In Vitro Synthesis of Type IV Procollagen*

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Total RNA was isolated from parietal endoderm cells of 13½-day mouse embryos that synthesize large amounts of type IV procollagen. In vitro translation of this RNA in the reticulocyte lysate supplemented with a ribonuclease inhibitor yielded two equally prominent polypeptides of $M_r = 165,000$ and 168,000, immunoprecipitable with anti-mouse type IV collagen serum. The $M_r = 165,000$ polypeptide was shown by one-dimensional peptide mapping to represent an unmodified chain of type IV procollagen. The $M_r = 168,000$ polypeptide, the in vitro synthesis of which was barely detectable in the absence of a ribonuclease inhibitor, most likely represents the other genetically distinct chain of type IV procollagen. Similar results to those described were also obtained using poly(A) + RNA prepared from murine F9 embryonal carcinoma cells induced to differentiate in vitro into parietal endoderm.

Several characteristics distinguish type IV collagen from the other known collagens. In particular, type IV collagen is more extensively modified post-translationally as shown by elevated levels of 3-hydroxyproline, and higher ratios of 4-hydroxyproline to proline and hydroxylysine (1). Furthermore, it appears to contain unusually long pepsin-resistant domains (2, 3), although partial sequence analysis has revealed discontinuities in the triple helical Gly-x-y sequence (4). By analogy with the interstitial collagens, type IV collagen is thought to be synthesized in the form of procollagen. However, at least in culture conditions, there is apparently no proteolytic processing of type IV procollagen chains into smaller polypeptides (2, 5). In addition, limited pepsin digestion results in only a small reduction in its molecular weight (2, 6), suggesting that the propeptide extensions, if any, are much shorter than those present in the interstitial procollagens. Finally, type IV collagen is deposited exclusively in basement membranes where it is not assembled into fibrils typical of the higher order structure of interstitial collagens (7).

Type IV procollagen isolated from organ or cell cultures is resolved by gel electrophoresis into two polypeptides (2, 5, 6, 8, 9), designated as proa1(IV) and proa2(IV) of $M_r = 185,000$ and 170,000, respectively, (8) that presumably are products of separate genes (2, 5). Although they have not yet been purified and characterized separately, the two polypeptides resolved by gel electrophoresis generate different one-dimensional peptide maps (5). Similarly, the two chains of type IV collagen of bovine kidney cortex, purified by carboxymethylcellulose chromatography, yielded different one-dimensional peptide maps after chemical or enzymatic cleavage. The amino acid composition of these chains was found to be very similar except for the 2-fold difference in their arginine content (10).

In cell cultures the ratio of synthesis of the two polypeptides can vary considerably, it was suggested that they belong to different triple helical molecules of type IV procollagen (5, 8). In contrast, however, the subunit structure of type IV collagen isolated from bovine kidney apparently is a heterotrimer of the two chains (10).

In this paper, we report experiments on the in vitro synthesis of type IV procollagen. In the presence of a ribonuclease inhibitor, in vitro translation of RNA isolated from cells synthesizing large amounts of type IV procollagen yielded two major polypeptides of $M_r = 165,000$ and 168,000 immunoprecipitable with anti-type IV collagen serum. One-dimensional peptide mapping showed that the $M_r = 165,000$ polypeptide represented an unmodified chain of type IV procollagen. The $M_r = 168,000$ polypeptide probably is the other genetically distinct chain synthesized in vitro.

