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Expression of Basement Membrane Proteins: Evidence for Complex Post-transcriptional Control Mechanisms

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Differentiated murine teratocarcinoma cell lines have been widely used as sources for the basement membrane proteins laminin and collagen IV. In order to understand the control of their expression, we have measured the transcription rates of the corresponding genes in nuclear run-on assays. The ratios of transcripts obtained from the five different genes of interest (for laminins A, B1, and B2 and α1(IV), α2(IV)) are rather different from the ratios of the corresponding mRNAs, which are again different from the protein levels needed. The gene for α2(IV) is transcribed at a higher rate than the one for α1(IV) and, similarly, the gene for laminin A is transcribed at a higher rate than the other two laminin genes, respectively. However, the α2(IV) and laminin A mRNA levels are lower than those for the other chains of the same molecule. The α1(IV) mRNA is 3- to 15-fold more abundant than the α2(IV) mRNA, depending on the cell line. At the protein level, the A chain seems to be limiting for the assembly of laminin, in accordance with its low mRNA level. The two collagen chains have variable pool sizes, but the triple helical molecules always seem to be composed of two α1(IV) and one α2(IV) chains. These results point to extensive control mechanisms at various stages of post-transcriptional events, some of which we could identify. © 1993 Academic Press, Inc.

INTRODUCTION

Basement membranes are thin extracellular matrices with ubiquitous distribution in the body and the animal kingdom. They are responsible for an orderly tissue structure, interact in many ways with different cell types, and function as a semipermeable filter [1]. As they are affected in many pathological conditions [2], the understanding of the regulation of their biosynthesis is one of our goals. We have focused on the two main components of basement membranes, i.e., collagen IV and laminin. Collagen IV is normally a heterotrimer, consisting of two α1(IV) and one α2(IV) chains [3, 4]. The best characterized form of laminin consists of three different chains, A, B1, and B2 (cf. [5, 6]).

For control of the biosynthesis of such complex, three-chain molecules several mechanisms can be envisioned. First, control steps could occur at one or more of several levels, e.g., during transcription of the corresponding genes, at the RNA level during processing of the primary transcripts, via stability of the primary or processed transcripts, during transport of the mRNA from the nucleus or during translation, and at the protein level via degradation of either the single chains or the assembled proteins. In the case of extracellular proteins, additional steps include secretion of the assembled proteins and remodeling of the basement membrane. Second, control at the various levels could be exerted for just one peptide chain with redundant synthesis and degradation of the others, or the biosynthesis of each chain could be strictly regulated at one or several levels.

For the heterotrimer collagen I ($(\alpha1(I))_2\ \alpha2(I)$), the main controlling step seems to be transcription, as the ratio of 2:1 for α1(I) and α2(I) is maintained from the transcription to the protein level [7, 8] with some variation [9]. In contrast, the ratios of the mRNAs for the constituent chains of collagen IV and laminin can vary considerably in different cells and tissues [10, 11], thus pointing to different regulatory mechanisms. We therefore surveyed transcription rates, steady-state mRNA levels, and protein levels for a number of murine cell lines, most of which are differentiated, teratocarcinoma-derived epithelial cells. Although most of these cells stem from the same tumor and are very closely related [12], they differ with respect to the amount of collagen IV synthesized [11]. Comparison of transcription rates and mRNA steady-state levels points to cell-specific differences in the regulatory potential of these cells.

MATERIALS AND METHODS

Cells and culture conditions. Cells were grown in tissue culture dishes at 37°C with 5% CO_2. They were seeded at 3×10^5 to 10^6 cells per dish and passaged when confluent (about 5×10^6–10^7 cells). Un-

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less otherwise stated, cell numbers refer to dishes with a 10 cm diameter (Falcon No. 3003). PYS-2 cells (P1, 3, 11, and 12) were fed with enriched DMEM [3]. All other cells were grown in DMEM with 0.2% glucose. The different cell lines are further characterized in [11–13]. PYS cells were originally described in [14] and PFHR-9 cells (HR9) in [15]. Cell line 8a was obtained from W. Schulz (cf. [16]) and F9 cells from Y. Yamada.

Metabolic labeling. For pulse chase experiments, the cells were preincubated in methionine-free medium with either 10% dialyzed fetal calf serum (FCS) or 0.5% FCS for 60 min prior to labeling with 20 (HR9 cells) or 125 μCi/ml (P1 cells) [^{35}S]methionine (NEN) for 17 or 15 min. The labeling medium was replaced by normal medium (with 10% FCS) or by medium with 0.5% FCS for the chase.

α,α'-Dipyridyl (Sigma) was dissolved in EtOH and used as a 100× stock (30 m*M*) in 10% EtOH/PBS. It was added to the methionine-free preincubation medium as well as to the chase medium. Dead cells were removed from the chase medium by centrifugation; the cell layer was washed two times with TBSE (50 m*M* Tris/HCl, pH 7.6, 150 m*M* NaCl, 1 m*M* EDTA) and scraped into 1 or 2 ml of TBSE with 1 m*M* NEM, depending on the size of the plate (6 or 10 cm diameter). The cell layer was kept frozen at −80°C until use while labeled medium was stored at 4°C to prevent precipitation of medium components and collagen IV. Frozen cells were lysed with 1% Triton X-100; the insoluble debris was removed by centrifugation and the supernatant was again stored frozen.

For analysis of the labeled proteins, 10 μl of cell extract or 20 μl of medium was loaded onto 5% SDS–polyacrylamide gels (17 cm long) run at 35 mA. Samples were reduced with 1 m*M* DTT; reduced and unreduced samples were boiled for 5 min prior to loading. The gels were impregnated with 25% PPO in DMSO before drying.

