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Isolation of cDNA clones specific for collagen IV and laminin from mouse teratocarcinoma cells

(F9 teratocarcinoma/basement membranes/hybrid selection/retinoic acid/cAMP)

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ABSTRACT **The synthesis of the proteins laminin and collagen IV is stimulated ≈20-fold in F9 mouse teratocarcinoma stem cells after treatment of the cells with retinoic acid and N^6, $O^{2'}$-dibutyryl-cAMP (Bt_2cAMP). A cDNA library from F9 cells treated with retinoic acid, Bt_2cAMP, and theophylline (F9-R+DBC cells) was constructed to isolate cDNA coding for collagen IV or laminin. The recombinant plasmids were screened by differential colony hybridization to cDNA synthesized from poly(A)$^+$ RNA isolated from F9 stem and F9-R+DBC cells. Differentially hybridizing plasmids were then used as probes to hybridize to RNA transfer blots to determine the size of their specific mRNA. Only plasmids containing cDNA sequences specific for high molecular weight mRNA were further analyzed. Studies by hybridization-selection, *in vitro* translation, and immunoprecipitation showed that a plasmid clone, pcI5, contains cDNA homologous to collagen IV (α2) mRNA, and another plasmid clone, pcI56, contains cDNA homologous to laminin B mRNA. By RNA blot analyses, the size of mRNA coding for collagen IV (α2) is 7.6 kilobases; the size of mRNA for laminin is 6.8 kilobases. Using the technique of RNA blot hybridization, we studied the time course of the increase in mRNA coding for collagen IV (α2) and laminin B in F9 cells after retinoic acid and Bt_2cAMP treatment. Both collagen IV (α2) and laminin B mRNAs are present in F9 stem cells. Collagen IV (α2) mRNA and laminin B mRNA levels increase slightly at approximately 12 hr after retinoic acid and Bt_2cAMP addition, with a dramatic increase between 12 and 24 hr after drug treatment.**

Mouse teratocarcinoma stem cells, which resemble embryonic stem cells in many aspects, provide a cell culture system for the study of some aspects of early embryogenesis (1). The mouse teratocarcinoma cell line F9, which does not spontaneously differentiate, can be induced to differentiate by retinoic acid (RA) or RA plus N^6,$O^{2'}$-dibutyryl-cAMP (Bt_2cAMP) (2–4). In monolayer culture, Strickland and colleagues (2, 3) demonstrated that RA alone caused F9 stem cells to differentiate into primitive endoderm-like cells. These primitive endoderm cells then differentiated into parietal endoderm cells in the presence of cAMP. In contrast to F9 stem cells, these differentiated parietal endoderm cells synthesized and secreted large quantities of the extracellular matrix proteins collagen IV and laminin (2). Thus, the level of these proteins is regulated by RA and Bt_2cAMP.

Laminin is an extracellular matrix glycoprotein that is found in most basement membranes of mammalian tissues (5, 6). It is a major component of Reichert's membrane, which is synthesized and secreted by parietal endoderm cells in the mouse embryo (7–9). Immunoprecipitated secreted laminin consists of three polypeptides of molecular masses of approximately 450,000 (A), 240,000 (B_1), and 230,000 (B_2) daltons (5, 10). A fourth component (C), or entactin (150,000 daltons), is sometimes also immunoprecipitated by antilaminin serum (10). The A, B_1, and B_2 chains of laminin are all glycosylated with N-linked oligosaccharides. Laminin binds to collagen IV and may serve as an attachment factor for epithelial and endothelial cells (11).

Collagen IV, a major component of basement membranes, can be distinguished from other collagens by its extensive post-translational modification (12). Hydroxylation of proline and lysine residues occurs; this hydroxylation step can be prevented by α,α′-dipyridyl, an inhibitor of prolyl and lysyl hydroxylases (12). Hydroxylysine can then be modified by attachment of glucose and galactose residues through *O*-glycosidic bonds prior to secretion. A tunicamycin-sensitive, asparagine-linked glycosylation step may also occur prior to secretion (13). Although collagen IV is structurally analogous to procollagens of other types, there is apparently no proteolytic processing of procollagen IV chains, at least in most tissues (13–16). Thus, we denote it as collagen IV. The molecular weight of newly synthesized secreted collagen IV is 185,000 for the proα1 (IV) polypeptide and 170,000 for the proα2 (IV) polypeptide (17). There is evidence that the two polypeptides are products of separate genes (18–20).