EXPERIMENTAL PROCEDURES

Materials—The following is a list of material and their sources used in the present study: amino acids, 2,2'-bipyridyl (British Drug House), 2-aminourine (Cambrian Chemicals), bacterial collagenase (Advanced Biofacturers), creatine kinase, creatine phosphate, dibutyl cyclic AMP, N-ethylmaleimide, phenylmethylsulfonyl fluoride, trans-retinoic acid, tunicamycin (Sigma), $\mathrm{CsCl}$ (Koch-Light Laboratories), dithiothreitol (Calbiochem), ENH'ANCE (New England Nuclear), glass fiber filters, GF/C (Whatman), gaunidinium thiocyanate (Fluka), isobutyl methylicanthine (Alrichr), $[^{14} \mathrm{C}]$methionine (specific activity 1000-1400 Ci/mmol), $[^{14} \mathrm{C}]$-labeled molecular weight markers (Amersham International), oligo(dT)-cellulose, type 2 (Collaborative Research Inc.), protein A-Sepharose 4B-CL (2 mg of protein A/ml of packed beads; Pharmacia), proteinase from Staphylococcus aureus V8 (Miles), tRNA from calf liver (Boehringer Mannheim), RNasin, 30,000 units/ml (BIOTEC/USA).

Cells—Mouse F9 embryonal carcinoma cells were grown on tissue culture dishes coated with 1% gelatin in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For induction of parietal endoderm differentiation (11), 5 x $10^{-3}$ M trans-retinoic acid, 10-1 M dibutyryl cyclic AMP, and 10-3 M isobutyl methylicanthine were added 4 days before RNA extraction or metabolic labeling. Parietal endoderm cells attached to Reichert's membrane were dissected from 13½-day mouse embryos, rinsed in Dulbecco's modified Eagle's medium and used immediately for RNA extraction or metabolic labeling (9).

Metabolic Labeling—Approximately 20 Reichert's membranes or confluent F9 cultures of about 4 x $10^7$ cells were rinsed twice with Dulbecco's modified Eagle's medium containing 1 μg/ml of methionine and incubated in this medium with $[^{35} \mathrm{S}]$methionine (30 μCi/ml) for 30 min±1 h. For metabolic labeling in the presence of tunicamycin (5 μg/ml) and 2,2'-bipyridyl (0.5 mM), cells or membranes were preincubated with these inhibitors for 2 h before the addition of $[^{35} \mathrm{S}]$ methionine. The presence of these drugs inhibited protein synthesis by about 50%, as did 5 μg/ml of tunicamycin alone. However, other studies on parietal endoderm cells had shown that this level of tunicamycin was required to completely block N-glycosylation.

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Radioactivity immunoprecipitated with preimmune rabbit serum was quantitatively immunoprecipitated with anti-type IV collagen serum and counted using toluene-based liquid scintillant.

To estimate type IV procollagen synthesis, cell cultures were labeled with \([35S]\)methionine were extracted in 0.4 M NaCl, 5 mM EDTA, 1 mM dithiothreitol. Under the standard conditions, protein translation contained 1000 units/ml of a ribonuclease inhibitor, RNAse inhibitor. Aliquots (0.1 ml) of this supernatant were added to 1 ml of buffer A containing 100 nM of anti-mouse type IV collagen serum and immunoprecipitated as above using 100 nM of protein A-Sepharose. The Sepharose beads, collected by centrifugation, were washed twice with 1 ml of buffer A, once with 1 ml of 10% Tris, pH 6.8, buffer A, containing 2 ml of anti-mouse type IV collagen serum, incubated at room temperature for 1 h, and mixed with 10 ml of protein A-Sepharose at +4 °C for 90 min. The Sepharose beads, collected by centrifugation, were washed twice with 1 ml of buffer A, once with 1 ml of 10% Tris, pH 6.8, and suspended in 20 ml of twice concentrated electrophoresis sample buffer, and kept at 100 °C for 5 min before analysis by NaDodSO4-polyacrylamide gel electrophoresis. To isolate type IV procollagen from cell cultures, about 4 x 10^6 cells, metabolically labeled with \([35S]\)methionine were extracted in 0.4 M NaCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, 0.05% Triton X-100, and 50 mM Tris, pH 6.8, buffer B, centrifuged at 10,000 x g for 10 min at +4 °C, and the supernatant, mixed with an equal volume of glycerol, was stored at -20 °C until use. Aliquots (0.1 ml) of this supernatant were added to 1 ml of buffer A containing 10 ml of anti-mouse type IV collagen serum and immunoprecipitated as above using 20-30 ml of protein A-Sepharose.

