The EMBO Journal vol.6 no.10 pp.2897–2905, 1987

Contact-dependent regulation of vinculin expression in cultured fibroblasts: a study with vinculin-specific cDNA probes

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Communicated by B.Geiger

Vinculin specific cDNA clones were isolated from chicken embryo fibroblast (CEF) cDNA library in λgt11. The clones, ranging in size from 2.8 to 5.0 kb, were initially selected by rabbit antibodies to vinculin. Their identity was further confirmed by their specific reactivities with a battery of different vinculin-specific monoclonal antibodies. Southern blot analysis of restriction enzyme digested chicken spleen DNA suggested that all the isolated cDNA clones correspond to the same gene(s). Northern blot hybridization revealed that the vinculin-specific cDNA clones react with a single 6.5 kb mRNA in total cellular RNA preparations of CEF, whole chicken embryos and chicken gizzard smooth muscle. Moreover, fractionation of CEF poly(A)$^+$ RNA by sucrose gradient centrifugation followed by translation in cell free system indicated that the mRNA coding for vinculin has a size of about 6.0–7.0 kb. The identity of these clones was finally confirmed by selection hybridization assay. The isolated vinculin-specific cDNA probes were subsequently used in order to study the effect of substrate adhesiveness on the expression of vinculin. We show here that cells cultured on highly adhesive substrate, such as endothelial extracellular matrix (ECM), form large vinculin-rich focal contacts, while cells grown on poorly adhesive substrate poly(2-hydroxyethyl methacrylate) [poly(HEMA)] contain only small distorted vinculin spots. These morphological differences were accompanied by over 5-fold reduction in vinculin synthesis in cells growing on poly(HEMA), compared to those cultured on the ECM and over 7.5-fold decrease in the levels of vinculin-specific mRNA. We thus suggest that cell contact formation induced by various substrates may regulate the expression of vinculin gene, thereby affecting the assembly of adherens type junction.

Key words: cell-adhesions/focal-contacts/gene expression/vinculin

Introduction

Vinculin is a 130 kd microfilament-associated protein which is ubiquitously present in the plaque domain of cell-to-cell and cell-to-substrate adherens type junctions (Geiger, 1979; Geiger *et al.*, 1980). In these sites vinculin interacts with additional junctional proteins forming transmembrane complexes which interconnect actin filaments to the cytoplasmic surfaces of the junctional membrane (Geiger, 1981a, 1983; Geiger *et al.*, 1985a). It is commonly believed that these cytoskeleton-bound cell contacts play a direct role in processes such as cell anchorage, motility and morphogenesis and may affect cell growth and cytodifferentiation (Geiger *et al.*, 1984a,b).

It is notable that in spite of a wealth of information on the distribution of vinculin, only little is known on its fine structure and molecular interactions. One of the sources of difficulty in such studies is the complexity of the junctional structures and their molecular heterogeneity. It has been shown that cell-matrix contacts also contain, besides vinculin, the unique components talin (Burridge and Connell, 1983a,b; Geiger *et al.*, 1985b) and integrin (Horwitz *et al.*, 1985, 1986; Hynes, 1987) which are apparentally absent from intercellular adherens junctions. The intercellular adherens junction display, however, specific constituents, such as A-CAM (Volk and Geiger, 1984a, 1986a,b), L-CAM (Boller *et al.*, 1985) or plakoglobin (Cowin *et al.*, 1986) which are not found in sites of contact to non-cellular matrices.

Another source of variability is the intrinsic heterogeneity of vinculin itself. It has been shown that vinculin exists as a group of antigenically indistinguishable isoelectrophoretic variants (Geiger, 1982). Studies on isoforms diversity indicated that the different vinculin isoforms display distinct subcellular localizations and their relative proportions may vary from one cell type to the other. In addition, it has been shown that muscle tissues contain a variant molecule with higher mol. wt (~150 kd) denoted metavinculin, which share similar peptide maps patterns and antigenic properties with vinculin, yet shows a distinct extraction profile (Feramisco *et al.*, 1982; Siliciano and Craig, 1982, 1987; Craig, 1985).

Another matter of interest concerns the role of vinculin in the cascade of events which lead to the formation of the adherens junctions. It has been proposed that the establishment of vinculin-rich plaques is locally initiated and regulated by the contact itself due to the reorganization of putative 'contact receptors' (Geiger, 1981b. For discussion of the molecular basis for junction dynamics, see Geiger *et al.*, 1984a,b). Interestingly, it has recently been reported that modulation of the extent of cell contacts by changing cell density or substrate adhesiveness, may affect the rate of vinculin synthesis. These cells, cultured under conditions which favored extensive cell contact formation expressed considerably higher levels of vinculin than sparsely plated cells or cells cultured on poorly adhesive substrate (Ungar *et al.*, 1986). Despite the apparent generality of this phenomenon, the molecular mechanism underlying such contact-mediated regulation of contact-related molecules remained unclear.

In order to gain insight into the fine molecular structure of vinculin and study the control of its synthesis, we have cloned the vinculin gene. We report here on the isolation and characterization of several vinculin-specific clones from chick embryo fibroblast cDNA library in λgt11. Using these probes we show that the effect of substrate adhesiveness on vinculin expression is manifested at the mRNA level.

Results

Isolation of vinculin immunoreactive cDNA clones

Screening of 5×10^5 independent recombinants of CEF cDNA library in λgt11 (see 'Materials and methods') with vinculin specific rabbit serum revealed four putative vinculin clones designated cVin1, cVin5, cVin6 and cVin13. The protein pro-

ducts expressed by the purified clones reacted specifically both with affinity purified vinculin antibodies and a battery of vinculin specific monoclonal antibodies. As shown in Figure 1, the immunoreactivity of the four clones obtained with the different antibodies was variable, suggesting distinct expression of the relevant epitopes on each of the fusion proteins.

Characterization of the vinculin cDNA clones

To further characterize the putative vinculin clones, the different recombinant λgt11 DNA preparations were digested with *Eco*RI. Examination of the digestion products separated on 1% agarose gel indicated that the apparent sizes of the cloned cDNAs are: cVin1, 2.8 kb; cVin5, 4.9 kb; cVin6 and cVin13, 3.2 kb.

