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Expression of human collagen type IV genes is regulated by transcriptional and post-transcriptional mechanisms

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The molecules of the basement membrane specific collagen type IV are heterotrimers consisting of two α1(IV) and one α2(IV) polypeptide chains. Comparison of the ratios of transcription by nuclear run-on analysis and mRNAs by RNase protection assay indicates the involvement of transcriptional as well as post-transcriptional events in the control of overall collagen type IV expression. The relative ratios of transcription of the respective genes COL4A1 and COL4A2 remained near 2:1 in most cells, whereas the ratio of mRNA steady-state levels α1(IV)/ α2(IV) varied from 0.3:1 to 1:1 and did not parallel the subunit structure of the protein. Nevertheless, secreted protein shows a 2:1 ratio of the subunit polypeptides. This indicates that post-translational processes during chain selection, aggregation and secretion finally determine the amount of secreted protein.

Basement membrane; Collagen type IV; Regulation

1. INTRODUCTION

Type IV collagen is the major structural component of all basement membranes [1] and the functional heterotrimeric molecule consists of two α1(IV) and one α2(IV) polypeptide chains [2,3]. The monomers are organized in a complex network optimally adapted to the various biological and physiological functions in the basement membrane [4]. In some specialized basement membranes minor amounts of additional chains, α3(IV), α4(IV) and α5(IV) [5–7] have been detected. Their impact to the structural organization of basement membranes is not yet clear, but their functional importance is shown by the involvement of α3(IV) in the pathogenesis of Goodpasture syndrome [8] or α5(IV) in Alport syndrome [9].

Regulation of type IV collagen gene expression is only poorly understood. The genes coding for the human and mouse α1(IV) and α2(IV) chains, COL4A1 and COL4A2, are located closely linked on chromosome 13q34 [10]. Both genes are orientated head-to-head and are transcribed in opposite directions [11,12]. Their transcription start sites are separated by a region of only 127 bp, comprising the two overlapping promoters for both genes. The promoter region and additional activating elements located in both genes are presently being analyzed in more detail.

Since the heterotrimeric collagen type IV molecule consists of two α1(IV) and one α2(IV) polypeptide chains, one would expect coordinated expression of the genes. Such a correlation has been found for the expression of the collagen type I genes. The genes COL1A1 and COL1A2 are transcribed in a ratio of 2:1 [13]. The same ratio was also observed at the steady-state level of mRNA [14], which is in accordance with the chain composition found in the secreted collagen type I molecule. Data on the transcription rates of collagen type IV genes are not yet available, mainly due to the much lower expression rate of collagen type IV genes in comparison to the collagen type I genes.

To gain a more detailed insight in the control of human collagen type IV gene expression, we compared the transcriptional rates of COL4A1 and COL4A2 with the steady-state amounts of the corresponding mRNAs in a variety of cells. Additionally, chain composition of secreted protein was determined after metabolic labeling. The ratio of transcription rates was found to be relatively constant (α1:α2 = 2:1), as was the subunit composition of the secreted molecules. The mRNA steady-state levels, however, showed significant variations.

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2. MATERIALS AND METHODS

2.1. *Cells and plasmids*

Human fibrosarcoma HT1080 [15], teratocarcinoma Tera2 [16], placenta-derived Hs723.P1, epithelial A431 and epidermoid HBL100 [15] were purchased from American Type Culture Collection. Human embryo fibroblasts (HE) were a kind gift of M. Aumailley. All cells were grown in Dulbecco's Modified Eagles Medium supplemented with 10% fetal calf serum.

For determination of transcription rates the following fragments were used: a 0.26 kb *Pst*I fragment from β-tubulin [17], a 0.45 kb *Eco*RI-*Bam*HI fragment of β-actin [18], 0.8 kb *Eco*RI fragment specific for $tRNA^{Val}$ [19], a 3.8 kb *Hin*dII-*Eco*RV fragment from a full length construct of α1(IV) mRNA and 4.2 kb *Eco*RI fragment from

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a full-length α2(IV) cDNA construct. The collagen type IV constructs were a gift of R. Nischt (Martinsried). For RNase protection assays were used: clone pBSHH containing a 147 bp *Hin*dII-*Hin*dIII fragment from human α1(IV) mRNA, corresponding to pos. 768–915 [20] and pBSES containing a 118 bp *Sma*I-*Eco*RI fragment, corresponding to pos. 241–358 [21] of α2(IV) mRNA. Fragments were cloned in pBSIISK- (Stratagene) and pUC-derived vectors.

