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Enhancer elements directing cell-type-specific expression of cytokeratin genes and changes of the epithelial cytoskeleton by transfections of hybrid cytokeratin genes

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The cytokeratins, which form the intermediate filaments (IFs) characteristic of epithelial cells, are encoded by a large family of genes whose members are differentially expressed in patterns different in the various kinds of epithelia. To identify possible *cis*-regulatory DNA elements involved in the cell-type-specific expression of these genes, we examined, in transfection assays, 5′ upstream sequence intercepts of a certain cytokeratin gene, i.e. that for bovine cytokeratin IV* (CKIV*), in combination with the coding portions of either the chloramphenicol acetyltransferase (CAT) gene or other cytokeratin genes. A 5′ upstream region located between the cap-site and nucleotide −605 was found to enhance the specific expression of these reporter genes in bovine mammary gland-derived BMGE+H cells, which express the endogenous gene, but not in bovine kidney epithelium-derived MDBK cells which synthesize cytokeratins other than IV*. This epithelium-type-specific expression was also observed in heterologous combinations, e.g. in murine keratinocytes, but not in other murine cell lines such as 3T3 fibroblasts. When a fragment located between −180 and −605 was coupled to the HSV-TK promoter it stimulated the expression of the reporter gene in a cell-type-specific manner. The enhancer character of this 425 nucleotide long region is also demonstrated. Moreover, the CKIV* promoter/enhancer complex was able to direct the expression of epidermal cytokeratins characteristic for suprabasal differentiation, i.e. bovine cytokeratins Ia and VIb, in cells that normally do not express these genes. We show that the newly synthesized cytokeratins integrate into the pre-existing cytokeratin IF system of the transfected cells and that the forced expression of one of these cytokeratins does not induce the endogenous gene encoding its normal pair partner.

Key words: Intermediate filaments/cytokeratins/gene expression/cell differentiation/cytokeratin gene enhancers

Introduction

Of the various cytoskeletal proteins, those forming the multigene family of intermediate filament (IF) and nuclear lamina proteins display a particularly high complexity and cell-type specificity of expression (for reviews see Franke *et al.*, 1982b; Osborn and Weber, 1983; Franke, 1987; Fuchs *et al.*, 1987; Steinert and Roop, 1988). (i) Most mesenchymally derived cells express only one type of IF protein, i.e. vimentin, a protein which can also occur, in addition to the specific IF of the given cell type, in some other kinds of cells, including certain epithelia, epithelium-derived tumours and cultured epithelial cells. (ii) Myogenesis is usually correlated with the synthesis of another IF protein, desmin. (iii) During astrocyte differentiation IFs containing the glial fibrillary protein, GFP, are formed, whereas (iv) neuronal differentiation is characterized by the expression of one or several members of the neurofilament polypeptides NF-L, NF-M and NF-H and/or the vimentin-related neuronal IF polypeptide ('peripherin'; Portier *et al.*, 1984; Leonard *et al.*, 1988). (v) Epithelial cells usually contain IFs formed by various combinations of 2−11 polypeptides out of a group of at least 19 cytokeratins, in patterns that are characteristic for a given epithelial cell type (Franke *et al.*, 1981a, 1982b; Moll *et al.*, 1982; Cooper *et al.*, 1985; Quinlan *et al.*, 1985). A special subgroup of epithelial cells, including the trichocytes of hair follicles, expresses various combinations of polypeptides of another set of eight major and two minor cytokeratins ('trichocytic', i.e. hair cell-type cytokeratins), instead of—or in combination with—epithelial cytokeratins (Crewther *et al.*, 1980; Heid *et al.*, 1986, 1988). In addition to these typical patterns of expression, several combinations of co-expressions of different IF proteins have been reported for certain cell types (for refs see Franke and Moll, 1987; Jahn *et al.*, 1987), resulting in complicated mosaics of IF protein complements.

The cytokeratins are the most complex group of IF proteins and, in contrast to the other groups, are obligatory heteropolymers (Lee and Baden, 1976; Steinert *et al.*, 1976; 1985; Hatzfeld and Franke, 1985; Eichner *et al.*, 1986) in that two polypeptides of members of each acidic (type I) and the basic (type II) subfamily are required to form the heterotypic tetramers representing the IF subunits (e.g. Quinlan *et al.*, 1984, 1985; Cooper *et al.*, 1985; Hatzfeld and Franke, 1985; Steinert *et al.*, 1985, 1988). The various types of epithelia, i.e. the 'simple' (one-layered), stratified (multilayered) and 'complex' (pseudostratified) ones, synthesize different kinds of cytokeratins, and such tissue-specific cytokeratin patterns are often retained in carcinomas and cultured epithelial cell lines (Moll *et al.*, 1982; Quinlan *et al.*, 1985). While the cytokeratin compositions of simple epithelia are simple, comprising cytokeratins 8 and 18 with or without either cytokeratin 7 and/or cytokeratin 19 (Moll *et al.*, 1982; Cooper *et al.*, 1985; Quinlan *et al.*, 1985), stratified or pseudostratified epithelia express several other cytokeratins (components 1−6 and 9−17), either with mutually exclusive or in addition to the simple epithelial cytokeratins (Moll *et al.*, 1982; Cooper *et al.*, 1985; Quinlan *et al.*, 1985). Among these some polypeptides such as components 1, 2, 9, 10 and 11 are specifically synthesized in superbasal layers of epidermis (e.g. Fuchs and Green,

