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Positive elements in the laminin γ1 gene synergize to activate high level transcription during cellular differentiation

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ABSTRACT

Transcription of the murine laminin γ1 gene is activated during retinoic acid/cAMP induced differentiation of F9 embryonal carcinoma cells. Positive transcription control elements associated with two DNase I hypersensitive regions in the large first intron of the gene have been identified which confer a differentiation response on the laminin γ1 promoter. However, the kinetics of transcriptional activation suggest each DNA region interacts with transcription factors appearing at different times during differentiation. Synergy between the two regions *in cis* causes high level activation.

INTRODUCTION

Basement membranes are thin sheets of extracellular matrix laid down during development or in response to the need for new tissue growth or remodeling. They are thought to have important functions in the formation and maintenance of many tissues (1) as a physical barrier to the movement of cells and molecules and because of the surface they provide upon which cells can migrate and differentiate. In adults, basement membrane integrity is maintained by a poorly understood process which may be compromised by injury or disease. For example, a thickening of the glomerular basement membrane is frequently associated with diabetes mellitus (2).

Laminin, the major glycoprotein constituent of basement membrane, promotes cell attachment through interaction with cell surface receptors (3). We are interested in the genetic mechanisms which render the synthesis of the subunits of laminin responsive to developmental events.

Basement membrane production during development of the mouse embryo starts in the newly differentiated parietal endoderm shortly after implantation of the blastocyst (4). Our limited understanding of the control of synthesis of basement membrane proteins has principally come from studies of F9 embryonal carcinoma cells (5). When adherent F9 cells are exposed to retinoic acid and agents which elevate intracellular levels of cAMP they differentiate into parietal endoderm-like cells (6). They synthesize and secrete basement membrane proteins (7–9) as well as tissue plasminogen activator (6) and Sparc(osteonectin/BM40) (10). During differentiation transcription is activated of genes encoding the α1, β1 and γ1 chains of laminin (11; Phillips,S.L. and Chung,A.E., unpublished results), entactin (Phillips,S.L. and Chung,A.E., unpublished results), the α1(IV) and α2(IV) chains of collagen (12–14), and perlecan (15). The fact that transcription of all of these genes except the perlecan gene (15) is coordinately induced during differentiation (11,16; Phillips,S.L. and Chung,A.E., unpublished results) suggests they respond to a common control mechanism. However, with the exception of a promoter proximal retinoic acid response element in the laminin β1 gene (17) and the retinoic acid receptor proteins which bind to it (18), the mechanism mediating transcriptional activation of these genes remains elusive.

Many genes expressed in highly differentiated cells are controlled by *cis*-acting elements a considerable distance from the transcription start site. Many of these elements were originally located because they bind transcription control proteins that locally alter chromatin structure (cf. 19,20). Thus, we have initiated exploration of the mechanism controlling transcription of the murine laminin γ1 gene by measuring the DNase I sensitivity of chromatin over 25 kb around its transcription start site in undifferentiated and differentiated F9 cells. This analysis has led to the discovery of two positive regulatory regions located ~5 and 12 kb to the right of the transcription start site within the large first intron. Each region independently confers different kinetics of differentiation response on the promoter of the laminin γ1 gene in F9 embryonal carcinoma cells. Together the two regions synergize to mediate a very high level of promoter activity.

MATERIALS AND METHODS

Isolation of clones containing laminin γ1 genomic DNA

A genomic DNA library was kindly provided by Dr Steven Weaver, University of Illinois at Chicago. The library contains *Mbo*I partial digests of BALB/cJ genomic DNA cloned into the *Bam*HI site of lambda vector L47.1. The library was screened for clones using standard techniques (21) with a 0.58 kb *Pst*I–*Eco*RI fragment from cDNA clone λ573 containing sequence from the 5′-end of the murine laminin γ1 mRNA (22). Two of the clones that overlap the beginning of the laminin γ1 transcription unit, Lam γ1 #100 and Lam γ1 #154, are shown in Figure 1. Restriction maps of genomic DNA within these clones are identical to the map published by Ogawa *et al.* (23) as well as the map predicted

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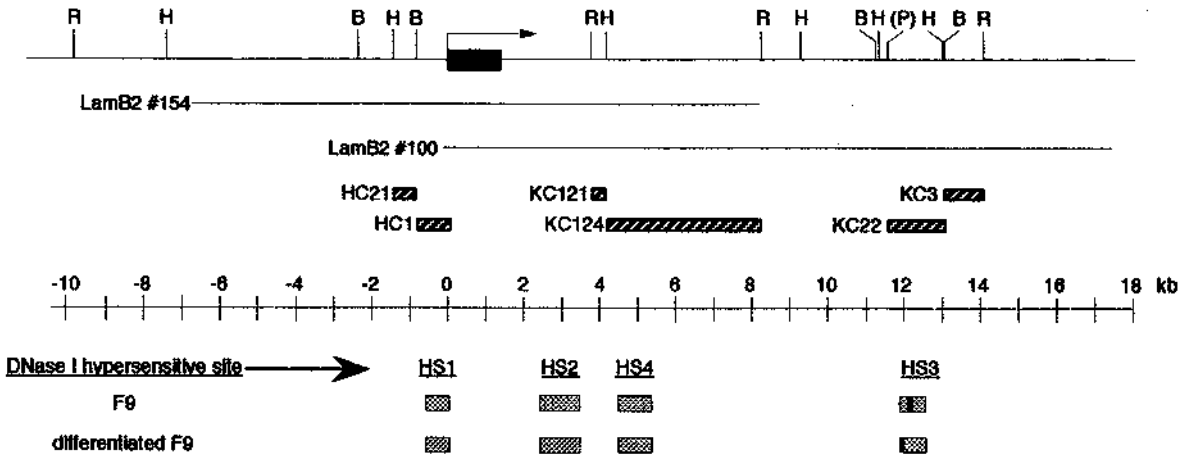

Figure 1. DNase I hypersensitive sites around the beginning of the laminin γ1 gene in undifferentiated and differentiated F9 embryonal carcinoma cells. **(Top)** A map of the 5′-end of the gene showing restriction sites used for mapping DNase I hypersensitive sites and subcloning; restriction enzymes used for the analysis were *Bam*HI (B), *Eco*RI (R), *Hin*dIII (H) and *Pst*I (P). Only one of the many *Pst*I sites is shown. Exon 1 is represented by a solid bar with the start site of transcription indicated by an arrow at position 0. The location of lambda genomic clones LamB2 #154 and LamB2 #100 used as a source of subclones for this investigation as well as three probes, HC21, KC121 and KC3, used for indirect end-labeling are shown under the restriction map. The location of fragments HC1, KC22 and KC124 containing the promoter and differentiation response elements, respectively, are also indicated. **(Bottom)** The location of DNase I hypersensitive sites in F9 control cells and cells induced to differentiate with retinoic acid and dibutyryl cAMP is indicated with regard to the distance in kb from the transcription start site. The approximate size of each hypersensitive region, determined from the breadth of the DNase I dependent bands in figures two through six, is indicated by the width of each box. No hypersensitive sites were detected upstream of the gene between –1 and –10 kb.

from restriction endonuclease digests of BALB/cJ genomic DNA and F9 embryonal carcinoma genomic DNA (Chang,H.S. and Phillips,S.L., unpublished results).