Run-on transcription assays. Cells were harvested for preparation of nuclei according to [17], but 0.2% Triton X-100 was used instead of 0.5%. Run-on transcription followed the protocol of [18]. Nuclei from 2×10^7 cells and 75–100 μCi [^{32}P]UTP (3000 Ci/mmol (NEN)) were used for each experiment. The newly synthesized RNA was hybridized to slot blots (Genescreen (NEN)) containing 1.5 μg of isolated, alkali-denatured cDNA fragments (crosslinked by uv light). After hybridization for 48 h and washing, the filters were incubated with RNase A (Pharmacia) to destroy the nonhybridizing introns. The filters were exposed for 3–7 days to preflashed XAR film (Kodak). The autoradiograms were evaluated as described below. The signals were normalized for U content of the newly synthesized mRNA that could hybridize to the corresponding cDNA fragment. The fragments used were obtained from cDNA clones from the 3′ parts of the mRNAs and covered between 1.3 and 1.9 kb.

RNA isolation and hybridizations. Total RNA was isolated according to either [19] or [20]. All platings of cells were done at least in duplicates; RNA was prepared separately from each dish. For quantitation of mRNA steady-state levels, each RNA preparation was loaded either four times in parallel on a slot blot or two times as serial dilutions. The same blots were hybridized successively with specific M13 probes. The last hybridization was always with a probe for 28S RNA, as this probe could not be quantitatively stripped from most blots. In order to obtain similar signal intensities for 28S compared to the laminin and collagen type IV mRNAs, the labeled 28S probe was diluted with cold material to obtain a 10-fold excess with respect to the calculated amount of 28S present on the membrane. The probes used for Northern and slot blot hybridizations have been listed in [11]. The blots were further treated as described under [21].

Estimation of half-lives. Cells were seeded the day before the experiment at a density of 1.3×10^6 per dish. After 17 h, the cells were fed and 1 h later 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Sigma) was added (0.1 m*M*). For each time point, three dishes each with and without DRB were used to isolate total RNA. The amounts for each mRNA represent the mean value from hybridizations for three parallel RNA preparations corrected for differences in the controls. The time course was followed for 8 h (one experiment) or 12 h (two experiments). Longer incubations were not useful as the cells started dying. Incorporation of [^{3}H]UTP was inhibited by 30% when cells were preincubated for 30 min with DRB prior to labeling in the presence of DRB for 4 h.

Actinomycin D (Sigma) was used under similar conditions at concentrations of 0.08 or 5 μg/ml. Incorporation of [^{3}H]UTP was inhibited to 97% with the higher dose. Ribosomal precursors were undetectable on Northern blots after 1 h incubation with 5 μg/ml or 3 h with 0.08 μg/ml.

Incubations with dexamethasone. P3 cells were seeded at a density of 1.5×10^6 per plate; 4 h later dexamethasone (Sigma) was added to half of the plates at a concentration of 10 μM. After 20 h, 0.1 m*M* DRB was added to half of the plates with and without dexamethasone in order to determine the half-life of the mRNAs. Total RNA was prepared at regular intervals from cells treated with dexamethasone and/or DRB and control cells. A concentration of 10 μM of dexamethasone had been previously determined to give maximal reduction of the mRNA levels for collagen IV and laminin after 22 h.

Standardizations and densitometric evaluations. Only preflashed Kodak XAR films were used for all exposures (at −70°C). Signals in the linear range of the film were scanned with a Bio-Rad densitometer (Model 620) and the relative amounts calculated from peak areas. mRNA amounts loaded on a slot blot were corrected according to the signal obtained with the probe for 28S rRNA (cf. [11]), as the calculations of mRNA concentrations from optical densities (260 nm) were often not accurate enough. For Fig. 1, all slot blots with total RNAs from the different cell lines were hybridized together. For the successive hybridizations, all probes were labeled to the same specific activities and exposed for the same time, so that the mRNA levels for all five mRNAs could be compared to each other. The signals for run-on hybridizations were standardized by including a large excess of a *DraI–PstI* fragment (5′ end of 18S rDNA, cf. [13]) divided on several slots as an internal control for the incorporation of [^{32}P]UTP. Results with different preparations of nuclei used in parallel were standardized according to those signals for 18S RNA.

RESULTS

We used several murine, differentiated teratocarcinoma-derived cell lines to study the individual steps during biosynthesis of the basement membrane proteins collagen IV and laminin. The cell lines have been described in detail in [11–13]. Of the PYS cells, the lines PYS-1 and PYS-2L do not synthesize collagen IV, while all PYS cells and PFHR-9 cells (HR9) produce roughly equal amounts of laminin [11, 12]. Undifferentiated F9 cells synthesize neither protein [22], while the transformed cell line 8a, a derivative of induced F9 cells (cf. [16]), makes both and resembles HR9 cells with respect to the amounts. All cell lines mentioned are derived from the same teratocarcinoma [12].

Previous results [11] had shown that the steady-state mRNA levels for the two chains of collagen IV (α1(IV), α2(IV)) and the three chains of laminin (A, B1, B2) do not agree with the ratios of the chains which are needed at the protein level (2:1 and 1:1:1, respectively). These steady-state levels could be predetermined by transcription rates and/or post-transcriptional events such as polyadenylation, processing of the primary transcripts, and transport from the nucleus or mRNA stabil-

