JOURNAL OF VIROLOGY, Dec. 1984, p. 1000–1004
0022-538X/84/121000-05$02.00/0

Vol. 52, No. 3

Mouse Mammary Tumor Virus-Related Sequences in Mouse Lymphocytes Are Inducible by 12-*O*-Tetradecanoyl phorbol-13-acetate

BYOUNG S. KWON* AND SHERMAN M. WEISSMAN

Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

Received 23 April 1984/Accepted 16 August 1984

cDNA libraries from EL-4 cells treated with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) were screened for TPA-inducible sequences by differential hybridization. The most abundant inducible species was a sequence similar to that of mouse mammary tumor virus (MMTV). Induction of the mRNA corresponding to the MMTV-related sequences was already evident 30 min after TPA treatment, whereas the maximum accumulation occurred after 20 h of exposure to TPA. TPA also increased levels of MMTV-related RNA in the normal spleen cells of BALB/c and C57BL/6 mice. The level of RNA expression corresponding to MMTV-related sequences, however, was markedly elevated in EL-4 cells as compared with normal spleen cells. Southern blots of EL-4 cell DNA showed that the MMTV-related sequences were inserted into multiple locations of the EL-4 genome. Sequence analysis revealed that the MMTV-related cDNA clones included a part of the *env* gene and the right long terminal repeat of MMTV. However, the cDNA sequences were substantially different from published MMTV proviral sequences, most notably because of a contiguous deletion of 491 base pairs in the open reading frame within the U_3 region.

The potent tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) accelerates development of tumors induced by primary carcinogens and has been shown to exert pleiotropic effects in a number of tumor systems (13, 26). Its effects seem to be mediated by specific cell membrane receptors which are copurified with protein kinase C (5, 28). However, the effect of TPA on the activation of gene expression is unknown, and it is not known whether the tumor promoter activity and the function of differentiation induction are mediated by the same pathway. The murine thymic leukemia T-cell line EL-4 produces T-cell growth factor (Il-2) constitutively in the absence of TPA but, upon the addition of TPA, increases the production of Il-2 (7) and other lymphokines such as colony-stimulating factor (11) and B-cell growth factor (12). To obtain insight into the mechanisms of action of TPA, we screened cDNA libraries of TPA-treated EL-4 for TPA-inducible clones by a differential hybridization procedure. Here, we report that the most abundant inducible species of polyadenylated RNA contained mouse mammary tumor virus (MMTV)-related sequences.

EL-4 cells were grown in RPMI 1640 medium containing penicillin and streptomycin (100 μg/ml each) supplemented with 100 mM sodium pyruvate, 10 mM nonessential amino acids, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.2]), 2 mM glutamine, and 5% fetal calf serum to a cell density of 1.5×10^6 cells per ml. The cells were collected, washed twice with phosphate-buffered saline, and treated with TPA (10 ng/ml) for various times. The stimulation was monitored by Il-2 assay (8) of the culture supernatant with Il-2-dependent CT-6 cells which had been maintained in RPMI 1640 medium supplemented with rat Il-2.