Vitamin A and its derivatives (retinoids) have been implicated in the control of differentiation in epithelial tissues and some other cell types (21). The mechanism by which RA induces differentiation of teratocarcinoma stem cells is not understood; RA may act in a manner analogous to steroid hormones (22). Because a molecular description of the action of RA on teratocarcinoma stem cells presumably depends on specific DNA probes, we have isolated recombinant plasmids containing laminin B and plasmids containing collagen IV (α2) cDNA sequences. These cDNA clones were then used to measure the collagen IV and laminin mRNA levels during the differentiation of F9 cells after the addition of RA and Bt_2cAMP.

MATERIALS AND METHODS

Materials. Tunicamycin was obtained from M. Suffness (Natural Products Branch, National Cancer Institute). Antisera against collagen IV and laminin were kindly provided by G. R. Martin (National Institutes of Health).

Cell Lines. F9 mouse teratocarcinoma cells, obtained from S. Strickland, were grown as described (2, 3). For time-course studies of differentiation, F9 cells were plated at 1×10^6 cells per 150 × 15 mm dish and grown overnight; RA (0.5 μM), Bt_2cAMP (0.5 mM), and theophylline (0.5 mM) were then added (F9-R+DBC cells; RA-, Bt_2cAMP-, and theophylline-treated F9 cells).

Radiolabeling of Intracellular and Secreted Proteins. Two days after treatment with RA, Bt_2cAMP, and theophylline, cells

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Abbreviations: RA, retinoic acid; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl-cAMP; kb, kilobase(s).

were treated overnight (14–18 hr) with or without tunicamycin (5–10 μg/ml). Cells were then radiolabeled on day 3 with [^{35}S]methionine (100 μCi/ml; 1 Ci = 3.7×10^{10} Bq; 200 μl per well) at 37°C for 2–3 hr in methionine-free media containing 1% dialyzed calf serum in the absence or presence of tunicamycin or α,α′-dipyridyl (1 mM). A 30-min pretreatment with α,α′-dipyridyl was performed before radiolabeling.

Immunoprecipitation. Cell extracts or *in vitro* translational solutions diluted with 3–5 vol of phosphate-buffered saline (P_i/NaCl) and 1% Triton-X 100 were reacted with 10 μl of polyclonal antibodies directed against collagen IV or against laminin at 4°C for 1 hr or overnight. Five to ten milligrams of protein A-agarose was then added to the reaction mixtures and rotated for 1 hr at 4°C. The protein A-agarose was then washed three times with P_i/NaCl and once with 10 mM Tris·HCl, pH 7.4/1 mM EDTA. The antigens, collagen IV or laminin, were then eluted by boiling in $NaDodSO_4$ sample buffer and later analyzed by $NaDodSO_4$ gel electrophoresis (23).

RNA Isolation and cDNA Cloning. Total RNA was isolated from F9 cells by a modification of the guanidine·HCl method of Cox (24) and Strohman *et al.* (25). Poly(A)$^+$ mRNA was prepared by passing total RNA through a poly(U)-Sephadex column twice (26). Double-stranded cDNA was synthesized from poly(A)$^+$ RNA prepared from F9-R+DBC cells according to the procedure of Wickens *et al.* (27) with some modifications. The double-stranded cDNA was then treated with nuclease S1, tailed with dCTP, and run through a Bio-Gel A-15m column. Only dC-tailed cDNA of sizes from 500 to 2,000 base pairs was annealed to *Pst* I-digested poly(G)-tailed PBR322 and used to transform *Escherichia coli* 600 (28). Colony hybridization was carried out by using ^{32}P-labeled cDNAs from poly(A)$^+$ RNA of F9 stem and F9-R+DBC cells (29).

***In Vitro* Translation.** The *in vitro* translation was carried out in a reticulocyte lysate system (Bethesda Research Laboratories). RNasin, 2,500 units/ml (Biotec, Madison, WI), and liver tRNA, 100 μg/ml, were included in all assays. When total RNA was translated, 100 μg of RNA per ml was used.

RNA Blot Analysis. Total RNA (5 μg) from F9 stem and F9-R+DBC cells was electrophoresed on 1% or 1.25% agarose/2.2 M formaldehyde gels and transferred to nitrocellulose filters (30). Plasmids were isolated by alkaline lysis and cesium chloride gradient centrifugation (31), nick-translated with [α-^{32}P]-dCTP (32), and hybridized to the RNA blots in the presence of 10% dextran sulfate (33).

Hybridization-Selection. The hybridization-selection procedure was performed by using established procedures, with some modifications (34, 35).