In order to determine the size of vinculin transcripts in cells and tissues, poly(A)$^+$ mRNA from primary cultures of chick embryo fibroblasts, or from 9-day-old chick embryos, were fractionated on a 1% agarose gel in 6% formamide. Hybridization of Northern blots of the RNA samples with each of the nick-translated vinculin clones revealed a single mRNA species of about 6.5 kb (Figure 2). Moreover, identical results were obtained by Northern blot analysis of RNA derived from a variety of chicken tissues, including gizzard smooth muscle (data not shown). Further substantiation for the identity of this transcript as vinculin mRNA was obtained by fractionation of poly(A)$^+$ mRNA of CEF on 15–30% sucrose gradient, followed by *in vitro* translation of the various fractions in reticulocytes cell free system. Immunochemical examination of the translation products indicated that the mRNA coding for vinculin migrates in a fraction corresponding to 6–7 kb (Figure 3).

In view of the close relationships of all four isolated probes (see above), we have used cVin5 DNA for selection hybridization analysis. An *Eco*RI digest of cVin5 DNA in λgt11 vector was immobilized to nitrocellulose and incubated with total CEF RNA. Filters were extensively washed and the hybridized RNA was eluted and translated in reticulocytes cell-free system. Examination of the translation products by SDS–PAGE indicated that the selected mRNA transcript specifically directed the synthesis of a 130 kd polypeptide (Figure 4), comigrating with vinculin. This protein product was not synthesized by RNA selected on λgt11 DNA.

Comparative restriction map analysis of the three vinculin-specific cDNA clones revealed extensive homology as shown in Figure 5. With the exception of the *Hin*dIII site found only in cVin13 and cVin5 and the *Ava*I site present exclusively at the 3′ end of cVin1, all other restriction sites within the overlapping regions were identical. The basis for the diversity of the *Hin*dIII and *Ava*I sites is not known yet. The possibility that it stems from allelic polymorphism is presently being examined.

Sequence analysis from the 5′ end of cVin5 disclosed a GC-

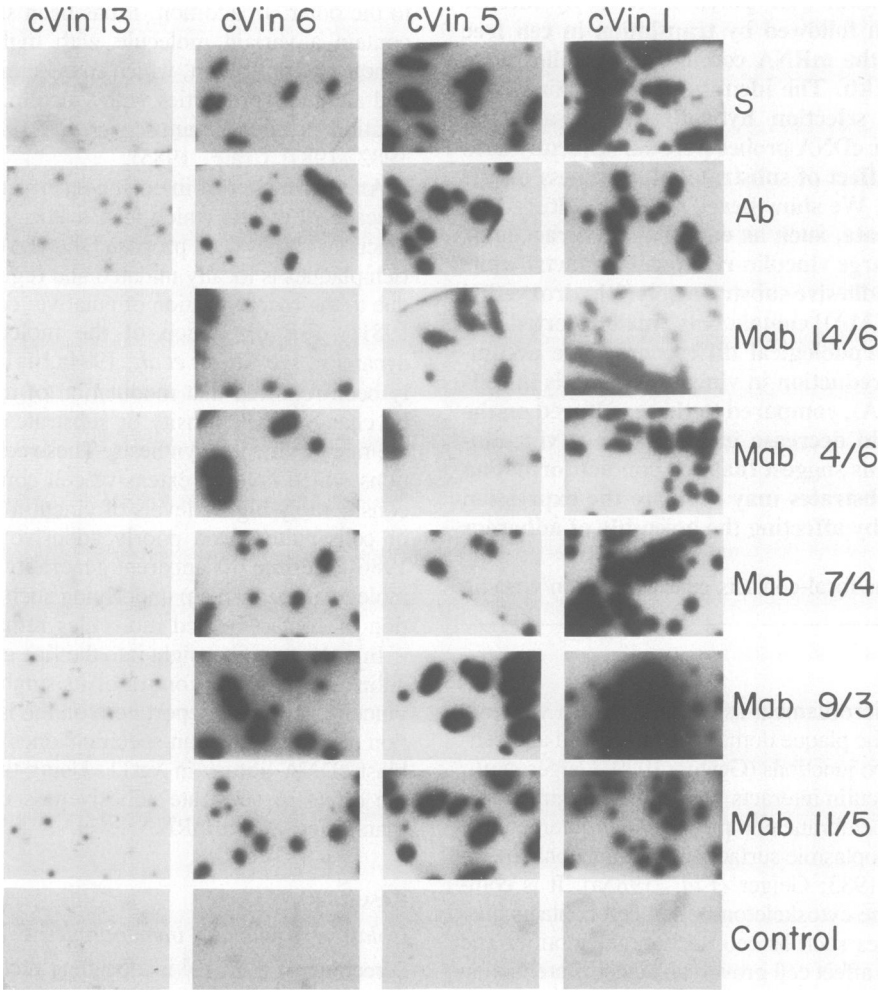

Fig. 1. Isolation and identification of vinculin cDNA clones by antibody screening. Immunoblotting analysis of vinculin-positive-λgt11 recombinant phages with vinculin-specific antiserum (S) affinity purified anti vinculin antibodies (Ab) and different vinculin specific monoclonal antibodies (Mab), as well as control, non relevant, monoclonal antibody (control).

rich region followed by the initiation ATG codon. Matching the nucleotide sequences obtained with those of Price *et al.* (1987) essentially pointed to an apparent identity. Moreover, the deduced *N*-terminal amino acid sequences were in line with those obtained by Vandekerckhove, Gimona and Small (personal communication). The cVin5 clone contained a stretch of 35 bp upstream to the initiation codon and cVin1 was shown to extend 210 bp further towards the 5′ end. Based on these results and on those obtained by Price *et al.* (1987) using primer extension we conclude that the 5′ non-coding region of the mRNA is ~250 bp long.

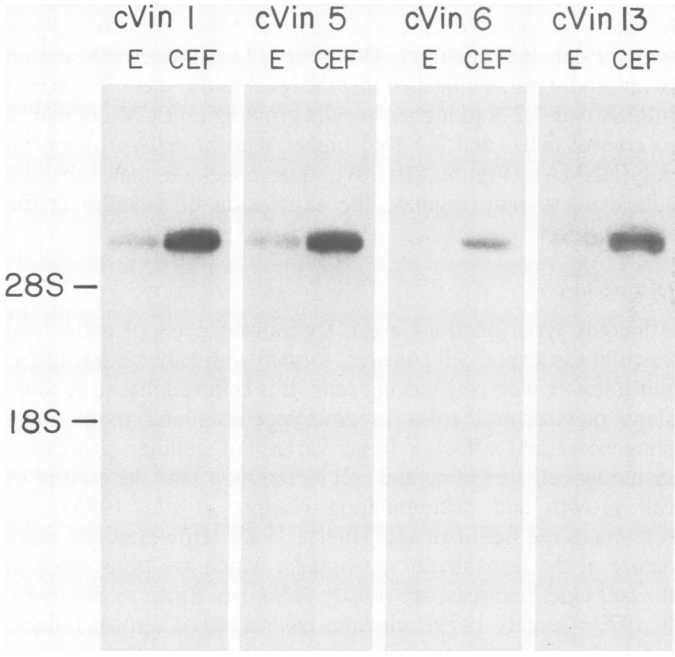

Fig. 2. Northern blot analysis with vinculin-cDNA of RNA from chick embryo fibroblasts (CEF) and from 9 days old whole chick embryo (E). Total cellular poly(A)$^+$ RNA (10 μg/lane) was separated on 1% agarose gel, blotted and hybridized with the different [^{32}P]vinculin-specific cDNA probes. The approximate size of the single mRNA species detected on the gel is 6.5 kb.