Cell culture

F9 embryonal carcinoma cells were kindly provided by Dr Albert E. Chung, Department of Biological Sciences, University of Pittsburgh. Cells were cultured in humidified 5% CO_2 in high glucose Dulbecco's modified Eagles medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum as has been described previously (24). Cells were seeded at a density of 2.5×10^5/100 mm plate and incubated overnight to permit attachment. Differentiation was initiated by a change to fresh medium containing 0.1 µM retinoic acid and 1 mM dibutyryl cAMP (Sigma Chemical Company, St Louis, MO). Forty eight and 72 h later the culture medium was replaced with fresh medium including the two drugs.

DNase I treatment of nuclei

Nuclei were prepared essentially as described by McKnight and Palmiter (25). All steps in the preparation were carried out at 4°C. Cells, cultured as described above, were washed once with ice cold phosphate-buffered saline (PBS), scraped into PBS with a rubber policeman and washed twice by centrifugation at 800 *g* for 1 min and resuspended in the same solution. Cells were recovered by centrifugation, washed once with 10 mM Tris–HCl pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and lysed by suspension at ~2×10^7 cells/ml in the same solution containing 0.5% Triton X-100. Nuclei were recovered by centrifugation at 800 *g* for 3 min, washed once with 10 mM Tris–HCl pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and resuspended at a DNA concentration of 1 mg/ml in 15 mM Tris–HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.5 mM DTT, 0.25 mM sucrose. 5 µl DNase I (Sigma Chemical Company, St Louis, MO), diluted to the appropriate concentration, was added to 95 µl suspensions of nuclei on ice, gently mixed and the reactions were initiated by transfer to 37°C. DNase I digestion was stopped after 3 min by the addition of 0.9 ml of 10 mM Tris–HCl pH 7.8, 5 mM EDTA, 0.5 % SDS to each reaction. DNA was purified as described by Wu (26).

Restriction endonuclease digests and hybridization analysis of DNA

Samples of purified DNA (10 µg) were digested to completion with restriction endonucleases as described by the supplier. DNA digests were subjected to electrophoresis through agarose gels in 89 mM Tris-borate buffer, pH 8.3, 1 mM EDTA containing 0.1 µg/ml ethidium bromide and transferred to GeneScreen Plus membrane (Du Pont) by capillary blotting under alkaline conditions (27,28). DNA was crosslinked to the membrane by UV irradiation (29). Purified probes were prepared from restriction digests of pBluescript II SK(–) subclones of Lamγ1 #100 or Lamγ1 #154 DNA. Map location of the three probes HC21, KC121 and KC3 used in the hybridization analyses are shown in Figure 1. Membranes were prehybridized for 2 h at 52°C in 50% formamide, 0.25 M NaCl, 0.25 M $NaHPO_4$, 1 mM EDTA, 100 µg/ml denatured salmon sperm DNA and 7% (w/v) SDS (30). Hybridization was carried out overnight in the same conditions at 43°C in the presence of 5 ng/ml denatured probe radiolabeled to a specific activity of at least 10^9 d.p.m./µg DNA by random primed synthesis (31). Membranes were washed twice at 52°C with 0.3 M NaCl, 0.03 M sodium citrate and 0.1% SDS, followed by two washes at 52°C with 25 mM sodium phosphate pH 7.2, 1 mM EDTA, 0.1% SDS and two additional washes under the same conditions with the SDS concentration increased to 1%. Radioisotope on dried membranes was visualized by autoradio-

graphy at –70°C using XAR film (Kodak, Rochester, NY) and intensifying screens (Lightning Plus, Du Pont).

Plasmid constructions

pHC1Luc. A fragment containing the promoter and transcription start site from base pairs (bp) –833 through +106 of the laminin γ1 gene (23) was prepared by the polymerase chain reaction according to published methods [Perkin Elmer Biotechnology Catalog Technical Information (1991), 21] using primers HSP001 (5′-gggagatctGGATCCAGTCCCTTGGACA-3′) and HSRP1020 (5′-gggagatctAGCCGGACGCAGAGCCGAT-3′) and genomic clone λlamB2 #154 as template. Each primer contained a *Bgl*II recognition sequence on the 5′-end. The 0.99 kb fragment was cloned into the *Bgl*II site of pXP2, a promoterless luciferase reporter gene (32), and the correct orientation identified by restriction analysis.

pKC22HC1Luc. The 1.5 kb KC22 *Pst*I–*Bam*HI fragment maps across hypersensitive site 3 from +11.7 to +13.1 kb of the laminin γ1 gene (Fig. 1). KC22 was subcloned into pUC18 to place a *Hin*dIII site adjacent to the *Pst*I site, removed by digestion with *Hin*dIII and cloned into the *Hin*dIII site of pHC1Luc to form pKC22HC1Luc. Clones containing both orientations of KC22 were identified by restriction analysis.

pKC21HC1Luc. The source of the 1 kb KC21 *Pst*I–*Sac*I fragment was pKC2 which contains the *Bam*HI fragment from +11.3 to +13.1 kb cloned into the *Bam*HI site of pBlueScript SKII(–). KC21 was subcloned from pKC2 into the *Pst*I/*Sac*I sites of pBlueScript SKII(–) to generate pKC21, removed from pKC21 by digestion with *Hin*dIII and *Sac*I and cloned into the *Hin*dIII/*Sac*I sites of pHC1Luc.

pKC211HCLuc1. The 0.2 kb *Rsa*I–*Sac*I fragment from pKC2 was subcloned into the *Sma*I/*Sac*I sites of pBlueScript SKII(–) to generate pKC211, removed from pKC211 by digestion with *Hin*dIII and *Sac*I and cloned into the *Hin*dIII/*Sac*I sites of pHC1Luc.

pKC212HC1Luc. The 0.8 kb PstI–RsaI fragment from pKC2 was subcloned into the *Pst*I/*Sma*I sites of pBlueScript SKII (–) to generate pKC212, removed from pKC212 by digestion with *Hin*dIII and *Sac*I and cloned into the *Hin*dIII/*Sac*I sites of pHC1Luc.