RESULTS

Construction of a cDNA Library from Differentiated Cell RNA. In F9 cells, both collagen IV synthesis (13) and laminin synthesis (36) were increased by ≈20-fold after stem cell differentiation in the presence of RA and Bt_2cAMP. Thus, the differential screening of a cDNA library with stem and RA/Bt_2cAMP cDNA probes should favor the isolation of cDNA specific for collagen IV or laminin. We constructed a cDNA library from F9 cells treated with RA and Bt_2cAMP. Approximately 300 bacterial colonies were screened and about 8 colonies showed increased hybridization with the ^{32}P-labeled cDNA probe synthesized from F9-R+DBC cell RNA. Plasmids of five different colonies (I5, I56, I69, J6, and J31) that exhibited an increased hybridization signal with the RA/Bt_2cAMP-specific ^{32}P-labeled cDNA probe were prepared and further analyzed.

RNA Blot Analysis. Because both collagen IV and laminin are large proteins, the mRNA coding for each of these proteins should be of high molecular weight. Thus, we next determined the size of the mRNA homologous to the cDNA sequence in each of the five recombinant plasmids. The I5 plasmid specifically hybridized to a 7.6-kilobase (kb) mRNA, plasmids I56 and I69 hybridized to a 6.8-kb mRNA, plasmid J31 hybridized to a 3.0-kb mRNA, and plasmid J6 hybridized to a 2.9-kb mRNA, as determined by comparison with mouse 18S rRNA (2,060 nucleotides) and 28S rRNA (5,303 nucleotides). Our data suggested that the colonies I56 and I69 probably contained the same recombinant plasmid. Because the I5 and I56 plasmids hybridized to high molecular weight mRNA, they could potentially contain cDNA inserts complementary to collagen IV or laminin mRNA. Therefore, the plasmids I5 and I56 (designated pcI5 and pcI56) were further characterized.

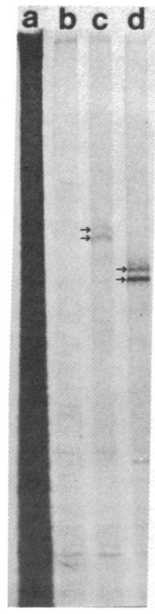

FIG. 1. Autoradiogram of [^{35}S]methionine-labeled proteins electrophoresed on a 5% $NaDodSO_4$/polyacrylamide gel. mRNA from F9 cells grown 3 days in RA and Bt_2cAMP was translated *in vitro*: lane a, total translation products; lanes b–d, translation products immunoprecipitated (see text) with preimmune serum (lane b), with antibodies directed against laminin (lane c), or with antibodies directed against collagen IV (lane d). Arrows indicate collagen IV and laminin bands.

***In Vitro* Translation of mRNA for Collagen IV and Laminin.** We first demonstrated that we could detect collagen IV and laminin in our *in vitro* translation system. When we translated F9-R+DBC cell RNA *in vitro*, we observed two polypeptide chains (155,000 and 145,000 daltons*), which were immunoprecipitated by collagen IV antiserum (Fig. 1, lane d), and two major chains, which were immunoprecipitated with laminin antiserum (Fig. 1, lane c). These laminin chains were approximately 200,000 and 190,000 daltons and thus presumably corresponded to unglycosylated laminin B chains. Occasionally, we detected other polypeptide chains that could be *in vitro* translated and immunoprecipitated with laminin antiserum; these minor *in vitro* translated products had molecular masses of 290,000, 260,000, 170,000, and 120,000 daltons (data not shown).

Hybridization-Selection and *in Vitro* Translation. pcI5 hybridized to a mRNA coding for a polypeptide that migrates on a $NaDodSO_4$/polyacrylamide gel with a molecular mass of ≈145,000 daltons (Fig. 2 *Left*, lanes b and c). We obtained a much better signal in the hybridization-selection procedure if we used nitrocellulose filters that had been used once before for hybridization of RNA to pcI5 or pcI56 DNA (Fig. 2 *Left*, lanes c and e, vs. lanes b and d). Immunoprecipitation of the

* Molecular masses were estimated by using noncollagenous marker proteins; because collagens migrate abnormally on $NaDodSO_4$/polyacrylamide gels, the estimates may be higher than the true molecular masses (37). The molecular masses we have assigned to collagen IV (α1) and (α2) chains are slightly different from those assigned by others (13, 17).

