellulose membranes, and hybridized to the entire BVL-1 plasmid (5) labeled with ^{32}P to a specific activity of 4×10^8 dpm/μg by nick translation. BVL-1 is a genomic clone containing the 3′ LTR and the complete intervening sequence of a single mouse VL30 element (5). Two of the VL30-infected isolates displayed an intense autoradiographic signal representing a 30S RNA species (RVL-1, lane 4, and RVL-2, lane 9). RNA obtained from uninfected Rat-1 cells (lane 6) displayed no evidence of hybridization to 30S RNA. Two smaller bands with faint homology to VL30 were identified in both uninfected and VL30-infected Rat-1 cells upon prolonged autoradiographic exposure. Each lane contained 10 μg of total cellular RNA.

DNA (10 μg) was digested with *Pst*I, size fractionated on agarose gels, transferred to nylon membranes, and hybridized with a ^{32}P-labeled (Polymeraid Kit; R & L Chemicals, Ltd.) VL30 LTR probe obtained by *Xho*I-*Xba*I digestion of BVL-1 (5). Mouse Psi-2 cells containing 50 to 200 copies of VL30 produced a smear of hybridization bands (Fig. 2, lane 11), while uninfected Rat-1 cells and six clonal lines of infected but unresponsive Rat-1 cells negative for VL30 RNA expression had no specific hybridizing bands (lanes 1 and 5 to 10). Each of the RNA-positive lines RVL-1, RVL-2, and RVL-3 displayed one or two discrete fragments hybridizing to the LTR probe, confirming that the VL30 DNA was integrated in these cells.

The hybridization patterns observed in RVL-1 and RVL-3 (Fig. 2, lanes 2 and 4) can be readily explained by the presence of one or more *Pst*I sites within the VL30 element, producing two LTR-containing fragments upon restriction digestion. Since the frequency of infection by VL30 pseudovirions was low in these experiments, it is most unlikely that any of the successfully infected lines contained more than one proviral copy of the element. The unique patterns of hybridization obtained for RVL-1, -2, and -3 confirm that each of these cell lines resulted from independent infection events. Similar results showing either one or two hybridizing fragments of 1 to 7 kilobases were obtained when digestions were performed with the restriction enzymes *Eco*RI and *Bam*HI (data not shown).

Having thus identified three successfully infected Rat-1 lines containing mouse VL30 DNA, we next tested the response of these individual elements to activators of protein kinase C. Nearly confluent monolayers of cells were exposed for 4 h to TPA or dioctanoylglycerol (DOG), and total cellular RNA was then extracted. The RNA was separated by electrophoresis on agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with the VL30 LTR probe. TPA treatment of RVL-2 produced a substantial increase in the level of VL30 RNA in RVL-2 cells (Fig. 3A, lanes 1 to 3), while similar treatment of uninfected Rat-1 cells produced no RNA species hybridizing to the LTR probe (lanes 4 to 6). The time course of TPA treatment was extended to 12 h (Fig. 3B, lanes 4 to 6). The level of RNA expressed from the single VL30 element in TPA-treated RVL-2 cells was similar to that seen in mouse AKR-2B cells stimulated with TPA (Fig. 3A, compare lanes 2 and 7). This is particularly interesting in view of the presence of 100 to 200 copies of VL30 DNA in the latter cells. This result may indicate that only a small subset of the multiple VL30 copies in AKR-2B cells was TPA responsive. Alternatively, increased expression of individual elements in rat cells may result from differing levels of specific transcriptional factors.

Clone RVL-3 was also tested in response to DOG, which has previously been shown to be an effective inducer of VL30 RNA in mouse cells (9). While RVL-3 exhibited low levels of VL30 before treatment and after exposure to the vehicular control dimethyl sulfoxide (DMSO; data not shown), it is apparent that both TPA and DOG induced VL30 RNA in RVL-3 (Fig. 3B, lanes 2 and 3, respectively), indicating that the single VL30 element in this cell line had the capacity to respond to both of these activators of protein kinase C.