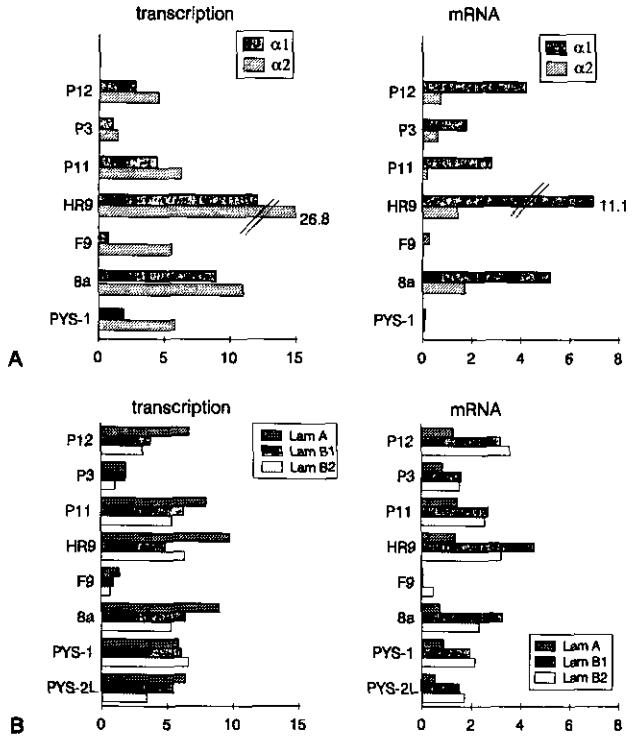

FIG. 1. Run-on transcription and steady-state mRNA levels. Confluent cells were used either to isolate nuclei for run-on experiments or for isolating total RNA, which was hybridized to probes specific for each of the five mRNAs for collagen IV (A) and laminin (B). The amounts of transcripts calculated after densitometric evaluation of the blots have been corrected for differences in content of uridine nucleotides in the transcribed and hybridizing parts for the run-on assays. The bars in (A) and (B) represent the relative amounts of transcripts for different cell lines standardized to 18S (run-on) or 28S rRNA (total RNA), respectively.

ity. Investigation of some of these processes has led to a more detailed picture of the post-transcriptional control steps.

Comparison of Transcription Rates and Steady-State mRNA Levels

We measured the transcription rates of the five genes of interest and determined in parallel the corresponding steady-state mRNA levels in confluent cells from different cell lines. The transcription rates and the mRNA levels in total RNA are depicted in Figs. 1A and 1B. The ratios of the transcripts for some cell lines are given in Table 1. The ratios of primary transcripts (hnRNA) in isolated nuclei per incubation period differed markedly from those for the processed transcripts (mRNAs). The primary transcripts for $\alpha 2$(IV) nearly always outnumber those for $\alpha 1$(IV). The opposite is found at the mRNA level. Similarly, for laminin, the primary transcripts for laminin A predominate, in contrast to the ratios at the mRNA level. This is also true for F9 cells, which transcribe all five genes, while the only mRNA present in reasonable amounts is the one for laminin B2, as has also been noted in [23, 24].

The run-on transcription rates did not change with different hybridizing cDNA fragments, independent of their position within the 3′ ends of the genes. For laminin A, a part of a genomic clone [25] containing about 500 bp from within the gene and 1 kb downstream from the 3′ end of the mRNA was used. As the signal was as strong as with cDNA fragments (not shown), it has to be concluded that transcription continues for at least 1 kb downstream of the end of the laminin A mRNA.

In some run-on assays, a cDNA probe for the basement membrane protein nidogen was also included (cell line P12). The signal was at background level, in accordance with the very low mRNA levels for nidogen in all cell lines tested [11].

Run-On Transcription in Different Growth Phases

We have shown that mRNA steady-state levels for the basement membrane proteins change in response to growth phase [11], reaching a minimum during exponential growth. This could be due to changes in transcription rates and/or changes in mRNA stability. Therefore, we investigated transcription rates in isolated nu-

TABLE 1

Ratios of Primary Transcripts Are Compared to the Ratios of mRNA Steady-State Levels for Alpha 1 (IV) and Alpha 2 (IV) and Laminin A, B1, B2, Respectively

		Ratio[a]			
		alpha 1/alpha 2		lam A/B1/B2	
Cell line[b]		Run-on	mRNA[c]	Run-on	mRNA[c]
P12	Lag	1.3	5.6	1.7/1/0.6	0.5/1/1.0
	Exp.	0.7	4.8	0.9/1/0.3	0.3/1/0.9
	Confl.	0.5	5.6	1.3/1/0.8	0.4/1/1.1
P3	Lag	0.7	2.2	1.2/1/0.3	0.5/1/1.0
	Exp.	0.7	3.4	1.3/1/0.4	0.3/1/1.0
	Confl.	0.7	2.9	0.5/1/0.2	0.5/1/1.0
P11	Lag	5.9	15.4	8.9/1/3.2	0.7/1/1.2
	Exp.	0.4	12.4	4.2/1/1.5	0.5/1/1.1
	Confl.	0.9	15.0	3.0/1/1.6	0.5/1/0.9
HR9	Lag	0.6	11.8	3.5/1/2.5	1.2/1/1.1
	Exp.	0.1	9.6	1.2/1/1.4	0.5/1/1.0
	Confl.	0.2	7.8	0.8/1/2.3	0.3/1/0.7
8a[d]	Confl.	0.9	3.0	2.3/1/0.6	0.2/1/0.7
F9[d]	Confl.	0.3	(14.3)	1.1/1/0.3	(1.0/1/5.0)
PYS-1[d]	Confl.	0.3	(1.6)	0.9/1/0.1	0.5/1/1.1

[a] The amounts for alpha 2 (IV) and laminin B1 have been arbitrarily set as 1.

[b] The growth phase of the cells is indicated (next column).

[c] Numbers in parentheses indicate very low mRNA levels.

[d] The values for these cell lines are taken from the experiments shown in Fig. 1.

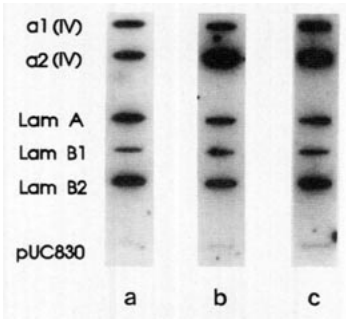

FIG. 2. Run-on transcription with nuclei of HR9 cells during different growth phases. Nuclei were isolated from cells in different growth phases (a, lag; b, exponential; c, stationary phase). An example of hybridizations for the run-on experiments is shown (overexposed). Treatment of the data was as in Fig. 1 and the ratios for primary transcripts and mRNAs are given in Table 1. The signal for laminin B2 appears too strong, as the part of the primary transcript being detected is particularly rich in U (cf. Table 1).

clei from cells in lag, exponential, and stationary growth phases and compared these rates to the corresponding steady-state mRNA levels. An example of slot blots hybridized with labeled transcripts is shown in Fig. 2; all results are summarized in Table 1. The influence of growth phase on transcription rates differs for the five genes. While in exponentially growing and confluent cells there is always an excess of transcripts for $\alpha 2$(IV) compared to $\alpha 1$(IV), in lag phase this ratio comes close to 1:1 or in some cell lines $\alpha 1$(IV) transcripts are in excess (Table 1).