pKC221HC1Luc. The 0.5 kb *Sac*I–*Bam*HI fragment from pKC22HC1Luc was removed by digestion with *Sac*I and cloned into the *Sac*I site of pHC1Luc to generate pKC221HC1Luc. The clone containing the correct orientation of the KC221 insert was identified by restriction analysis.

pKC222HC1Luc. The 0.5 kb *Sac*I fragment from pKC22HC1Luc was cloned into the *Sac*I site of pKC211HC1Luc. The clone containing the correct orientation of the insert was identified by restriction analysis.

pKC223HC1Luc. The 0.5 kb *Sac*I fragment from pKC22HC1Luc was cloned into the *Sac*I site of pKC212HC1Luc. The clone containing the correct orientation of the insert was identified by restriction analysis.

pKC224HC1Luc. The 1.25 kb KC224 fragment was prepared by the polymerase chain reaction using primers HS3P3 (5′-gggaagcttCTGTGCAGCAAGGAGTG-3′) and HS3P5 (5′-gggggtaccCGCCCCTGCCCCTCTGC-3′) and pKC2 as template. HS3P3 and HS3P5 contain *Hin*dIII and *Kpn*I linkers, respectively, on the 5′-end. The fragment was digested with *Hin*dIII and *Kpn*I, purified by electrophoresis and cloned into the *Hin*dIII/*Kpn*I sites of pHC1Luc.

pKC225HC1Luc. The 1.12 kb KC225 fragment was prepared by PCR using pKC2 as template and primers HS3P3 and HS3P6 (5′-gggggtaccAAGCCTCGTGTCTCTGA-3′) which contains a 5′ *Kpn*I linker. The fragment was digested with *Hin*dIII and *Kpn*I, purified by electrophoresis and cloned into the *Hin*dIII/*Kpn*I sites of pHC1Luc.

pKC227HC1Luc. The 0.88 kb KC227 fragment was prepared by PCR using pKC2 as template and primers HS3P6 and HS3P4 (5′-gggaagcttGTTTGTTTGTTTGTCTG-3′) which contains a *Hin*dIII linker on its 5′-end. The fragment was digested with *Hin*dIII and *Kpn*I, purified by electrophoresis and cloned into the *Hin*dIII/*Kpn*I sites of pHC1Luc.

pKC228HC1Luc. A 0.22 kb fragment was prepared by PCR using pKC2 as template and primers HS3P3 and HS3P15 (5′-gggaagcttCCCAGAGCCCTCCAGGC-3′) which contains a *Hin*dIII linker on its 5′-end. The fragment was digested with *Hin*dIII, purified by electrophoresis and cloned into the *Hin*dIII sites of linear pKC222HC1Luc prepared by partial digestion with *Hin*dIII. The clone containing the fragment inserted at the correct location and orientation was identified by restriction analysis.

pKC124HC1Luc. The 4.1 kb KC124 *Hin*dIII–*Eco*RI fragment maps across hypersensitive site 4 from +4.2 to +8.3 kb. The source of KC124 was pKC12 which contains the 4.5 kb *Eco*RI fragment from +3.8 to +8.3 kb cloned into the *Eco*RI site of pBlueScript SKII(–). KC124 was purified by electrophoresis from a *Hin*dIII digest of pKC12 and cloned into the *Hin*dIII site of pHC1Luc to form pKC124HC1Luc. Clones containing both orientations of the fragment were identified by restriction analysis.

pKC22/124HC1Luc. Fragment KC124 was prepared as described and cloned into the *Hin*dIII sites in pKC22HC1Luc linearized by partial digestion with *Hin*dIII. Clones containing both orientations of KC124 in the *Hin*dIII site between the KC22 and HC1 fragments were identified by restriction analysis.

Transfection protocols and assay of reporter gene activity

Transfections were carried out by modifications of published procedures (33,34). Cells were seeded at 1×10^5 cells/35 mm plate in 2 ml Dulbecco's modified Eagles medium containing 10% fetal bovine serum, cultured overnight and provided with fresh medium before transfection. Each calcium phosphate/DNA co-precipitate contained 0.5 pmol test plasmid, 0.2 µg pRL.*neo* and pUC18 DNA added to bring the final DNA mass to 5 µg. pRL.*neo* was kindly provided by Dr Justus B. Cohen (Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, PA). The DNA, in a volume of 90 µl, was mixed with 100 µl 2× HEPES phosphate buffer (136 mM NaCl, 5 mM KCl, 11.2 mM glucose, 208 mM HEPES, 1.4 mM Na_2HPO_4). 10 µl 2.5 M $CaCl_2$ was dripped into the solution, gently mixed and held at room temperature for 30 min until a finely suspended precipitate was

evident. The calcium phosphate/DNA suspension was slowly dripped into the culture dish and the dish returned to the incubator after gentle mixing. Twenty-four hours after addition of the co-precipitate, cells were passaged into a 100 mm dish containing 10 ml DME medium supplemented with 10% fetal bovine serum and G418 at 1 mg/ml. The medium was changed every 3 days. Approximately 1 week after transfection the G418 concentration was lowered to 400 µg/ml. Following an additional week individual or pooled clones were recovered and grown in medium containing G418 at 200 µg/ml. The yield of G418 resistant clones was similar for all test plasmids. Mixed clone preparations contained at least 50 independent clones.

The luciferase assay was essentially as described (35; Luciferase assay procedure, Technical bulletin #101, Promega Corporation). For experiments lasting no more than 2 days cells were seeded at 10^5 per 35 mm plate. Differentiation was induced as described above. Following removal of the medium the attached cells were rinsed twice with PBS, lysed by the addition of 0.25 ml of a solution containing 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 1 mM EDTA, 0.1% Triton X-100 and 10% glycerol. After incubation at room temperature for 10 min, the lysate was clarified by centrifugation for 1 min in a microcentrifuge at room temperature and the supernatant stored at –70°C. Luciferase activity was measured in a Picolite (Packard Instruments) or AutoLumat 930 (EG&G Berthold) luminometer set at 25°C. Light output was integrated over 30 s. Each reaction contained 10 µl clarified lysate and 100 µl reagent containing 20 mM Tricine pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM luciferin and 530 µM ATP. Protein content was measured using the DC-protein assay kit (BioRad Laboratories).

RESULTS

Transcription of the laminin γ1 gene is activated when F9 embryonal carcinoma cells are induced to differentiate by treatment with retinoic acid and cAMP (11). A number of putative *cis*-acting elements are upstream of the transcription start site (23). Our approach to understanding control of the gene was first to map DNase I hypersensitive sites in chromatin containing the gene and then to use this information to guide our search for differentiation response elements.