cDNA libraries were prepared with the membrane-associated and cytoplasmic polyadenylic acid-containing [poly(A)$^+$] mRNA fraction of TPA-treated EL-4 cells. Lambda phage BV-2 (16) was used as a vector to facilitate the differential screening because plaques were uniform in size and easier to manipulate than bacterial colonies. The recombinant phages were plated and transferred to duplicate filters (3). One set of filters was hybridized to a ^{32}P-labeled cDNA probe prepared with poly(A)$^+$ membrane or cytoplasmic mRNA of induced cells and the other, to a ^{32}P-labeled cDNA probe prepared with poly(A)$^+$ membrane or cytoplasmic mRNA of uninduced cells. The hybridization intensities of each plaque with the two probes were compared for the occurrence of TPA-inducible clones. Figure 1 shows an example of the differential screening. The sensitivity of the plaque hybridization for screening was estimated by two reconstruction experiments (see the legend to Fig. 1), which indicated that an input probe of ca. 1×10^3 cpm of ^{32}P-labeled cDNA was necessary to detect a specific sequence, and under the conditions used for screening, a sequence corresponding to as little as 0.02% of total mRNA could be detected. In initial screening of ca. 20,000 cytoplasmic and 10,000 membrane-associated cDNA inserts, 200 potential induction-specific clones were selected. The clones were plaque purified and rescreened twice. Finally, phage DNAs were prepared, fixed on to two copies of nitrocellulose filters (14), and hybridized separately to the induced and uninduced probes. Only 15 clones from membrane-cDNA libraries and 2 clones from cytoplasmic cDNA libraries were consistently scored as inducible. The inserts from 10 of these clones were excised and subcloned into pBR322. By cross-hybridization among the clones, the clones were classified into six different groups. One group of clones appeared five times: four times from membrane libraries and once from a cytoplasmic library, thus representing the most abundant inducible species. In initial RNA blotting analysis, inserts from these clones hybridized strongly to a 24S RNA species and weakly to a 35S RNA species, suggesting to us that the insert sequences were related to a retrovirus genome. We chose the longest insert of the clones (pKR1101) for further analysis. Cytoplasmic RNA of EL-4 cells was extracted at the

* Corresponding author.

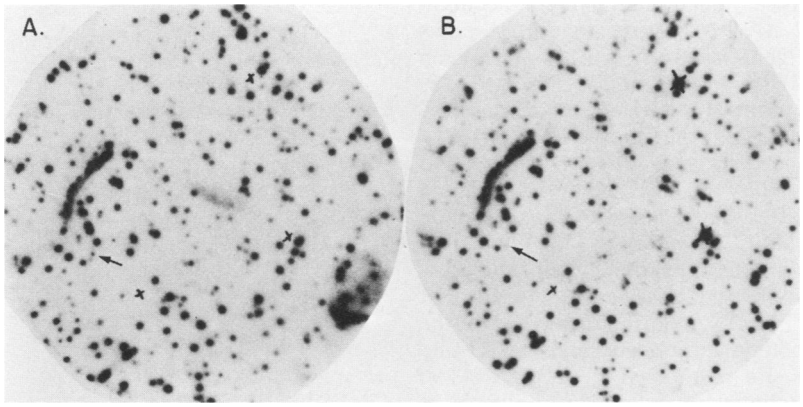