Antiserum—Preparation and specificity of the anti-mouse type IV collagen sera used here have been described (8, 15) and were gifts of Drs. Eileen Adams and George R. Martin. Identical results were obtained with both antisera. The antisera react with both chains of type IV procollagen from cell cultures, about 4 x 10^6 cells, metabolically labeled with \([35S]\)methionine and isolated from tunicamycin and 2,2'-bipyridyl-treated cells.

Peptide Mapping—Type IV procollagen synthesized in vitro or produced by tunicamycin and 2,2'-bipyridyl-treated cells was isolated by immunoprecipitation. Aliquots (about 10^4 cpm) of the immunoprecipitates were treated with 30 μg of S. aureus V8 proteinase at 37 °C in 30 μl of 65 mM Tris, pH 6.8, 0.5% NaDodSO4, for 1 h at 37 °C. The reaction was stopped by adding 30 μl of 10% trichloroacetic acid-treated electrophoresis sample buffer and analyzed by NaDodSO4-polyacrylamide gel electrophoresis.

Gel Electrophoresis—Samples dissolved in 65 mM Tris, pH 6.8, 2% NaDodSO4, 10% glycerol, 10 mM dithiothreitol (electrophoresis sample buffer) were kept at 100 °C for 5 min and analyzed by 6% NaDodSO4-polyacrylamide gel electrophoresis as described (17). Molecular weight standards were [35S]methionine, containing 2 x 10^4 cpm, bovine serum albumin, M, 68,000, ovalbumin, M, 46,000, carbonic anhydrase, M, 30,000, and myoglobin, M, 15,000. After electrophoresis, gels were stained in 0.1% Coomassie blue, 10% trichloroacetic acid, 5% mercaptoethanol, pH 5.0, dried under vacuum onto filter paper, and exposed to Kodak SB-5 films at -70 °C. To prevent cracking of the gel, 12% and 15% polyacrylamide gels were incubated for 30 min in 1% glycerol before drying.

RESULTS

Fig. 1 (lane 3) shows that the type IV procollagen synthesized by mouse embryo parietal endoderm cells migrates on NaDodSO4 gel electrophoresis as two distinct polypeptides of M, 200,000 and 195,000, denoted here as the proc1(1V) and proc2(1V) chains, respectively. When cells are incubated with 2,2'-bipyridyl, an inhibitor of hydroxylation of prolyl and lysyl residues of collagenous proteins (18), the type IV procollagen chains migrate on gel electrophoresis significantly faster than the chains made by control cells (lane 4). In addition, the ratio of the two chains was changed suggesting that relatively less of the presumably unhydroxylated pro1(1V) is made by the 2,2'-bipyridyl-treated cells when compared to control cells (lane 3). When tunicamycin, which specifically inhibits N-glycosylation (18, 20), is added together with 2,2'-bipyridyl, there is a further small increase in the migration of type IV procollagen (lane 5) suggesting that it contains N-linked oligosaccharide side chains. It is to be noted that the unmodified type IV procollagen samples (lanes 4 and 5) are also devoid of the glycosylactosylactosyl residues which normally would be linked to hydroxylysyl residues of type IV collagen (1). The finding that only one major polypeptide of the unmodified type IV procollagen is usually recovered from parietal endoderm cells treated with 2,2'-bipyridyl and tunicamycin is also evident in Fig. 3 (lane 4) and Fig. 5 (lane 10). The same result was also noted in other experiments using murine F9 embryonal carcinoma (Fig. 3, lane 5), F9 parietal endoderm, STO and ST3 cells, and hamster N1L cells (data not shown), all of which produce detectable amounts of type IV procollagen.

