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Polyadenylylated RNA complementary to a mouse retrovirus-like multigene family is rapidly and specifically induced by epidermal growth factor stimulation of quiescent cells

(differential gene expression/peptide growth factors/specific abundant polyadenylylated mRNA/cell proliferation)

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ABSTRACT **Complementary DNA probes prepared from total polysomal poly(A)$^+$RNA populations were used to identify clones of mouse DNA containing sequences whose expression is specifically enhanced after epidermal growth factor (EGF) stimulation of quiescent mouse embryo cells in culture. Three such clones were isolated and used to study changes in the levels of clone-specific poly(A)$^+$RNA in the polysomes of cells after mitogenic stimulation by EGF. RNA complementary to sequences present in these clones increased ≈10-fold as a fraction of the total poly(A)$^+$RNA by 6 hr after stimulation. All three clones were found by hybridization criteria to contain sequences related to the class of mouse retrovirus or transposon-like elements termed VL30. These VL30-related sequences were further found to be complementary to EGF-inducible poly(A)$^+$RNAs and enhanced expression was detectable as early as 1 hr after EGF stimulation. In contrast, nine additional clones, including an AKR-type murine leukemia provirus DNA clone, contained no detectable VL30 sequence elements and were complementary to poly(A)$^+$RNA species whose relative concentration was essentially constant in quiescent and EGF-stimulated cells. Therefore, VL30 sequence elements appear distinct in that they encompass members whose expression is specifically regulated in response to a defined peptide growth factor.**

The growth of many types of cells in culture is regulated, in part, by essential serum growth factors. When cells deplete these factors, they enter a quiescent state sometimes termed G_0 (1, 2). The binding of peptide growth factors such as epidermal growth factor (EGF) to specific membrane receptors initiates a complex series of biochemical events leading to renewed cell proliferation (3).

On the molecular level, generalized increases in the rates of RNA and protein synthesis are readily observed prior to the initiation of DNA synthesis (4–6). In addition, dramatic alterations in the abundance of a few specific proteins have been reported (7–10). However, similar attempts at identifying the induction of specific mRNA sequences, generally have proven less rewarding (11, 12). Thus, it is not clear whether peptide growth factors are capable of differentially regulating specific genes in a manner analogous to steroid and other hormones, or whether they simply act in a generalized fashion to increase the overall rates of RNA synthesis or processing, or both.

In this study we utilized replica screening of a recombinant DNA library to isolate genomic clones corresponding to polysomal poly(A)$^+$RNA sequences whose abundance is enhanced after short-term stimulation of cultured mouse embryo cells with EGF. These studies demonstrate that mitogenic stimulation of quiescent cells by EGF results in a rapid and selective induction of at least one class of specific polyadenylylated RNA sequences. These RNAs were found to be closely related by hybridization criteria to a class of repeated 5.2-kilobase (kb) elements that have structural characteristics analogous to both integrated retrovirus proviruses and certain classes of transposable genetic elements.

MATERIALS AND METHODS

Cell Culture. The cell culture conditions used have been described in detail (13, 14). Nongrowing cultures of AKR-2B mouse embryo cells were produced by allowing the cells to grow to confluency in 490-cm^2 plastic roller bottles (about 5 days) and then shifting the serum concentrations to 0.5% for 48 hr. These nongrowing cells were stimulated to begin traversing the cell cycle by the addition of EGF (10 ng/ml). Cells were harvested 1, 4, or 6 hr after stimulation. The growth state of all experimental cells was verified both prior to and 20 hr after EGF stimulation by measuring the rate of [^{3}H]thymidine incorporation in cell aliquots as described (12). Cell fractionation, purification of RNA, and oligo(dT)-cellulose chromatography have been described in detail (12).

Synthesis of Double-Stranded cDNA. Reactions were performed essentially as described by Wickens *et al.* (15). All double-stranded cDNA probes were preparatively treated with S1 nuclease to remove hairpin loops and prevent immediate strand reassociation after denaturation.

Kinase End-Labeled RNA. Polysomal poly(A)$^+$RNA (1–2 μg) was subjected to mild alkaline hydrolysis by the method of Maizels (16) and then was end-labeled with T4 polynucleotide kinase as described (17). The specific activity of end-labeled RNA was 5–6 × 10^7 dpm/μg.