FIG. 1. Differential screening of cDNA libraries: hybridization pattern of plaques containing cloned cDNAs with either induced (A) or uninduced (B) ^{32}P-labeled cDNA probes. Double-stranded cDNA from membrane-associated or cytoplasmic poly(A)$^+$ mRNA of TPA-treated EL-4 was prepared by standard procedure (17, 31). *Bam*HI linkers were ligated to both ends of the cDNA. The cDNAs were inserted into a unique *Bam*HI site of lambda phage, BV-2 (16). The recombinant phage DNA was packaged in vitro. *Escherichia coli* LE392 cells were infected by the recombinant phages and plated such that ca. 400 phages containing cDNA inserts were present per 150-mm petri dish. Phage DNAs were transferred to two nitrocellulose filters and fixed to the filters. The sensitivity of the plaque hybridization for screening was estimated by two reconstruction experiments with λKT137 (K. Takeshida, personal communication), which contains a human β-globin gene. λKT-137 plaques were mixed with charon 4A plaques and plated such that 4 to 30 λKT137 plaques appeared among 1,000 charon 4A plaques in a plate. The phage DNAs were transferred to duplicate filters. One set of filters was hybridized to a ^{32}P-labeled β-globin intron and the other was hybridized to 10-fold serial dilutions of ^{32}P-labeled β-globin cDNA (ranging from 10^6 to 10 cpm per filter). An input of 1×10^3 cpm of β-globin cDNA could detect λKT137 plaques. In other experiments, β-globin mRNA was mixed with EL-4 mRNA such that β-globin mRNA represented 2, 0.2, 0.02, or 0.002% of the total RNA. ^{32}P-labeled cDNA probes from the mixture were prepared and hybridized to the filters as above, with an input probe of 5×10^6 cpm per filter. When 0.02% of β-globin mRNA was present, the cDNA probe could detect λKT137 plaques. ^{32}P-labeled cDNA probes (specific activity, 2×10^8 cpm/μg) were prepared from induced and uninduced EL-4 cells with oligodeoxythymidylic acid 12-18 as a primer. One set of filters was hybridized to 5×10^6 cpm of the induced [^{32}P]cDNA per filter. The duplicate set of filters was hybridized to 5×10^6 cpm of the uninduced [^{32}P]cDNA per filter. cDNA probes from the membrane-associated and cytoplasmic poly(A)$^+$ mRNA were used to screen membrane and cytoplasmic cDNA libraries, respectively. Filters were hybridized in a solution containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution, 10% dextran sulfate, and herring sperm DNA (250 μg/ml) at 68°C for 24 h. Filters were washed extensively at 68°C in each of 3× SSC, 2× SSC, and 1× SSC and exposed to X-ray film (Fuji RX) with an intensifying screen for 4 days at −70°C. An arrow indicates a clone that hybridized only to the induced cDNA probe. X's indicate registration marks.