2.2. *Nuclear run-on analysis*

Cells were grown until late exponential phase and aliquots of 1–2 × 10^7 cells, were used for preparation of nuclei [22]. Nuclear run-on analysis was performed exactly as described in [22]. The labeled RNA was purified, fragmented by treatment with NaOH, precipitated with ethanol and hybridized to nitrocellulose filters with bound fragments (3 μg of isolated insert per slot) in 10 mM TES, 0.2% SDS, 10 mM EDTA, pH 7.4, 300 mM NaCl at 65°C for 36–48 h. Filters were washed with 2 × SSC at 55°C, incubated with RNase A (10 μg/ml) at 37°C and washed with 2 × SSC at 55°C. Autoradiographs were analyzed by densitometry and direct determination of radioactivity. Values are expressed in comparison to the signal of β-tubulin set as 100%. The molar ratios were calculated by correcting for the length of the probes used.

2.3. *Preparation of RNA and RNase protection assay*

RNA was isolated from the cytoplasmatic supernatants after preparation of nuclei for nuclear run-on analysis [23]. Riboprobes of identical specific activity were generated from linearized pBSHH and pBSES by T7 RNA polymerase following the procedure of the supplier (Stratagene). Full-length transcripts were isolated and 1 × 10^5 cpm of each riboprobe were hybridized with 20 μg RNA and RNase protection assays were performed according to [22]. Protected bands were separated on sequencing gels and autoradiographs were analyzed by densitometry and relative amounts were calculated after correction for the number of labeled bases in the hybridizing fragments.

2.4. *Protein analysis*

HT1080 cells were plated at a density of 2 × 10^5 cells per 75 cm^2, grown for 3, 27 and 51 h and metabolically labeled with [^{35}S]methionine (20 μCi/ml; 1000 Ci/mmol) for 24 h in methionine-free RPMI-medium. Aliquots of the media, representing identical numbers of cells, were separated by SDS-gel electrophoresis on 5% polyacrylamide gels and the labeled proteins were visualized by fluorography. The intensity of bands was determined by densitometry and the amounts were calculated after correction for the content of methionine in α1(IV) and α2(IV) chains.