Construction of the AKR-2B *Eco*RI Partial Digest Library. Partial *Eco*RI restriction digests of mouse AKR-2B cellular DNA were used for cloning in the *Escherichia coli* phage λ vector Charon 4A as described by Maniatis *et al.* (18).

Differential Plaque Filter Hybridization. The technique used was a modification of that described by Benton and Davis (19) and St. John and Davis (20) and has been described (17).

Filters were processed for hybridization by using essentially the procedure of Jeffries and Flavell (21). Selected clones were plaque purified and propagated in liquid culture, and the DNA was purified by banding the phage in CsCl, followed by digestion with proteinase K, extraction with phenol, and precipitation with ethanol.

Dot Hybridization. The procedure of Kafatos *et al.* (22) was

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Abbreviations: EGF, epidermal growth factor; kb, kilobase(s); LTR(s), long terminal repeat(s).

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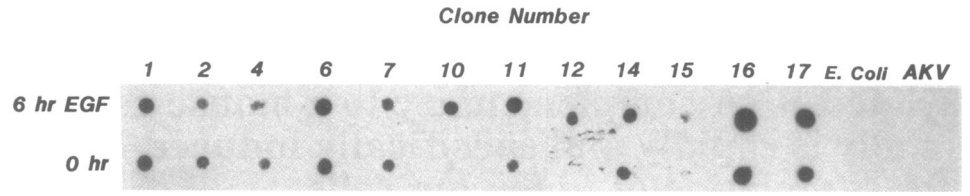

FIG. 1. Filter dot hybridization. DNAs from 12 individual genomic clones, *E. coli* DNA, and AKV proviral DNA were denatured, immobilized on filters, and hybridized to 2 μg of kinase ^{32}P-labeled polysomal poly(A)$^+$mRNA from either quiescent ("0" hr) AKR-2B cells or cells stimulated by EGF for 6 hr.

used with modifications as described (17). Kinetic hybridizations were carried out essentially as described (17, 22) by using Millipore HAWP 13-mm filters or the Bethesda Research Laboratories Hybridot Manifold (Bethesda, MD). Duplicate filters were removed and processed for liquid scintillation spectrometry after 0, 2, 4, and 6 hr of hybridization in sealed scintillation vials.

Southern Blots. The method of Jeffries and Flavell (21) was used with modifications as described (17). Nick-translation was performed by the method of Rigby *et al.* (23).

Expression Blots. The methods were essentially those as described by Courtney *et al.* (17).

RESULTS

Selection of DNA Clones Encoding Differentially Expressed Poly(A)$^+$RNA Sequences. Twelve clones were originally selected for study by replica screening of ≈10^5 plaques from a Charon 4A/AKR-2B genomic library with cDNA probes prepared from polyribosome-associated poly(A)$^+$RNA isolated from quiescent AKR-2B cells and from AKR-2B cells 6 hr after EGF stimulation (data not shown). Seven of these were selected on the basis of enhanced representation in poly(A)$^+$RNA from EGF-stimulated cells, but only three proved to be positive for differential expression in ensuing experiments.

Levels of Clone-Specific Poly(A)$^+$RNAs in Different Cell Types. The dot hybridization procedure of Kafatos *et al.* (22) was used as a rapid and semiquantitative means of determining the relative concentration of clone-specific poly(A)$^+$RNAs in quiescent and EGF-stimulated cells. DNA from each of the 12 clones was immobilized on filters and hybridized to *in vitro* ^{32}P-labeled polysomal poly(A)$^+$RNA from new cell preparations. Fig. 1 shows a series of such dot hybridizations. Independent preparations of cells were tested in a second experiment with identical results (not shown). Of the 12 clones, 9 were observed to hybridize similar amounts of the two different probes, indicating that the relative concentrations of these clone-specific sequences were essentially independent of the growth state of the cells.

Clones 10, 11, and 12 appeared clearly different. RNA species complementary to these three clones appeared to be present in considerably higher relative concentrations in poly(A)$^+$RNA from 6-hr EGF-stimulated cells than from quiescent cells. Fig. 1 also illustrates control hybridizations to *E. coli* DNA and to a cloned AKR murine leukemia virus proviral DNA genome, designated AKV. No selective enhancement of AKV-related RNA levels was observed after stimulation with EGF.