According to the number of amino acids in the vinculin molecule [based on amino acid analysis (Geiger, 1980; Jockush and Isenberg, 1981)], the expected length of the coding region is approximately 3.6 kb. This implies a long (~2.6–2.7 kb) non-coding 3′ region as shown in Figure 5.

Southern blot analysis of chicken genomic spleen DNA

High molecular weight chicken spleen DNA was completely digested with various restriction enzymes including *Eco*RI, *Pst*I, *Bam*HI, *Hae*III and *Hin*fI. The restriction fragments were electrophoretically separated, blotted and hybridized to the different ^{32}P-labeled cVin DNA probes.

As visualized in Figure 6, all the cDNA clones tested revealed a very similar hybridization pattern with the digestion products of both frequent and rare cutters. cVin5 displayed the broadest spectrum of recognition as cVin5 detected all the fragments reacted with cVin13 and cVin1. Moreover, all our cDNA clones reacted identically with a series of restriction fragments, including 9.5, 4.5 and 4.3 kb *Bam*HI fragments, 4.6, 3.0 and 1.75 kb *Eco*RI fragments, 3.2 kb *Pst*I fragment, 2.7 kb *Hae*III and 2.3 kb *Hin*fI fragment. However, some differences among the various vinculin probes were manifested; for example, cVin1 does not react with 6.5 kb *Pst*I fragment, detected by cVin5 and cVin13, while cVin13 does not react with 1.6, 2.3 and 5 kb *Eco*RI fragments recognized by cVin1 and cVin5. We, therefore, conclude that all the isolated vinculin clones recognize various segments of the same gene(s).

Contact dependent regulation of vinculin expression

In order to study the role of substrate adhesiveness on the expression of vinculin, we have plated chick embryo fibroblasts

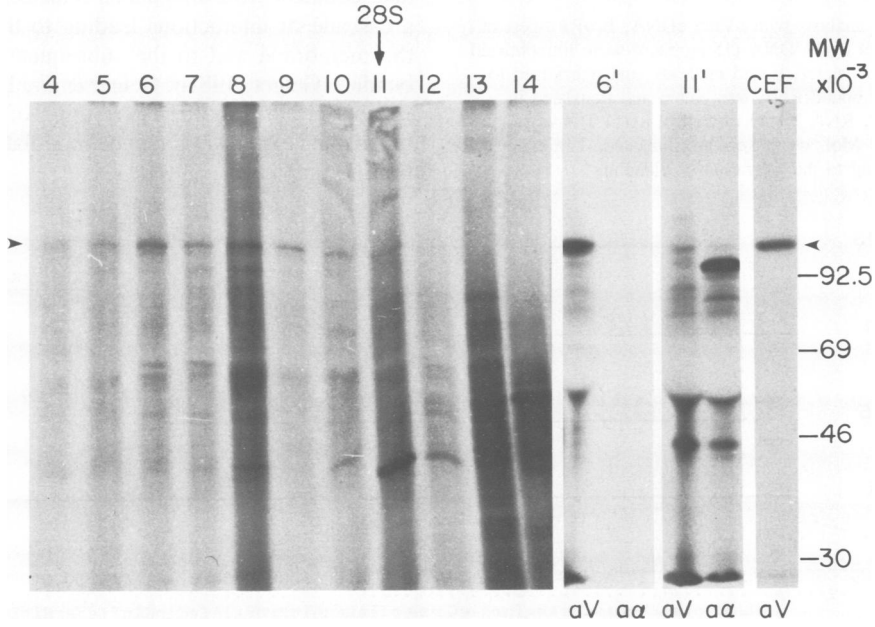

Fig. 3. Fractionation of poly(A)$^+$ RNA from chick embryo fibroblasts on sucrose gradient. Poly(A)$^+$ RNA (100 μg) was fractionated on 15–30% sucrose gradient, as detailed in 'Materials and methods' and its various fractions were collected from the bottom tube. The translation products of each fraction were immunoprecipitated with anti vinculin (aV) or anti α-actin (aα). Vinculin was detected mostly in fraction No. 6 (6′), while α-actin was present in fraction 11 (11′). The immunoprecipitated vinculin comigrated with metabolically labeled vinculin from chick embryo fibroblast (CEF). Mol. wt markers are indicated, as well as the location of the vinculin band. Based on the relative migration of 28A and 18S rRNA, the peak of vinculin transcript appears to migrate in fraction corresponding to 6–7 kb RNA.

at subconfluency on endothelial extracellular matrix (ECM) or on poly(2-hydroxyethyl methacrylate) [poly(HEAM)]-coated plates, as well as on control, untreated tissue culture dishes. Microscopic examination revealed remarkable differences in the morphology of the cells growing on the different substrates. Cells plated on the ECM coated dish formed a flat monolayer with the cells often displaying an epithelial morphology (Figure 7A). Immunofluorescent labeling of these cells revealed many large, vinculin-rich focal contacts along the ventral cell membrane. In contrast, cells plated on poly(HEMA) were mostly rounded or spindle-shaped and contained only few, distorted, vinculin spots (Figure 7C). To study the effect of the various substrates on the rate of vinculin synthesis, cells were metabolically labeled with [^{35}S]methionine and samples containing equal amount of TCA precipitable counts, subjected to immunoprecipitation. Densitometric scanning of the autoradiograms indicated that the level of vinculin synthesis in the well-spread cells, growing on ECM, was 2.3- and 5-fold higher than that found in control cells or in cells growing on poly(HEMA), respectively (Figure 8A). These results are consistent with the previous results indicating that vinculin synthesis correlates with the extent of cell spreading (Ungar *et al.*, 1984).