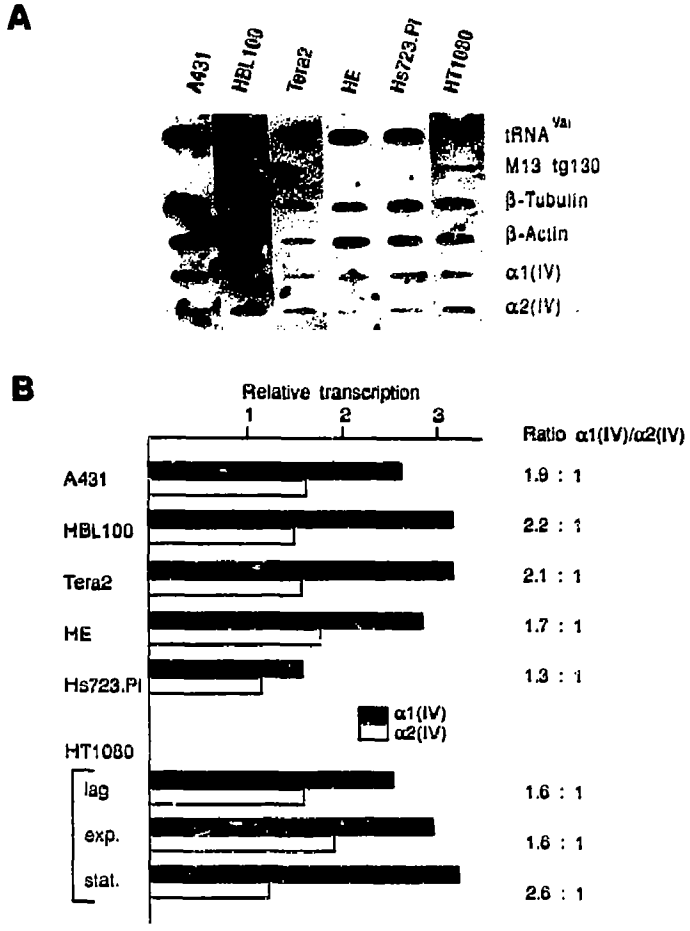

Fig. 1. Determination of transcription rates of collagen type IV genes in different human cells. (A) Representative nuclear run on experiments using nuclei of the cell lines indicated. Nascent labeled RNA was hybridized to filter bound probes as shown. (B) Amounts of α1(IV) and α2(IV) specific primary transcripts were determined by densitometry of autoradiographs and expressed in comparison to β-tubulin set arbitrarily as 100. The calculated relative molar ratios of α1(IV) and α2(IV) mRNAs are indicated.

3. RESULTS

3.1. *Relative transcriptional rates of COL4A1 and COL4A2 genes*

To determine the relative transcription rates of the COL4A1 and COL4A2 genes, nuclear run-on transcription experiments were performed (Fig. 1A). Nuclei were isolated from human cell lines known to produce collagen type IV, such as fibrosarcoma HT1080, epithelial line HBL100 or epidermoid carcinoma line A431 [15], the teratocarcinoma Tera2 [16], embryo fibroblasts and the placenta derived cells Hs723.Pi. For standardization and as internal controls, probes specific for β-tubulin, β-actin and t-RNA^{Val} were used. Because of the low levels of expression of the collagen type IV genes, cDNA probes corresponding to almost the entire α1(IV) or α2(IV) mRNA had to be used. The specificity of the probes was shown before by Northern blotting (data not shown). The filters were treated with RNase A after hybridizing to get a direct correlation of the signal intensity with the length of the probe and the amount of bound RNA. This was especially important for the probes detecting α1(IV) and α2(IV) mRNAs, due to the large number of introns within the corresponding genes and primary transcripts [27]. The amount of bound RNA was determined and the relative ratios were calculated by considering the length of the probes and the number of incorporated labeled nucleotides (Fig. 1B). In all cells, high levels of transcription of the tRNA^{Val} gene, β-tubulin and β-actin mRNAs were detected. Transcripts from the collagen type IV genes were detected at much lower levels. Compared to the synthesis of β-tubulin, only less than 0.21% of collagen type IV mRNAs synthesized, when calculated on a molar basis. In most cells the COL4A1 gene was tran-