In order to more quantitatively assess the impressions drawn from the simple dot procedures, measurements were made of the kinetics of hybridization of poly(A)$^+$RNA from both cell populations to selected cloned DNAs. Under the conditions utilized in these experiments (cloned DNA excess), the rate of hybridization is proportional to the relative concentration of clone specific sequences and independent of the specific activity of the probe (22). The results of these experiments are shown in Fig. 2. RNA hybridizing to clone 16 showed no more than a 2-fold difference in relative concentration in probes from either cell type. In contrast, RNA species specific for clones 10, 11, and 12 were present in ≈5- to 12-fold increased relative concentrations in the poly(A)$^+$RNA population of 6-hr EGF-stimulated cells than in that of quiescent cells. The actual increase per cell in absolute sequence mass would be larger by a factor of 4 or 5 owing to the overall increased mass of poly(A)$^+$RNA in EGF-stimulated AKR-2B cells (12).

Shared Sequence Elements in Cloned DNAs. Individual cloned DNAs were further analyzed by restriction enzyme and cross-hybridization analyses. However, clone 16 was omitted from these determinations because of difficulties in preparing DNA from this clone. Agarose gel electrophoresis of the fragments produced by digesting each clone with *Eco*RI revealed mouse DNA inserts ranging in size from 11.5 to >20 kb (not shown). Each clone gave rise to a distinctive set of fragments, although several fragments of similar mobility were noted.

In order to test for the presence of common sequence ele-

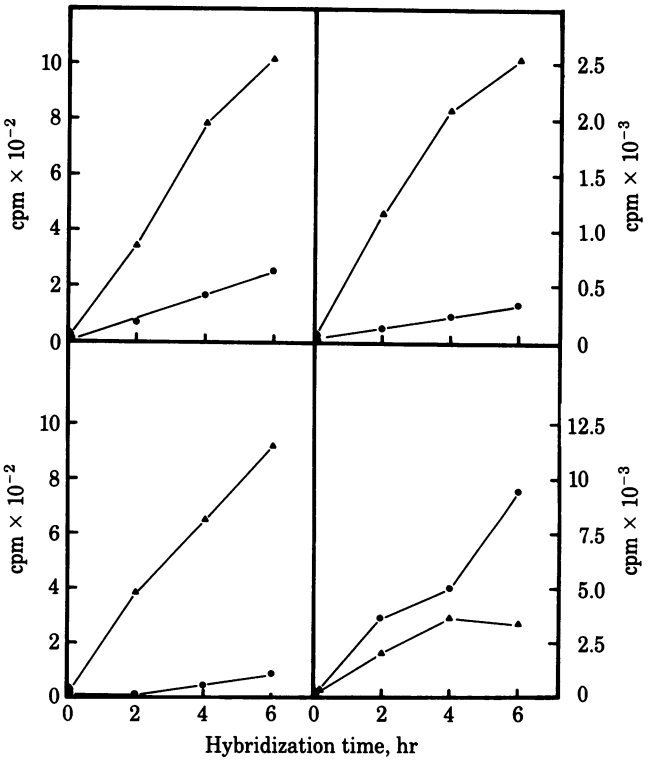

FIG. 2. Kinetics of hybridization of poly(A)$^+$mRNA to excess filter-bound cloned DNAs. DNAs from clones 10 (*Upper Left*), 11 (*Upper Right*), 12 (*Lower Left*), and 16 (*Lower Right*) were denatured and spotted onto filters in duplicate at 2.5 μg per dot. The DNA dots were hybridized to 2 μg of kinase ^{32}P-labeled polysomal poly(A)$^+$mRNA from either quiescent AKR-2B cells (●) or cells stimulated with EGF for 6 hr (▲).