Mapping DNase I hypersensitive sites around the beginning of the laminin γ1 transcription unit

Our primer extension analysis confirmed the published position (23) of the transcription start site of the murine laminin γ1 gene (data not shown). Figure 1 shows the map location of genomic DNA in λ phage clones Lamγ1 #100 and Lamγ1 #154 which overlap the 5′-end of the murine BALB/C laminin γ1 transcription unit and were used as the source of indirect end-labeling probes for these experiments. Nuclei from undifferentiated and differentiated F9 cells were digested with increasing concentrations of DNase I. Figure 2 demonstrates that the promoter lies within a broad hypersensitive region in undifferentiated and differentiated F9 cells. This region, which we have designated HS1, appears to contain the transcription start site and covers ~600 bp of upstream DNA. DNA prepared from nuclei not treated with DNase I and DNase I digestion of naked DNA showed no distinct subfragments in this analysis (data not shown). Subtle differences in the fine structure

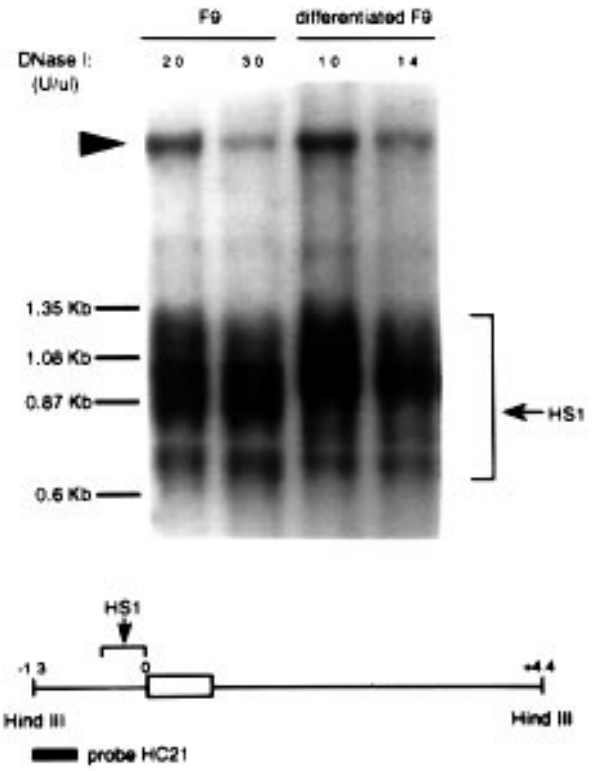

Figure 2. Mapping a DNase I hypersensitive region at the 5′-end of the laminin γ1 gene. The mapping strategy and location of the indirect end-labeling probe is displayed at the bottom of the figure. Map coordinates from Figure 1 are shown above the ends of the *Hin*dIII fragment. F9 cells were induced to differentiate by exposure to 0.1 µM retinoic acid and 1 mM dibutyryl cAMP for 4 days. Nuclei were prepared and incubated with the indicated concentrations of DNase I. 10 µg of purified DNA from each sample was digested to completion with *Hin*dIII, separated by electrophoresis in a 1.5% agarose gel, and subjected to blot-hybridization analysis using radiolabeled HC21 as probe. The positions of DNA standards are indicated along the left side of the autoradiogram as well as the location of the *Hin*dIII band that is the only one present in the absence of DNase I (▶).

of HS1 appeared as a consequence of differentiation. For example, a distinct site within the region revealed by a 0.9 kb subfragment in the digests of DNA from F9 cells, was not evident in digests of DNA from differentiated F9 cells (Fig. 2). The complexity of hypersensitivity in this region is not surprising since it contains a number of probable *cis*-acting elements including Sp1-like sequences and one cAMP response element (23). These results suggest that the laminin γ1 gene is not silent in F9 cells because the promoter is in a closed chromatin configuration where access of transcription factors or the basal transcription complex is occluded by nucleosomes. Rather the gene may be silent because of the presence of bound repressor proteins or the absence of interaction with factors bound at distant *cis*-acting elements. No additional DNase I hypersensitive regions were detected in 9.8 kb of DNA between the transcription start site and the upstream *Eco*RI site in either undifferentiated or differentiated F9 cells (data not shown).

A search for hypersensitive sites in chromatin directly downstream of the transcription start site was carried out in *Hin*dIII digests using the KC121 probe. DNase I treatment generated a prominent 0.9 kb subfragment and three larger subfragments due to

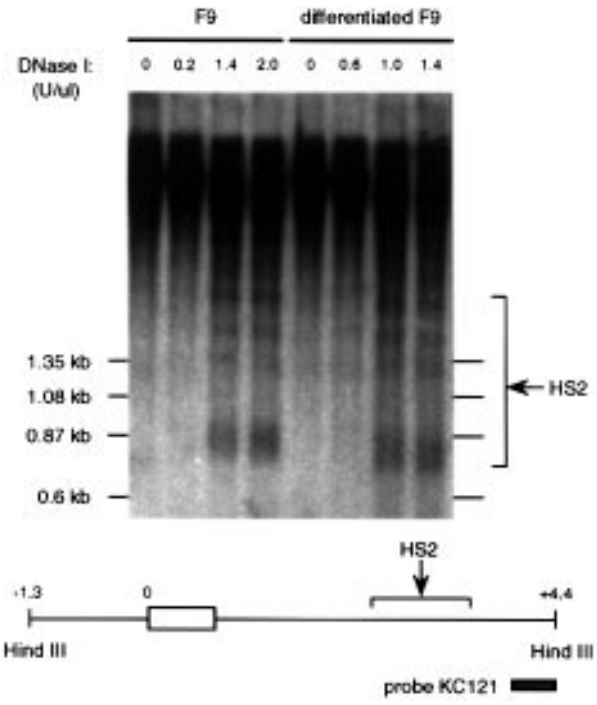

Figure 3. Mapping DNase I hypersensitive site 2 (HS2) in the laminin γ1 gene. The mapping strategy and location of the indirect end-labeling probe is displayed at the bottom of the figure. Map coordinates from Figure 1 are shown above the ends of the *Hin*dIII fragment. Nuclei were isolated from cells treated as in Figure 2 and incubated with the indicated amounts of DNase I. 10 µg purified DNA from each sample was digested with *Hin*dIII, separated by electrophoresis in a 1.2% agarose gel and subjected to blot-hybridization analysis using radiolabeled KC121 as probe. The positions of DNA standards are indicated along the sides of the autoradiogram.