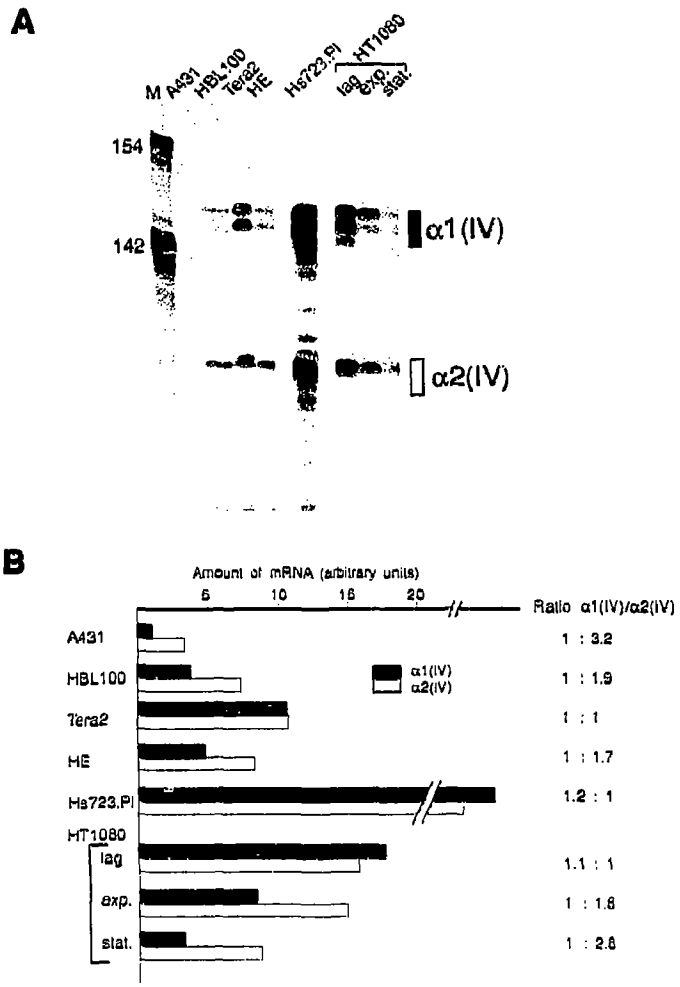

Fig. 2. Determination of levels of α1(IV) and α2(IV) mRNA in different cells by a ribonuclease protection assay. (A) Ribonuclease protection assay of equal amounts (10 μg) of cytoplasmatic mRNA from different cells using α1(IV) and α2(IV) specific riboprobes. The protected fragments were separated on a sequencing gel and the specific bands of 144–148 bases for α1(IV) and 114 bases for α2(IV) are indicated. RNA was isolated in parallel from the same cells used for nuclear run-on analysis (Fig. 1). (B) Quantitation of relative molar amounts of mRNAs after densitometry and correction for the number of incorporated labeled bases. Values are expressed in arbitrary units and molar ratios are indicated.

scribed more efficiently than the COL4A2 gene, leading to a relative transcription ratio of approximately 2:1, with the exception of Hs723.Pl where the two genes were transcribed with almost equal rates.

To detect for changes during cultures of cells, HT1080 cells were analyzed during lagphase (lag), exponential growth (log) and after reaching confluency (stat; Fig. 1B). Although the absolute levels of transcription did not change dramatically, an increase of the excess of COL4A1 transcription from 1.6:1 in freshly plated cells to 2.6:1 in confluent cells could be observed. Thus, cell type and culture conditions seem to have only little influence on the ratio of transcription rates of COL4A1 and COL4A2.

Table 1

Comparison of relative rates of transcription and mRNA levels of human α1(IV) and α2(IV) in different cells (Values represent molar ratios of α1(IV):α2(IV)).

Cells	Transcription	mRNA level
A431	1.9:1	0.3:1
HBL100	2.2:1	0.5:1
HE	1.7:1	0.6:1
Hs723.Pl	1.3:1	1.2:1
Tera2	2.1:1	1.0:1
HT1080/lag	1.6:1	1.1:1
HT1080/exp	1.6:1	0.5:1
HT1080/stat	2.6:1	0.4:1

3.2. *Steady-state levels of collagen type IV mRNAs*

To allow an accurate measurement of molar amounts, the steady-state levels of α1(IV) and α2(IV) mRNAs were determined by a ribonuclease protection assay of cytosolic RNA isolated from the cells used for nuclear run-on analysis. Riboprobes of identical specific activity and specific for short regions of both mRNAs, were mixed for RNase protection with equal amounts of isolated RNA from different cells. Fragments of 147 and 118 bases were resistant against digestion with ribonuclease, specific for the α1(IV) and α2(IV) mRNA, respectively (Fig. 2A). The highest levels of mRNAs were found in the placenta-derived cell Hs723.Pl, whereas the established cell lines showed significantly lower levels of mRNAs (Fig. 2B). In Tera2, Hs723.Pl and HT1080 cells about equal amounts of each mRNA were detected. An excess of α2(IV) mRNA was found in A431, HBL100 and HE cells. Thus the ratio of steady-state levels of mRNAs did not correspond to the ratios of transcription rates of the collagen type IV genes (see Fig. 1B). The influence of cell growth was found to be more pronounced at the mRNA level than at the transcriptional level. The ratio of α1(IV):α2(IV) mRNAs changed from 1.1:1 in freshly plated HT1080 cells to 0.4:1 in confluent cells, whereas the ratio of transcription rates increased from 1.6:1 to 2.6:1 (see Table I).