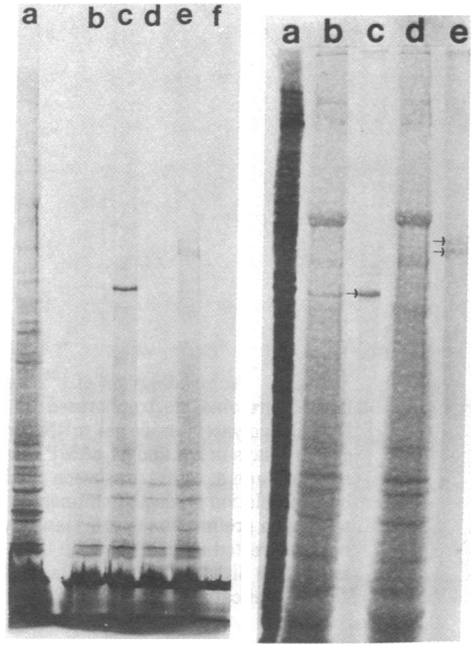

FIG. 2. Identification of cDNA clones for laminin and collagen IV by hybridization-selected translation. Recombinant plasmid DNAs immobilized on nitrocellulose filters were used to select mRNAs from total RNA isolated from F9-R+DBC cells. The hybridized mRNAs were released and translated *in vitro*, and the [^{35}S]methionine-labeled translation products were analyzed before (*Left*) and after (*Right*) immunoprecipitation on 5% NaDodSO$_4$/polyacrylamide gels. (*Left*) Lanes: a, translation products from 1 μg of total RNA from F9-R+DBC cells; b, proteins synthesized by mRNA selected by clone I5 (fresh nitrocellulose paper); c, proteins synthesized by mRNA selected by clone I5 (paper reused); d, proteins synthesized by mRNA selected by clone I56 (fresh nitrocellulose paper); e, proteins synthesized by mRNA selected by clone I56 (paper reused); f, proteins synthesized by mRNA selected by PBR322 DNA without any cDNA inserts. (*Right*) Lanes: a, not relevant to these experiments; b, proteins synthesized by mRNA selected by clone I5 (fresh paper); c, proteins synthesized by mRNA selected by clone I5 (reused paper) immunoprecipitated with antibodies against collage IV; d, proteins synthesized by mRNA selected by clone I56 (fresh nitrocellulose paper); e, proteins synthesized by mRNA selected by clone I56 (reused paper) immunoprecipitated with antibodies against laminin. Arrows indicate collagen IV and laminin bands.

pcI5-specific polypeptide with anticollagen IV serum indicated that it was one of the two collagen IV polypeptides (Fig. 2 *Right*, lane c).

pcI56 hybridized to mRNAs coding for two polypeptides that migrated on a NaDodSO$_4$/polyacrylamide gel with molecular masses of 200,000 and 190,000 daltons, respectively (Fig. 2 *Left*, lanes d and e). These proteins were immunoprecipitated by antilaminin serum (Fig. 2 *Right*, lane e).

We attempted to further identify the protein, shown in Fig. 2 *Left*, lane c, that was *in vitro* translated by the mRNA that hybridized to pcI5. This protein could be immunoprecipitated with anticollagen IV serum (Fig. 2 *Right*, lane c), so we knew that the protein was one of the collagen IV chains. We then demonstrated that the mobility on NaDodSO$_4$/polyacrylamide gels of the hybrid-selected, *in vitro* translated collagen IV chain (Fig. 3, lane c) was identical to that of *in vitro* translated, immunoprecipitated collagen IV (α2) from total RNA isolated from F9-R+DBC cells (Fig. 3, lane a). Thus, the cDNA contained in pcI5 codes for collagen IV (α2).

Similar experiments were performed to further identify the proteins, shown in Fig. 2 *Left*, lane e, that were *in vitro* translated by the mRNA that hybridized to pcI56. We knew that these proteins could be immunoprecipitated with antilaminin serum

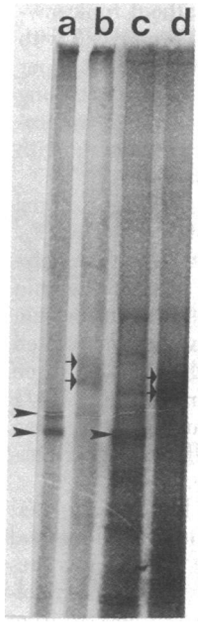

FIG. 3. Identification of cDNA clones for laminin and collagen IV. Comparison of total [^{35}S]methionine-labeled *in vitro* translation products (from mRNA isolated from F9-R+DBC cells) immunoprecipitated with either anticollagen IV serum or antilaminin serum with the *in vitro* [^{35}S]methionine-labeled translation products of the mRNAs that were hybrid-selected by pcI5 and pcI56. Lanes: a, total F9-R+DBC RNA, *in vitro* translated and immunoprecipitated with anticollagen IV serum; b, total F9-R+DBC RNA, *in vitro* translated and immunoprecipitated with antilaminin serum; c, proteins synthesized by mRNA selected by pcI5; d, proteins synthesized by mRNA selected by pcI56. Autoradiogram of 5% NaDodSO$_4$/polyacrylamide gel. Arrows and arrowheads denote the positions of the relevant protein bands.