For the laminin genes, there is nearly always an excess of transcripts from the laminin A gene, while at the mRNA level laminin B1 predominates. The ratios of transcription rates for the three laminin genes change with growth phase and depend on the particular cell lines. With the exception of cell lines P11 and HR9, the laminin B2 gene is transcribed least, while B2 mRNA levels reached similar values as those for B1. Between different experiments, the greatest variability in transcription rates and mRNA levels was observed with laminin B2 (cf., [11]).

mRNA Stability and Half-Lives

One reason for the deviation between transcription rates and mRNA steady-state levels could be different half-lives for the different mRNAs. We therefore investigated different methods for determination of their stability.

1,10-Phenanthrolin has been used successfully in yeast [26], but we found it to be too toxic for our cells. A pulse–chase method with [^{3}H]uridine and prior depletion of the internal uridine pool [27, 28] gave too low a signal to be evaluated.

Actinomycin D has been widely used to measure mRNA half-lives in many species. We incubated several of the cell lines (P11, P12, HR9) for up to 10 h with 0.08 or 5 μg/ml actinomycin D and isolated total RNA at regular intervals. When applying equal amounts of RNA to slot blots or Northern blots, a small increase in mRNA for $\alpha 1$(IV) instead of a decrease was seen during the first 4 h followed by a decline to the original level by 8 h after which the level remained nearly constant until 10 h (not shown). The cells looked well during this time. According to these results, the $\alpha 1$(IV) mRNA would be extremely stable. However, this seemed unlikely to us, particularly as actinomycin D has been reported to prolong the lifetime of some mRNAs [29].

Another widely used inhibitor of only RNA polymerase II (pol II) is 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, which has been shown to inhibit elongation of transcripts *in vitro,* but is supposed to have no effect on initiation [29]. This substance also proved quite toxic to the cells, limiting incubation times to 12 h. For cell line P3, mRNA levels for all five chains declined as expected within 12 h. Three examples of semilogarithmic plots for calculation of half-lives are given in Fig. 3. As can be seen from Fig. 3, as with actinomycin D, the mRNA levels rose or did not change during the first 4 h. Therefore, the first three points were omitted when calculating the regression curve. The half-lives of the five mRNAs under investigation are given in Table 2. As the corresponding slot blots were standardized with a probe for 28S rRNA, the calculated half-lives may be slightly overestimated. For the mRNAs for $\alpha 1$(I) and $\alpha 2$(I) collagen, half-lives of 8–10 h have been determined [7, 31, 32]; this is about twice as long as for most of the collagen IV and laminin mRNAs. It is possible that the evaluation of half-lives worked for the P3 cells because they have the lowest level of transcription of all collagen IV producing cell lines (see Discussion). For other cell lines, the problems described for the experiments with actinomycin persisted with DRB, especially for $\alpha 1$(IV).

Influence of Dexamethasone on Expression

The influence of the glucocorticoid dexamethasone on the expression of collagen IV and laminin was also investigated. Dexamethasone has been shown to decrease the biosynthesis of many collagens in tissue culture [7, 32, 33], but for collagen IV, the mechanism is not evident. Therefore, we performed run-on experiments with nuclei from P3 cells treated for 22 h with dexamethasone. While the transcription rates from the two collagen IV genes were essentially unchanged, there was an increase in transcription of about 20–40% for the three laminin genes (data not shown). At the mRNA level, the amounts of all collagen IV and laminin mRNAs were reduced by 30 to 50% after 24 h, but by 48 h they had risen back to about starting levels for collagen IV mRNAs and to even higher than the starting levels for the laminin mRNAs (not shown). The half-lives of all mRNAs dropped by about 30% after 22 h treatment

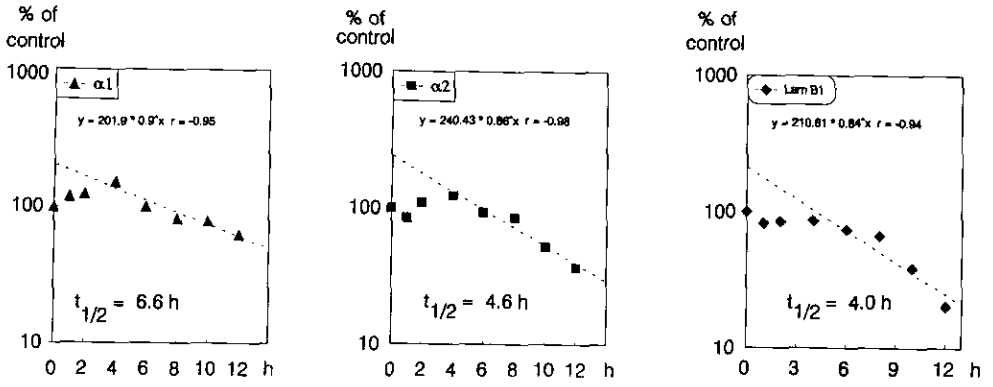

FIG. 3. Estimation of the half-lives of the mRNAs for α1(IV), α2(IV), and laminin B1. Cells were incubated with 0.1 m*M* DRB for the times indicated. The signals obtained after hybridization were plotted on a logarithmic scale against time. The first three points were omitted when fitting the data with an exponential regression curve. The equations for the regression curves and the correlation coefficients are shown in the graphs.

with dexamethasone (Table 2), indicating a reduced stability.