In order to determine whether the changes in vinculin expression are due to alteration in the level of vinculin mRNA, or to post-transcriptional events, we analysed vinculin mRNA directly, using our newly isolated vinculin specific cDNA probe. Total RNA extracted from cells growing on either ECM, poly(HEMA) or control untreated dish was subjected to Northern blots analysis with ^{32}P-labeled vinculin cDNA probe. Densitometric scanning of autoradiograms indicated that the level of the specific vinculin mRNA was 3.2-fold higher in cells growing on ECM compared to control cells, and 7.5-fold higher than in cells growing on poly(HEMA) (Figure 8B). We thus conclude that substrate adhesiveness may regulate the expression of vinculin at the mRNA level.

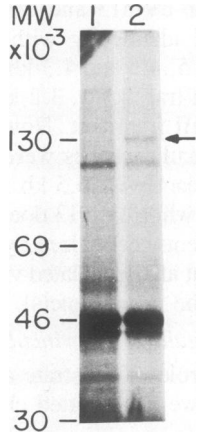

Fig. 4. Selection hybridization analysis with cVin5 cDNA. *Eco*RI digest of cVin5 DNA in λgt11 or control λgt11 DNA (15 μg each) were immobilized on nitrocellulose, as detailed in 'Materials and methods' and hybridized to total CEF RNA (100 μg). The bound RNA was eluted and translated in reticulocyte cell free system. 1, RNA bound to control λgt11 DNA, 2, RNA selected by cVin5 DNA. Mol. wt makers are indicated. The arrow points the position corresponding to the migration of vinculin.

Discussion

Adherens type junctions are a specialized class of actin- and vinculin-associated cell contacts, formed with either extracellular substrates or with neighboring cells. It is believed that such adhesions play central roles in embryogenesis and tissue morphogenesis and affect a large variety of cellular processes, including cell spreading and cell locomotion, and the control of cell growth and differentiation (Geiger *et al.*, 1985a; for references see Edelman and Thiery, 1985). However, the exact nature of the transmembrane signals, which are transmitted in the cell junction areas, are mostly unknown. Since its discovery in 1979 (Geiger, 1979), vinculin has served as a most reliable molecular landmark for adherens type junctions. Dissection of these cell-contacts into their constituting subdomains indicated that vinculin is a component of the membrane-bound junctional plaque (Geiger *et al.*, 1981, 1985b). Moreover, we have shown that the membrane-bound vinculin maintains a dynamic equilibrium with a soluble cytoplasmic pool (Geiger *et al.*, 1984a; Kreis *et al.*, 1984). We also proposed that the formation of surface contacts with exogenous matrices or membranes triggers a cascade of interactions leading to the binding of vinculin to the membrane and to the subsequent local assembly of actin bundles (Geiger, 1981b; Geiger *et al.*, 1984b). As will be discussed below, not only the distribution of vinculin within the cells but also its expression may be modulated by cell contact formation (Ungar *et al.*, 1986).

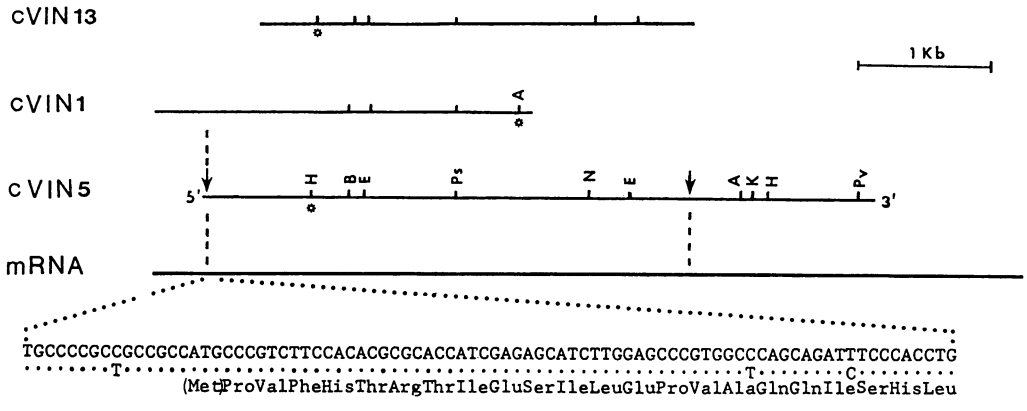

Fig. 5. Restriction map analysis of the three vinculin-specific cDNA clones (cVin13, cVin1 and cVin5) and their alignment along the vinculin mRNA. The restriction enzymes used included *Hin*dIII (H), *Bam*HI (B), *Eco*RV (E), *Pst*I (Ps), *Nco*I (N), *Ava*I (A), *Kpn*I (K) and *Pvu*II (Pv). Notice that one *Hin*dIII site is present on cVin13 and cVin5 (asterisks) but not on cVin1 and the reverse is true for the *Ava*I site on cVin1 (asterisk). Partial nucleotide sequence of cVin5 is presented, matched with the sequence of a corresponding region in a clone of Price *et al.* (1987). Few discrepancies are marked, as well as the deduced *N*-terminal amino acid sequence. Direct protein sequence analysis suggests that the *N*-terminal amino acid is proline (see text). The arrows and aligned broken lines indicate the sites of initiation and predicted termination.

Unfortunately, despite many biochemical and immunohistochemical studies, which supplied considerable information on the general properties of vinculin and its patterns of organization, the detailed structure of the molecule and its functional domains are not known yet. It has thus become evident that further information, regarding the structure of vinculin and its controlled expression, should be obtained through the use of vinculin-specific nucleic acid probes. In this article we describe the isolation of such vinculin specific cDNA clones and their application for the study of the effect of contact formation on the expression of vinculin specific mRNA.

Using an immunochemical screening procedure we selected four vinculin immunoreactive cDNA clones of CEF cDNA library in λgt11. The fusion protein products of all the isolated clones reacted with both poly and monoclonal vinculin antibodies, but the degree of immunoreactivity of the isolated clones with different antibodies was not uniform. For example, the protein product of cVin1 showed the highest immunoreactivity with all antibodies, while cVin13 showed the lowest immunoeractivity with vinculin antiserum and hardly reacted with Mabs 4/6 and 4/16. cVin5 showed an intermediate immunoreactivity. These differences do not reflect the relative sizes of the cloned cDNA since the latter (cVin5) is actually the largest and includes cVin13 and the majority of cVin1 (Figure 5). It is thus likely that conformational variations among the fusion proteins affect the antigenic behavior. It is anticipated that when the complete primary structure coded for by the different clones is available, it will become possible to relate the particular antigenic reactivity to specific segments of the vinculin molecule.