(Fig. 2 *Right*, lane e). We then demonstrated that the molecular masses of the hybrid-selected, *in vitro* translated laminin chains on NaDodSO$_4$/polyacrylamide gels (Fig. 3, lane d) were very similar to that of the major *in vitro* translated, immunoprecipitated laminin B chains (200,000 and 190,000 daltons) from total RNA isolated from F9-R+DBC cells (Fig. 3, lane b). Thus, the cDNA contained in pcI56 codes for laminin B chains.

Comparison of *in Vitro* Translated Laminin and Collagen IV with Laminin and Collagen IV Synthesized *in Vivo*. F9-R+DBC cells were radiolabeled with [^{35}S]methionine after treatment with α,α'-dipyridyl and tunicamycin (38), which should prevent all of the known posttranslational modifications of laminin and collagen IV. The radiolabeled intracellular proteins from F9-R+DBC cells, immunoprecipitated with anticollagen IV or antilaminin sera, were then compared with the *in vitro* translation products on NaDodSO$_4$/polyacrylamide slab gels.

Collagen IV, immunoprecipitated from the cells labeled *in vivo* with [^{35}S]methionine in the presence of the drugs, migrated as two bands, α1 and α2, of 147,000 and 140,000 daltons, respectively (Fig. 4, lane a). The collagen IV, immunoprecipitated after *in vitro* translation of F9-R+DBC mRNA, migrated as two bands with molecular masses of approximately 155,000 and 145,000 daltons, respectively (Fig. 4, lane b). This result suggests that the two collagen IV chains contain signal peptides that are cleaved *in vivo* but that are not cleaved in the *in vitro* translation system.

Laminin, immunoprecipitated from cells labeled *in vivo* with [^{35}S]methionine in the presence of α,α'-dipyridyl and tunicamycin, migrated as three bands of 400,000, 190,000, and 160,000 daltons (Fig. 4, lane c), which correspond to intracellular unmodified laminin A, B_1, and B_2 chains, respectively. (When intracellular laminin is immunoprecipitated from F9 cells labeled with [^{35}S]methionine *in vivo*, collagen IV coprecipitates (Fig. 4, lane c); this does not occur when secreted laminin is immunoprecipitated.) The laminin that was immunoprecipitated after *in vitro* translation of F9-R+DBC mRNA migrated in this experiment as three major bands of 200,000, 190,000, and 170,000 daltons (Fig. 4, lane d), although two faint bands of higher molecular mass presumably related to laminin A could also be seen. The relationship of these three major *in vitro* translated immunoprecipitated chains (200,000, 190,000, and 170,000 daltons) to the laminin B_1 and B_2 chains of 190,000 and

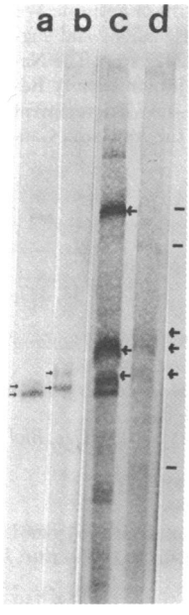

FIG. 4. Comparison of total F9-R+DBC RNA, *in vitro* translated and immunoprecipitated, with *in vivo* [^{35}S]methionine-labeled, immunoprecipitated proteins. Lanes: a, intracellular *in vivo* [^{35}S]methionine-labeled proteins immunoprecipitated with anticollagen IV serum [see arrows pointing to collagen IV (α1) and (α2)]. F9-R+DBC cells were treated with α,α'-dipyridyl and tunicamycin (see text) prior to incubation in medium containing [^{35}S]methionine; b, total F9-R+DBC RNA, *in vitro* translated and immunoprecipitated with anticollagen IV serum (see arrows to left of lane); c, intracellular *in vivo* [^{35}S]methionine-labeled proteins immunoprecipitated with antilaminin serum. Arrows at right point to laminin A, B_1, and B_2 chains. Cells were treated as in lane a for *in vivo* [^{35}S]methionine labeling; d, total F9-R+DBC RNA, *in vitro* translated and immunoprecipitated with antilaminin serum. Arrows at right point to three major *in vitro* translated products at 200,000, 190,000, and 170,000 daltons. Other minor *in vitro* translated products, indicated by lines at right, can be seen on this gel. Autoradiogram of a 5% NaDodSO$_4$/polyacrylamide gel.

160,000 daltons seen by *in vivo* labeling is unclear, although these data suggest that the laminin B_1 band at 190,000 daltons (Fig. 4, lane c) may actually consist of two polypeptides. As we have shown, pcI56 hybridizes to mRNAs that code for both the 200,000- and the 190,000-dalton *in vitro* translated laminin bands but not the 170,000-dalton band (Fig. 2 *Right*, lane e).