By making use of the ability of Psi-2 cells to package VL30 RNA into infectious particles, we transferred individual mouse VL30 elements to rat cells in the absence of accompanying mouse genomic DNA. Three independent clonal lines of VL30-infected cells were isolated, each of which contained at least one integrated VL30 element and also produced VL30 transcripts in response to TPA. Since these

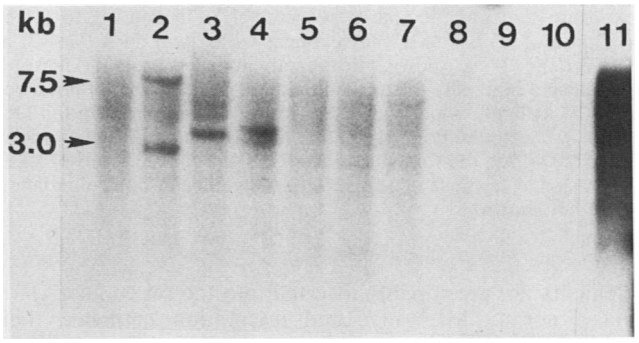

FIG. 2. Presence of mouse VL30 LTR sequences in genomic DNA from responsive Rat-1 subclones. Genomic DNA was extracted from confluent 10-cm plates, purified (7), and size fractionated by electrophoresis on 1% agarose gels before transfer to nylon membranes. The 550-base-pair *Xho*I-*Xba*I fragment of BVL-1 containing the 3′ LTR of a single VL30 element (5) was labeled with ^{32}P to a specific activity of 10^8 dpm/μg by random hexamer extension and used as a hybridization probe. Genomic DNA from the Psi-2 mouse cell line displayed a broad smear of hybridizing bands consistent with the presence of several hundred VL30 units per genome (lane 11). The three TPA-responsive Rat-1 subclones RVL-1, RVL-2, and RVL-3 (lanes 2, 3, and 4, respectively) showed a pattern of 1 or 2 distinct bands hybridizing to the VL30 LTR probe. None of the nonresponsive Rat-1 subclones (lanes 5 to 10) displayed any bands hybridizing to the LTR probe. No LTR-hybridizing bands were evident in lane 1, which contained a threefold excess of DNA from uninfected Rat-1 cells (30 μg compared with 10 μg of DNA in lanes 2 to 11). kb, Kilobases.

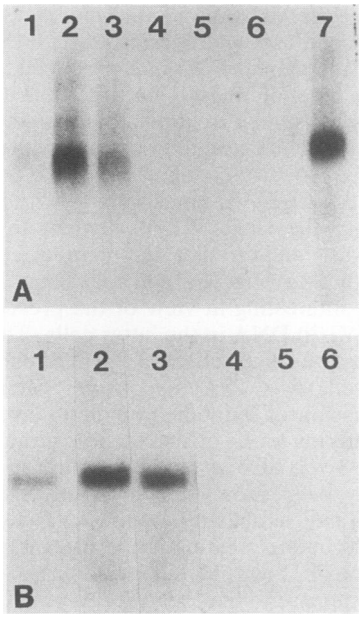

FIG. 3. Induction of VL30 RNA expression by activators of protein kinase C in VL30-infected Rat-1 cells. Northern blots were constructed by using total cellular RNA extracted from confluent 10-cm plates after serum deprivation and treatment with either TPA or DOG (9). (A) Results obtained with RVL-2 (lanes 1 to 3) and uninfected Rat-1 cells (lanes 4 to 6) are shown. After a 4-h exposure to TPA (lane 2), RVL-2 expressed a 30S VL30 transcript at levels comparable to those seen in the mouse AKR-2B cell line after TPA stimulation (lane 7). VL30 transcripts were not evident in untreated RVL-2 cells (lane 1) and were only slightly induced by 4 h of exposure to DMSO (lane 3). VL30 30S RNA was not detectable in the uninfected Rat-1 cells at 0 h or after 4 h of exposure to either TPA or DMSO (lanes 4, 5, and 6, respectively). (B) Uninfected Rat-1 cells remained unresponsive to TPA after 4, 8, or 12 h of exposure (lanes 4, 5, and 6, respectively). In lanes 1 to 3, the response of RVL-3 to a 4-h treatment with either TPA (lane 2) or DOG (lane 3) is shown. Both activators of protein kinase C produced a markedly greater induction of VL30 than that produced by 0.1% ethanol, which was used as the vehicular control for TPA (lane 1). Two other controls, serum deprivation alone and exposure to 0.1% DMSO, the solvent used for DOG, produced levels of VL30 RNA similar to those observed with the vehicular control in lane 1 (data not shown).

elements are presumably inserted into the rat cellular DNA via a normal MLV-mediated integration pathway, their positions in the genome are almost certainly different in each case. The induction of VL30 RNA levels by TPA stimulation in all three cases therefore strongly suggests that the ability to respond to activation of protein kinase C is a property intrinsic to the VL30 elements transferred.