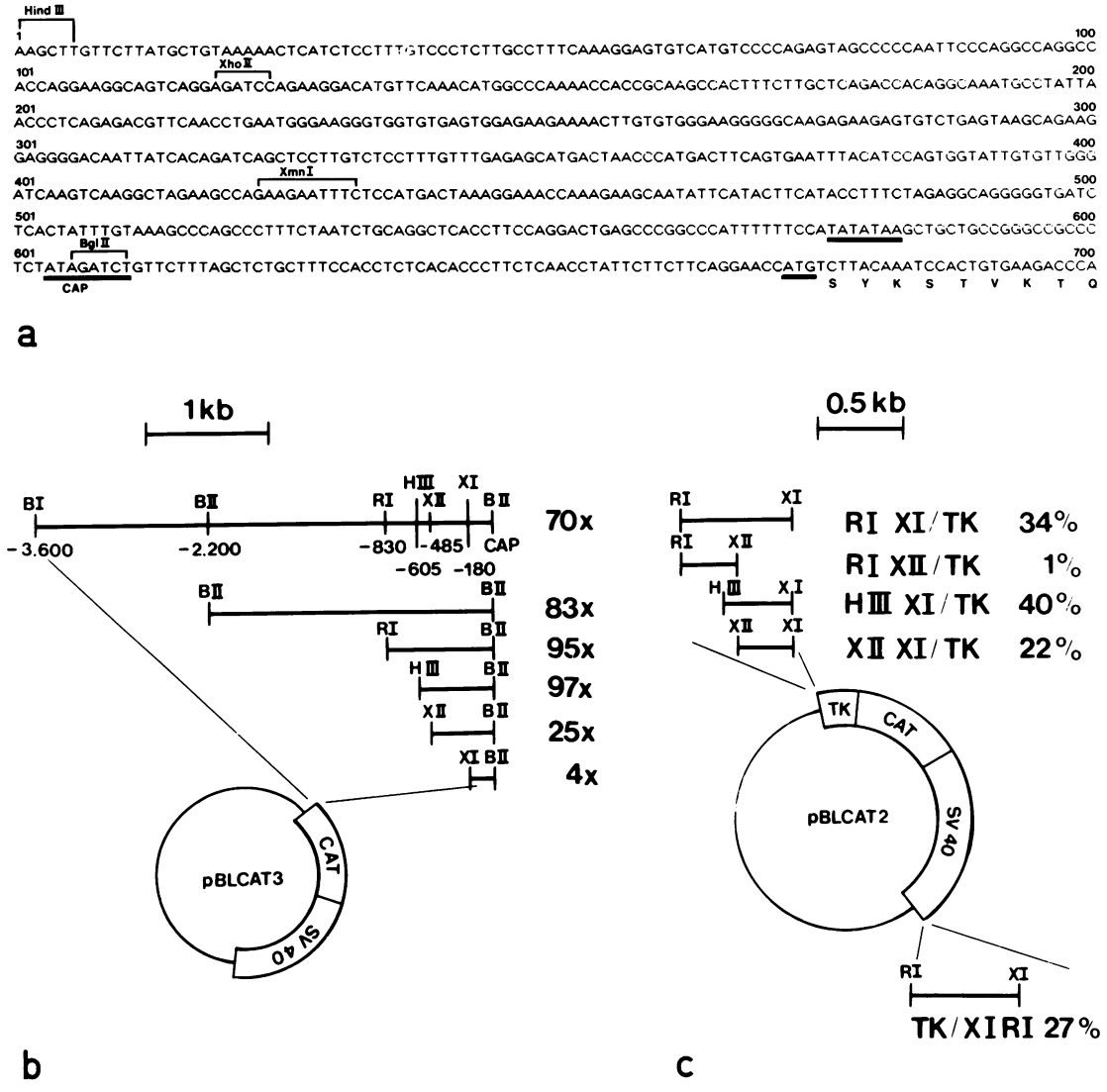

Fig. 1. Sequence organization and transcriptional function of the 5′-upstream region of bovine cytokeratin gene IV*. (**a**) Nucleotide sequence of 605 nt preceding the cap-site. TATA-box, cap-site and translation-initiation codon are underlined. Restriction enzyme recognition sites used in the analysis of transcriptional activity are denoted. (**b,c**) Maps of the constructs used in the analyses of 5′-upstream elements of cytokeratin gene IV*. (**b**) Restriction fragments of the region analysed, extending from the cap-site to various upstream restriction enzyme recognition sites (relative nucleotide positions are given as numbers) were brought into pBLCAT3 (Luckow and Schütz, 1987) in a position 5′ to the CAT gene and the small t intron and the polyadenylation site of SV40. The specific factor by which CAT-activity is stimulated in transfected BMGE+H cells above background, i.e. pBLCAT3 vector alone, is indicated on the right margin. (**c**) Restriction fragments spanning various regions from −180 to −830, relative to the cap-site, were cloned into pBLCAT2 (Luckow and Schütz, 1987) 5′ to the HSV TK promoter or 3′ to the SV40 small t polyadenylation site (lower part). The specific CAT activity in transfected BMGE+H cells is given as percent, setting the construct pTKCAT14A (TK promoter in combination with the two 72 bp repeats of the SV40 enhancer) as 100%, after subtraction of background stimulation with pBLCAT2 only. Restriction enzymes: BI, *Bam*HI; BII, *Bgl*II; HIII, *Hin*dIII; RI, *Eco*RI; XI, *Xmn*I; XII, *Xho*II.

1980; Moll *et al.*, 1982; Sun *et al.*, 1985; Huszar *et al.*, 1986; Knapp *et al.*, 1986; Fuchs *et al.*, 1987; cf, Roop, 1987) and some other squamous epithelia, including gingiva, exocervix and vagina (Moll *et al.*, 1983; Achtstätter *et al.*, 1985; Quinlan *et al.*, 1985; Ouhayoun *et al.*, 1985), and thus represent hallmarks of advanced vertical differentiation within these tissues. In addition to the correlation of cytokeratin expression with cell and tissue differentiation programs there are also influences on cytokeratin synthesis by environmental factors such as external concentrations of certain hormones (e.g. Schmid *et al.*, 1983b; Roop, 1987), growth factors, and vitamin A-related compounds (cf. Fuchs and Green, 1981; Gilfix and Eckert, 1985; Kim *et al.*, 1987; Kopan *et al.*, 1987; Roop *et al.*, 1987).

Analyses of mRNAs for IF proteins in various cell lines and tissues, either by *in vitro* translation or by *in situ* hybridization, have shown that in most cases the mRNA pattern correlated with the protein pattern, and it has been suggested that the cell-type-specific expression of IF proteins of this multigene family is primarily at the transcriptional level (e.g. Fuchs and Green, 1980; Capetanaki *et al.*, 1984; Jorcano *et al.*, 1984a; Lilienbaum *et al.*, 1986; Fuchs *et al.*, 1987; Pieper *et al.*, 1987; Roop, 1987; for an exception indicative of translational control see Tyner and Fuchs, 1986). Apparently, the various IF protein genes, despite their remarkable sequence homologies and similar exon−intron patterns (e.g. Lehnert *et al.*, 1984; Marchuk *et al.*, 1984; Fuchs *et al.*, 1987; Steinert and Roop, 1988), contain

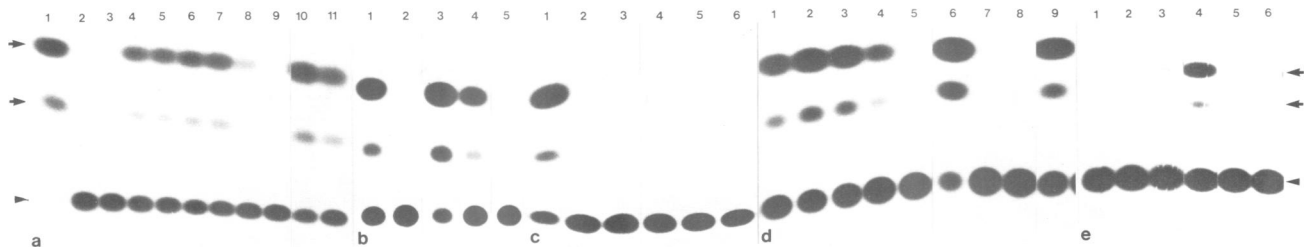

Fig. 2. Analyses of CAT activities in extracts of bovine BMGE+H (**a,b**), MDBK cells (**c**), as well as murine AT5 keratinoyctes (**d**) and 3T3 fibroblasts (**e**), after transfection with the following clones: (**a**) pTKCAT14A (**lane 1**), pBLCAT3 (**lane 2**), pBLCAT2 (**lane 3**), compared with derivatives of pBLCAT3 containing the following CKIV* fragments inserted 5′ to the CAT gene: 3.6 kb *Bam*HI/*Bgl*II (**lane 4**), 2.2 kb *Bgl*II/*Bgl*II (**lane 5**), 830 bp *Eco*RI/*Bgl*II (**lane 6**), 605 bp *Hin*dIII/*Bgl*II (**lane 7**), 485 bp *Xho*II/*Bgl*II (*lane 8*), and 180 bp *Xmn*I/*Bgl*II (**lane 9**). Results obtained with derivatives of pBLCAT2 containing the 650 bp *Eco*RI/*Xmn*I fragment cloned 5′ to the HSV TK promoter (**lane 10**) or 3′ to the SV40 small t polyadenylation site (**lane 11**). (**b**) Analyses of CAT activities using derivatives of pBLCAT2 containing the following fragments 5′ to the HSV TK promoter: 650 bp *Eco*RI/*Xmn*I region (**lane 1**), 345 bp *Eco*RI/*Xho*II region (**lane 3**), 425 bp *Hin*dIII/*Xmn*I region (**lane 3**), 305 bp *Xho*II/*Xmn*I region) (**lane 4**), in comparison with pBLCAT2 alone (**lane 5**). (**c**) CAT activities in extracts of MDBK cells transfected with the following constructs: pTKCAT14A (**lane 1**), pBLCAT3 (**lane 2**), pBLCAT2 (**lane 3**), pBLCAT3 with the 2.2 kb *Bgl*II/*Bgl*II (**lane 4**) and with the 180 bp *Xmn*I/*Bgl*II fragment (**lane 5**) inserted 5′ to the CAT gene, and pBLCAT2 containing the 650 bp *Eco*RI/*Xmn*I fragment cloned 5′ to the HSV TK promoter (**lane 6**). (**d**) Cells were transfected with derivatives of pBLCAT3 containing the following fragments: 2.2 kb *Bgl*II/*Bgl*II (**lane 1**), 830 bp *Eco*RI/*Bgl*II (**lane 2**), 605 bp *Hin*dIII/*Bgl*II (**lane 3**), 485 bp *Xho*II/*Bgl*II (**lane 4**), 180 bp *Xmn*I/*Bgl*II (**lane 5**), all cloned 5′ to the CAT-gene. Their activities are compared with those obtained after transfection with pTKCAT14A (**lane 6**), pBLCAT3 alone (**lane 7**), pBLCAT2 (**lane 8**) and a derivative of pBLCAT2 in which the 650 bp *Eco*RI/*Xmn*I fragment was cloned 5′ to the HSV TK promoter (**lane 9**). (**e**) 3T3 fibroblasts were transfected with derivatives of pBLCAT3 containing the 2.2 kb *Bgl*II/*Bgl*II (**lane 1**) and the 180 bp *Xmn*I/*Bgl*II (**lane 2**) fragments, both inserted 5′ to the CAT-gene, in comparison with transfections of a derivative of pBLCAT2 with the 650 bp *Eco*RI/*Xmn*I fragment, cloned 5′ to the HSV TK promoter (**lane 3**), pTKCAT14A (**lane 4**), pBLCAT3 alone (**lane 5**), and pBLCAT2 alone (**lane 6**). The amount of protein used was the same for all samples in a given set of experiments (**a,b,c,d** or **e**). Triangles indicate the position of the substrate chloramphenicol, arrows denote the products 1-acetate chloramphenicol and 3-acetate chloramphenicol.