various times after treatment of the cells with TPA, fractionated on a 1.4% formaldehyde gel, transferred to a nitrocellulose filter, and hybridized to nick-translated, ^{32}P-labeled pKR1101 (Fig. 2). An increase in 24S RNA was already evident at 30 min after TPA treatment. The maximum accumulation of pKR1101 cRNA occurred after 20 h of exposure to TPA, and levels of RNA remained at an induced level for at least 92 h. The enhancement of expression was up to eightfold over the level of uninduced control (Fig. 2, inset). Similar sequences were also inducible in the normal lymphoid cells. As shown in Fig. 3, TPA induced pKR1101 cRNA expression in normal spleen cells of BALB/c and C57BL/6 mice (parental strain of EL-4 cells). The level of cRNA expression, however, was markedly elevated in EL-4 cells as compared with that in normal spleen cells. The level of pKR1101 cRNA expression after TPA treatment in BW5147 cells, another thymoma T-cell line, was similar to

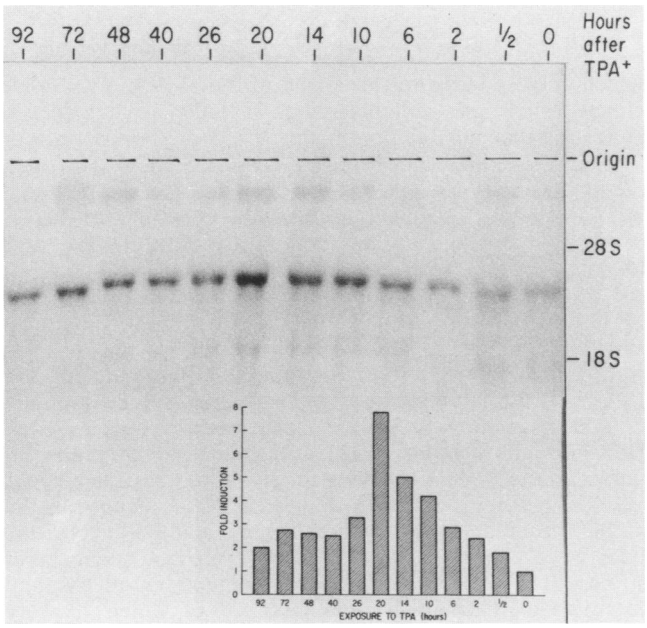

FIG. 2. Time course of TPA effect on pKR1101 cRNA induction. Total RNA of EL-4 was extracted at the various times after treatment with TPA. Total RNAs (50 μg) from TPA-treated or untreated EL-4 were fractionated on 1.4% formaldehyde denaturing gel and transferred to nitrocellulose filter (30). ^{32}P-labeled, nick-translated pKR1101 (specific activity, 5×10^8 cpm/μg) was hybridized to the filter at 42°C in a solution containing 5× SSC, 50% formamide, 50 mM sodium phosphate buffer (pH 7.4), 1× Denhardt solution, 0.1% sodium dodecyl sulfate, and salmon sperm DNA (250 μg/ml) for 24 h. Filters were then washed three times for 5 min each in 2× SSC and 0.1% sodium dodecyl sulfate at room temperature and three times at 42°C in 0.1× SSC and 0.1% sodium dodecyl sulfate. The filter was exposed to X-ray film (Fuji RX) without an intensifying screen for 36 h at −70°C. Inset: relative intensities of the bands at the 24S position, as measured by a Beckman DU-8B UV/Vis spectrophotometer at 500 nm. The numbers were normalized with the 0-h control.

that in normal spleen cells, although the maximum accumulation occurred after 72 h of exposure. Figure 4 shows Southern blot analysis of genomic DNA of EL-4 and spleen cells of C57BL/6 and BALB/c. The gene for pKR1101 was inserted into multiple locations of the EL-4 genome and was inserted in normal spleen cells in four to five locations.

The nucleotide sequence of the five cDNA clones related to pKR1101 was determined by the dideoxynucleotide chain termination method (27) after the fragments were subcloned into the M13 vector mp8 (20). Figure 5 shows the nucleotide sequence of the insert from clone pKR1101, with the positions at which MMTV proviral sequences of C3H (4, 18), GR (15, 23), or both differ. The cDNA started at the end of the R sequence of the right long terminal repeat (LTR) and extended to the *Bam*HI site of the *env* gene of MMTV. The cDNA contained putative polyadenylation signals of MMTV and the TATA box, which read AGTAAA and TATAAAAGA, respectively. The cDNA also contained the entire coding region for viral gp36 and part of the gp52 sequence. The cDNA, however, had a 491-nucleotide deletion within the U_3 sequence. The deletion spanned from the base position −180 to −671, from the end of R sequence which corresponded to the 3′ end of the cDNA. Two other clones which spanned this region had the same deletion, indicating that the deletion might be a consistent pattern of the MMTV-related sequences expressed in EL-4. The open reading frame (ORF) of U_3 sequence had a coding potential of 216 amino acids, spanning from the beginning of the U_3 sequence to 21 nucleotides upstream of the TATA box, followed by three stop codons in phase. The amino-terminal 181 codons of the ORF were the same as those of the other MMTV (18) except for 11 amino acids differences due to single-base changes. The carboxy-terminal 35 amino acids were completely different from the reported sequence (4, 18). Two out of five putative steroid-binding sites within the LTR (22) were present, but three other regions were missing. However, the deletion might not affect the sequences involved in the glucocorticoid regulation of downstream genes (19). In addition, there were 63 single-base changes in the pKR1101 coding region of the *env* gene and the ORF of the LTR when compared with the MMTV proviral sequence of strain C3H (1, 4): 38 changes were silent substitutions, and 25 resulted in 22 amino acid changes, of which 13 were