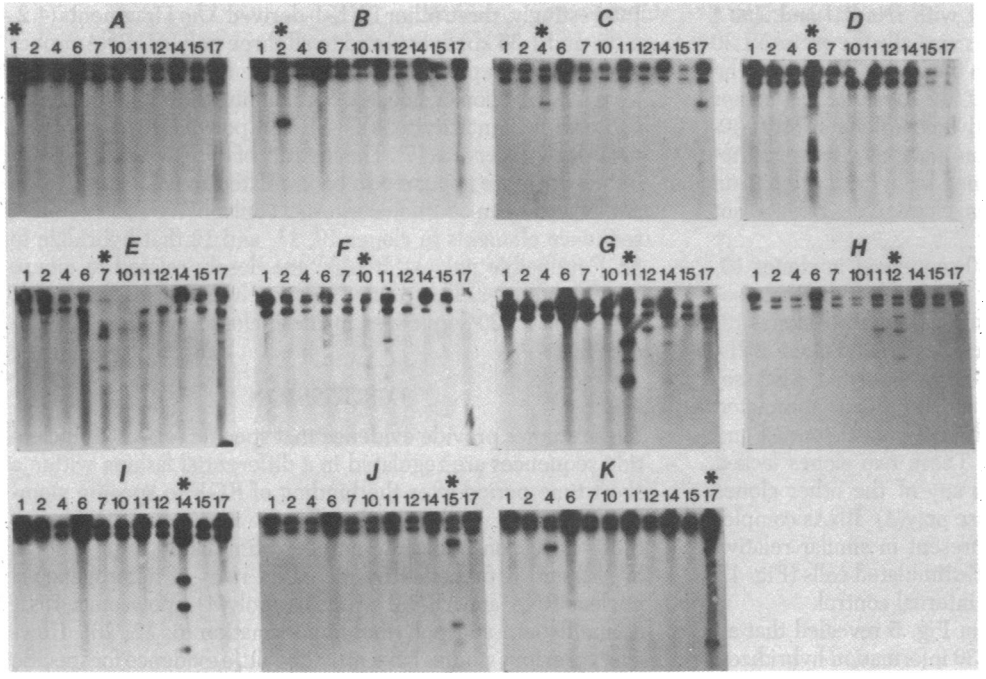

FIG. 3. Cross-hybridization analysis of cloned DNAs *Eco*RI-digested DNAs from 11 individual genomic clones were electrophoresed on 11 different 0.7% agarose gels and then transferred to nitrocellulose paper. Each filter was hybridized to one of 11 nick-translated probes corresponding to a respective cloned DNA (the clone-specific probe is denoted by an asterisk). Hybridization was followed by washes of increased stringency. All filters corresponding to the autoradiograms shown were washed with 0.015 M NaCl/0.0015 M Na citrate, pH 7, at 65°C. The strongly hybridizing bands at the top of the gels correspond to the 19- and 12-kb separated arms and the 31-kb hybridized arms of the phage λ vector. Clones 4 and 10 contain *Eco*RI-derived inserts between the 12- and 19-kb phage λ arms.

ments, similar *Eco*RI digests were blotted onto nitrocellulose and hybridized to nick-translated aliquots of each cloned DNA (Fig. 3). Clones 1, 2, 6, 7, 14, and 15 were judged to contain little sequence homology with any other clone, although a few faint cross-hybridizing bands could be observed in several instances. Because these bands were generally more pronounced prior to stringency washing of the blots (see legend to Fig. 3), it is likely that they represent members of a family of related, but not identical, highly repetitive DNA sequence elements. This conclusion was strengthened by the fact that each cloned probe gave rise to a background smearing pattern of varying intensities when blotted against *Eco*RI digests of mouse genomic DNA (not shown).

Two examples of much stronger sequence homology were detected. A single 5.1-kb *Eco*RI fragment of clone 4 strongly hybridized with a similar-size fragment derived from clone 17 (Fig. 3 *C* and *K*). In addition, two fragments (4.6 and 1.9 kb) derived from clone 11 showed homology with a 6.0-kb fragment from clone 12 (Fig. 3 *G* and *H*). Some homology was also observed between clones 10 and 11 (Fig. 3*F*), although this was more difficult to assess due to a similar mobility of the *Eco*RI fragments of clone 10 and the strongly hybridizing Charon 4A arms. These results suggested that clones 4 and 17 and clones 11 and 12, and possibly clone 10, might represent multiple isolates of related gene families. Alternatively, they could represent overlapping segments of a single gene or different genes associated with closely related repetitive sequences.