different regulatory elements that are responsible for their differential, often mutually exclusive, cell-type-specific expression. Even genes encoding very closely related IF proteins such as desmin and vimentin (Ngai *et al.*, 1985; Quax *et al.*, 1985) or certain cytokeratin genes of the same subfamily, including closely neighbouring ones (Blessing *et al.*, 1987; Romano *et al.*, 1988; Rosenberg *et al.*, 1988), contain elements that allow their differential expression. As a first attempt to elucidating the principles of regulation of (i) the expression of different cytokeratin genes in different epithelia, (ii) the synthesis of near-stoichiometric amounts of certain pairs of type I and type II cytokeratins, and (iii) the absolute quantities of a given IF protein, we have tried to identify *cis*-regulatory elements that may be involved in the epithelium-specific synthesis of cytokeratins. In the present study, we report the localization of a cytokeratin gene-specific enhancer element and its use to experimentally change the cytokeratin pattern of an epithelial cell type by transfection with hybrid cytokeratin gene constructs.

Results

Choice of genes

The genes encoding bovine epidermal cytokeratins Ia and VIb are specifically expressed in keratinocytes of suprabasal layers of muzzle epidermis and related tissues (cf. Franke *et al.*, 1981b; Jorcano *et al.*, 1984a,b; Cooper and Sun, 1986), as the homologous human cytokeratins 1 and 10/11 are synthesized in suprabasal layers of epidermis (e.g. Fuchs and Green, 1980; Cooper *et al.*, 1985; Roop, 1987). These cytokeratins are not detected in simple and complex glandular epithelia, including those of the mammary gland, and in cell cultures derived therefrom (Moll *et al.*, 1982; for bovine cells see Schmid *et al.*, 1983a,b; Franke *et al.*, 1981a, 1982a; Jorcano *et al.*, 1984a). In contrast, only simple epithelial cytokeratins are synthesized in culture lines of simple epithelial cells such as bovine kidney epithelial MDBK cells, which produce the equivalents to human cytokeratins 8 and 18 (Franke *et al.*, 1981a, 1982b); and combinations of simple epithelial cytokeratins with certain cytokeratins characteristic of complex epithelia, such as bovine cytokeratins III and IV (corresponding to human cytokeratins 5 and 6; cf. Blessing *et al.*, 1987), are produced in cells of the mammary-gland-derived culture line, BMGE+H (Schiller *et al.*, 1982; Schmid *et al.*, 1983b; Jorcano *et al.*, 1984a; Blessing *et al.*, 1987). In both BMGE+H and MDBK cells, the genes encoding typical stratified epithelial cytokeratins such as bovine components Ia and VIb are inactive as shown by Northern blot (cf. Jorcano *et al.*, 1984a) and 'nuclear run on' (data not shown) experiments. We therefore used these two cell lines for our studies on the regulation of specific cytokeratin genes and concentrated, first, on one of the two genes encoding a cytokeratin IV polypeptide, termed gene IV* (cf. Blessing *et al.*, 1987), which is expressed in BMGE+H (Blessing *et al.*, 1987) but not in MDBK cells. The portion of this gene that is relevant to the present study, i.e. 700 nt immediately preceding the coding region (Figure 1a), contains several elements common to diverse differentially expressed genes, including other cytokeratin genes (for review see Fuchs *et al.*, 1987). A typical TATA box is followed by a cap-site 28 nt downstream.

CAT assays

To identify elements responsible for the cell-type-specific expression of the cytokeratin gene IV* we coupled various regions preceding the cap-site to the CAT gene (Figure 1b) and analysed them, by transient transfection assays, in BMGE+H and MDBK cells. The results (Figures 1b and 2a,c) showed that an element of 605 nucleotides was sufficient to stimulate CAT synthesis effectively in BMGE+H cells, by >90-fold. Upon further size reduction

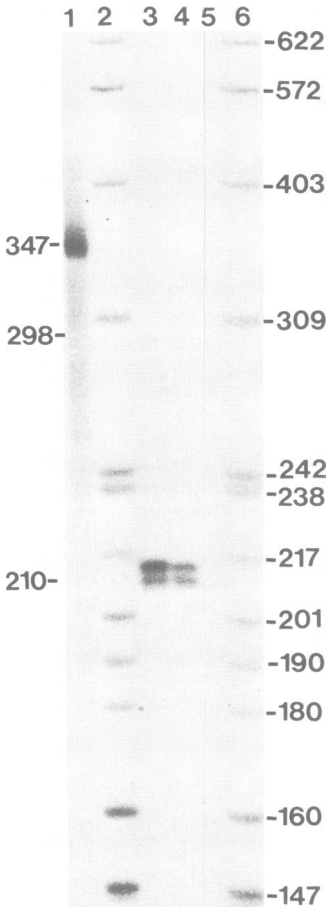

Fig. 3. Correct transcription initiation from the stimulated TK promoter as demonstrated by RNase protection assay using a uniformly labelled 347 nt antisense RNA probe derived from the 298 bp *Eco*RI/*Pvu*II fragment of pTKCAT (Miksicek *et al.*, 1986). **Lane 1**: Probe used; **lane 2**: *Hpa*II-digested and end-labelled pBR322 DNA; **lane 3**: protection assay with RNA from BMGE+H cells transfected with pTKCAT14A; **lane 4**: protection assay with RNA from BMGE+H cells transfected with a derivative of pBLCAT2 containing the 650 bp *Eco*RI/*Xmn*I fragment of the CKIV* gene, cloned 5′ to the HSV TK promoter; **lane 5**, protection assay with RNA from BMGE+H cells transfected with pBLCAT2 alone; **lane 6**, as lane 2. Correctly initiated RNA protects a fragment of ~210 nt as is the case upon stimulation of the TK promoter with the SV40 enhancer (lane 3) as well as with the 650 bp *Eco*RI/*Xmn*I fragment of the CKIV* gene (lane 4).