Analysis of Collagen IV mRNA Levels and Laminin mRNA Levels After Treatment of F9 Cells with RA and Bt_2cAMP. Total RNA from F9 cells isolated at different times after RA and Bt_2cAMP treatment was analyzed. Both collagen IV and laminin mRNA were present in F9 stem cells (Figs. 5 and 6). There were slight increases in the levels of both pcI5- (collagen IV) and pcI56- (laminin) specific mRNA at ≈12 hr after RA and Bt_2cAMP treatment, and dramatic increases between 12 and 24 hr after drug treatment. A further increase in the amount of both mRNAs occurred 48 hr and 72 hr after drug treatment (Figs. 5 and 6). We have also observed a reproducible decrease in mRNA levels for both collagen IV and laminin between 3 and 6 hr after addition of the drugs; the significance of this decrease is unclear. F9 stem cells treated with RA alone (without Bt_2cAMP) exhibited a similar time course of increase of both pcI5- and pcI56-specific mRNAs, but the increases were not as large as those seen in the presence of RA plus Bt_2cAMP (data not shown).

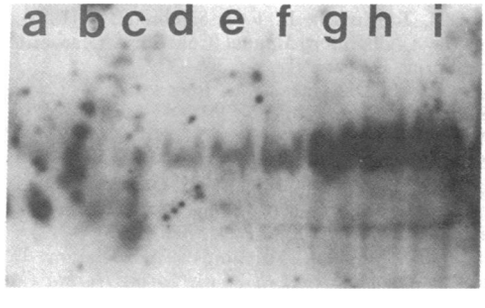

FIG. 5. RNA blot hybridization analysis of collagen IV (α2) mRNA levels from cells harvested at different times after the addition of RA with Bt_2cAMP. Five micrograms of each total RNA preparation was separated on a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled nick-translated DNA from clone I5. Time after treatment with RA and Bt_2cAMP is indicated as follows. Lanes: a and b, control stem cell RNA; c, 6 hr; d, 12 hr; e, 18 hr; f, 24 hr; g, 48 hr; h, 72 hr; i, 96 hr.

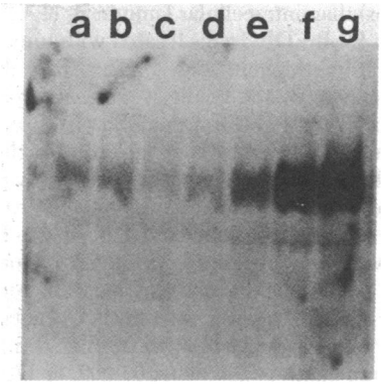

FIG. 6. RNA blot hybridization analysis of laminin B mRNA levels from cells harvested at different times after the addition of RA with Bt_2cAMP. Five micrograms of each total RNA preparation was separated on a 1.25% agarose/formaldehyde gel, transferred to nitrocelluose filters, and hybridized with ^{32}P-labeled nick-translated DNA from clone I56. Time after treatment with RA and Bt_2cAMP is indicated as follows. Lanes: a, control stem cell RNA; b, 3 hr; c, 6 hr; d, 12 hr; e, 24 hr; f, 48 hr; g, 72 hr.

DISCUSSION

We describe the isolation of the plasmids that contain cDNA sequences complementary to mRNAs that code for the glycoproteins collagen IV (α2) and laminin B. The cDNA insert in pcI5 was identified as a sequence homologous to collagen IV (α2) mRNA, and the cDNA insert in pcI56 was identified as a sequence homologous to laminin B mRNA by hybridization-selection and *in vitro* translation.

When mRNA that specifically hybridized to pcI5 DNA was *in vitro* translated, one polypeptide with a molecular mass of 145,000 daltons was synthesized. This polypeptide was immunoprecipitated by anticollagen IV serum (Fig. 2 *Right*). Two chains of collagen IV were synthesized *in vitro* or *in vivo*. F9-R+DBC cells labeled *in vivo* with [^{35}S]methionine in the presence of α,α'-dipyridyl and tunicamycin synthesized two intracellular chains of collagen IV with molecular masses of 147,000 and 140,000 daltons, designated as collagen IV (α1) and collagen IV (α2), respectively (Fig. 4). Because the pcI5-specific polypeptide comigrated on gels with the lower molecular mass collagen IV synthesized *in vitro* (145,000 daltons) (Fig. 3), pcI5 contains cDNA specific for mRNA coding for the lower molecular mass collagen IV polypeptide, collagen IV (α2). The difference between the molecular weights of *in vitro* translated collagen IV (α2) (145,000 daltons) and unmodified collagen IV (α2) synthesized *in vivo* (140,000 daltons) may be the result of a signal peptide (39).