For in vitro protein synthesis, total cellular RNA was prepared from parietal endoderm cells and translated in the nuclease-treated rabbit reticulocyte lysate. Immunoprecipitation of the in vitro synthesized proteins with anti-type IV collagen serum recovered a major polypeptide of M, 165,000 (Fig. 1, lane 2, arrowhead), that closely comigrates on gel electrophoresis with the major unmodified type IV procollagen chain isolated from tunicamycin and 2,2'-bipyridyl-treated parietal endoderm cells (Fig. 1, lane 5).
collagen synthesized by parietal endoderm cells. Lane 1, pro-
thionine-labeled in vitro translation product and type IV pro-
collagen immunoprecipitated from 2,2'-bipyridyl- and tunicamycin-
standard proteins; lane 3, M, = 165,000 polypeptide synthesized in
undifferentiated F9 cells treated with 2,2'-bipyridyl and
tunicamycin. All samples were digested with S. aureus V8 proteinase
for 1 h at 37°C, and the peptides were analyzed on a 12% gel. The
peptides generated from all three samples. The
pro-al chain from parietal endoderm cells appeared
slightly faster than those shown in lane 1,
but cells incubated with 2,2'-bipyridyl;
lane 5, as for lane 3 but cells
cultured with 2,2'-bipyridyl and tunicamycin; lane 6, 3H-labeled
protein M, standards (see under “Experimental
Procedures”). All lanes are from the same 6% polyacrylamide gel, but lanes 1 and 2 are
from one exposure to x-ray film, and lanes 3-6 are from a second
shorter exposure. Note the close comigration of the M, = 165,000
peptide maps of unmodified
and synthesized in vitro by the reticulocyte lysate supplemented with total
RNA isolated from parietal endoderm cells; lane 2, M, = 165,000
collagen immunoprecipitated from the in vitro translation reac-
tion with anti-type IV collagen serum (provided by E. Adamson);
lane 3, type IV procollagen chains immunoprecipitated with the same
antisera from control parietal endoderm cells; lane 4, as for lane 3
but cells incubated with 2,2'-bipyridyl; lane 5, as for lane 3 but cells
incubated with 2,2'-bipyridyl and tunicamycin; lane 6, 3H-labeled
polypeptide immunoprecipitated from the in vitro translation reac-
tion with anti-type IV collagen serum (provided by E. Adamson);
lane 3, type IV procollagen chains immunoprecipitated with the same
antisera from control parietal endoderm cells; lane 4, as for lane 3
but cells incubated with 2,2'-bipyridyl; lane 5, as for lane 3 but cells
incubated with 2,2'-bipyridyl and tunicamycin; lane 6, 3H-labeled
protein M, standards (see under “Experimental Procedures”). All
lanes are from the same 6% polyacrylamide gel, but lanes 1 and 2 are
from one exposure to x-ray film, and lanes 3-6 are from a second
shorter exposure. Note the close comigration of the M, = 165,000
pro-al chain from parietal endoderm cells appeared
slightly faster than those shown in lane 1 (data not shown).

Fig. 1. (left) Comparison by gel electrophoresis of [35S]me-
thionine-labeled in vitro translation product and type IV pro-
collagen synthesized by parietal endoderm cells. Lane 1, pro-
tehiones synthesized in the reticulocyte lysate supplemented with total
RNA isolated from parietal endoderm cells; lane 2, M, = 165,000
polypeptide immunoprecipitated from the in vitro translation reac-
tion with anti-type IV collagen serum (provided by E. Adamson);
lane 3, type IV procollagen chains immunoprecipitated with the same
antisera from control parietal endoderm cells; lane 4, as for lane 3
but cells incubated with 2,2'-bipyridyl; lane 5, as for lane 3 but cells
incubated with 2,2'-bipyridyl and tunicamycin; lane 6, 3H-labeled
protein M, standards (see under “Experimental Procedures”). All
lanes are from the same 6% polyacrylamide gel, but lanes 1 and 2 are
from one exposure to x-ray film, and lanes 3-6 are from a second
shorter exposure. Note the close comigration of the M, = 165,000
pro-al chain from parietal endoderm cells appeared
slightly faster than those shown in lane 1 (data not shown).