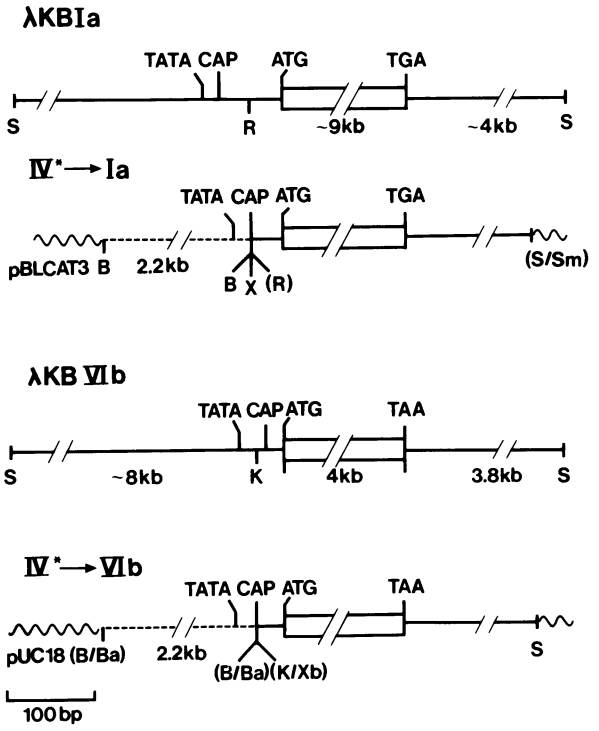

Fig. 4. Cytokeratin hybrid gene constructs used for transfections. **λKBIa:** Genomic clone for cytokeratin Ia (cf. Lehnert *et al.*, 1984; Blessing *et al.*, 1987). **IV*→Ia:** cytokeratin Ia gene under the control of the 2.2 kb *Bgl*II/*Bgl*II promoter/enhancer fragment of the CKIV* gene. The fragment was cloned in the correct orientation in the *Bgl*II site of pBLCAT3, followed by replacement of the CAT gene and the SV40 processing signals by the 13 kb *Rsr*II/*Sal*I fragment of λKBIa containing the gene for cytokeratin Ia. **λKBVIb:** Genomic clone for cytokeratin VIb (cf. Rieger *et al.*, 1985). **IV*→VIb:** cytokeratin VIb gene under the control of the 2.2 kb *Bgl*II/*Bgl*II promoter/enhancer fragment of the cytokeratin IV* gene. The 7.8 kb *Kpn*I/*Sal*I fragment of λKBVIb containing the gene for cytokeratin VIb was cloned in the *Xba*I/*Sal*I sites of pUC18. Then the 2.2 kb *Bgl*II promoter/enhancer fragment of the cytokeratin IV* gene was cloned into the *Bam*HI site of the construct and checked for the correct orientation. TATA-boxes, cap-sites, translation initiation and translation stop codons are indicated. The boxes denote the structural genes for cytokeratins Ia and VIb, the dotted lines represent the 2.2 kb promoter/enhancer fragment of the cytokeratin IV* gene and the wavelines indicate vector sequences. Restriction nuclease cleavage sites are indicated: B, *Bgl*II; Ba, *Bam*HI; K, *Kpn*I; R, *Rsr*II; S, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. Names of restriction enzymes in brackets indicate that the originally intact recognition sequence was destroyed during the cloning procedures.

of this element, e.g. by only 120 nt (i.e. to −485), CAT synthesis was clearly reduced. No significant stimulation of CAT expression from these constructs was observed in MDBK cells (Figure 2c).

When the potential enhancer activities of various upstream elements were examined in CAT constructs containing the thymidine kinase (TK) promoter of herpes simplex virus (HSV1), in comparison with the SV40 enhancer, remarkable activity was observed for the *Hin*dIII−*Xmn*I fragment containing nucleotides −180 to −605 (Figures 1c and 2b). On further reduction by 120 nt (i.e. in the *Xho*II−*Xmn*I fragment) this activity decreased by about half (Figures 1c and 2b). Significant activity was also noted when this element was positioned downstream from the CAT gene and the SV40 t-antigen polyadenylation site (Figures 1c and 2a, lanes 10 and 11), and in an inverted orientation, thus demonstrating its enhancer character. Again, this stimulation was specific for BMGE+H cells and was not observed in MDBK cells (Figure 2c). The correct transcription initiation from the stimulated TK promoter was determined by RNase mapping (Figure 3) according to the method described by Miksicek *et al.* (1986). The correctly initiated RNA protected a 210 nt long fragment whereas unspecific initiation in the vector would have resulted in the protection of a 298 bp fragment.

The stimulatory effects of these upstream elements, including the enhancer function, were also observed in heterologous transfections, e.g. in cultured murine keratinocytes of line AT5 (Figure 2d). In contrast, no stimulation of the bovine gene promoter was observed in non-epithelial murine cells such as 3T3 fibroblasts, and in this cell line the enhancer effects on the TK promoter were only minimal (Figure 2e).

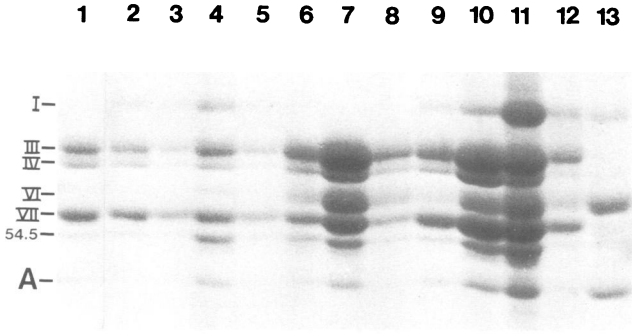

Fig. 5. Coomassie-blue staining of SDS−PAGE separated cytokeratin preparations from BMGE+H cells that had been stably transfected with the neomycin resistance marker plasmid pAG60 alone (**lane 1**; clone was name N1), with pAG60 and the construct IV*→Ia (**lanes 2, 3, 4** and **5**; clones were named Ia-3, Ia-4, Ia-13, Ia-14), with pAG60 and the construct IV*→VIb (**lanes 6, 7** and **8**; clones were named VI-1, VI-5, VI-13) and with pAG60 in combination with both constructs IV*→Ia and IV*→VIb (**lane 9, 10, 11, 12** and **13**; clones were named Ia/VI-2, Ia/VI-5, Ia/VI-8, Ia/VI-13, Ia/VI-3). Roman and Arabic numerals denote epidermal and mammary gland cytokeratin polypeptides (cf. Schiller *et al.*, 1982; Schmid *et al.*, 1983a,b). A, residual actin. Note the transfection-forced synthesis of epidermal cytokeratins Ia (lanes 2−5 and 9−13) and VIb (lanes 6−8 and 9−13) in this mammary gland cell line. In some clones the amounts of the foreign cytokeratins exceed the amounts of the endogenous cytokeratins (e.g. lanes 11 and 13).

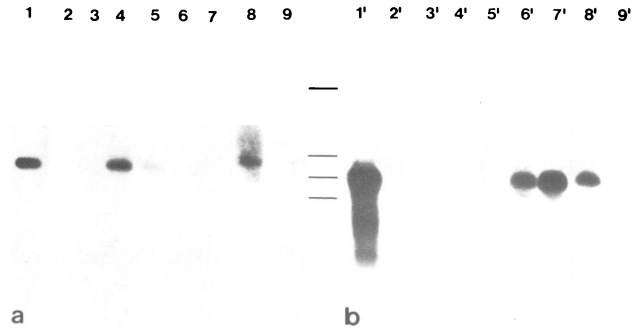

Fig. 6. Northern blot analyses of 20 μg of total RNA extracted from bovine snout epidermis (**lanes 1, 1'**), two different pAG60-transfected BMGE+H cell clones (**lanes 2, 2'** and **3, 3'**) clone Ia-13 (**lanes 4** and **4'**), clone Ia-14 (**lanes 5** and **5'**), clone VI-1 (**lanes 6** and **6'**), clone VI-5 (**lanes 7** and **7'**), clone Ia/VI-5 (**lanes 8** and **8'**), in comparison with normal BMGE+H cells (**lanes 9** and **9'**). The filters were hybridized with a riboprobe corresponding to exon I of cytokeratin genes Ia (**a**) and VIb (**b**), respectively. The horizontal bars indicate the positions of the 28S, 23S, 18S and 16S rRNAs. Note that mRNAs specific for cytokeratin Ia and/or VIb is exclusively present in cell clones, transfected with the corresponding constructs IV*→Ia and/or IV*→VIb.