All the isolated vinculin cDNAs recognize, under stringent hybridization and washing conditions, a single 6.5 kb mRNA species in both total and poly(A)$^+$ RNA preparations of chick embryo fibroblasts, embryonic tissues and chicken gizzard smooth muscle. The authenticity of the 6.5 kb mRNA as a vinculin transcript was independently confirmed by fractionation of mRNA on a sucrose gradient followed by translation in a cell-free system and immunoprecipitation. As shown in Figure 3, vinculin-specific mRNA migrated in fractions corresponding to 6−7 kb mRNA. this size of vinculin mRNA is considerably larger than the 3.6 kb mRNA expected, on the basis of protein molecular weight and amino acid composition of the vinculin molecule. We cannot yet provide an explanation for the significance of the large size of vinculin transcript. We anticipate that when detailed information is available on the primary structure of the vinculin gene and on the entire nucleotide sequence of the 3′ non-coding region of the mRNA, this aspect will be clarified.

Since only a single transcript is detected by Northern blot analysis, one may conclude that all vinculin isoforms are coded for by the same mRNA species and thus the differences among the isoforms are a result of post-translational modifications. Another matter of interest concerns the molecular relationships between vinculin and ~150 kd protein metavinculin which is present in muscle cells. It has previously been shown that the two proteins share similar peptide map and immunoreactivity (Geiger, 1982; Feramisco *et al.*, 1982; Siliciano and Craig, 1982, 1987; Craig, 1985; Volberg *et al.*, 1986). However, at the level of mRNA, we did not detect additional transcripts, besides the vinculin mRNA, in chicken gizzard RNA which might code for metavinculin. We do not know whether the cDNA probes used here have the capacity to hybridize with metavinculin mRNA or else both proteins are coded for by the same mRNA species, which is ap-

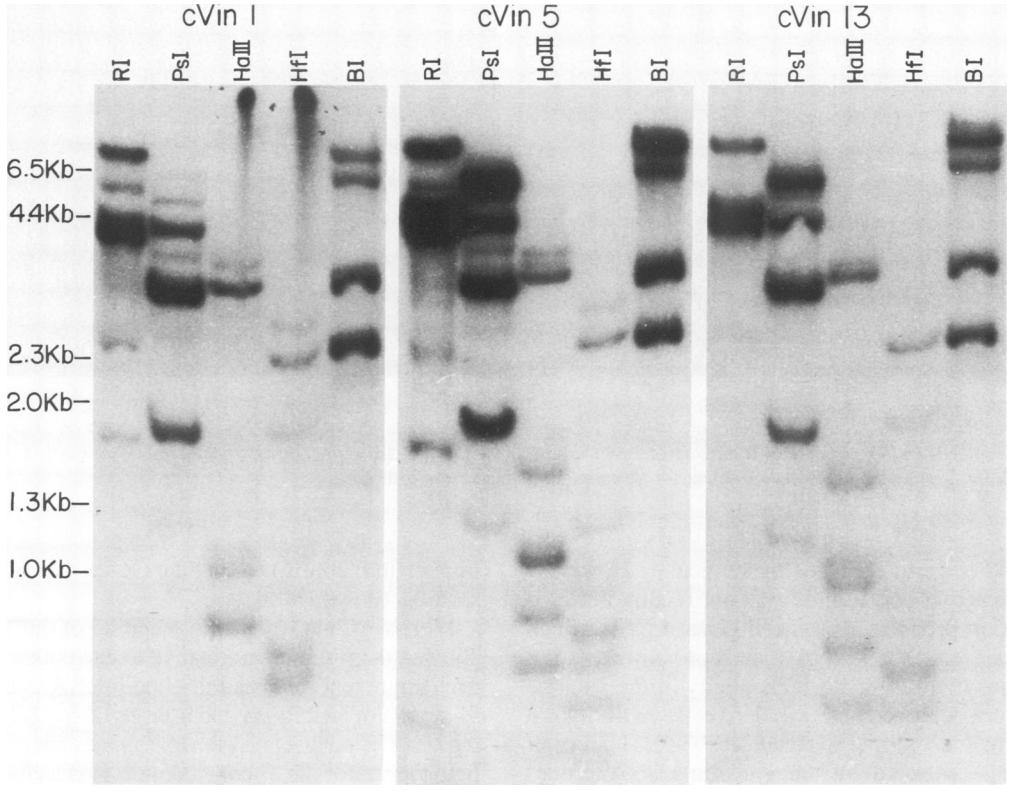

Fig. 6. Southern blot analysis of genomic chick spleen DNA. Chicken spleen DNA (10 μg) was digested with *Eco*RI (RI) or *Pst*I (PsI) or *Hae*III (HaIII) or *Hin*fI (HfI) or *Bam*HI (BI) and fractionated on 1% agarose gel, blotted onto nitrocellulose and hybridized to each of the ^{32}P-labeled cVin probes (cVin1, cVin5, cVin13). Molecular weight (M.W.) of the DNA fragments were determined by comparison with the migration of *Hin*dIII digests of λ phage and *Hae*III digest of φX174.