Because the collagen IV chains have a high percentage of common amino acids (12), it is surprising that mRNA coding for collagen IV (α2), but not collagen IV (α_1), is hybrid-selected by pcI5 DNA (Figs. 2 and 3). One possible explanation is that the cDNA insert in pcI5 DNA is small (660 base pairs) and may contain a high percentage of noncoding sequences that may be nonhomologous between the two collagen IV mRNAs. Alternatively, the mRNA coding for collagen IV (α1) may be more susceptible to RNase, as observed by Kurkinen *et al.* (13), and may be lost during the hybridization procedure. The isolation of a cDNA clone that is specific for collagen IV (α1) should answer this question.

pcI56, a cDNA clone specific for laminin B (insert size, 1,300 base pairs), hybridizes to mRNAs coding for two chains of laminin, which, as *in vitro* translation products, migrate on NaDodSO$_4$/polyacrylamide gels slightly slower (200,000 and

190,000 daltons) than intracellular laminin B_1 (190,000 daltons) synthesized by F9-R+DBC cells treated with tunicamycin and labeled with [^{35}S]methionine (Fig. 4). This slight difference in molecular mass may be the result of a signal peptide cleaved *in vivo* but not *in vitro*. Because pcI56 hybridizes to mRNAs coding for two laminin chains, we conclude that these mRNAs contain some homologous sequences. A less likely possibility is that one species of mRNA can be translated *in vitro* to yield two polypeptide chains of different molecular mass. If pcI56 hybridizes to two distinct mRNAs, the mRNAs coding for the 200,000- and 190,000-dalton proteins must be very similar in size, because only one band is seen on RNA blots (Fig. 6).

No mRNA coding for the *in vitro* translated immmunoprecipitated laminin bands at 290,000, 260,000, or 170,000 daltons is hybrid-selected by pcI56 (Fig. 2 *Right*, lane e). Again, there may be no homology between the mRNAs coding for these chains and the mRNAs coding for the 200,000- and 190,000-dalton chains, or the mRNAs coding for these chains may be unstable during the hybridization-selection and *in vitro* translation procedures. The three major protein bands seen after *in vitro* translation and immunoprecipitation with antilaminin serum (200,000, 190,000, and 170,000 daltons) are related to the laminin B_1 (190,000 daltons) and B_2 (160,000 daltons) chains observed by *in vivo* radiolabeling of tunicamycin-treated F9 cells, because they have similar molecular masses and are immunoprecipitated by laminin antiserum. Because the exact relationship is unclear, we currently define the pcI56 clone as hybridizing to laminin B mRNA.

By RNA blot hybridization analyses, pcI5 hybridizes to mRNA of ≈7.6 kb. The minimal mRNA size estimated from the size of the *in vitro* translated collagen IV (α2) 145,000-dalton polypeptide is 4.0 kb. Therefore, mRNA of collagen IV (α2) has ≈3.2 kb of noncoding sequences. The pcI56 probe hybridizes to a mRNA of 6.8 kb. The minimal sizes of the laminin B mRNAs are estimated to be 5.4 and 5.2 kb. The high content of noncoding sequences in collagen IV (α2) mRNA and laminin B mRNA may play an important role in the regulation of the transcription or processing of these messages.

The time course of the accumulation of collagen IV (α2) or laminin B mRNA in response to RA and Bt_2cAMP treatment was studied by RNA blot hybridization. There is a slight initial decrease in the levels of both collagen IV (α2) and laminin B mRNAs between 3 and 6 hr after the drug treatment, followed by a significant increase in the mRNA levels for both collagen IV (α2) and laminin B over the next 12–72 hr (Figs. 5 and 6). The initial decrease in these mRNA levels is not a general phenomenon because the level of actin mRNA is unchanged during this 3- to 6-hr time period after drug addition (unpublished data). Addition of RA without Bt_2cAMP causes a smaller increase in collagen IV (α2) and laminin B mRNA levels, over the same 12- to 72-hr time period, whereas addition of Bt_2cAMP without RA results in no increase in these mRNA levels over those in undifferentiated F9 stem cells (unpublished data). Thus, cAMP enhances the action of RA by an unknown mechanism. We do not yet know whether the induction of collagen IV (α2) or laminin B mRNA (or both) is a primary or secondary event after RA and Bt_2cAMP addition. Nevertheless, we have shown that the increase in the level of mRNAs that code for collagen IV (α2) and laminin B proteins, either because of increased transcription or increased stability of these mRNAs, results in the increased collagen IV (α2) and laminin protein content of F9-R+DBC cells. More detailed studies must be performed to dissect the mechanism of action of RA and Bt_2cAMP on these teratocarcinoma stem cells. The collagen IV (α2) and laminin B cDNA clones will be very useful probes for these continuing studies.