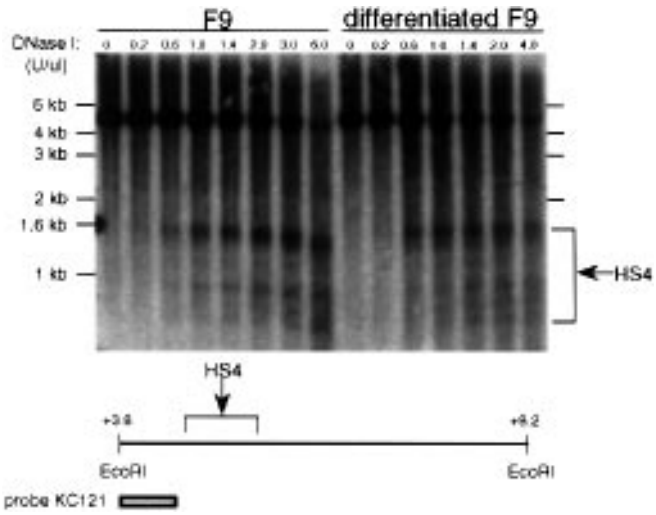

Figure 4. Mapping DNase I hypersensitive site 4 (HS4) in the laminin γ1 gene. The mapping strategy and location of the indirect end-labeling probe is displayed at the bottom of the figure. Map coordinates from Figure 1 are shown above the ends of the *Eco*RI fragment. Nuclei were isolated from cells treated as in Figure 2 and incubated with the indicated amounts of DNase I. 10µg purified DNA was digested with *Eco*RI, separated by electrophoresis in an 0.8% agarose gel and subjected to blot-hybridization analysis using radiolabeled KC121 as probe. The positions of DNA standards are indicated along the sides of the autoradiogram.

a hypersensitive region mapping from ~2.4 to 3.5 kb beyond the transcription start site (Fig. 3). This region which we designate HS2 was present in undifferentiated as well as differentiated F9 cells. Lower exposure of the autoradiogram in Figure 3 revealed the 4.4 kb subfragment caused by HS1 at the promoter (data not shown).

Mapping DNase I hypersensitive sites further downstream into intron one was carried out in *Eco*RI digests of DNA using fragments KC121 and KC3 as indirect end-labeling probes. The analysis using probe KC121 demonstrated the presence of a group of DNase I dependent subfragments ~0.7–1.5 kb long (Fig. 4). The DNase I hypersensitive sites causing these fragments map to a region from ~4.5 to 5.3 kb downstream of the transcription start site which we designate HS4. HS4 was present in undifferentiated F9 cells and differentiated F9 cells (Fig. 4).

Using probe KC3 in the analysis revealed a 600 bp region of hypersensitivity we designate HS3 mapping ~12.5 kb downstream of the transcription start site (Fig. 5). The most interesting feature of this region is the change in DNase I sensitivity of several sites within the region associated with differentiation. For example, close inspection of Figure 5 reveals a prominent 1.8 kb subfragment in the DNA from undifferentiated cells which is much less abundant in the DNA from differentiated cells. Furthermore, a 2.1 kb subfragment appears with differentiation. This change in chromatin structure with differentiation suggests that HS3 contains differentiation response elements.

A differentiation responsive enhancer is associated with hypersensitive site 3

To address whether DNA sequences in hypersensitive region 1 (HS1) and hypersensitive site 3 (HS3) contain differentiation response elements chimeric genes were constructed using a promoterless luciferase reporter plasmid (32) and DNA fragments HC1 and KC22 (Fig. 1) which overlap these hypersensitive sites. HC1 maps from –833 to +106 bp and contains the transcription start site and promoter. Fragment KC22 spans HS3 from the *Pst*I site at +11.6 kb to the *Hin*dIII site at +13.1 kb. Transient expression transfection assays of plasmids pHC1Luc and pKC22HC1Luc demonstrated HC1 functions as an active promoter in the correct orientation in undifferentiated as well as differentiated F9 cells. HC1 is several fold more active than the RSV LTR. Furthermore, the activity is unaffected by the presence of KC22 in either orientation in the reporter gene (data not shown).

To take into account the possible role chromatin plays in the regulation of transcription, cells containing stably integrated reporter genes were tested. Mixed clone populations of cells stably transfected with pHC1Luc or pKC22HC1Luc were prepared as described in the Materials and Methods and treated for 36 h with retinoic acid and dibutyryl cAMP. Figure 6 shows that pHC1Luc activity was quite low in F9 cells and only weakly responsive to the presence of the two drugs. However, pKC22HC1Luc very strongly responded to the drugs. KC22 showed insignificant promoter activity in either orientation but conferred a 3-fold response on stable integrants of reporter genes driven by the minimal (–81 to +52) HSV1 thymidine kinase promoter as well as in pKC22(–)HC1Luc, containing KC22 in the reverse orientation (data not shown). These results demonstrate KC22 contains a differentiation responsive enhancer. Table 1 shows that

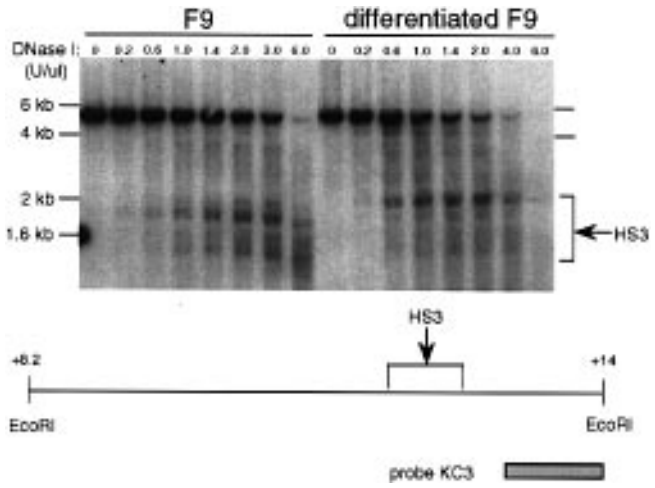

Figure 5. Mapping DNase I hypersensitive site 3 (HS3) in the laminin γ1 gene. The mapping strategy and location of the indirect end-labeling probe is displayed at the bottom of the figure. Map coordinates from Figure 1 are shown above the ends of the *Eco*RI fragment. Nuclei were isolated from cells treated as in Figure 2 and incubated with the indicated amounts of DNase I. 10 µg purified DNA was digested with *Eco*RI, separated by electrophoresis in an 0.8% agarose gel and subjected to blot-hybridization analysis using radiolabeled KC3 as probe. The positions of DNA standards are indicated along the sides of the autoradiogram.