Identification of VL30-Related Sequence Elements in Highly EGF-Inducible Clones. Studies from our laboratory have demonstrated that the expression of a class of retrovirus-like or transposon-like sequence elements termed VL30 (24) is selectively enhanced in AKR-2B cells continuously cultured for many generations in the presence of EGF (17). To test for the possible presence of VL30 sequence elements in the clones studied here, Southern blots of cloned DNAs were hybridized with a VL30-specific probe derived from a clone previously characterized in our laboratory (17). This clone, termed BVL-1, contains a complete 5.2-kb VL30 sequence including long terminal repeats (LTRs) and ≈7 kb of unrelated flanking DNA. Several restriction fragments derived from clones 10, 11, and 12 but no others specifically hybridized to the VL30 probe (Fig. 4*A*). In order to eliminate the possibility that the observed hybridization was simply due to minor contamination of the gel-purified probe with non-VL30-flanking sequences, ^{32}P-labeled aliquots of clones 10, 11, and 12 were hybridized to diagnostic restriction enzyme digests of clone BVL-1. All three probes specifically hybridized to four internal restriction fragments that

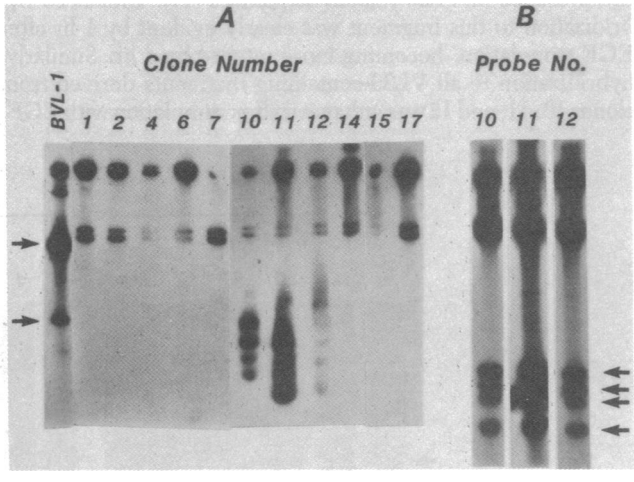

FIG. 4. Analysis of selected clones for homology to VL30 genes. (*A*) DNA from 11 of the 12 clones discussed in the text were digested with *Eco*RI, *Hin*dIII, and *Xba* I. Clone BVL-1 was digested with *Eco*RI and *Xho* I, and all digests were electrophoresed on a 1.0% agarose gel, transferred to nitrocellulose paper, and hybridized to a nick-translated VL30-specific probe derived from clone BVL-1 (4.5×10^7 cpm). The filter corresponding to the autoradiogram shown was washed with 0.015 M NaCl/0.0015 M Na citrate, pH 7, at 65°C. The top four bands are derived from the phage λ vector. The topmost arrow indicates the position of a 4.7-kb fragment derived internally from the VL30 sequence in clone BVL-1, whereas the lower arrow denotes the position of an ≈2-kb junction fragment containing both VL30 and flanking DNA sequences (17). (*B*) BVL-1 DNA was digested with *Eco*RI, *Hin*dIII, and *Xho* I, electrophoresed on a 1.0% agarose gel, transferred to nitrocellulose paper, and probed with nick-translated DNA from clone 10, 11, or 12. Arrows indicate the position of four fragments internal to the VL30 sequence in clone BVL-1 (see text).

resulted from digesting clone BVL-1 with *Hin*dIII and *Xho* I (Fig. 4*B*). These fragments encompass virtually the entire VL30 sequence in clone BVL-1 (17). Within the limits imposed by the lengths of these restriction fragments, we conclude that clones 10, 11, and 12 contain a representative complement of VL30-specific information. This information includes sequences homologous to a VL30 LTR as determined by hybridization to an LTR-specific subclone derived from clone BVL-1 (data not shown).