Fig. 2. (right) One-dimensional peptide maps of unmodified
type IV procollagen synthesized by parietal endoderm cells
and synthesized in vitro by the reticulocyte lysate using parai-
etal endoderm RNA. Lane 1, [35S]methionine-labeled type IV pro-
collagen immunoprecipitated from 2,2'-bipyridyl- and tunicamycin-
treated mouse embryo parietal endoderm cells; lane 2, 3H-labeled M,
standard proteins; lane 3, M, = 165,000 polypeptide synthesized in
the reticulocyte lysate from RNA isolated from parietal endoderm
cells; lane 4, as for lane 2; lane 5, type IV procollagen immunoprecip-
itated from differentiated F9 cells treated with 2,2'-bipyridyl and
tunicamycin. All samples were digested with S. aureus V8 proteinase
for 1 h at 37°C, and the peptides were analyzed on a 12% gel. The
closed arrowheads mark the characteristic M, = 84,000 and 89,000
peptides generated from all three samples. The open arrowheads
mark faint peptides clearly visible in lane 5 on the original autoradi-
ogram.

Further evidence that the in vitro synthesized M, = 165,000
polypeptide represents a full length but unglycosylated and
unhydroxylated translation product of type IV procollagen
mRNA was provided by peptide mapping experiments. The
unmodified type IV procollagen produced by tunicamycin and
2,2'-bipyridyl-treated parietal endoderm cells (material simi-
lar to that shown in Fig. 1, lane 5), and the in vitro synthesized
M, = 165,000 polypeptide (Fig. 1, lane 2), labeled with [35S]
methionine, were isolated by immunoprecipitation, and
approximately equal amounts of radioactivity were digested with
S. aureus V8 proteinase, followed by gel electrophoresis. Fig. 2
shows that practically all the peptides obtained from the in
vitro synthesized M, = 165,000 polypeptide (lane 3) were also
generated from the unmodified type IV procollagen chain
(lane 1), indicating that the chemical structures of both sam-
pies were very similar, if not identical.

We next investigated whether translatable type IV procoll-
gen mRNA could be isolated from cultures of differentiated mouse F9 cells. F9 embryonal carcinoma cells cultured with
retinoic acid and cyclic AMP undergo cellular differentiation
into parietal endoderm-like cells and synthesize increased
amounts of type IV procollagen compared with the control
cultures which retain their undifferentiated properties and
have only low level of type IV procollagen synthesis (11). Under the culture conditions used here, differentiated F9 cells
were estimated as described under “Experimental Proce-
dures” to be synthesizing approximately 1.0% of their total
proteins as type IV procollagen. This compares with 6.8%
calculated for normal parietal endoderm and 0.07% for undif-
fferentiated F9 cells. As shown in Fig. 3 (lane 3) the differen-
tiated F9 cell cultures used here produced the two type IV
procollagen chains in a ratio similar to that synthesized by
normal parietal endoderm cells (lane 2). The procollagen chains
of both samples closely comigrated on gel electrophoresis, but
the procollagen chain from parietal endoderm cells appeared
slightly larger than that from the F9 cells. The same result
was obtained also with a different preparation of anti-mouse
type IV collagen serum (lanes 7 and 8). However, when
immunoprecipitated from 2,2'-bipyridyl- and tunicamycin-
treated cells, type IV procollagen of both cell types appeared
as one major comigrating polypeptide of M, = 165,000 on
NaDodSO4-gel electrophoresis (Fig. 3, lanes 4 and 5). One-
dimensional peptide mapping of the unmodified chains sug-
gested that the type IV procollagens produced by differen-
tiated F9 cells and mouse embryo parietal endoderm cells are
similar (Fig. 2, compare lanes 1 and 5). The low level of type
IV procollagen synthesis by undifferentiated F9 cells is evident
from Fig. 3 (lane 6).