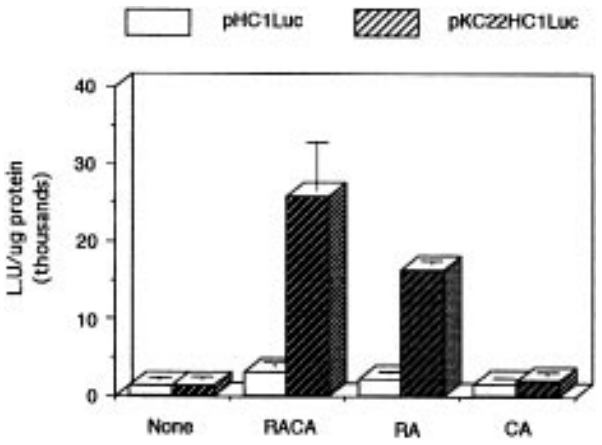

Figure 6. Response of stable integrants of pHC1Luc and pKC22HC1Luc in mixed clones of F9 cells to retinoic acid or dibutyryl cAMP. Mixed clones of G418 resistant F9 cells containing pHC1Luc or pKC22HC1Luc were prepared as described in the Materials and Methods. Cells (1×10^5) were seeded into 35 mm plates, allowed to attach for ≥6 h at 37°C in DMEM + 10% fetal bovine serum and treated with 0.1 µM retinoic acid (RA), 1 mM dibutyryl cAMP (CA) or both (RACA) in DMEM + 10% fetal bovine serum for 36 h. Control cells (None) were incubated for 36 h in the absence of either drug. Luciferase activity and protein content were measured in clarified lysates as described in the Materials and Methods. Each value is the mean of duplicate assays with the range shown by the error bars. The activity of stable integrants of pXP2 the parent plasmid containing the promoterless, enhancerless reporter gene was 100–200 light units (L.U.)/µg protein.

pKC22HC1Luc in seven pure clonal lines also responded to the drugs even though there were dramatic quantitative differences in basal and induced activity in each line which is thought to be due to the chromatin environment at different sites of integration of the reporter gene and/or to copy number. No response to the drugs was detected in three clones.

Table 1. Induction of pKC22HC1Luc in individual clones of F9 cells

Clone	Activity (L.U./µg protein)	
	none	RACA
2	44	11 408
3	11	5 197
6	10	2 025
7	235	55 300
8	849	38 800
9	31	22 200
10	86	16 700

Individual clonal cell lines containing stably integrated pKC22HC1Luc were obtained as described in Materials and Methods. Cells were assayed for induction of luciferase activity as described in Figure 6. Data are the mean of duplicate assays.

Strickland and co-workers (36) demonstrated that adherent F9 cells differentiate into primitive endoderm in the presence of retinoic acid and that further differentiation into parietal endoderm-like cells occurs in the presence of cAMP. Differentiation of the cells does not occur in the presence of cAMP alone. To address whether pKC22HC1Luc responds to only one of the drugs, the mixed clone population of F9 cells was also treated with each drug alone. As shown in Figure 6, transcriptional activation of pKC22HC1Luc was caused predominantly by retinoic acid whereas cAMP caused little if any activation.

The differentiation responsive enhancer in KC22 is a complex regulatory region

Deletions were generated and assayed to map the regulatory element(s) within KC22. Figure 7 presents the structure of the reporter genes containing the designated subfragments of KC22 and assays of their response to retinoic acid/dibutyryl cAMP as stable integrants in mixed clone preparations of F9 cells. pKC227HC1Luc contains the smallest contiguous sequence within KC22 that retained substantial activation on treatment of the cells with the drugs. Further deletions within this 877 bp fragment indicate that the enhancer is a complex regulatory region composed of at least several distinct cis-acting elements within the regions we designate A, B and C in Figure 7. For example, deletion of 208 bp between the *Rsa*I and *Sac*I sites (B region) abolished enhancer activity (see pKC223HC1Luc) indicating that the B region is essential for enhancer activity. However, the B region itself displayed no enhancer activity (see pKC211HC1Luc). In addition, when the *Sac*I–*Hin*dIII fragment was removed, which includes the C region, enhancer activity was lost (see pKC21HC1Luc) but this region was not sufficient for significant enhancer activity by itself (see pKC221HC1Luc). Furthermore, deletion of fragments containing the A region abolished significant enhancer activity (see pKC222HC1Luc and pKC228HC1Luc) indicating that the A region was essential for enhancer activity. However, the A region was not sufficient for enhancer activity without regions B and C (compare pKC212HC1Luc with pKC21HC1Luc and pKC223HC1Luc).

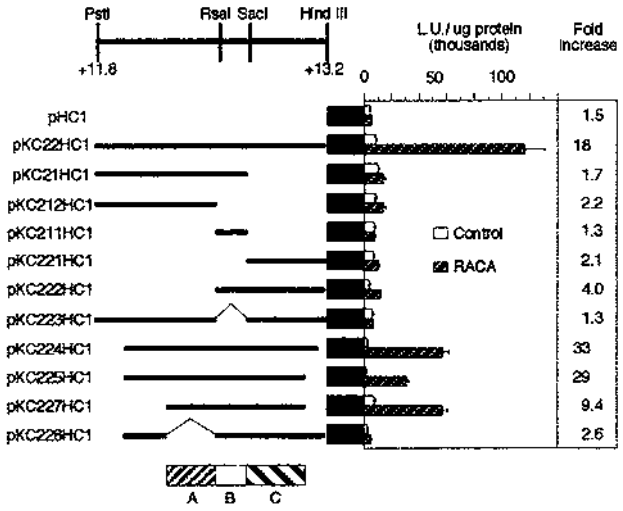

Figure 7. Deletion analysis of the differentiation responsive enhancer in KC22. Mixed clones of G418 resistant cells containing stably integrated pKC22HC1Luc or deletions within the KC22 region in the same construct were assayed for activity as described in the legend to Figure 6. A restriction map of KC22 including map coordinates of the *PstI* and *Hind*III cleavage sites is shown in the upper left of the figure. The region of KC22 included in each construct is indicated by a horizontal line under the restriction map adjacent to the name of the plasmid. The fragment HC1 (–0.8 to +0.1) which contains the laminin γ1 promoter, transcription start site and associated 5′ elements is present in each construct at the indicated location. Reporter gene activity is presented as the mean of triplicate assays +/– the standard deviation. The fold induction of the luciferase reporter gene in each construct by retinoic acid plus dibutyryl cAMP is indicated in the column on the right side of the figure. The boxes labeled A, B and C at the bottom of the figure indicate the three essential domains of the enhancer inferred from the data.