In order to determine if the VL30 sequences in clones 10, 11, and 12 were related to the highly EGF-inducible RNA species that hybridized to these clones, similar digests of clones 10, 11, and 12 and the previously characterized VL30 clone BVL-1 were blotted and hybridized to ^{32}P-labeled poly(A)$^+$RNA isolated from quiescent AKR-2B cells and from cells stimulated with EGF for 1 and 4 hr, respectively. Digests of clones 1 and 14 were also included in the assay. These two clones lacked detectable sequence homology with any of the other clones utilized in these experiments. Because poly(A)$^+$RNAs complementary to these two clones were present in similar relative concentrations in quiescent and EGF-stimulated cells (Fig. 1), their inclusion serves as a necessary internal control.

An analysis of the data displayed in Fig. 5 revealed that all restriction fragments that contain VL30 information hybridized only weakly to poly(A)$^+$RNA from quiescent cells. It is especially important to note that these restriction fragments included a 4.7-kb *Xho* I fragment derived from clone BVL-1 (17, 25). Recent sequence determinations have established that the *Xho* I sites delineating this fragment lie completely within the LTR sequence (unpublished data). Thus, this fragment contains a complete VL30 sequence, with the exception of one copy of the LTR, and lacks non-VL30-flanking sequences. Therefore, hybridization of poly(A)$^+$RNA to this fragment is diagnostic of the presence of VL30-related sequences (17). Enhanced hybridization to this fragment was clearly evident by 1 hr after EGF stimulation, becoming more intense by 4 hr. Similarly, hybridization to all VL30-containing fragments derived from clones 10, 11, and 12 was enhanced after stimulation with EGF. Interestingly, three other BVL-1-derived *Xho* I fragments (4.2, 2.05, and 1.35 kb) hybridized to all three poly(A)$^+$RNA probes in an EGF-independent fashion. These fragments are derived from a 7-kb region of mouse DNA flanking the VL30 sequence, and complementarity to AKR-2B cell poly(A)$^+$RNA has been previously observed (17). The identity of this RNA is not known but its presence appeared to be unrelated to EGF stimulation and served as an additional internal control. We conclude that sequence elements in clones 10, 11, and 12 that hybridize to EGF-inducible poly(A)$^+$RNA(s) are closely related to mouse VL30 sequences. These data do not exclude the possibility that other non-VL30 sequences in these clones are also EGF inducible.

DISCUSSION

These studies provide evidence that specific classes of nucleotide sequences are regulated in a differential fashion within a short time period after the binding of EGF to specific membrane receptors. In AKR-2B cells, the initiation of DNA synthesis in response to acute mitogen stimulation requires ≈12 hr (12) and is preceded by increased rates of heterogeneous nuclear RNA and rRNA synthesis, poly(A)$^+$ polysomal RNA accumulation, and polyribosome formation (6, 12, 26). However, previous studies have provided little evidence for specific regulation of individual sequences (12).

The physiological significance of VL30 genes is not known. They are a dispersed class of moderately repetitive sequence elements (≈100 copies per haploid genome), which are 5.2 kb long, contain 0.5-kb-LTRs, and encode a major 30S RNA transcript of unknown function (24, 27–29). Recent studies from our laboratory have shown that VL30 genes are arranged in a generally polymorphic fashion in the genomes of all mice examined, irrespective of species or geographic origin, and are genetically related to a similar class of sequences in the rat genome (25). Therefore, VL30 genes appear to have arisen in rodent genomes prior to *Mus* speciation. They may represent either defective retrovirus genomes acquired early in rodent evolution or a class of retrovirus-like cellular transposable elements sim-