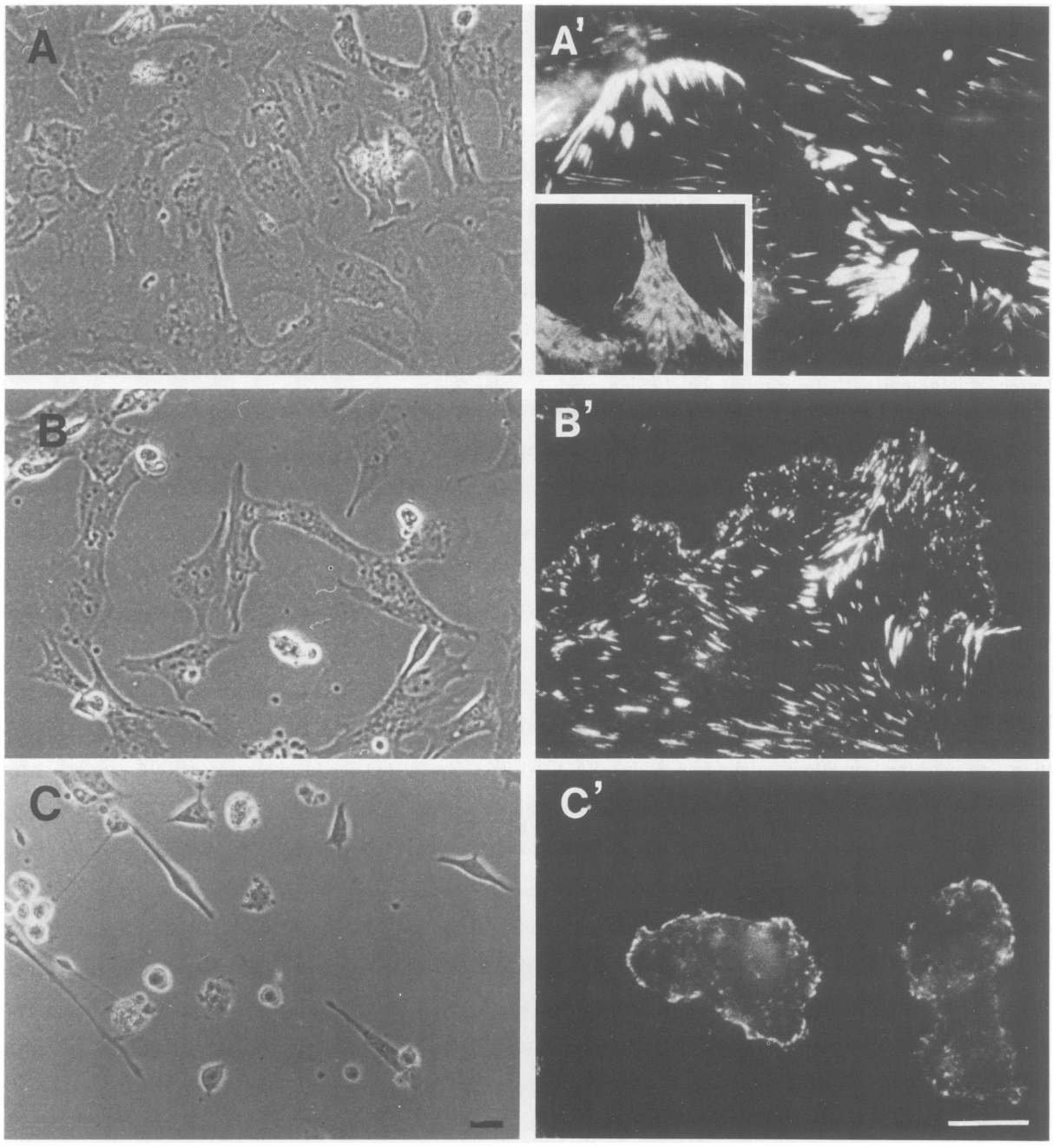

Fig. 7. Effect of substrate adhesiveness on cell morphology (**A−C**) and vinculin organization (**A′−C′**). CEF grown for 2 days on extra cellular matrix from bovine corneal endothelial cells (**A,A′**), CEF grown on control tissue culture dish (**B,B′**), CEF grown on poly(HEMA) (60 $\mu g/cm^2$) (**C,C′**). **A−C**, phase contract microscopy; **A′−C′**, immunofluorescence labeling for vinculin. Bars indicate 10 μM.

parently sufficiently large to code for metavinculin. However, a 150 kd immunoreactive polypeptide was not readily detected among the translation products of chicken gizzard RNA which were hybrid selected on cVin5 DNA (data not shown).

Our most rigorous criteria for the authenticity of the putative vinculin cDNA clones described here was derived from the hybrid selection experiment and a partial sequence analysis. The former confirmed that RNA selected by the vinculin cDNA probes directed the synthesis of vinculin in cell-free translation system. Matching of the 5′ sequences of cVin5 with those of Price *et al.* (1987) as well as with authentic *N*-terminal sequences of the protein (Vandekerckhove, Gimona and Small, personal communication) confirmed that the clones described here are indeed specific for vinculin.

Moreover, the restriction mapping of our three cDNA clones allowed their alignment relative to each other and to the mRNA. This alignment indicated that the mRNA contains a non-coding 5′ region of ~250 bp and a large (2.6−2.7 kb) 3′ non-coding region. The poly(A) tail, however, does not contribute significantly to the size of the 3′ non-coding region as found by ribonuclease H analysis of vinculin mRNA hybridized to oligo-dT.

To study the interrelationships between our isolated cDNA clones, we have compared their differential reactivities with restriction fragments of chicken genomic DNA. The closely

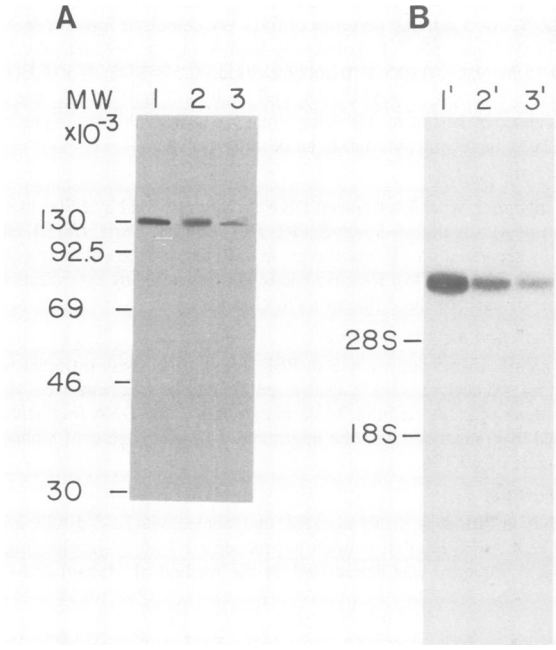

Fig. 8. Effect of substrate adhesiveness on vinculin protein and RNA expression. (**A**) Immunoprecipitation of metabolically labeled vinculin from chick embryo fibroblasts cultured on (1) ECM, (2) normal culture dish and (3) poly(HEMA) coated dish. Aliquots of equal amounts of TCA precipitable counts (3×10^5) were immunoprecipitated. Densitometric scanning indicated that the relative amounts of vinculin in slots 1,2,3 are: 2.3/1/0.46, respectively; (**B**) Northern blot analysis of total RNA (20 μg/lane) extracted from cell cultures as in **A**. [(1′)-ECM, (2′)-normal dish, (3′)-poly(HEMA)]. RNA was fractionated on 1% agarose in 6% formamide, blotted and quantitated by hybridization with ^{32}P-labeled cVin5 DNA. The relative amounts of vinculin specific mRNA in slots 1′,2′,3′ are: 3.2/1/0.43, respectively.

similar hybridization patterns obtained with the digestion products of both frequent (*Hae*III, *Hin*fI) and rare cutters (*Eco*RI, *Pst*I, *Bam*HI) indicated that all the vinculin probes react with the same gene or small, closely related, gene family. The minor differences among the clones could be attributed to the sequence variation of the different probes.