Expression of hybrid epidermal genes in non-epidermal cells

To examine possible dominant negative regulatory effects of downstream elements involved in the differential expression of cytokeratin genes, we dissected the transcribed region of the suprabasally expressed genes of bovine epidermal cytokeratins Ia (a type II cytokeratin) and VIb (a type I cytokeratin) and coupled them either to the SV40 promoter-enhancer element or to the 2.2 kb *Bgl*II 5′ upstream fragment of cytokeratin gene IV* (Figure 4; see also Figure 1b). When BMGE+H and MDBK cells were transfected with the constructs described in Figure 4 cell-type-specific expression was observed in BMGE+H cells, both in transient expression assays and in stable transfectants, but not in MDBK cells. For example, Figure 5 shows SDS−PAGE of the cytokeratins present in cytoskeletal preparations from a series of BMGE+H cell clones permanently transfected with IV*→Ia and/or IV*→VIb gene constructs. The relative proportions of the experimentally introduced cytokeratins were variable in different clones and, remarkably, some of them synthesized large amounts of products of the transfected gene constructs comparable to—or even exceeding—the endogenous cytokeratins (e.g. Figure 5, lanes 11 and 13). These findings also indicate that no dominant negative regulatory elements exist in regions downstream of the initiation sites of the genes for cytokeratins Ia and VIb.

To examine whether the synthesis of a certain cytokeratin polypeptide induces the gene of its partner, i.e. the complementary cytokeratin with which it is normally co-expressed and complexed (for refs see Introduction; cf. Giudice and Fuchs, 1987), we performed Northern blot analyses of RNAs extracted from several transfectant BMGE+H cell clones. All clones examined (Figure 6) expressed only the RNA derived from the transfected constructs, i.e. either cytokeratin Ia or VIb, thus indicating that the endogenous gene encoding the corresponding normal partner was not induced.

For a more detailed analysis of the polypeptides newly synthesized in the transfected cells we compared, by two-dimensional gel co-electrophoresis, cytoskeletal proteins from bovine snout epidermis with [^{35}S]methionine-labelled proteins of the transfected cells (Figure 7). The labelled component VIb from the cells transfected with IV*→VIb constructs comigrated with authentic cytokeratin VIb from bovine snout. However, the metabolically labelled cytokeratin Ia newly expressed in cells containing IV*→Ia constructs showed a distribution of isoelectric variants that was different from the pattern seen in the authentic tissue protein: most of it was recovered in more acidic variants and little, if any, appeared in the position of the unlabelled epidermal protein (Figure 7a−f). This suprising result indicated that cytokeratin Ia synthesized in BMGE+H cells was post-translationally modified in a mode different from that existing in native epidermal tissue. Indeed, comparison of the pattern of the epidermal cytokeratin Ia variants with that of the [^{32}P]phosphate-labelled polypeptides from IV*→Ia transfected cells (Figure 7g and h) showed that the more acidic variants synthesized in BMGE+H cells were phosphorylated, and some of them appeared in positions similar to those of the ^{32}P-labelled protein from epidermal tissue (cf. Schiller *et al.*, 1982).

The lack of induction of the complementary 'pair partner' cytokeratin was also evident at the protein level. Cells transfected with IV*→Ia expressed the exogenous Ia gene but not the endogenous VIb gene (Figure 7a and b), and *vice versa* (Figure 5, lanes 6−8). Moreover, two-dimensional gel electrophoresis revealed only a single polypeptide spot comigrating with component VIb from bovine snout epidermis (not shown), indicating that the closely related polypeptides VIa and VIb (Franke *et al.*, 1981b; Schiller *et al.*, 1982) are products of different genes or different alleles of the same gene, but not differently modified products from the same gene.

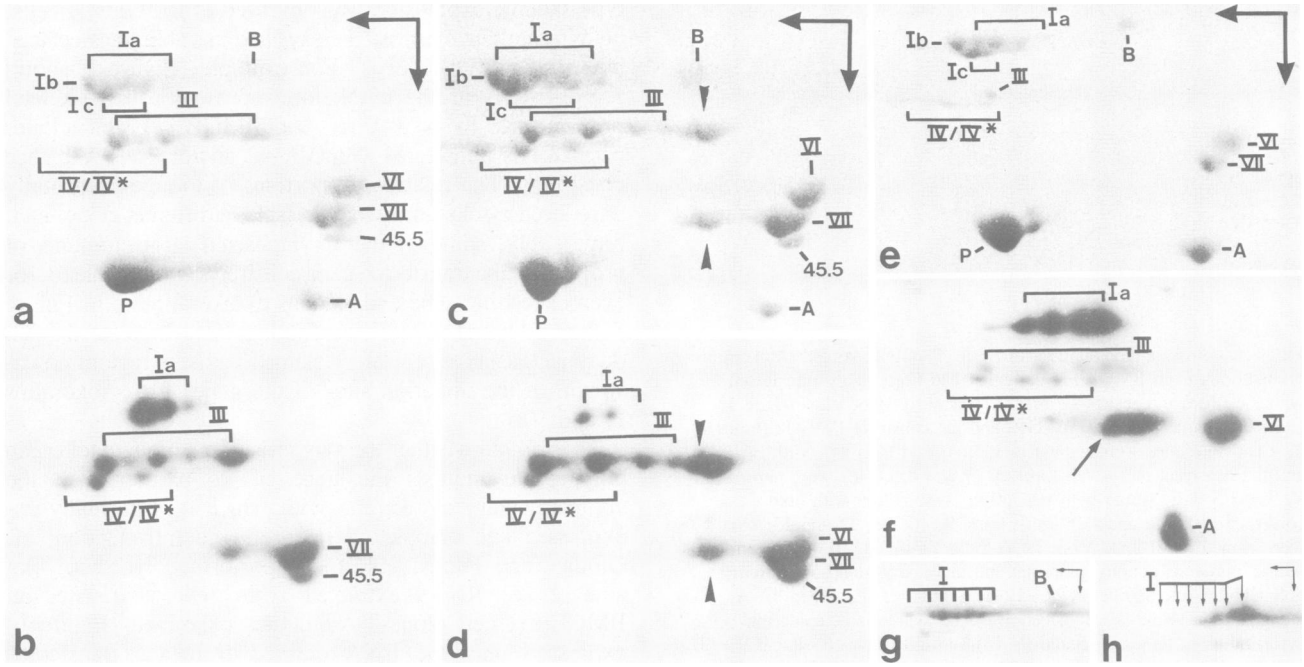

Fig. 7. Two-dimensional gel electrophoresis (horizontal arrows, direction of first dimension non-equilibrium pH gradient electrophoresis; vertical arrows, direction of SDS−PAGE) of unlabelled cytokeratins extracted from bovine snout epidermis (**a**,**c**,**e**,**g**) and [^{35}S]methionine-labelled cytokeratins (**b**) of clone Ia-13 (cf. lane 4 in Figure 5), (**d**) of clone Ia/VI-5 (cf. lane 10 in Figure 5), (**f**) of clone Ia/VI-3 (cf. lane 13 in Figure 5) and (**h**) [^{32}P]phosphate-labelled cytokeratins of clone Ia/VI-3 (cf. lane 13 in Figure 5). (**a**,**c**,**e**,**g**) Coomassie-blue staining; (**b**,**d**,**f**,**h**) fluorography. Cytokeratin designations as in Figure 5. A, α-actin; B, BSA; P, phosphoglycerokinase. Arrowheads in (**c**) and (**d**) denote positions of complexes between cytokeratins III and VII. The arrow in (**f**) denotes an as yet unidentified cytoskeletal component. Note the absence of cytokeratin VIb in (**b**), showing that transfection and expression of a single cytokeratin Ia gene does not induce the endogenous cytokeratin VIb gene. Note also the synthesis of relatively small amounts of cytokeratins Ia and VIb in clone Ia/VI-5 (**d**), compared to the accumulation of large amounts of these cytokeratins in the transfected cell clone Ia/VI-3 (**f**). Remarkably, in most transfected cell clones, a larger proportion of component Ia than normal is recovered in acidic variants (**b**,**d**,**e**). Comparisons of unlabelled epidermal (**g**) and [^{32}P]phosphate-labelled component Ia extracted from clone Ia/VI-3 (**h**) indicates that the positions of the more acidic variants (multiple brackets in **g**) correspond to phosphorylated forms (arrowed brackets in **h**).