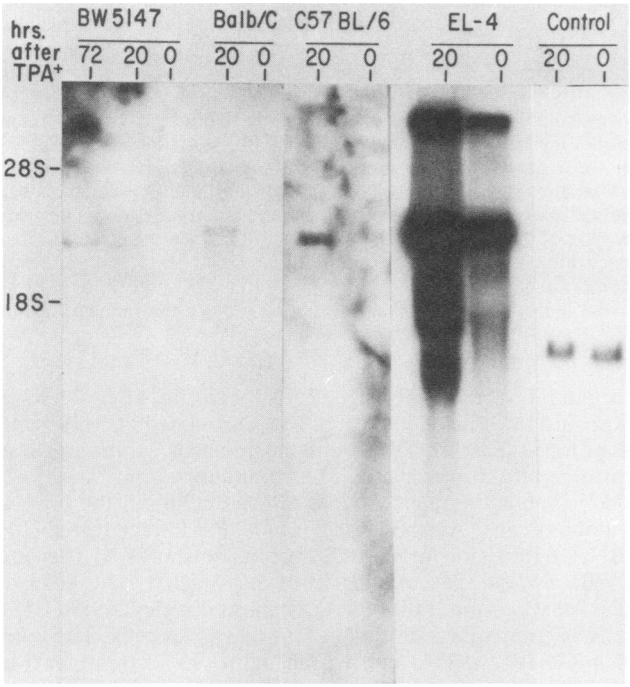

FIG. 3. Effect of TPA on pKR1101 cRNA induction in normal mouse spleen cells. Spleens were removed from BALB/c and C57BL/6 mice. Splenocytes were squeezed out under a stainless steel net and washed twice with RPMI 1640 medium supplemented with antibiotics and 5% fetal calf serum. Cells were suspended in the same medium as above. Cells were incubated with TPA (10 ng/ml) up to 72 h. Untreated control was incubated the same length of time in the same medium without TPA. Total RNA was extracted from both TPA-treated and untreated cells at 20 and 72 h of incubation. Total RNAs (50 μg) from TPA-treated or untreated normal mouse splenocytes, BW5147, or EL-4 cells were fractioned on 1.4% formaldehyde gel, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled, nick-translated pKR1101 as described in the legend to Fig. 2. The filter was exposed to X-ray film (Fuji RX) with an intensifying screen for 48 h at −70°C. Positions of the ribosomal RNAs are indicated. A clone which appeared to be uninducible was selected arbitrarily and used as a control for an amount of RNA loaded (control lanes). A darkening at the top of 72-h lane of BW5147 was an artifact which did not appear in repeated experiments.

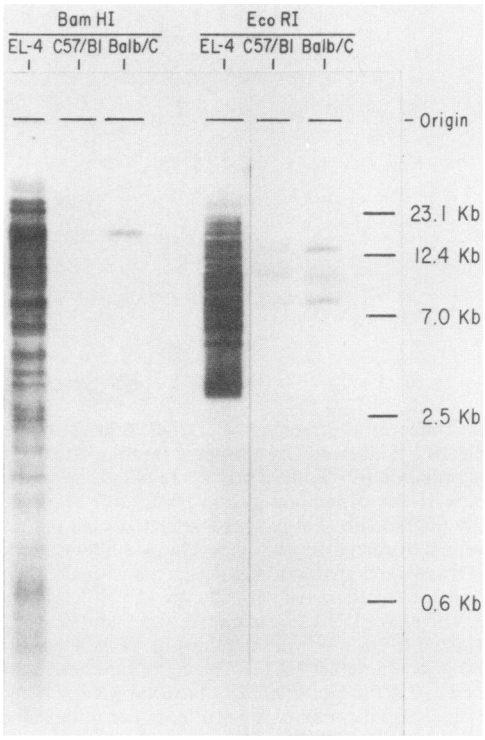

FIG. 4. High-molelcular-weight DNAs were isolated from splenocytes of normal C57BL/6 and BALB/c mice or from EL-4 cells by a method described by Gross-Bellard et al. (9). The DNA samples were digested to completion with either *Eco*RI or *Bam*HI. Digested DNA samples (10 μg) were run on horizontal, submerged 0.8% agarose gels (0.7 cm thick) in standard Tris-EDTA-acetate buffer (pH 7.8) at 1.5 V/cm for ca. 16 h. Molecular weight markers were prepared by mixing an *Ava*I- and *Kpn*I-digested phage charon 28 DNA and a *Bam*HI-digested phage charon 28 DNA. The DNA was transferred to the nitrocellulose filter, hybridized to ^{32}P-labeled pKR1101, and washed essentially as described by Southern (29) and Herr and Gilbert (10). The filters were autoradiographed at −70°C with an intensifying screen for 48 h. The labeled DNA had a specific activity of 5×10^8 cpm/μg.