One question not addressed by these experiments is whether all VL30 elements integrated in the mouse genome are equally responsive to induction by activators of protein kinase C. In fact, recent evidence indicates that at least one VL30 element, that represented in the BVL-1 plasmid used as a probe in these experiments, does not convey responsiveness to EGF or TPA when used in a CAT expression vector (L. J. Schmidt, C. L. French, M. O'Byrne, and M. J. Getz, J. Cell Biol. **103**:479a, 1986). The use of the pseudovirion-mediated transfer protocol described in this study may have facilitated the identification of TPA-responsive VL30 elements, as both the Psi-2 packaging cell line and the recipient Rat-1 cells were exposed to TPA during the transfer procedure. Whether the ability to respond to activators of protein kinase C is a unique characteristic of the particular VL30 element(s) transferred or a general property of most VL30 elements cannot be determined from the data presented here. However, isolation of these individual TPA-inducible elements should facilitate the identification of the VL30 DNA sequences that respond to activators of protein kinase C and should contribute to an understanding of the role of flanking genomic sequences in modulating VL30 expression.

We gratefully acknowledge the technical assistance of Thanh-Hoai Dinh, Alison Gammie, and Shall Jue. We thank Julie Robinson for assistance in the preparation of the manuscript.

This work was supported by Public Health Service grant CA39360 to B.E.M. from the National Institutes of Health and a grant from the Medical Research Foundation of Oregon to K.D.R. A.M.C.B. is a Special Fellow of the Leukemia Society of America.

LITERATURE CITED

1. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. **107**:303–307.
2. **Courtney, M. G., L. J. Schmidt, and M. J. Getz.** 1982. Organization and expression of endogenous virus-like (VL30) DNA sequences in nontransformed and chemically transformed mouse embryo cells in culture. Cancer Res. **42**:569–576.
3. **Dragani, T. A., G. Manenti, G. Della-Porta, S. Gattoni-Celli, and I. B. Weinstein.** 1986. Expression of retroviral sequences and oncogenes in murine hepatocellular tumors. Cancer Res. **46**:1915–1919.
4. **Foster, D. N., L. J. Schmidt, C. P. Hodgson, H. L. Moses, and M. J. Getz.** 1982. Polyadenylylated RNA complementary to a mouse retrovirus-like multigene family is rapidly and specifically induced by epidermal growth factor stimulation of quiescent cells. Proc. Natl. Acad. Sci. USA **79**:7317–7321.
5. **Hodgson, C. P., P. K. Elder, T. Ono, D. N. Foster, and M. J. Getz.** 1983. Structure and expression of mouse VL30 genes. Mol. Cell. Biol. **3**:2221–2231.
6. **Keshet, E., and A. Itin.** 1982. Patterns of genomic distribution and sequence heterogeneity of a murine "retrovirus-like" multigene family. J. Virol. **43**:50–58.
7. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 280–282. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
9. **Rodland, K. D., S. Jue, and B. E. Magun.** 1986. Regulation of VL30 gene expression by activators of protein kinase C. J. Biol. Chem. **261**:5029–5033.
10. **Scolnick, E. M., W. C. Vass, R. S. Howk, and P. H. Duesberg.** 1979. Defective retrovirus-like 30S RNA species of rat and mouse cells are infectious if packaged by type C helper virus. J. Virol. **29**:964–972.
11. **Sherwin, S. A., U. R. Rapp, R. E. Benveniste, A. Sen, and G. J. Todaro.** 1978. Rescue of endogenous 30S retroviral sequences from mouse cells by baboon type C virus. J. Virol. **26**:257–264.