Some clones of cells transfected with both constructs, IV*→Ia and IV*→VIb, were characterized by the constitutive expression of very high levels of products of the 'foreign' genes (e.g. Figure 7e and f). Remarkably, this was often correlated with a drastic reduction of the expression of the endogenous cytokeratins III, IV and IV*, and the endogenous type I cytokeratins designated VII and 45.5 kd (cf. Schmid *et al.*, 1983b) were hardly detectable at all (Figure 7e and f).

Localization of products of transfected genes

The distribution of the cytokeratins synthesized from the transfected genes was examined by immunolocalization techniques using antibodies specific for a certain cytokeratin. For example, antibodies to cytokeratin VIb that did not bind to cytokeratin fibrils of normal BMGE+H cells (data not shown) was used to follow the appearance of this protein in the transfected cells. The amounts of 'new' cytokeratins produced in transfected cells were usually large enough to be detected by immunfluorescence microscopy, both in transient and stable expression. For example, Figure 8 shows the expression of cytokeratin VIb in incompetent cells, i.e. in MDBK cells transfected with the VIb gene under the control of the SV40 early enhancer/promoter. When competent cells i.e. those of the BMGE+H line, were transfected with the same SV40-hybrid gene construct or with the 5′-upstream 2.2 kb CKIV* enhancer/promoter element driving the genes for cytokeratins Ia and VIb extensive IF arrays containing cytokeratins Ia and VIb were seen (not shown).

In some transfected cell clones, most of the newly added cytokeratin VI material appeared in perinuclear aggregates, probably representing transient forms of cytokeratin IF accumulation (data not shown). Therefore, we examined whether the newly added cytokeratin synthesized from the transfection construct formed structures different from the endogenous ones or whether they were integrated into a 'mixed fibril' system. Double immunolabelling microscopy (Figure 9) showed practically coincident patterns of fluorescence, indicative of mixing of the new cytokeratins with the 'old' ones and their integration into the common IF cytoskeleton of the transfected cell. In some of the clones, in which the 'new' cytokeratins Ia and VIb were particularly abundant, exceeding even the endogenously synthesized cytokeratins (e.g. Figure 7f), the new cytokeratins did not exclusively appear in extended fibrillar arrays but in the form of short whiskers and granular bodies (Figure 10). Surprisingly, however, the relative fequency of fibrillar and granular arrays varied in these clones from cell to cell (e.g. Figure 10c).

Discussion

Our results show that a relatively short element of the 5′ upstream region of a certain cytokeratin gene contains information sufficient to promote transcription in one type

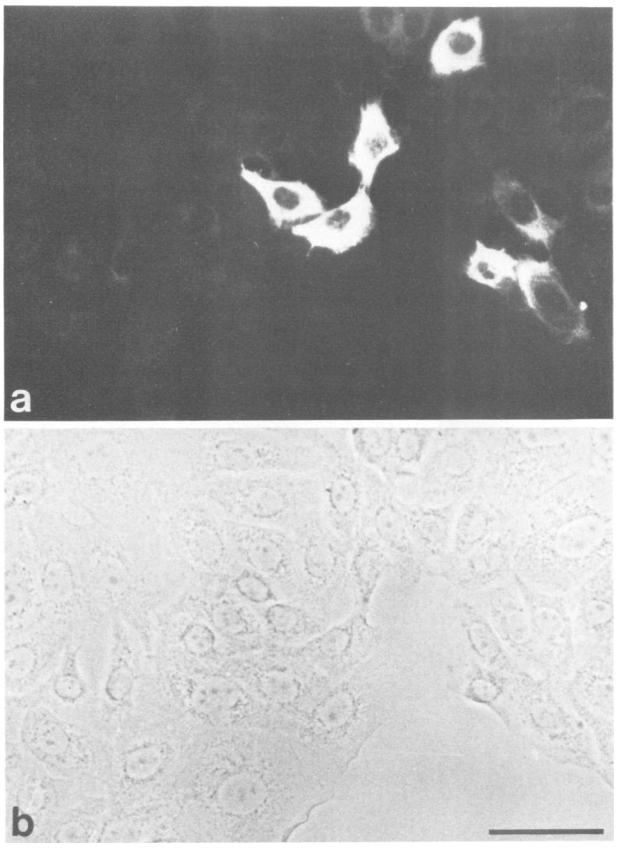

Fig. 8. Immunofluorescence microscopy of the cytokeratin VI specific antibody K_s8.60 (**a**; phase contrast picture of the same field is shown in **b**), showing kidney epithelial cells of line MDBK two days after transfection with the bovine cytokeratin genes Ia and VIb under the control of the SV40 promoter/enhancer. The length of the bar 50 μm. The group of cells showing positive reaction for cytokeratin VIb demonstrates that this epidermal cytokeratin gene can be expressed in simple epithelial type cells when coupled to a functional promoter. No positive cells were found when MDBK cells were transfected with the constructs IV*→Ia and IV*→VIb (data not shown).

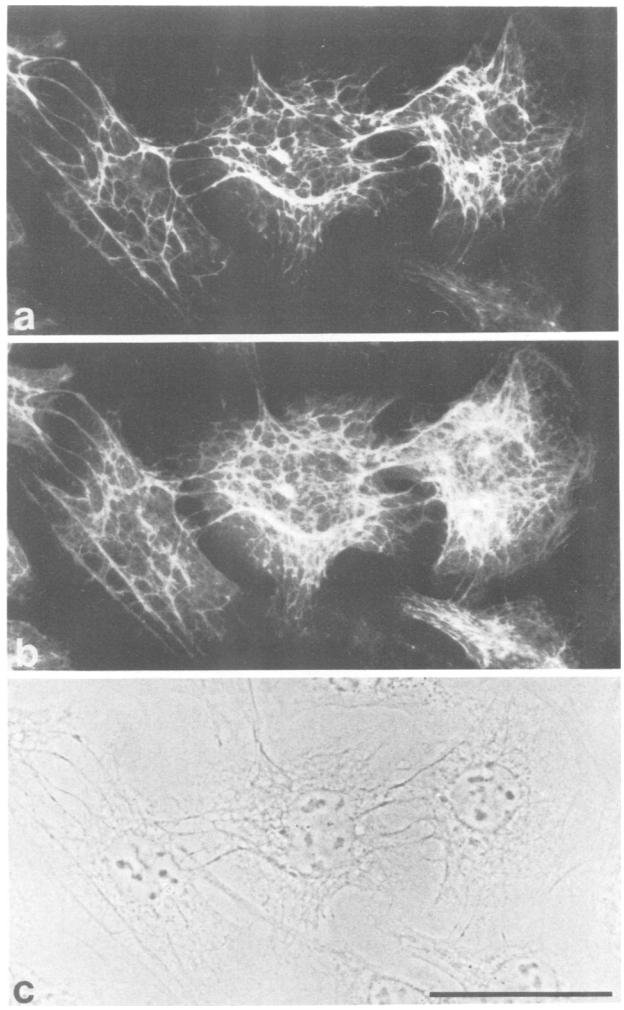

Fig. 9. Double label immunofluorescence microscopy, using murine monoclonal antibody K_s8.60 specific for component VIb (**a**) and broad range guinea pig cytokeratin antibodies (**b, c**, phase contrast picture corresponding to **b**) of BMGE+H cell stably transfected with the constructs IV*→Ia and IV*→VIb (clone Ia/VI-5; cf. lane 10 in Figure 5 and Figure 7d). Bar, 50 μm. The coincidence of structures labelled with the two different antibodies demonstrates that the newly synthesized cytokeratins integrate into a common IF system.

of epithelial cell, i.e. mammary gland epithelium-derived BMGE+H cells, but not in other kinds of epithelial cells such as kidney epithelial MDBK cells. Transcription-promoting activity of this upstream element is also evident when it is placed downstream of the reporter gene and on the other strand, thus justifying its classification as an 'enhancer' (DeVilliers and Schaffner, 1983; Weiher *et al.*, 1983).