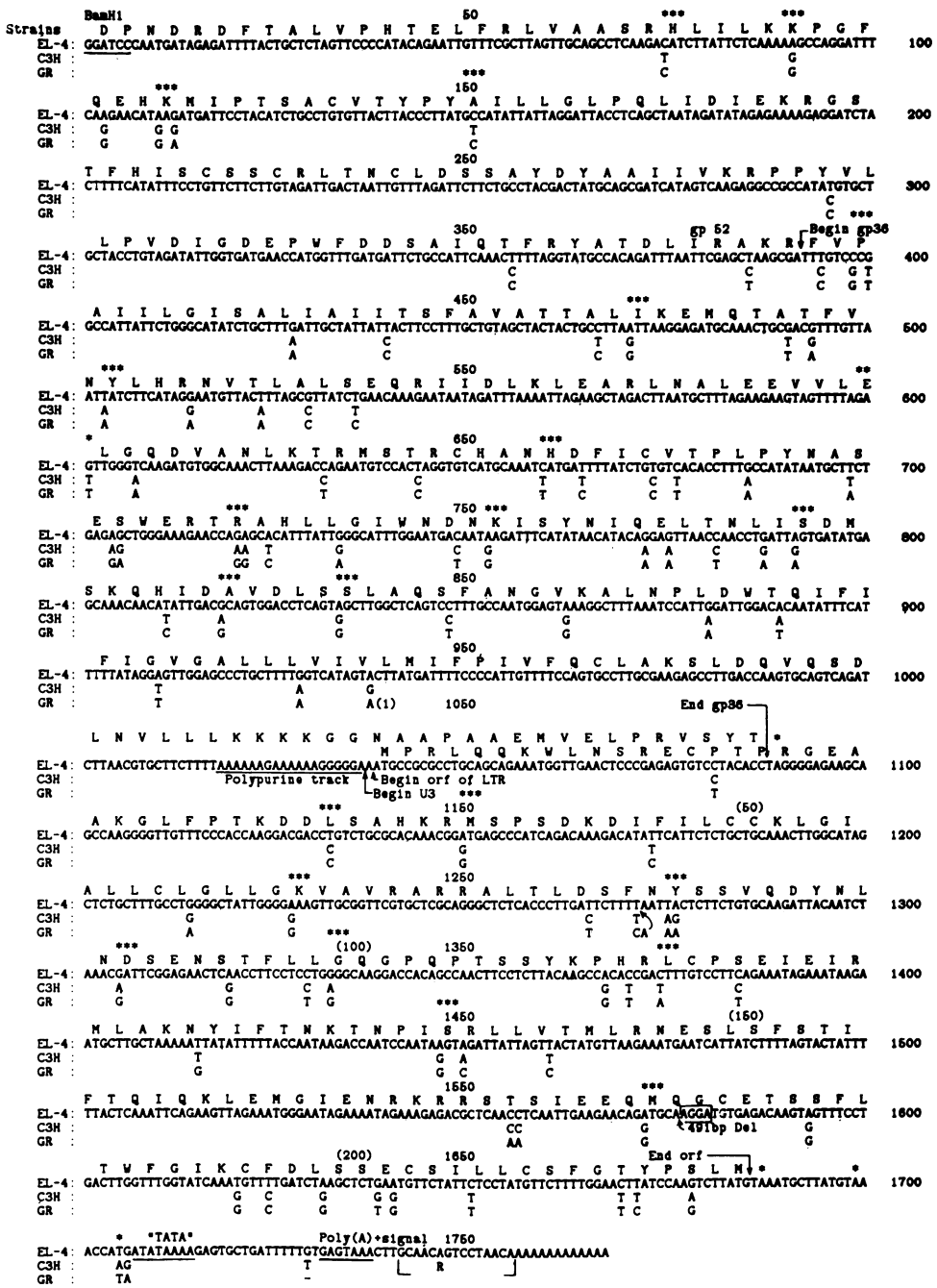

FIG. 5. Nucleotide sequence and deduced amino acid sequence of pKR1101 cDNA insert and comparison of the sequence with published sequences of other strains, C3H and GR. Numbers below each line refer to nucleotide position from the *Bam*HI site of the *env* gene of MMTV. Numbers above each line of the LTR ORF refer to amino acid position from the first methionine residue. Functional landmarks such as the polypurine tract, the TATA box, and polyadenylation signals are indicated. Positions at which the pKR1101 MMTV sequence of EL-4 varies from the proviral sequence of MMTV GR and C3H are shown under the pKR1101 sequence. Boundaries for U_3 and R sequences of LTR and for gp52 and gp36 of viral glycoprotein are indicated by referring to prior publications (1, 4, 18). Single asterisks (*) refer to stop codons. Sets of three asterisks (***) indicate that the amino acids below the marks are different between pKR1101 and C3H. AGGA appeared at the other end of the deleted sequence.