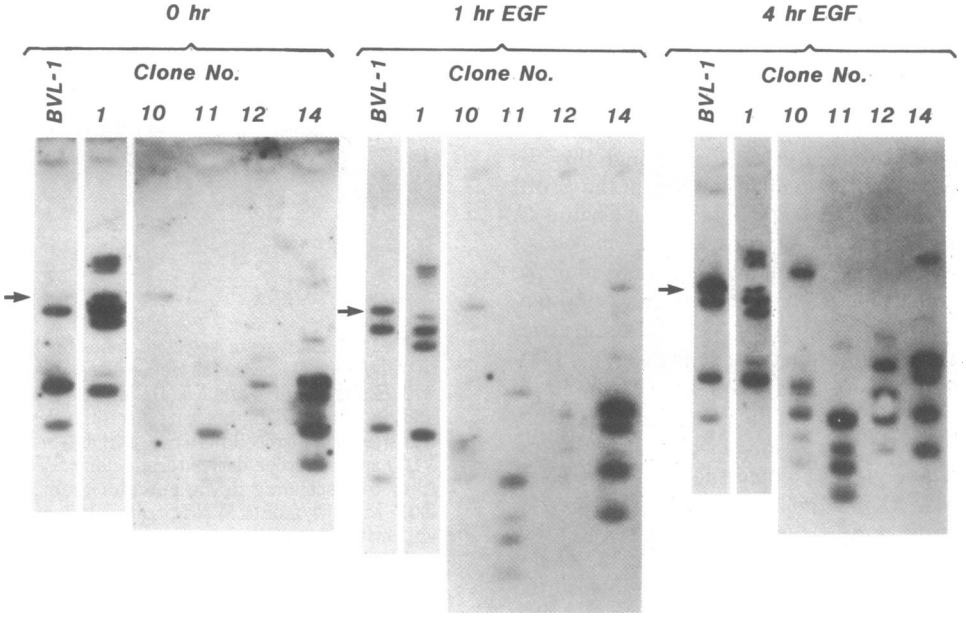

FIG. 5. "Expression" blot analysis of cloned DNAs. BVL-1 DNA digested with *Eco*RI and *Xho* I and DNA from clones 1, 10, 11, 12, and 14 digested with *Eco*RI, *Hin*dIII, and *Xba* I were electrophoresed on three 1.0% agarose gels, transferred to nitrocellulose paper, and hybridized to 2 μg of kinase ^{32}P-labeled polysomal poly(A)$^+$mRNA from AKR-2B cells treated with EGF for 0, 1, or 4 hr. The blots shown have been washed with 0.015 M NaCl/0.0015 M Na citrate, pH 7, at 65°C. The arrows indicate the position of the VL30-specific 4.7-kb *Xho* I fragment of clone BVL-1.

ilar to the *copia* sequences of *Drosophila* and the Ty-1 sequences of yeast (30, 31).

The finding that clones 10, 11, and 12 represent independent isolates of VL30 gene sequences was initially surprising because no criteria other than complementarity to EGF-inducible poly(A)$^+$RNA was applied during selection. However, several earlier studies have suggested an association between VL30 gene expression and the proliferative capacity of cells. Enhanced accumulation of VL30 RNA has been shown to occur in a wide variety of rodent cell lines that display growth characteristics of transformed cells (24, 32). Howk *et al.* have reported further that the levels of VL30 RNA in several clones of NIH 3T3 cells correlates with the differing ability of the clones to grow in soft agar (33). In addition, studies in our laboratory have shown that enhanced VL30 gene expression occurs in two chemically transformed cell lines and in nontransformed AKR-2B cells continuously cultured in the presence of EGF (17). In the latter case, however, it could not be determined whether the enhancement was directly related to EGF or indirectly associated with long-term metabolic changes induced by chronic mitogen stimulation. The present studies clarify this issue and raise the possibility that enhanced VL30 gene expression in transformed cells may be related to the secretion of and possible autostimulation by growth factor-like compounds known to be produced by certain transformed cells and tumors (34–36).

The variable locations of VL30 elements in the chromosomes of different mice makes it unlikely that they encode a gene product important in normal cell growth control. It is possible, however, that they have acquired structural features, perhaps specific nucleotide sequences, that facilitate EGF-dependent regulation by as yet undetermined mechanisms. Precedent for this possibility is afforded by the regulation of mouse mammary tumor virus RNA synthesis by glucocorticoid hormones (37). In this case, specific sequences present in the mouse mammary tumor provirus LTR are most likely responsible for conferring hormone responsiveness (38). In this regard, we have recently determined the nucleotide sequence of a large fraction of a VL30 LTR (to be published elsewhere). In addition to putative promoter and polyadenylylation signals, this sequence contains several pairs of internal tandem direct repeats. One of these, a 36-base-pair tandem repeat, is partially homologous to a region of the 72-base-pair tandem repeat sequence found in the LTR of the murine leukemia virus-related Moloney sarcoma virus (39). It will be of interest to determine whether this is indicative of functional similarities, particularly the transcriptional enhancer activity associated with the Moloney virus direct repeat (39) and whether these sequences have significance in EGF-dependent regulation of expression.

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