Protein Levels and Pulse–Chase Experiments

Steady-state mRNA levels may not necessarily correlate with protein levels. Some mRNAs in the cytoplasm may be inefficiently or even not translated, possibly due to features of the primary sequence or secondary structure. In addition, degradation may reduce the amounts of the protein of interest. In order to address these questions, we performed pulse–chase experiments with the cell lines HR9 and P1. Examples of such experiments are seen in Figs. 4 and 5. Comparison of the two cell lines revealed differences in the relative rates of labeling of laminin and collagen IV. While for both cell lines the laminin A chain was labeled more quickly than the B chains (cf. Figs. 4 and 5B), labeling and secretion of laminin was retarded with respect to collagen IV in HR9 cells (Fig. 4). The labeling was repeated on several consecutive days with cells all seeded at the same time; and it was seen (Figs. 4 and 5) that the pattern of secretion varied according to growth phase, exponentially growing cells (Day 2) synthesizing more basement membrane proteins per cell than confluent cells (Day 3), and that there are cell-specific differences in the rate of labeling of different chains.

Not much is known about intracellular degradation of newly synthesized chains of collagen IV and laminin. As unhydroxylated single chains of collagen IV should be more susceptible to degradation than chains in triple helical molecules, we inhibited hydroxylation with α,α′-dipyridyl while doing pulse–chase experiments. For the cell line 8a, which produces the highest amounts of collagen IV of all cell lines tested, as well as cell line P1, the unhydroxylated chains remained within the cell. No substantial decrease of unhydroxylated chains was detected during a chase of up to 8 h (not shown). Small amounts of un(der)hydroxylated chains were detected

TABLE 2

Half-Lives of mRNAs for Basement Membrane Proteins (Cell Line P3)

	Half-life[a]			
mRNA		−dex		+dex
α1(IV)		6.6	7.3	4.6
α2(IV)		4.6	4.6	3.1
Laminin A	4.0[b]	4.3	4.6	3.3
Laminin B1	6.6[b]	4.0	4.3	2.9
Laminin B2		4.6	4.6	3.1

[a] The values in the last two rows were determined with parallel plates.

[b] Values from an experiment with incubation for 8 h instead of 12 h.

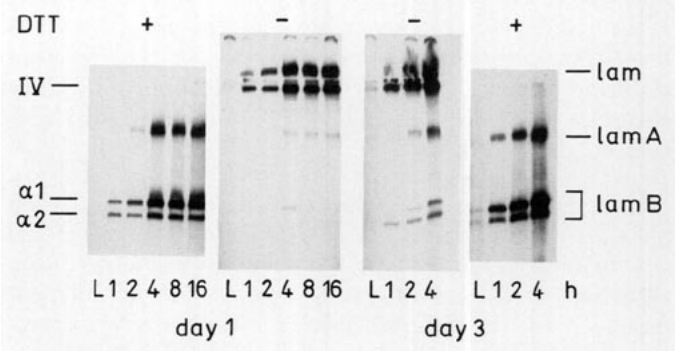

FIG. 4. Pulse–chase metabolic labeling of HR9 cells. HR9 cells were seeded at a density of 3×10^5/dish. Fifty hours later (1.2×10^6 cells/dish) the first pulse–chase experiment was performed. The second experiment started 48 h later (8.5×10^6 cells/dish). The cells were fed on the day between the two experiments. Only the upper half of the gels (with and without DTT) with the bands for laminin and collagen IV is shown. With these particular gels, unreduced laminin did not enter the separating gel, but remained in the stacking gel, probably due to aggregation of laminin. L, labeling medium; lam, laminin; IV, triple helical collagen IV.