conservative and 9 were nonconservative (five in *env* and four in the LTR ORF). It appeared that the coding functions of glycoproteins and the first 181 amino acids of the LTR ORF were well maintained, even though multiple cycles of amplification and reintegration had occurred in EL-4 cells. The functional significance of the altered coding potential of the LTR ORF in pKR1101 requires further investigation. When the pKR1101 sequence was compared with that of strain GR (15, 23), the divergence of *env* sequences was similar to that found for C3H: 48 single-base changes resulted in 13 amino acid changes, of which 5 were nonconservative. However, there was a single-base addition and deletion

in addition to 31 single-base changes in the LTR which would result in a long frame shift.

The rapid induction of MMTV-related mRNA expression by TPA indicated that its effect on the gene expression might be directed via the TPA-receptor complex as in the case of steroid stimulation. However, the half-time for the maximal accumulation of MMTV RNA by TPA was considerably longer than that reported for steroid induction (24). This might indicate that the TPA induction process required a recruitment of other inducers or that the half-life of induced RNA was longer in lymphoid cells than in the mouse mammary cells (25). The increase of MMTV mRNA expression after TPA treatment in EL-4 was very similar to that in Mm5mt/c cells reported by Arya (2). Michalides et al. (21) have noticed that acquisition of MMTV coincides with the onset of leukemogenesis in GR mice. Very recently, Dudley and Risser (6) reported that MMTV genomes were amplified in several transplantable T-cell lymphomas of C57BL/6, A/J, and BALB/c origin. If the multiple insertions of the pKR1101 sequences accounted for the elevated expression of the mRNA, many of the MMTV sequences inserted in the EL-4 genome might be transcriptionally active. This might be a factor involved in the aberrant regulation of growth or lymphokine production by EL-4 cells.

We thank G. Fathman and J. J. Farrar for suggestions and providing EL-4 and Il-2-dependent T-cells, B. Forget and J. Eisenstadt for providing human β-globin mRNA and a Beckman DU-8B spectrophotometer, P. Lebowitz for a critical reading, J. Kim for technical assistance, and Ann M. Mulvey for preparing this manuscript.

This research was supported by Public Health Service grant CA 30938-03 from the National Institutes of Health. B.S.K. acknowledges financial support from the Swebilius Cancer Research Award.