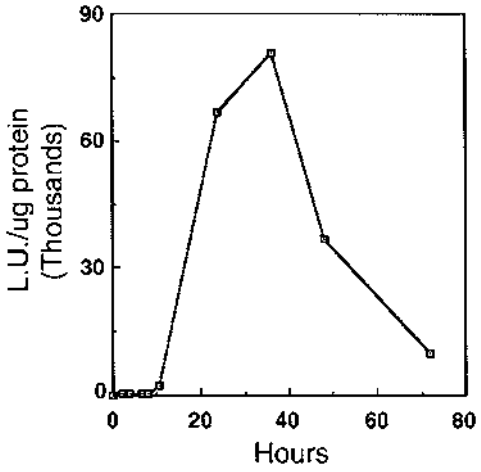

Figure 8. Kinetics of induction of luciferase activity from stably integrated pKC22HC1Luc in G418 resistant F9 clone #9. Cells were seeded in the evening and allowed to attach overnight. To assure similar cell densities at the time of assay and guard against overcrowding, the cells were seeded in 35 mm plates at the following densities; 2×10^5 for assays between 2 and 24 h, 1×10^5 for assays at 36 and 48 h and 0.5×10^5 for assays at 72 h. Induction was started by a change to medium including 0.1 μM retinoic acid and 1 mM dibutyryl cAMP. Control cells were seeded at 10^5 per 35 mm plate and assayed 36 h later. Lysates were prepared as described in the Materials and Methods, quickly frozen in crushed dry ice and stored at –80°C until luciferase and protein assays were performed.

The enhancer in KC22 confers a late but transitory activation onto the laminin γ1 promoter in response to differentiation

The laminin γ1 gene is a member of the family of late response genes in F9 cells (37). Transcription of the laminin genes during retinoic acid/cAMP induction of differentiation occurs following a lag of 12–24 h (15,11). If pKC22HC1Luc contains the essential *cis*-acting elements required for a differentiation response we reasoned that its kinetics of activation would at least qualitatively resemble those of the endogenous gene. Figure 8 displays the kinetics of activation of stably integrated pKC22HC1Luc in F9 clone #9 (Table 1). Similar kinetics were observed in mixed clones of cells (data not shown). Rapid transcriptional activation followed a lag of ~10 h after exposure to retinoic acid/dibutyryl cAMP. However, we were very surprised to find that luciferase activity peaked between 30 and 40 h and declined back towards basal activity. This is in distinct contrast to the endogenous gene which continues to be transcribed at a high rate in fully differentiated F9 cells (24,11).

A positive response element in KC124 synergizes with the KC22 region to direct a high response to differentiation

Previous work in our laboratory demonstrated DNA fragments overlapping HS4 such as KC124 and subclones thereof have strong enhancer activity in reporter genes driven by the minimal HSV1 thymidine kinase promoter when transiently transfected into F9 cells (Chang,H.S. and Filler,T., unpublished results). Therefore, we tested whether KC124 could confer a sustained response to differentiation when place *in cis* to the laminin γ1 promoter. Reporter genes were constructed containing KC124 in front of HC1 (pKC124HC1Luc) or between KC22 and HC1 (pKC22/124HC1Luc) and their kinetics of activation examined as stable integrants in mixed clones. KC124 also confers a differentiation response on the promoter (Fig. 9B). However, activation followed a much longer lag of ~72 h than observed with KC22 (compare Fig. 9A and B). Basement membrane gene transcription is nearly fully activated at this time and the cells are undergoing the morphological change to parietal endoderm-like cells. KC124 contains no detectable promoter activity in transient transfection assays (data not shown). Thus, it appears KC124 contains at least one positive element that responds to differentiation. When both KC22 and KC124 were included in the reporter gene a very high transcriptional activation was observed, at least 30 times the sum of the activities of the reporter containing a single fragment (Fig. 9C). Changes in KC124 orientation and position with respect to KC22 and the HC1 driven luciferase transcription unit remain to be evaluated.

DISCUSSION

We have discovered several regions in the first intron of the laminin γ1 gene which contain positive transcription control elements. One group of elements referred to as the KC22 enhancer maps in the first intron ~12.5 kb from the transcription start site and confers a response to differentiation onto transcription driven by the promoter of the laminin γ1 gene in F9 embryonal carcinoma cells. Deletion analysis revealed at least several distinct *cis*-acting elements are required for KC22 enhancer activity.

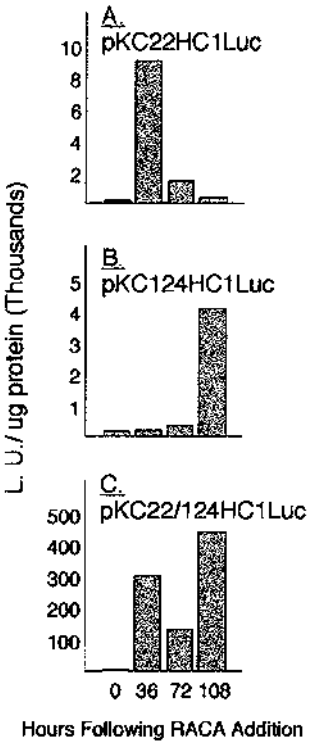

Figure 9. Kinetics of induction of luciferase activity from stable integrants of pKC22HC1Luc, pKC124HC1Luc and pKC22/124HC1Luc. Mixed clones of G418 resistant F9 cells containing pKC22HC1Luc (**A**), pKC124HC1Luc (**B**) or pKC22/124HC1Luc (**C**) were prepared as described in the Materials and Methods and treated as described in Figure 8.

The KC22 enhancer was initially located by mapping DNase I hypersensitive regions in F9 cell chromatin containing the gene. Figure 1 displays the location of DNase I hypersensitive regions we mapped in the laminin γ1 transcription unit. Each region contains a number of hypersensitive sites. Changes in the fine structure of HS1 and HS3 during F9 differentiation strongly suggest different proteins bind the promoter and the KC22 enhancer in differentiated cells. The sequence of KC22 (unpublished data) reveals a number of putative control elements including an octamer consensus (38) in region A, a number of near matches to retinoic acid response elements (39) with core motifs separated by 1, 2 or 5 bp in the A and B domains as well as consensus BOX DNA sequences (40) in domain C. Our preliminary electrophoretic mobility shift assays support the hypothesis that new KC22 DNA binding activities appear in differentiated cells. Work in progress will reveal which sequences are essential for KC22 activity.

A novel feature of the KC22 enhancer is its decline in activity after 36 h exposure of F9 cells to retinoic acid/dibutyryl cAMP. We reasoned that if the KC22HC1Luc reporter gene contains the essential *cis*-acting elements for response to differentiation then its kinetics of induction should resemble those of the endogenous gene. This turned out to be the case with regard to the lag characteristic of late genes. However, loss of enhancer activity at a time when the rate of transcription of the endogenous gene continues to increase suggests that other *cis*-acting elements are required to sustain high level transcription. Our discovery of the KC124 positive element which displays very late induction kinetics and synergy with the KC22 enhancer supports this hypothesis. In our laboratory induction of the laminin γ1 gene in F9 cells occurs following 1 day of exposure to retinoic acid/dibutyryl cAMP. Morphogenesis to parietal endoderm-like cells occurs 2–3 days later when cells are secreting large amounts of basement membrane proteins. Different kinetics of KC22 and KC124 mediated activation suggests each interacts with factors active at different times during this process. Perhaps factors leading to KC22 mediated transcriptional activation are necessary for turning on the endogenous gene and those leading to KC124 mediated transcriptional activation are required for maintaining its expression. Interestingly, the presence of both regions in the reporter gene leads to a substantially higher activity than the sum of their independent activities. This synergism suggests that *in cis* the activity of each region is influenced by the presence of the other. Whether this is due to increased binding or *trans*-activation through the interaction of factors able to independently bind to each region, or to the recruitment of additional factors that bridge between the two regions or directly modify chromatin structure but have no independent transactivation properties remains to be worked out.