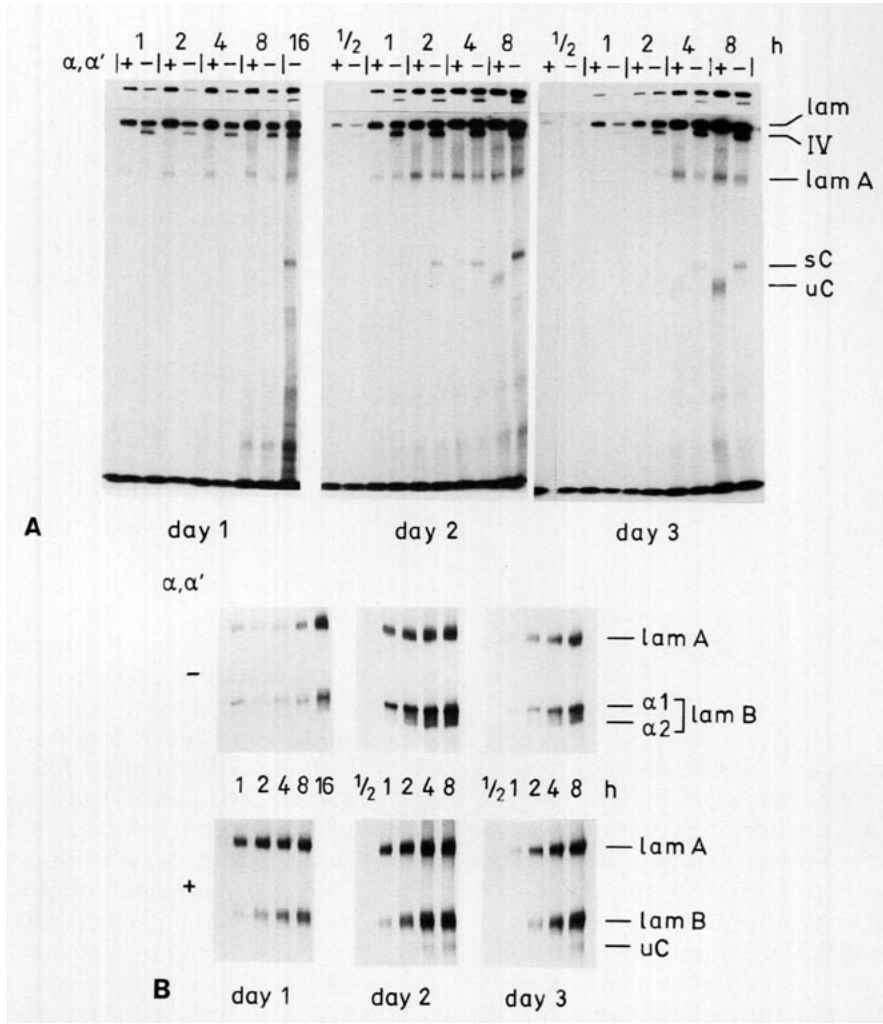

FIG. 5. Pulse–chase metabolic labeling of P1 cells. Cells were seeded at a density of 3.3×10^5 per plate. The first pulse–chase experiment started 37 h later (1.4×10^6 cells/plate), the second and third 24 and 48 h after the first (4.8×10^6 and 9.9×10^6 cells/plate). Cells for the third experiment were fed 16 h earlier. Labeling with and without α,α'-dipyridyl was performed in parallel; the same labeling medium was used for 15 min on all three days. (A) Gels showing the chase medium (unreduced) from all three experiments (exposed for the same time). The upper part presents the bands for laminin (lam) and triple helical collagen IV (IV) from a shorter exposure of the gels in order to indicate the differences in the amounts. (B) Part of the gels with reduced samples showing the bands for laminin A chain and the B chains which are not separated from each other and the two collagen IV chains (incubations without α,α'-dipyridyl). lam, laminin; IV, triple helical collagen IV; sC, single collagen IV chains; uC, un(der)hydroxylated collagen IV chains.

in the medium for both cell lines at the end of the chase period (Fig. 5, uC). These chains migrate slower than the unhydroxylated chains within the cell, but faster than the single collagen IV chains (sC) which are sometimes secreted into the medium (cf. [34]). Thus, the lower amount of collagen IV synthesized by P1 cells and the other PYS cell lines, compared to HR9 or 8a cells [12], is probably not due to enhanced intracellular degradation.

DISCUSSION

As the genes for the two chains of collagen and the three chains of laminin are rather large, quick changes of protein levels cannot be obtained by modulating the rates of transcription, only by post-transcriptional mechanisms. This can be inferred from Fig. 6, which gives minimal estimates for the time needed to synthesize these basement membrane proteins.

While the overall transcription rates of the genes for collagen IV and laminin roughly correlate with protein levels, we have provided ample evidence for the need for, and occurrence of, post-transcriptional regulatory mechanisms. In the following we discuss some of the most striking features.

Transcription Rates and Steady-State mRNA Levels

The two genes for α1(IV) and α2(IV) collagen are located head to head on the same chromosome [36, 37] in

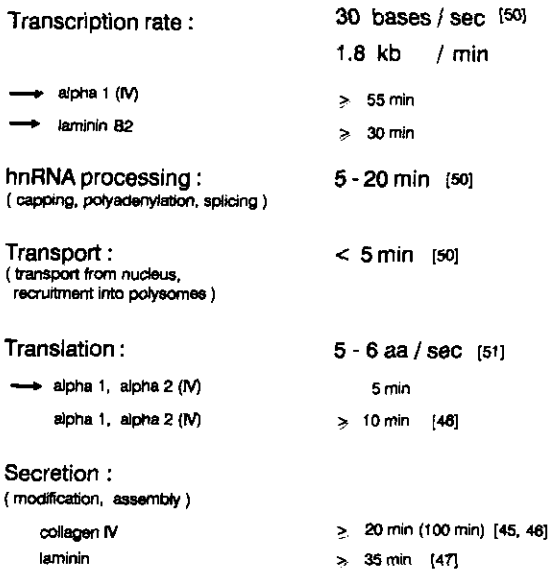

FIG. 6. Time schedule for expression of basement membrane proteins. Arrows indicate calculated values for basement membrane proteins.

mouse and human and have a short, shared promoter region. Therefore, similar transcription rates for both genes were expected. But the rate of transcription of the gene for α2(IV) nearly always exceeds that for the α1(IV) gene in mouse (Figs. 1 and 2; Table 1). This result is not restricted to teratocarcinoma-derived cell lines, as an excess of α2(IV) transcripts was also found for the epithelial cell line GZ (not shown) which is derived from a murine tumor of the choroid plexus [35]. In contrast, in human cell lines, transcription of the α1(IV) gene always exceeds that of the α2(IV) gene [38]. There are several possible explanations for these species-specific differences: While the structure of the promoter is very similar in mouse and man, an enhancer for both genes has up to now only been detected in mouse [37], whereas for the human genes an unidirectional negative element was identified (A. Haniel, U. Welge-Lüssen, E. Pöschl, and K. Kühn, in preparation).

Another known difference between the genes and mRNAs for α2(IV) of mouse and man is the insertion of a retroposon (B1 element) in the minus orientation into the murine 3′ untranslated region (3′ UTR) [39]. Retroposons acting as enhancers or silencers have been identified [40, 41]. Even though the B1 element in the 3′ UTR is only 74% identical to the B1 consensus sequence, it might exert a function as enhancer.

At the mRNA level, the α1(IV) transcript is more abundant than the α2(IV) mRNA in mouse, whereas the opposite is true in several human cell lines tested [38]. Therefore different control mechanisms have to be postulated in mouse compared to human. The longer half-life of α1(IV) mRNA compared to α2(IV) in mouse may play a role (see Table 2). Another postulated effect could again depend on the retroposon in the 3′ UTR of α2(IV) mRNA. It occurs in the minus orientation, which is assumed to be destroyed in the nucleus [42 and references therein]. This could take place via degradation of double-stranded RNA, as for other inverted B1 sequences in primary transcripts. Degradation might be inefficient for the α2(IV) mRNA because of the relatively low homology of the retroposon with the B1 consensus sequence, so that some transcripts survive. The two opposite effects, enhanced transcription and enhanced degradation of primary transcripts, may roughly cancel out.

The 3′ UTRs of the α2(IV) mRNAs of mouse and man not only differ with respect to the retroposon, but also in the remainder of the 3′ UTR, as the two sequences are only homologous in a short region into which the retroposon was inserted [39]. In contrast the 3′ UTRs of the α1(IV) mRNAs are highly conserved (78%) between mouse and man [43].