LITERATURE CITED

1. **Arthur, L. O.,T. D. Copeland, S. Oroszlan, and G. Schochetman.** 1982. Processing and amino acid sequence analysis of the mouse mammary tumor virus *env* gene product. J. Virol. **41**:414–422.
2. **Arya, S. K.** 1980. Phorbol ester-mediated stimulation of the synthesis of mouse mammary tumor virus. Nature (London) **284**:71–72.
3. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180–182.
4. **Donehower, L. A., A. L. Huang, and G. L. Hager.** 1981. Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. J. Virol. **37**:226–238.
5. **Driedger, P. E., and P. M. Blumberg.** 1980. Specific binding of phorbol ester tumor promoters. Proc. Natl. Acad. Sci. U.S.A. **77**:567–571.
6. **Dudley, J., and R. Risser.** 1984. Amplification and novel locations of endogenous mouse mammary tumor virus genomes in mouse T-cell lymphomas. J. Virol. **49**:92–101.
7. **Farrar, J. J., J. Fuller-Farrar, P. K. Simon, M. L. Hilfiker, B. M. Stadler, and W. L. Farrar.** 1980. Thymoma production of T-cell growth factor interleukin -2. J. Immunol. **125**:2555–2558.
8. **Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith.** 1978. T-cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. **120**:2027–2032.
9. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high-molecular weight DNA from mammalian cells. Eur. J. Biochem. **36**:32–38.
10. **Herr, W., and W. Gilbert.** 1983. Somatically acquired recombinant murine leukemia proviruses in thymic leukemias of AKR/J mice. J. Virol. **46**:70–82.
11. **Hilfiker, M., R. N. Moore, and J. J. Farrar.** 1981. Biologic properties of chromatographically separated murine thymoma-derived interleukin -2 and colony-stimulating factor. J. Immunol. **127**:1983–1987.
12. **Howard, M., J. J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. G. Paul.** 1982. Identification of a T-cell-derived B-cell growth factor distinct from interleukin -2. J. Exp. Med. **155**:914–923.
13. **Huberman, E., and M. F. Callahan.** 1979. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc. Natl. Acad. Sci. U.S.A. **76**:1293–1297.
14. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. **7**:1541–1552.
15. **Kennedy, N., B. Knedlitschek, N. E. Groner, P. Hynes, K. Herrlich, R. Michalides, and A. J. van Ooyen.** 1982. Long terminal repeats of endogenous mouse mammary tumor virus contain a long open reading frame which extends into adjacent sequences. Nature (London) **295**:622–624.
16. **Klein, B., and K. Murray.** 1979. Phage lambda receptor chromosomes for DNA fragments made with restriction endonuclease I of Bacillus amyloliquefaciens H. J. Mol. Biol. **133**:289–294.
17. **Land, H., M. Grez, H. Hauser, W. Lindenmaier, and G. Schutz.** 1981. 5'-terminal sequences of eucaryotic mRNA can be cloned with high efficiency. Nucleic Acids Res. **9**:2251–2266.
18. **Majors, J. E., and H. E. Varmus.** 1983. Nucleotide sequencing of an apparent proviral copy of *env* mRNA defines determinants of expression of the mouse mammary tumor virus *env* gene. J. Virol. **47**:495–504.
19. **Majors, J. E., and H. E. Varmus.** 1983. A small region of the mouse mammary tumor virus long terminal repeat confer glucocorticoid hormone regulation on a linked heterologous gene. Proc. Natl. Acad. Sci. U.S.A. **80**:5866–5870.
20. **Messing, J. B., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–316.
21. **Michalides, R., A. Wagenaar, J. Hilkens, J. Hilgers, B. Groner, and N. E. Hynes.** 1982. Acquisition of proviral DNA of mouse mammary tumor virus in thymic leukemia cells from GR mice. J. Virol. **43**:819–829.
22. **Payvar, R., D. DeFranco, G. L. Firestone, B. Edgar, O. Wrangs, and K. R. Yamamoto.** 1983. Sequence-specific binding of glucocorticoid receptor to MMTV DNA at sites within and upstream of the transcribed region. Cell **35**:381–392.
23. **Redmond, S. M. S., and C. Dickson.** 1983. Sequence and expression of the mouse mammary tumor virus *env* gene. EMBO J. **2**:125–131.
24. **Ringold, G. M.** 1979. Glucocorticoid regulation of mouse mammary tumor virus gene expression. Biochim. Biophys. Acta **560**:487–508.
25. **Ringold, G. M., K. R. Yamamoto, J. M. Bishop, and H. E. Varums.** 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. Proc. Natl. Acad. Sci. U.S.A. **74**:2879–2883.
26. **Rovera, G., T. G. O'Brien, and L. Diamond.** 1977. Tumor promoters inhibit spontaneous differentiation of Friend erythroleukemia cells in culture. Proc. Natl. Acad. Sci. U.S.A. **74**:2894–2898.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463–5467.
28. **Shoyah, M., and G. J. Todaro.** 1981. Specific high affinity cell membrane receptors for biologically active phorbol and ingenol esters. Nature (London) **288**:451–455.
29. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
30. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
31. **Wickens, M. P., G. N. Buell, and R. T. Shimke.** 1978. Synthesis of double-stranded DNA complementary to lysozyme, ovomucoid and ovalbumin mRNAs. J. Biol. Chem. **253**:2483–2495.