3.3. *Chain composition of secreted collagen type IV molecules*

The chain composition of secreted type IV was analyzed by metabolic labeling of HT1080 cells grown to different cell densities and SDS-gel electrophoresis (Fig. 3). The chain composition of secreted collagen type IV remained constant and showed a ratio of about 2:1 for the amounts of α1(IV) and α2(IV) subunits in all cases. From these experiments, it became clear that the composition of the secreted collagen type IV molecules is independent of the variations in the relative steady-state levels of mRNAs.

Fig. 3. Separation by SDS-PAGE of secreted proteins from HT1080 cells after metabolic labelling with [^{35}S]methionine. Cells were grown for 8 (lag), 40 (exp) or 72 h (stat) before labeling and aliquots of the medium representing identical numbers of cells were fractionated by SDS-PAGE and labeled proteins visualized by fluorography. Two representative experiments are shown and the collagen type IV chains are indicated.

4. DISCUSSION

Type IV collagen isolated from different basement membranes always revealed heterotrimeric molecules of the subunit structure $\alpha1(IV)_2\alpha2(IV)$ [1,2,24]. On the other hand, there are reports that the steady-state levels of mRNAs from different tissues in rat [25] or from skin fibroblasts from donors of different ages [26] can vary to a large extent. Because only little information was available about the coordinated regulation of these genes, we investigated the variations at different levels of expression.

The transcription rates for the COL4A1 and COL4A2 genes were found to be very low for the human cell lines investigated. The amount of specific transcripts was less than 0.2% of the level of β-tubulin transcription, when calculated on a molar basis. Although the low levels of expression and the complexity of the probes made it difficult to measure transcription rates of the collagen type IV genes as exact as those of β-tubulin and β-actin, the ratio of transcription rates of COL4A1 and COL4A2 could be determined in sufficient accuracy in several experiments. They were found to be relatively constant and most cells revealed a ratio of about 2:1. Additionally, the relative transcription rates changed only slightly in HT1080 cells during cell growth. We assume that the unique organisation of the collagen type IV genes in a bidirectional transcription unit is responsible for this coordination at the transcriptional level.

In contrast to the relatively stable transcription rates, analysis of the steady-state levels of mRNAs revealed higher variability. The amounts and ratios of $\alpha1(IV)$ and $\alpha2(IV)$ mRNAs differed between different cell lines, even though no striking variations had been seen at the transcriptional level. Generally, in most cases the amount of $\alpha2(IV)$ mRNA was higher than that of $\alpha1(IV)$. Only in cells with high mRNA content, like Hs723.Pl and HT1080, the two mRNA species were found in about equal amounts. The higher variability of the steady-state levels of collagen type IV mRNAs is in contrast with the relative constant transcription rates and this may be due to an additional control mechanism used for fine tuning of expression by limiting the amount of translation of one subunit. From our experiments we cannot deduce which post-transcriptional events are involved in the modulation of the mRNA ratio. For rabbit corneal endothelial cells it was shown that the half-life of $\alpha2(IV)$ mRNA changes significantly under different culture conditions [28]. Complex post-transcriptional regulatory mechanisms were also detected for different murine teratocarcinoma-derived cell lines [29].

It is interesting to compare the gene regulation of type IV collagen with that of the fibre-forming type I collagen molecule, also representing a heterotrimer of the subunit structure $[\alpha1(I)_2\alpha2(I)]$. It has been noted that the collagen type I genes are strictly regulated to a transcription ratio of 2:1, although the two genes COL1A1 and COL1A2 are located on different chromosomes [30]. Similar *cis*-acting elements found in both promoters of the two genes may be responsible for this effect [31]. Different from type IV collagen the ratio of $\alpha1(IV)$ and $\alpha2(IV)$ mRNAs remained relatively constant at about 2:1 [32].

In contrast to the variation of relative ratio of $\alpha1(IV)$ and $\alpha2(IV)$ mRNAs the secreted type IV collagen molecules always contained two $\alpha1(IV)$ and one $\alpha2(IV)$ chains. This ratio seems to be controlled by the assembly of the subunit chains before formation of the triple helix. During this step the subunits associate via their C-terminal domains into a complex with the typical 2:1 stoichiometry. Single chains not incorporated into a triple helical molecule may be degraded as shown for collagen type I [33]. Thus, the final composition of secreted functional molecules appears to be determined during the step of chain assembly which also compensates for imbalances at earlier stages of expression.

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