For the laminin genes, no comparative transcription rates for all three genes have been available before now. Surprisingly the laminin A gene was transcribed at the highest rate, while the laminin A mRNA levels are only 25–50% of those for laminin B1 (cf. Fig. 1B). As the half-life of all three mRNAs is similar (cf. Table 2), an additional regulatory step has to be postulated to adjust the amounts of primary transcripts to the mRNA levels. The 3′ UTR of the laminin A mRNA contains a noncanonical polyadenylation signal (AUUAAA). Although this sequence is the most frequently occurring natural mutation of the canonical polyadenylation signal AAUAAA, it has been shown to be processed inefficiently, thereby leading to reduced expression of the corresponding protein (cf. [44]). This also seems to be the case for laminin A, as its 3′ UTR lowered the expression of an indicator gene threefold, compared to constructs with the canonical polyadenylation signal (C. Gottschling, J. Huber, and I. Oberbäumer, submitted for publication).

Estimation of Half-Lives

The determination of half-lives for the collagen IV and laminin mRNAs led to unexpected problems. The rise in mRNA levels and/or lack of decay after addition of inhibitors of transcription could be due to several reasons. Shutdown of transcription may not immediately and uniformly affect all genes, with some genes continuing to be transcribed for a while without the usual competition for scarce transcription factors. As transcription and processing may take several hours (cf. Fig. 6), such trailing production of mRNAs may outpace decay for some time. Slow processing or transport is probably not a reason for the increase in mRNA levels, as partially processed molecules should also be detected

by slot blots, unless they are solubilized inefficiently during RNA preparation. Such precursors would, however, go undetected on Northern blots, due to size heterogeneity. In addition, at the moment we cannot exclude that the collagen mRNAs are much more stable in other cells than in the cell line P3.

Tissue culture conditions may also play a role. The cells used for measuring half-lives were always seeded the day before the experiment and fed before the addition of the drug and were therefore at the end of lag phase or the beginning of exponential phase. Perhaps the increase in mRNA levels at the beginning of incubation could be an indirect effect of a special, labile protein(s) related to growth phase, which would normally have a destabilizing effect during exponential growth, in accordance with the decrease of all basement membrane mRNA levels at this time [11]. Therefore, we are now studying half-lives under different growth conditions.

For laminin B1, a half-life of about 6.6 h was determined in an additional experiment with P3 cells, while the half-life of laminin A mRNA remained 4 h, so there may also be some variations in the stability of laminin mRNAs, again presumably depending on growth state (Table 2).

Pulse–Chase Labeling

The pulse–chase experiments indicate changing ratios of the two α chains during different growth phases. During exponential growth of HR9 cells, labeled α1(IV) and α2(IV) chains are seen with roughly equal intensities at the beginning of the chase, within the cell as single polypeptides (not shown) as well as secreted into the medium as triple helical molecules (Fig. 4, Day 1, 1 h), while the single secreted chain migrates as an α1(IV) chain, presumably indicating an excess of α1(IV) chains compared to the newly synthesized α2(IV) chains, in agreement with secretion of single α1(IV). A large pool of free, unlabeled α1(IV) chains would lead to dilution of the label in α1(IV) in triple helical molecules compared to α2(IV). Closer to the stationary phase, the α1(IV) chains may be limiting, as a single collagen chain with the mobility of α2(IV) is secreted first (Fig. 4, Day 3). In some labeling experiments, no clear band for α2(IV) was detected at all (cf. Fig. 5B, Day 1). At the moment, it is not known whether the single chains are normal α1(IV) and α2(IV) chains, whether they are modified, or whether they represent additional collagen IV chains (cf. [34]). In confluent cultures, the ratio of labeled chains in triple helical molecules is roughly 2:1 (Fig. 4).

In suspension cultures of HT-1080 cells (human) and HR9 cells, the first secreted collagen IV molecules were detected after about 80 and 100 min, respectively [45, 46]. Our pulse–chase experiments show that this time is markedly shorter for attached cells. While Cooper *et al.* [47] detected the first labeled molecules of laminin in the culture medium after 35 min, our results show that collagen is secreted at least as quickly as laminin, although the time point of detection of the different labeled peptide chains varies for different cell lines (cf. Figs. 4 and 5). The labeling medium had been used repeatedly (3–4 times) for 17-min labeling periods, as it was concluded from the literature that no collagen IV or laminin was secreted during this time span. As Fig. 4 shows, small amounts of both proteins can be detected in this labeling medium, part of this material probably resulting from lysed cells, since some smaller proteins were also detected at higher levels than in the 60-min chase medium. For P1 cells, laminin and collagen IV can clearly be detected after a 15-min pulse and a 30-min chase (cf. Fig. 5A). Dhawan *et al.* [48] showed that the mRNAs for collagen I in suspended fibroblasts have a reduced half-life compared to adherent fibroblasts. The same effect could be responsible for the differences in secretion time between our adherent cells and suspended cells [45, 46].

It is surprising that more protein is synthesized per cell in the exponential growth phase than in the stationary phase, although mRNA levels are higher in the stationary phase. But the stability of the mRNAs and the efficiency of their translation may be increased in the exponential phase, due to longer poly(A) tails of the mRNAs (cf. [49]; H. Germaier, C. Speth, and I. Oberbäumer, in preparation).

Figure 5 also suggests that biosynthesis of laminin and collagen IV influence one another, as there is more laminin synthesized in P1 cells when hydroxylation and secretion of collagen IV is blocked by α,α'-dipyridyl. Similar results were also obtained with cell line 8a (not shown).

These data point to additional translational and post-translational control mechanisms as part of the many post-transcriptional regulatory steps.

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REFERENCES

1. Timpl, R. (1989) *Eur. J. Biochem.* **180,** 487–502.
2. Timpl, R., and Dziadek, M. (1986) *Int. Rev. Exp. Pathol.* **29,** 1–112.
3. Oberbäumer, I., Wiedemann, H., Timpl, R., and Kühn, K. (1982) *EMBO J.* **1,** 805–810.
4. Trüeb, B., Gröbli, B., Spiess, M., Odermatt, B. F., and Winterhalter, K. H. (1982) *J. Biol. Chem.* **257,** 5239–5245.
5. Beck, K., Hunter, I., and Engel, J. (1990) *FASEB J.* **4,** 148–160.
6. Sanes, J. R., Engvall, E., Butkowski, R., and Hunter, D. D. (1990) *J. Cell Biol.* **111,** 1685–1699.