When poly(A) + RNA isolated from differentiated F9 cells
was translated in vitro, followed by immunoprecipitation with
anti-type IV collagen serum, one major M, = 165,000 poly-

Fig. 3. Synthesis of modified and unmodified type IV pro-
collagen by parietal endoderm cells and differentiated F9 em-
byonal carcinoma cells. Lane 1, 3H-labeled protein M, standards;
lane 2, type IV procollagen immunoprecipitated from control mouse
embryo parietal endoderm cells with antisera provided by E. Ad-
son; lane 3, as for lane 2 but using control differentiated F9 cells;
lane 4, as for lane 2 but using parietal endoderm cells incubated with
2,2'-bipyridyl and tunicamycin; lane 5, as for lane 3 but using differ-
entiated F9 cells incubated with 2,2'-bipyridyl and tunicamycin; lane
6, as for lane 3 but using undifferentiated F9 cells. In both cases the
immunoprecipitation was carried out using extracts containing
the same amount of incorporated radioactivity so that the difference
between lanes 3 and 6 directly relates to the difference in the biosyn-
thesis of type IV procollagen by the two cell types. Lanes 7 and 8, as
for lanes 2 and 3 but using antisera provided by G. R. Martin.
peptide was recovered that comigrated on gel electrophoresis both with the type IV procollagen chain synthesized in vitro from mouse embryo parietal endoderm RNA and with the major unmodified type IV procollagen chain isolated from differentiated F9 cells treated with tunicamycin and 2,2'-bipyridyl (data not shown). The $M_r = 165,000$ polypeptide synthesized in vitro from differentiated F9 cell RNA was also sensitive to digestion with collagenase, as described under "Experimental Procedures," and its immunoprecipitation was blocked by exogenous type IV collagen purified from the culture medium of PYS parietal endoderm cells (data not shown). Finally, one-dimensional peptide mapping of the in vitro synthesized $M_r = 165,000$ polypeptide and the major unmodified type IV procollagen immunoprecipitated from differentiated F9 cells treated with 2,2'-bipyridyl and tunicamycin produced almost identical patterns from both samples (Fig. 4).