The fact that stable integrants of reporter constructs were required to detect KC22 and KC124 differentiation responsive activity supports the notion that chromatin plays a role in regulating the laminin γ1 gene. Demonstrating that changes in DNase I hypersensitivity also occur in chromatin containing the stably integrated KC22HC1Luc and KC22/124HC1Luc reporter genes will strengthen this view. A requirement that genes must be assayed as stable integrants to respond to differentiation of F9 cells was also observed for reporter genes containing the α1(IV) collagen promoter and its cell-type specific and differentiation responsive enhancers (41). Furthermore, chromatin containing these enhancers also display DNase I hypersensitive regions that change during F9 differentiation (unpublished data). We wonder whether an analysis of chromatin containing other late response genes in F9 cells would reveal elements with properties similar to the KC22 and KC124 differentiation responsive elements.

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REFERENCES

1 Hay,E.D. (1991) *Cell Biology of Extracellular Matrix.* Second Edition, Plenum Press, New York, NY.
2 Martinez-Hernandez,A. and Aminta,P.S. (1983) *Lab. Invest.*, **48**, 656–677.
3 Ruoslahti,E. (1991) *J. Clin. Invest.*, **87**, 1–5.
4 Hogan,B.L.M., Cooper,A.R. and Kurkinen,M. (1980) *Dev. Biol.*, **80**, 289–300.
5 Bernstine,E.G., Hooper,M.L., Grandchamp,S. and Ephrussi,B. (1973) *Proc. Natl. Acad. Sci. USA*, **79**, 3899–3903.
6 Strickland,S., Smith,K.K. and Marotti,R. (1980) *Cell* **21**, 347–355.
7 Carlin,B.E., Durkin,M.E., Bender,B., Jaffe,R. and Chung,A.E. (1983) *J. Biol. Chem.* **258**, 7729–7739.
8 Cooper,A.R., Tayor,A. and Hogan,B.L.M. (1983) *Dev. Biol.* **99**, 510–516.
9 Marotti,K.R., Brown,G.D. and Strickland,S. (1985) *Dev. Biol.*, **108**, 26–31.
10 Mason,I.J., Taylor,A., Williams,J.G., Sage,H. and Hogan,B.L.M. (1986) *EMBO J.*, **5**, 1465–1472.
11 Gudas,L.J., Grippo,J.F., Kim,K.-W., Larosa,G.J. and Stoner,C.M. (1990) *Ann. N. Y. Acad. Sci.*, **580**, 245–251.

12 Burbelo,P.D., Bruggeman,L.A., Gabriel,G.C., Klotman,P.E. and Yamada,Y. (1991) *J. Biol. Chem.*, **266**, 22297–22302.
13 Burbelo,P.D., Martin,G.R. and Yamada,Y. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9679–9682.
14 Tanaka,S., Kaytes,P. and Kurkinen,M. (1993) *J. Biol. Chem.*, **268**, 8862–8870.
15 Chakravarti,S., Hassell,J.R. and Phillips,S.L. (1993) *Dev. Dynamics*, **197**, 107–114.
16 Kleinman,H.K., Ebihara,I., Killen,P.D., Sasaki,M., Cannon,F.B., Yamada,Y. and Martin,G.R. (1987) *Dev. Biol.*, **122**, 373–378.
17 Vasios,G.W., Gold,J.D., Petkovich,M., Chambon,P. and Gudas,L.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9099–9103.
18 Vasios,G., Mader,S., Gold,J.D., Leid,M., Lutz,Y., Gaub,M.-P., Chambon,P. and Gudas,L. (1991) *The EMBO J.*, **10**, 1149–1158.
19 Grosveld,F., van Assendelft,G.B., Greaves,D.R. and Kollias,G. (1987) *Cell*, **51**, 975–985.
20 Liu,J.-K., Bergman,Y. and Zaret,K.S. (1988) *Genes Dev.*, **2**, 528–541.
21 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual.* Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY.
22 Durkin,M.E., Bartos,B.B., Liu,S., Phillips,S.L. and Chung,A.E. (1988) *Biochemistry*, **27**, 5198–5204.
23 Ogawa,K., Burbelo,P.D., Sasaki,M. and Yamada,Y. (1988) *J. Biol. Chem.*, **263**, 8384–8389.
24 Durkin,M.E., Phillips,S.L. and Chung,A.E. (1986) *Differentiation*, **32**, 260–266.
25 McKnight,G.S. and Palmiter,R.D. (1979) *J. Biol. Chem.*, **258**, 2802–2807.
26 Wu,C. (1989) *Methods Enzymol.*, **170**, 269–289.
27 Chomczynski,P. and Qasba,P.K. (1984) *Biochem. Biophys. Res. Commun.*, **122**, 340–344.
28 Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
29 Church,G.M. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
30 Amasino,R.M. (1986) *Anal. Biochem.*, **152**, 304–307.
31 Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.
32 Nordeen,S.K. (1988) *Biotechniques*, **6**, 454–457.
33 Chen,C.A. and Okayama,H. (1988) *Biotechnique,*. **6**, 632–638.
34 Darrow,A.L., Rickles,R.J. and Strickland,S. (1990) *Method. Enzymol.*, **190**, 110–117.
35 Brasier,A.R., Tate,J.E. and Habener,J.F. (1989) *Biotechniques*, **7**, 1116–1122.
36 Strickland,S. (1981) *Cell*, **24**, 277–278.
37 Wang,S.Y. and Gudas,L.J. (1988) *J. Cell. Physiol.*, **136**, 305–311.
38 Staudt,L.M., Singh,J., Sen,R., Wirth,T., Sharp,P.A. and Baltimore,D. (1986) *Nature*, **323**, 640–643.
39 Umesono,K., Murakami,K.K., Thompson,C.C. and Evans,R.M. (1991) *Cell*, **65**, 1255–1266.
40 Kihara-Negishi,R., Tsujita,R., Negishi,Y. and Ariga,H. (1993) *Mol. Cell. Biol.*, **13**, 7747–7756.
41 Burbelo,P.D., Horikoshi,S. and Yamada,Y. (1990) *J. Biol. Chem.*, **265**, 4839–4843.