7. Hämäläinen, L., Oikarinen, J., and Kivirikko, K. I. (1985) *J. Biol. Chem.* **260,** 720–725.
8. Vuust, J., Sobel, M. E., and Martin, G. R. (1985) *Eur. J. Biochem.* **151,** 449–453.
9. Olsen, A. S., and Prockop, D. J. (1989) *Matrix* **9,** 73–81.
10. Boot-Handford, R. P., Kurkinen, M., and Prockop, D. J. (1987) *J. Biol. Chem.* **262,** 12475–12478.
11. Oberbäumer, I., and Speth, C. (1992) *Cell Tissue Res.* **268,** 439–445.
12. Speth, C., Epplen, J. T., and Oberbäumer, I. (1991) *In Vitro Cell. Dev. Biol.* **27A,** 646–650.
13. Oberbäumer, I. (1992) *Nucleic Acids Res.* **20,** 671–677.
14. Lehmann, J. M., Speers, W. C., Swartzendruber, D. E., and Pierce, G. B. (1974) *J. Cell Physiol.* **84,** 13–28.
15. Chung, A. E., Estes, L. E., Shinozuka, H., Braginski, J., Lorz, C., and Chung, C. A. (1977) *Cancer Res.* **37,** 2072–2081.
16. Schulz, W. A., and Gais, G. (1989) *Biochim. Biophys. Acta* **1013,** 125–132.
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (Eds.) (1987) Current Protocols in Molecular Biology, Wiley-Interscience, New York.
18. White, J. D., and La Gamma, E. F. (1989) *Methods Enzymol.* **168,** 681–690.
19. Birnboim, H. C. (1988) *Nucleic Acids Res.* **16,** 1487–1497.
20. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
21. Oberbäumer, I., and Speth, C. (1990) *Anal. Biochem.* **185,** 77–79.
22. Strickland, S., Smith, K. K., and Marotti, K. R. (1980) *Cell* **21,** 347–355.
23. Grover, A., Edwards, S. A., Bourdon, M., and Adamson, E. D. (1987) *Differentiation* **36,** 138–144.
24. Seebacher, T., Manske, M., Geimer, P., and Bade, E. G. (1991) *Exp. Cell Res.* **196,** 66–71.
25. Huber, J. (1991) Thesis, University of Munich.
26. Santiago, T. C., Purvis, I. J., Bettany, J. E., and Brown, A. J. P. (1986) *Nucleic Acids Res.* **14,** 8347–8360.
27. Scholtissek, C. (1971) *Eur. J. Biochem.* **24,** 358–365.
28. Levis, R., and Penman, S. (1977) *Cell* **11,** 105–113.
29. Wisdom, R., and Lee, W. (1991) *Genes Dev.* **5,** 232–243.
30. Chodosh, L. A., Fire, A., Samuels, M., and Sharp, P. A. (1989) *J. Biol. Chem.* **264,** 2250–2257.
31. Raghow, R., Gossage, D., and Kang, A. H. (1986) *J. Biol. Chem.* **261,** 4677–4684.
32. Cockayne, D., Sterling, K. M., Shull, S., Mintz, K. P., Illeyne, S., and Cutroneo, K. R. (1986) *Biochemistry* **25,** 3202–3209.
33. Oikarinen, A., Salo, T., Ala-Kokko, L., and Tryggvason, K. (1987) *Biochem. J.* **245,** 235–241.
34. Engvall, E., Bell, M. L., Carlsson, R. N. K., Miller, E. J., and Ruoslahti, E. (1982) *Cell* **29,** 475–482.
35. van Dyke, M. E., Finlay, C., Miller, D., Marks, J., Lozano, G., and Levine, A. J. (1987) *J. Virol.* **61,** 2029–2032.
36. Pöschl, E., Pollner, R., and Kühn, K. (1988) *EMBO J.* **7,** 2687–2695.
37. Burbelo, P. D., Martin, G. R., and Yamada, Y. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9679–9682.
38. Schmidt, C., Pollner, R., Pöschl, E., and Kühn, K. (1992) *FEBS Lett.*, in press.
39. Oberbäumer, I., and Magdolen, U. (1988) *Nucleic Acids Res.* **16,** 7181.
40. Oliviero, S., and Monaci, P. (1988) *Nucleic Acids Res.* **16,** 1285–1293.
41. Saffer, J. D., and Thurston, S. J. (1989) *Mol. Cell. Biol.* **9,** 355–364.
42. Rogers, J. H. (1985) *Int. Rev. Cytol.* **93,** 231–279.
43. Myers, J. C., Brinker, J. M., Kefalides, N. A., Rosenbloom, J., Wang, S.-Y., and Gudas, L. J. (1986) *Nucleic Acids Res.* **14,** 4499–4517.
44. Sheets, M. D., and Wickens, M. (1989) *Genes Dev.* **3,** 1401–1412.
45. Pihlajaniemi, T., Myllylä, R., Alitalo, K., and Kivirikko, K. I. (1981) *Biochemistry* **20,** 7409–7415.
46. Fessler, L., and Fessler, J. (1982) *J. Biol. Chem.* **257,** 9804–9810.
47. Cooper, A. R., Kurkinen, M., and Hogan, B. L. M. (1981) *Eur. J. Biochem.* **119,** 189–197.
48. Dhawan, J., Lichtler, A. C., Rowe, D. W., and Farmer, S. R. (1991) *J. Biol. Chem.* **266,** 8470–8475.
49. Jackson, R. J., and Standart, N. (1990) *Cell* **62,** 15–24.
50. Darnell, J., Lodish, H., and Baltimore, D. (Eds.) (1990) Molecular Cell Biology, Scientific American Books, New York.
51. Hershey, J. W. B. (1991) *Annu. Rev. Biochem.* **60,** 717–755.

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