The first application of the vinculin cDNA probes described here was for the analysis of the effect of substrate adhesiveness on the expression of vinculin specific mRNA in cultured cells. For that purpose we have modified the tissue culture substrate so as to increase or decrease its adhesiveness. As the highly adhesive substrate, we have selected plates coated with endothelial ECM, and as the poorly adhesive substrate, culture dishes coated with poly(HEMA). The former was shown to greatly facilitate cell attachment and spreading of a large variety of cells, including cells which hardly attach to normal tissue culture substrates (Gospodarowicz *et al.*, 19878, 1989; Gospodarowicz, 1984; Vlodavsky *et al.*, 1980). Poly(HEMA) coating of culture dishes was shown to markedly restrict cell spreading and, at high concentration, to block cell attachment altogether (Folkman and Moscona, 1978). Moreover, the first indication that substrate adhesiveness may affect vinculin expression was recently reported, showing that fibroblastic cells cultured on poly(HEMA) synthesize ~7-fold less vinculin than those cultured on normal culture dish (Ungar *et al.*, 1986).

In our present study we found that chick embryo fibroblasts cultured on ECM-coated plates were flatly spread and tightly attached to the substrate through many large vinculin-containing focal adhesion plaques. This was in contrast to the sparse and distorted vinculin spots displayed by the cells growing on poly(HEMA). Immunoprecipitation of vinculin from metabolically labelled cells growing on the different substrates showed that the level of vinculin synthesis in the cells growing on ECM was 5-fold higher than that of the cells plated on poly(HEMA). Northern blot analysis indicated that the level of vinculin mRNA in cells growing on ECM was 7.5-fold higher than that found in cells growing on poly(HEMA) with the cells growing or regular plates expressing intermediate levels.

It should be mentioned that increase in vinculin synthesis in cells cultured on ECM is probably not universal since it was reported that in granulosa cells plated on such substrates the rate of vinculin synthesis is actually decreased (Ben-Zeev and Amsterdam, 1986). It may, nevertheless, be argued that the effect observed in that system is attributable to the differentiation of the cultured cells induced under these conditions and not to spreading *per se*. This issue is presently under direct investigation.

These findings indicate that environmental signals which can modulate the extent of cell adhesion and spreading, affect vinculin expression at the mRNA level: under culture conditions which favor establishment of extensive cell contact, the relative levels of vinculin mRNA increase considerably. Unfortunately, no direct information is yet available on the possible mechanism of such transcriptional control. In relation to this issue, it is interesting to consider the regulation of expression of the cytoskeletal protein, tubulin. It had been reported that the levels of soluble (unpolymerized) cytoplasmic tubulin down-regulate the transcription of its own gene in cells (Ben-Zeev *et al.*, 1979; Cleaveland *et al.*, 1981). Thus, general reduction in tubulin levels or extensive polymerization will lead to an enhanced transcription and therefore increased tubulin synthesis. In the case of vinculin, the possibility that a similar mechanism operates should be considered: it has been shown before that cellular vinculin may either be immobilized on the membrane of contact sites or diffusely distributed throughout the cytoplasm. Moreover, we have shown that the two pools constantly exchange components between them (Geiger, 1981b; Geiger *et al.*, 1984b). It may, therefore, be proposed that the recruitment of a large proportion of the cellular vinculin to newly formed contacts induced by an adhesive substrate will efficiently deplete the diffusable cytoplasmic pool and thereby stimulate vinculin synthesis. The contrary may be true for cells plated on poorly adhesive substrates. There is still no direct support to the hypothesis that the diffusible cytoplasmic vinculin autoregulates its own gene expression. Furthermore, it may be postulated that other elements, modulated by contact formation, function as second messenger and regulate vinculin expression. The availability of vinculin specific nucleic and antibody probes, as well as similar probes directed against other cell-contact constituents, may enable us to directly approach this aspect. This will shed light on the molecular mechanisms by which specific cell contacts can modulate structure and dynamics of cells and affect their growth and differentiation.

Materials and methods

Enzymes

All enzymes were purchased from New England Biolabs Inc., USA, unless otherwise indicated.

Immunochemical reagents

Rabbit serum directed against chicken gizzard vinculin was prepared and affinity purified according to Geiger (1979). Goat anti mouse Fab and goat anti rabbit IgG were affinity purified on the respective immobilized antigens and iodinated by the chloromine T method (Hunter, 1973). Conjugation of lissamine–rhodamine B sulfonyl chloride to antibodies was carried out as described (Brandtzaeg, 1973).

Cells

Chick embryo fibroblasts (CEF) were prepared from 5- to 9-days-old decapitated chicken embryos subjected to extensive trypsinization.

5×10^5 CEF were plated on 35 mm tissue culture dishes coated with 60 $\mu g/cm^2$ poly(2-hydroxyethyl methacrylate) [poly(HEMA)], Hydron Laboratories, New Brunswick, USA, as described (Folkman and Moscona, 1978), or on plates coated with extracellular matrix (ECM) from bovine corneal endothelial cells (kindly supplied by I.Vlodavsky of The Hebrew University in Jerusalem). The cells were maintained in Dulbecco Modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) and examined 2 days following plating.

Biosynthetic cell labeling and immunoprecipitation

Cells grown on different substrates were pulse-labeled for 1 h with [^{35}S]-methionine (Amersham, 50 μCi/ml, 1200 Ci/mmol) in methionine-free DMEM, containing 2% dialyzed FCS. Cells were washed twice with PBS at 4°C and extracted with RIPA buffer (50 mM Tris−HCl, 150 mM NaCl, 0.1% w/v SDS, 1% w/v Na-deoxycholate, 1% v/v Triton X-100, pH 7.2) in the presence of protease inhibitors: aprotinin (0.5 TIU/ml) and phenyl-methylsulfonyl-fluoride (PMSF) (2 mM). The extract was clarified by 5 min centrifugation at 10 000 *g* and aliquots corresponding to 3×10^5 trichloroacetic acid (TCA) precipitable counts were preadsorbed on *Staphylococcus aureus* Protein A (IgG-Sorb, The Enzyme Center, Inc., USA) and then immunoprecipitated with affinity purified vinculin antibodies and analyzed on SDS acrylamide gel. Fluorographed gels were exposed to X-ray film. The intensities of the vinculin bands of the fluorographs was measured with a Bio-Rad model 620 video densitometer.