In the course of the above experiments, in vitro translation of type IV procollagen mRNA consistently yielded one major $M_r = 165,000$ polypeptide. This result was unexpected in view of the previous data indicating two genetically distinct polypeptides for type IV procollagen. However, in further experiments when in vitro protein synthesis was carried out in the presence of a ribonuclease inhibitor (RNasin) followed by immunoprecipitation with anti-type IV collagen serum, two equally prominent polypeptides of $M_r = 165,000$ and 168,000 were detected (Fig. 5, lanes 6, 7, and 9). Using the same RNA preparation but without supplementation with the ribonuclease inhibitor, only one major polypeptide of $M_r = 165,000$ can be immunoprecipitated (Fig. 5, lane 8). The effect of the ribonuclease inhibitor RNasin was to increase the overall synthesis of total proteins and also more specifically to enhance the synthesis of large molecular weight proteins within the nuclease-treated reticulocyte lysate and the same volume of total reticulocyte lysate supplemented with RNasin, of poly(A) + RNA from differentiated F9 teratocarcinoma cells; lane 2, as for lane 1 but reticulocyte lysate not supplemented with RNasin; lane 3, total protein synthesis in reticulocyte lysate supplemented with RNasin, from total cell RNA of parietal endoderm cells; lane 4, as for lane 3, but reticulocyte lysate not supplemented with RNasin; lane 5, endogenous synthesis in the nuclease-reticulocyte lysate. The samples analyzed in lanes 1 to 5 were obtained from one experiment, and each translation was performed in similar volumes using the same concentration of RNA for each cell type; equal volumes were then analyzed. Lane 6, $M_r = 165,000$ and 168,000 polypeptides immunoprecipitated with anti-type IV collagen serum (provided by G. R. Martin, National Institutes of Health, Bethesda, MD) from reticulocyte lysates supplemented with RNasin, of poly(A) + RNA from differentiated F9 teratocarcinoma cells; lane 7, as for lane 6 but using total cell RNA obtained from parietal endoderm cells; lane 8, as for lane 7 but immunoprecipitated from a reticulocyte lysate not supplemented with RNasin; lane 9, a repeat of the experiment analyzed in lane 7 but included here to show that the relative synthesis of the $M_r = 165,000$ and 168,000 polypeptides can vary within this translation system. All immunoprecipitations were performed using the same batch of type IV collagen antiserum and the same volume of total reticulocyte lysate. Lane 10, the major unmodified polypeptide and ladder of collagenase-sensitive peptides (suggested by pulse-chase experiments to be prematurely terminated polypeptides, data not shown) which are immunoprecipitated with anti-type IV collagen serum from parietal endoderm cells treated with tunicamycin and 2,2'-bipyridyl. Note that when these samples are in adjacent lanes the in vitro synthesized $M_r = 165,000$ and 168,000 polypeptides migrate slightly behind the unmodified major type IV procollagen isolated from cells (lanes 9 and 10); Lane 11, type IV procollagen chains immunoprecipitated from control parietal endoderm cells. This figure is a composite made from four separate 6% NaDodSO4-polyacrylamide gels; lanes 1 to 5 were from one gel, lanes 6 to 11 from a second, and lanes 6 and 7 from two further gels. The molecular weight markers as defined in Fig. 1 are indicated by arrowheads.

![Fig. 4. One-dimensional peptide maps of unmodified type IV procollagen synthesized by differentiated F9 cells and synthesized in vitro with the reticulocyte lysate using F9 poly(A) + RNA. Lane 1, $[^{35}S]$methionine-labeled $M_r = 165,000$ in vitro translation product of poly(A) + RNA isolated from differentiated F9 cells and immunoprecipitated with anti-type IV collagen serum (provided by E. Adams); lane 2, undigested type IV procollagen from treated cells; lane 3, $[^{35}S]$methionine-labeled unmodified type IV collagen immunoprecipitated from differentiated F9 cells with 2,2'-bipyridyl and tunicamycin. Samples in lanes 1 and 3 were digested with S. aureus V8 proteinase for 15 min, and the peptides were analyzed on a 15% polyacrylamide gel. The major peptides of $M_r = 64,000$, 80,000, 43,000 (lane 1, 42,000), and 41,000 were generated from both samples. The position of the molecular weight standard proteins is shown on the left-hand side.](image-url)