While *cis*-regulatory elements enhancing cell-type-specific expression have been identified in similar near-promoter upstream positions, in other cytoskeletal protein multigene families (e.g. for actin genes see Bergsma *et al.*, 1986; Walsh and Schimmel, 1988), information on regulatory elements involved in the cell-type-specific expression of IF proteins is scarce. Julien *et al.* (1987) have shown that a large (~21.5 kb) fragment harbouring the human gene for neurofilament protein NF-L contains the information for the neuron-specific expression of this gene in a heterologous combination, i.e. in transgenic mice. With respect to the expression of desmin the literature is puzzling. The same group (Quax *et al.*, 1985; Pieper *et al.*, 1987; Van den Heuvel *et al.*, 1987) has reported that hamster desmin gene constructs containing an ~3.5 kb upstream region are permissively transcribed, upon transfection, in certain cultured cells that do not express their endogenous desmin gene, whereas a relatively short upstream intercept of this gene (from −89 to +25) should be sufficient to direct its expression in murine myogenic cells derived from pluripotential mouse teratocarcinomas. The regulation of the expression of avian and mammalian vimentin genes appears to be particularly complex. The upstream region of this gene seems to contain various positive and negative regulatory elements which, however, have not been studied with respect to cell-type specificity of expression but only in transfection assays using vimentin-producing cells such as mouse 3T3 fibroblasts (Rittling and Baserga, 1987) and erythroleukaemia MEL cells (cf. Ngai *et al.*, 1987). On the other hand, Krimpenfort *et al.* (1988) have recently reported that a region ~3.1 kb upstream of the transcription initiation site of the hamster vimentin gene directs, in transgenic mice, the

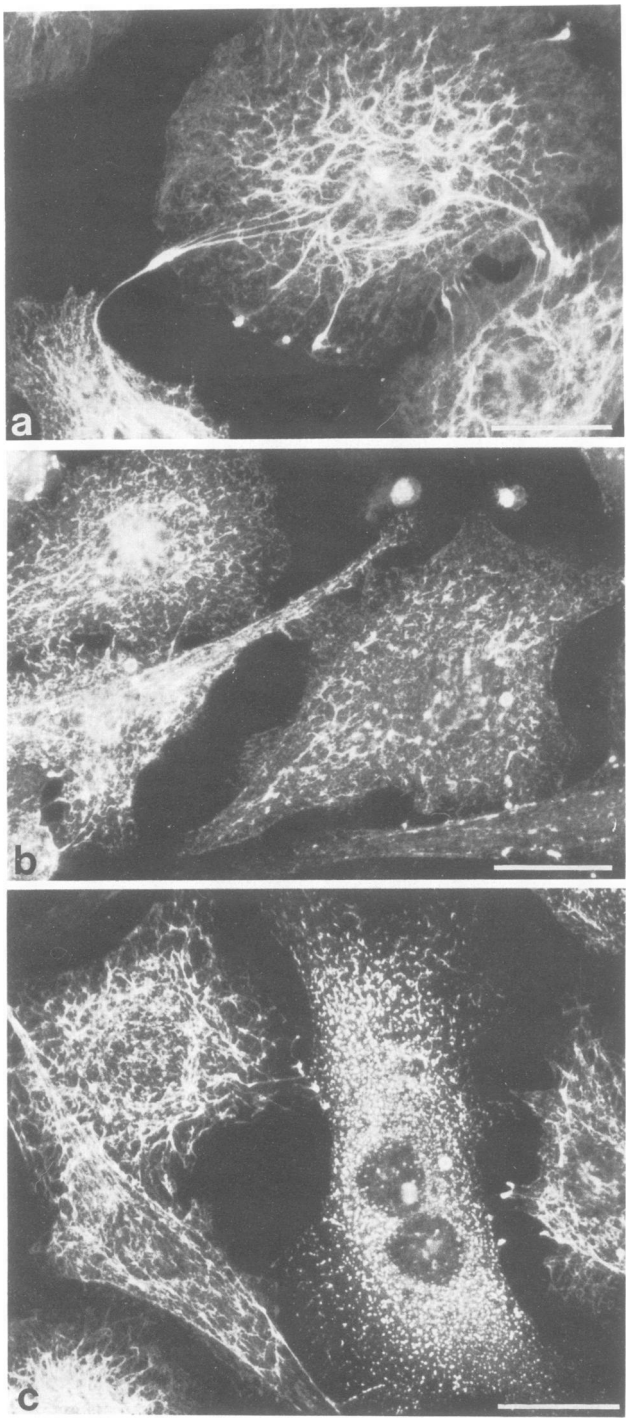

Fig. 10. Immunofluorescence microscopy, using antibody K_s8.60 specific for cytokeratin VI, of BMGE+H cell clones stably transfected with the constructs IV*→Ia and IV*→VIb, showing various amounts of 'new' cytokeratins derived from the transfected genes: (**a**) Clone Ia/VI-5 (lane 10 in Figure 5); (**b**) clone Ia/VI-3 (lane 13 in Figure 5); (**c**) clone Ia/VI-8 (lane 11 in Figure 5). Bars, 25 μm. The structures formed by the newly synthesized epidermal cytokeratins seems to vary somewhat with the amount—and probably type—of synthesized protein. The cytokeratin IF network of clones synthesizing low amounts of epidermal cytokeratins, compared to the endogenously synthesized cytokeratins, display the normal BMGE+H arrays (**a**) whereas cell clones that produce amounts of epidermal cytokeratins comparable to—or even exceeding—the endogenous cytokeratins show a disturbed IF distribution, resulting in the appearance of short and thin fibrils (**b**) or even granules (central cell in **c**).

expression of hybrid genes to tissues that also express their own vimentin gene. Finally, Kulesh and Oshima (1988) have reported that the transfected human cytokeratin 18 gene with ~2.4 kb upstream sequence is permissively transcribed in various cell lines, including murine fibroblasts, that do not express their endogenous cytokeratin 18 gene, indicating that this region does not contain *cis*-acting elements sufficient for the cell-type-specific inactivation of the transfected gene. Hence, our demonstration of a 605 nt long promoter plus enhancer element directing the cell-type-specific expression of the bovine cytokeratin IV* gene, in both homologous and heterologus combinations, presents the first case of an identified enhancer contributing to the expression of an IF protein. At present we cannot offer a convincing explanation why the situation of this bovine cytokeratin gene appears to be so much more simple as far as its cell-type-specific expression is concerned. Moreover, our experiments do not allow the exclusion of contributions by other positive or negative regulatory element (for examples of both kinds of elements in vimentin genes see Rittling and Baserga, 1987; Pieper *et al.*, 1987; Zehner *et al.*, 1987), including downstream sequences as they have been identified in several other multigene families, the best studied examples being the globins and the actins (e.g. Trudel and Constantini, 1987; DePonti-Zilli *et al.*, 1988; Reitman and Felsenfeld, 1988), In fact, recent findings of extensive sequence homologies in certain introns and other non-coding regions of some orthologous cytokeratin genes such as those encoding cytokeratins 10, 11 and 19 (Rieger and Franke, 1988; Bader *et al.*, 1988) might suggest the existence of important regulatory elements in these introns. Transgenic mice experiments of the kind described by Brinster *et al.* (1988) may be necessary to examine the possible existence of such elements in introns or downstream of the polyadenylation site.