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1. Martin, G. R. (1980) *Science* **209,** 768–776.
2. Strickland, S. & Mahdavi, V. (1978) *Cell* **15,** 393–403.
3. Strickland, S., Smith, K. K. & Marotti, K. R. (1980) *Cell* **21,** 347–355.
4. Hogan, B. L., Taylor, A. & Adamson, E. (1981) *Nature (London)* **291,** 235–237.
5. Timpl, R., Rohde, H., Robey, P., Rennard, S., Foidart, J. M. & Martin, G. R. (1979) *J. Biol. Chem.* **254,** 9933–9937.
6. Leivo, I., Vaheri, A., Timpl, R. & Wartiovaara, J. (1980) *Dev. Biol.* **79,** 100–114.
7. Howe, C. & Solter, D. (1980) *Dev. Biol.* **77,** 480–487.
8. Hogan, B. L. M. (1980) *Dev. Biol.* **76,** 275–285.
9. Smith, K. & Strickland, S. (1981) *J. Biol. Chem.* **256,** 4654–4661.
10. Cooper, A., Kurkinen, M., Taylor, A. & Hogan, B. (1981) *Eur. J. Biochem.* **119,** 189–197.
11. Terranova, V., Rohrbach, D. & Martin, G. R. (1980) *Cell* **22,** 719–726.
12. Bornstein, P. & Sage, H. (1980) *Annu. Rev. Biochem.* **49,** 957–1003.
13. Kurkinen, M., Foster, L., Barlow, D. & Hogan, B. L. (1982) *J. Biol. Chem.* **257,** 15151–15155.
14. Minor, R., Clark, C., Strause, E., Koszalka, T., Brent, R. & Kefalides, N. (1976) *J. Biol. Chem.* **251,** 1789–1794.
15. Heathcote, J., Sear, C. & Grant, M. (1978) *Biochem. J.* **176,** 283–294.
16. Foidart, J., Tryggvason, K., Robey, P., Liotta, L. & Martin, G. R. (1981) *Collagen Rel. Res.* **1,** 137–150.
17. Kleinman, H., McGarvey, M., Liotta, L., Robey, P., Tryggvason, K. & Martin, G. R. (1982) *Biochemistry* **21,** 6188–6193.
18. Crouch, E., Sage, H. & Bornstein, P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 745–749.
19. Alitalo, K., Vaheri, A., Krieg, T. & Timpl, R. (1980) *Eur. J. Biochem.* **109,** 247–255.
20. Tryggvason, K., Robey, P. & Martin, G. R. (1980) *Biochemistry* **19,** 1284–1289.
21. Lotan, R. (1980) *Biochim. Biophys. Acta* **605,** 33–91.
22. Elias, P. M. & Williams, M. (1981) *Arch. Dermatol.* **117,** 160–180.
23. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
24. Cox, R. A. (1968) *Methods Enzymol.* **12,** 120–129.
25. Strohman, R. C., Moss, P. S., Eastwood, J. M. & Spector, D. (1977) *Cell* **10,** 265–273.
26. Haff, L. A. & Bogorad, L. (1976) *Biochemistry* **15,** 4110–4115.
27. Wickens, M. P., Buell, G. N. & Schimke, R. T. (1978) *J. Biol. Chem.* **263,** 2483–2495.
28. Dagert, M. & Ehrlich, S. D. (1979) *Gene* **6,** 23–28.
29. Thayer, R. E. (1979) *Anal. Biochem.* **98,** 60–63.
30. Seed, B. (1982) *Genet. Eng.* **4,** 91–102.
31. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 368–369.
32. Rigby, P. W., Dieckmann, M., Rhoades, C. & Laemmli, U. K. (1977) *J. Biol. Chem.* **113,** 237–251.
33. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3683–3688.
34. Miller, J. S. & Roberts, B. E. (1982) *Genet. Eng.* **4,** 103–116.
35. Protter, A., Wang, S.-Y., Shelness, G. S., Ostapchuk, P. & Williams, D. L. (1982) *Nucleic Acids Res.* **10,** 4935–4950.
36. Hogan, B., Kurkinen, M., Cooper, A. & Barlow, D. (1983) in *Teratocarcinoma Stem Cells, Cold Spring Harbor Conf. on Cell Proliferation*, eds. Silver, L., Martin, G. R. & Strickland, S. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 10, pp. 520–525.
37. Freytag, J. W., Noelken, M. E. & Hudson, B. G. (1979) *Biochemistry* **18,** 4761–4768.
38. Rothman, J., Katz, R. N. & Lodish, H. F. (1978) *Cell* **15,** 1447–1454.
39. Blobel, G. & Sabatini, D. (1971) in *Biomembranes*, ed. Manson, L. A. (Plenum, New York), Vol. 2, pp. 193–195.