![Fig. 5. Effect of a ribonuclease inhibitor (RNasin) on the in vitro translation products and type IV procollagen synthesis in reticulocyte lysate. Lane 1, total protein synthesis in reticulocyte lysate supplemented with RNasin, from poly(A) + RNA of differentiated F9 teratocarcinoma cells; lane 2, as for lane 1 but reticulocyte lysate not supplemented with RNasin; lane 3, total protein synthesis in reticulocyte lysate supplemented with RNasin, from total cell RNA of parietal endoderm cells; lane 4, as for lane 3, but reticulocyte lysate not supplemented with RNasin; lane 5, endogenous synthesis in the nuclease-reticulocyte lysate. The samples analyzed in lanes 1 to 5 were obtained from one experiment, and each translation was performed in similar volumes using the same concentration of RNA for each cell type; equal volumes were then analyzed. Lane 6, $M_r = 165,000$ and 168,000 polypeptides immunoprecipitated from a reticulocyte lysate not supplemented with RNasin; lane 7, as for lane 6 but using total cell RNA obtained from parietal endoderm cells; lane 8, as for lane 7 but immunoprecipitated from a reticulocyte lysate not supplemented with RNasin; lane 9, a repeat of the experiment analyzed in lane 7 but included here to show that the relative synthesis of the $M_r = 165,000$ and 168,000 polypeptides can vary within this translation system. All immunoprecipitations were performed using the same batch of type IV collagen antiserum and the same volume of total reticulocyte lysate. Lane 10, the major unmodified polypeptide and ladder of collagenase-sensitive peptides (suggested by pulse-chase experiments to be prematurely terminated polypeptides, data not shown) which are immunoprecipitated with anti-type IV collagen serum from parietal endoderm cells treated with tunicamycin and 2,2'-bipyridyl. Note that when these samples are in adjacent lanes the in vitro synthesized $M_r = 165,000$ and 168,000 polypeptides migrate slightly behind the unmodified major type IV procollagen isolated from cells (lanes 9 and 10); Lane 11, type IV procollagen chains immunoprecipitated from control parietal endoderm cells. This figure is a composite made from four separate 6% NaDodSO4-polyacrylamide gels; lanes 1 to 5 were from one gel, lanes 6 to 11 from a second, and lanes 6 and 7 from two further gels. The molecular weight markers as defined in Fig. 1 are indicated by arrowheads.](image-url)
inhibitor. The $M_r = 168,000$ polypeptide has not yet been characterized by peptide mapping, but we believe it to represent the other in vitro synthesized chain of type IV procollagen.

**DISCUSSION**

In view of the available data indicating two genetically distinct polypeptides of type IV procollagen (2, 5, 10), in vitro synthesis of type IV procollagen might be expected to generate two separate unmodified chains. The results presented here suggest that this is indeed the case. In most cases two equally prominent polypeptides of $M_r = 165,000$ and 168,000 were recovered by immunoprecipitation with anti-type IV collagen serum after in vitro translation of RNA isolated from cells synthesizing large amounts of the two chains of type IV procollagen. This result, obtained both with the total RNA of parietal endoderm cells and poly(A) + RNA of differentiated F9 cells, was consistently seen when the in vitro synthesis reaction was supplemented with a ribonuclease inhibitor. Without this inhibitor, however, compared to the $M_r = 165,000$ polypeptide, only trace amounts of the $M_r = 168,000$ polypeptide were synthesized and only visible in the original autoradiograms. These data suggest that the mRNA encoding the $M_r = 168,000$ polypeptide is unstable in the nucleasewashed rabbit reticulocyte system used in the present study and may well explain our failure to detect this polypeptide in the earlier in vitro translation experiments that were carried out in the absence of a ribonuclease inhibitor.

As shown by one-dimensional peptide mapping using V8-proteinase, the in vitro synthesized $M_r = 165,000$ polypeptide represents an unmodified chain of type IV procollagen (Figs. 2 and 4). In these experiments, unmodified type IV procollagen, isolated by immunoprecipitation from cells treated with 2,2'-bipyridyl and tunicamycin, appeared quite unexpectedly as one major chain that migrated slightly faster in NaDodSO$_4$ gel electrophoresis than the in vitro synthesized $M_r = 165,000$ polypeptide (Figs. 1, 3, and 5). This result has consistently been observed also in cell culture systems other than parietal endoderm and F9 teratocarcinoma cells. Although various concentrations of 2,2'-bipyridyl and tunicamycin have been tested, we have been unable to visualize by gel electrophoresis two distinct unmodified chains of type IV procollagen isolated by immunoprecipitation from the treated cells. Because of this complication, whether the $M_r = 165,000$ polypeptide represents an in vitro synthesized proa2(IV) or proa2(IV) chain remains to be determined. Although the other in vitro synthesized polypeptide of $M_r = 168,000$, identified here by immunoprecipitation with anti-type IV collagen serum, has not yet been peptide mapped, it probably represents the other in vitro synthesized and genetically distinct chain of type IV procollagen.

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**REFERENCES**