Immunofluorescent labeling

Cells cultured on different substrates were permeabilized with Triton X-100 and fixed with 3% formaldehyde. Immunofluorescent labeling with affinity-purified vinculin antibodies was carried out as described (Geiger *et al.*, 1984a).

Isolation of cDNA clones

5×10^5 recombinants of large inserts − CEF cDNA library in λgt11, kindly provided by R.Hynes and J.T.Tamkum were screened according to Juynh *et al.* (1986). Phages were adsorbed to $CaCl_2$ treated *Escherichia coli* strain Y1090 plated at a density of 50 000 p.f.u./15 cm-plate and grown at 42°C for 3 h. 1 h after transfer of plates to 37°C, nitrocellulose filters (Schleicher and Schuell), previously soaked in 10 mM isopropylthiogalactoside (IPTG), were overlaid on plaques and further incubated for 7−8 h at 37°C, after which a second filter was applied for ~12 h. Filters were marked and transferred to 10% low fat milk (Tnuva, Israel), 0.05% Tween 20 in PBS. The two filters were washed and positive clones identified by crude rabbit antiserum or mouse monoclonal anti vinculin followed by ^{125}I-goat anti rabbit IgG or ^{125}I-rabbit anti mouse Fab (1×10^6 c.p.m./ml in milk−PBS solution). Positive clones were picked and plaque-purified λgt11 DNA containing positively cDNA clones was digested with *Eco*RI and inserts were size-fractionated on agarose gel. Insert cDNA was then used for subcloning into PBR.

Isolation and characterization of RNA

Total cell RNA was prepared by the LiCl/urea technique (LeMeur *et al.*, 1981) from 10^8 primary CEF or 9-day-old decapitated chick embryos. Cells or tissue were washed in phosphate buffered saline (PBS) and homogenized for 1 min in a Waring Blender in 3 M LiCi, 6 M urea, 0.1% SDS, 10 mM Na acetate pH 5.0 and 200 μg/ml heparin. After 16 h at 4°C, the pellet was recovered by centrifugation at 10 000 *g* for 30 min, washed twice in 4 M LiCl, 8 M urea and dissolved in 0.1% SDS, 0.1 M Na acetate, pH 5.0.

For Northern blot analysis, 10 μg poly(A)$^+$ RNA obtained by purification on oligo-dT cellulose or 20 μg of total RNA were subjected to electrophoresis on 1% agarose gel in 6% formaldehyde. RNA was transferred onto a nitrocellulose sheet and hybridized to 10^7 c.p.m. of each of the different cVin probes, radiolabeled by nick-translation to a specific activity of 1.3×10^8 c.p.m./μg.

RNA fractionation on sucrose gradient

100 μg of poly(A)$^+$ RNA were fractionated in 15−30% sucrose density gradient in 10 mM Tris acetate, 100 mM NaCl, 0.1 mM EDTA, 0.5% SDS pH 7.4 for 3 h at 20°C (45 000 r.p.m. in SW 50.1 rotor) fractions of 200 μl were collected, ethanol precipitated and translated in reticulocyte lysates.

Translation of RNA in reticulocytes lysate cell free system

0.1−0.4 μg poly(A)$^+$ RNA was incubated in a mixture of reticulocytes lysate, prepared as described by Jackson and Hunt (1983), in the presence of [^{35}S]methionine (~45 μCi, 1200 Ci/mmol) creatine kinase (20 μg/ml) creatine phosphate (1 mM), $MgCl_2$ (50 μM), KAc (8 mM), spermidine (35 μM) and Hepes buffer (2.4 mM) pH 7.5. Following incubation at 30°C for 1 h, 2 mM unlabeled methionine (Sigma) was added and the translation products analyzed either by immunoprecipitation (as described above) or directly by SDS acrylamide gel electrophoresis.

Selection hybridization

Equal amounts of cVin5 DNA in λgt11 or λgt11 DNA (15 μg) were *Eco*RI digested, incubated at 95°C then immediately ice-cooled and applied to nitrocellulose paper. The bound DNA was hybridized with 100 μg total CEF RNA at 50°C for 3 h in the presence of 65% v/v deionized formamide, 0.4 M NaCl, 20 mM Pipes, pH 7.4, extensively washed in 1 × SSC (standard saline citrate: 0.15 M NaCl, 0.015 M trisodium citrate) 0.5% SDS, 1 mM EDTA at 60°C, then in 1 × SSC, 1 mM EDTA, and finally in 2 mM EDTA. The bound RNA was eluted by heating to 100°C for 1 min in a water bath, ethanol-precipitated and translated in reticulocyte lysate as described above.

Genomic DNA analysis

15 μg of high mol. wt chicken spleen DNA (kindly provided by I.Shechter from our department) was digested with *Eco*RI, *Pst*I, *Bam*HI, *Hin*fI, *Hae*III, electrophoretically separated on 1% agarose gel in TBE and blotted onto nitrocellulose membrane (Southern, 1975). Reactive bands were identified by hybridization with 10^7 c.p.m. of each of the different cVin clones.

Partial restriction enzyme mapping

DNA of cVin1, cVin5 and cVin13 in PBR was digested with different restriction enzymes and analyzed on 1% agarose gel. Restriction fragments were visualized by ethidium bromide staining. Molecular weight of the DNA fragments were determined by comparison with the migration of *Hin*dIII digests of λ phage and *Hae*III digest of φX174.

DNA sequencing

cVin5 DNA in PBR was *Eco*RI digested, end-labeled with [α-^{32}d]ATP and was further digested with *Bam*HI, in order to produce a 1.1 kb, 5′-labeled fragment. Nucleic acid sequence was determined by the method of Maxam and Gilbert (1980).

Acknowledgements

We would like to express our gratitude to R.Hynes for providing us with his remarkable cDNA expression library, to A.Ben-Zeev for most valuable interactions and discussions, and to A.Avivi who was most helpful in the initial stages of this study. We extend thanks to I.Schechter for chicken DNA samples and to I.Vlodovsky for ECM-coated plates. R.B. is a fellow of the Israel Cancer Research Fund. B.G. is an E.Neter Professor in Cell and Tumor Biology. This study was supported by a grant of the Muscular Dystrophy Association and the Rockefeller−Weizmann Foundation.

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Received on April 27, 1987; revised on June 19, 1987