The dominant regulatory upstream element promoting the expression of the bovine cytokeratin IV* gene in mammary gland epithelium but not in kidney epithelial cells also presents an example of the individuality of the control of cytokeratin gene expression in relation to specific programmes of differentiation (see also Blessing *et al.*, 1987). However, it is also important to remember that the expression of a number of cytokeratin genes is not only determined by differentiation programs but is also under the regulatory influence of certain hormones and growth factors and of vitamin A (for refs see Introduction). We have not yet observed such influences for the bovine cytokeratin IV* gene.

The identification of an important upstream enhancer element directing the cell-type-specific expression of a certain cytokeratin has allowed us to design constructs introducing other cytokeratins into a cell that normally does not co-express them. As a particularly spectacular example we have brought the bovine genes for cytokeratins Ia and VIb, equivalent to human cytokeratins 1 and 10, under the control of cytokeratin IV* gene upstream elements and have shown that, upon transfection, these genes are expressed in the mammary gland cells that do not express their own genes for these proteins. The effective expression of these genes which resulted, in several cell clones, in high concentrations of the 'foreign' protein, is remarkable as these two cytokeratins normally appear only in suprabasal layers of

epidermis—and some similar squamous epithelia—where they seem to be part of a vertical differentiation programme usually resulting in cell death and desquamation. The experimentally forced expression of these epidermal cytokeratins in a permanently proliferating cell line shows that the accumulation of these proteins is perfectly compatible with the viability of an epithelial cell.

Our transfection results also show that the synthesis of a given cytokeratin does not necessarily result in the activation of the endogenous gene encoding its normal complementary 'pair partner'. Consequently, a generalization of the hypothesis that the synthesis of a type II cytokeratin leads to the expression of the gene encoding the corresponding type I cytokeratin (Giudice and Fuchs, 1987) is not possible. However, it should be kept in mind that our study differs from that of Giudice and Fuchs (1987) in that we have used epithelial cells that already express some other type I cytokeratins.

Our observation that the forcibly introduced epidermal cytokeratins Ia and VIb mix with the endogenous mammary gland cytokeratins and form a common IF system is not surprising, considering the results of our earlier experiments using mRNA microinjection (Franke *et al.*, 1984) and the transfection results of Giudice and Fuchs (1987). However, an unexpected finding of our experiments is that the total amounts of cytokeratins in the cytoskeletons of the transfected cells can considerably increase so that the introduced new cytokeratins may even exceed the old ones. This shows that a given kind of epithelial cell, here the BMGE+H line, can accommodate much more cytokeratin than it normally contains. Another unexpected observation in certain BMGE+H cells loaded with the 'foreign' cytokeratin Ia and VIb is the appearance of disordered IF structures such as heaps of 'whisker-shaped' and small globular particles. Although these disordered IF arrays superficially resemble other non-fibrillar forms of cytokeratins such as those found during mitosis (e.g. Franke *et al.*, 1982a; Lane *et al.*, 1982; Geiger *et al.*, 1984; Tölle *et al.*, 1987) or after certain drug treatments (for review see Knapp and Bunn, 1987), a detailed structural analysis will be necessary to compare the two states. Clearly, our gel electrophoretic results show that the cytokeratin polypeptides Ia and VIb are largely intact in the transfected cells, and therefore, the structural perturbations are not related to the similar looking non-IF structures formed by deletion mutants of certain cytokeratins (Albers and Fuchs, 1987). It may be that the conspicuous hyperphosphorylation of cytokeratin Ia (e.g. Figure 7) is responsible, at least in part, for these abnormal structures. On the other hand, the cells containing these short comma-like IF protein structures might represent certain cell clones characterized by anomalous IF assemblies that are present as a minor population, in the original BMGE+H cells (Schmid *et al.*, 1983a).

The identification of relatively short and simple-structured upstream regulatory elements directing cell-type specificity of cytokeratin gene expression also provides important information for designing experiments to elucidate the possible functions of specific cytokeratins and of the cells expressing them. In particular, such cytokeratin enhancer elements will be valuable tools to address certain other gene products to a specific type of epithelial cell, be it in culture or in transgenic animals.

Materials and methods

Plasmids

The plasmids pBLCAT2, pBLCAT3 and pTKCAT14A were a gift from B.Luckow (this Institute; cf. Luckow and Schütz, 1987). Plasmid pTKCAT14A was derived from pTKCAT (Miksicek *et al.*, 1986) by inserting the two 72 bp repeats of the SV40 enhancer into the *Bam*HI site 5′ to the HSV-TK promoter of pTKCAT (R.Miksicek, unpublished results). Sequence analysis was performed according to Maxam and Gilbert (1980).

Cell culture and transfections

The established bovine cell line BMGE+H (Schmid *et al.*, 1983b) and the mouse keratinocyte line AT5 (kindly provided by Dr A.Balmain, Beatson Institute of Cancer Research, Glasgow, UK) were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 20% fetal calf serum. MDBK cells from bovine kidney epithelium were grown as described (cf. Franke *et al.*, 1982a). Mouse 3T3 fibroblasts were cultured in DMEM containing 10% fetal calf serum. Cells were plated 20 h prior to transfection at 20% density. Five microgrammes of plasmid DNA were used for transfection of cells equivalent to 50 mm culture dish area, using the calcium phosphate co-precipitation method (Graham and van der Eb, 1973; De Villiers and Schaffner, 1983). For stable transfections, the G-418 resistance marker plasmid pAG60 (Colbère-Garapin *et al.*, 1981) was co-transfected with the plasmid constructs IV*→Ia and/or IV*→VIb at a molar ratio of ~1:3. Twenty-four hours after transfection the cells were split at 1:4 dilution and exposed to selective medium (700 μg G-418/ml). After 2 weeks single clones were picked and grown in the presence of 500 μg G-418/ml. Established stably transfected clones were then grown, alternatively, in media lacking G-418.

Cells were labelled for ~12 h with [^{35}S]methionine (~300 μCi/6 ml; Amersham International, Amersham, UK) in medium of reduced methionine content (cf. Franke *et al.*, 1981a) or with 500 μCi/6 ml [^{32}P]phosphate (in phosphate-free medium).

Chloramphenicol acetyltransferase (CAT) assays

CAT assays were performed using extracts prepared from cells 40 h after transfection essentially as described by Gorman *et al.* (1982).

RNase protection and Northern blot analysis assays

Total cellular RNA was isolated from cells 40 h after transfection as described (Kreis *et al.*, 1983). To prove the correct initiation of RNA synthesis, the same probe and conditions were used as described by Miksicek *et al.* (1986).

Northern blot analyses were performed as described (cf. Blessing *et al.*, 1987). The probes used to detect cytokeratin Ia and VIb mRNA corresponded to exon 1 of the specific genes.

Gel electrophoresis of proteins

Conditions for SDS−PAGE two-dimensional gel electrophoresis and processing for autoradiography were as described (e.g. Franke *et al.*, 1981a,b; Schiller *et al.*, 1982; Kreis *et al.*, 1983).

Antibodies and immunolocalization

Monoclonal murine antibody specific for human cytokeratin 10 and its bovine counterpart, cytokeratin VI (K_s8.60; for characterization see Huszar *et al.*, 1986), was obtained from Bio-Makor (Rehovot, Israel). Broad range guinea pig antibodies against cytokeratins were prepared as described (Franke *et al.*, 1978; cf. Schmid *et al.*, 1983a,b). Secondary antibodies (anti-mouse IgG coupled to Texas red and anti-guinea pig IgG coupled to fluorescein-isothiocyanate) were purchased from Dianova (Hamburg, FRG). Single and double label immunofluorescence microscopy on cells grown on cover slips was performed essentially as described (e.g. Kreis *et al.*, 1983; Franke *et al.*, 1984).

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