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Increased abundance of a normal cell mRNA sequence accompanies the conversion of rat mammary cuboidal epithelial cells to elongated myoepithelial-like cells in culture

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SUMMARY

Rat mammary cuboidal epithelial cell lines in culture convert to elongated myoepithelial-like cells. This conversion is accompanied by the appearance of a 9,000 molecular weight acidic polypeptide (p9ka), abundant in the elongated convertants, but which is hardly detectable in the cuboidal epithelial cells. A cDNA library corresponding to a low-molecular-weight fraction of poly(A)- containing RNA from a myoepithelial-like cell line, has been constructed. Recombinant plasmids containing cDNA complementary to p9ka mRNA have been identified by hybrid-selected translation. The mRNA for p9ka has been identified by Northern blotting and is found to be at least five-times more abundant in cultured myoepithelial-like rat mammary cells when compared to the cuboidal epithelial cells. This cytoplasmic mRNA sequence, which is present in increased abundance in cultured mammary myoepithelial-like cells, is also present, at lower levels, in normal rat tissues, including the mammary glands.

INTRODUCTION

The formation of malignant, metastasising tumours in the mammary glands of rats and humans is usually accompanied by a disappearance of fully-differentiated myoepithelial cells which are found in the normal glands (1, 2). To study this phenomenon in detail, cell culture has been used to isolate rat mammary epithelial stem cells and one such example is Rama 25 (3). Under one set of culture conditions, Rama 25 cells form structures which resemble the ducts found in early developmental phases of the mammary gland (4); under another set of culture conditions alveolar-like cells are formed (3), and under a third set of culture conditions Rama 25 cells give rise to elongated, myoepithelial-like cells at a low frequency (3). The duct, alveolar and myoepithelial cells represent the three major cell types of mammary parenchyma.

The conversion process to elongated myoepithelial-like cells is not specific for one cell line, but has been found in other epithelial cell lines derived from non-malignant tumours and normal mammary glands of rats and mice

(5,6,7,8,9,10). This conversion is thought to resemble a normal differentiation process that is occurring in ductal termini in vivo (11,12). In contrast, cell lines from malignant metastasising tumours fail to convert to the elongated myoepithelial-like cells in culture (13,14). In order to understand the reason for the failure of malignant cell lines to give rise to elongated cells, it has first been necessary to identify molecular markers for the elongated myoepithelial-like cells in culture and then to find out how their synthesis is controlled at a molecular level.

The elongated myoepithelial-like cells derived from the mammary epithelial cells in culture possess several polypeptides which are suitable candidates for molecular markers of this conversion (15). One acidic polypeptide of 9,000 molecular weight (p9ka) is present in abundance in extracts from the elongated cells, but is hardly detectable in extracts of the epithelial cells when they are analysed by two-dimensional gel electrophoresis (15). This protein was chosen as a molecular marker for the study of the conversion process in culture. Experiments with the cloned cell line, Rama 29 (3), derived from epithelial Rama 25 cells, showed that the dramatic increase in synthesis of p9ka depends on an increased amount of translatable mRNA (16). Here we describe the isolation and characterisation of a cDNA sequence complementary to part of the p9ka mRNA cloned into a bacterial plasmid. This cDNA sequence is used to study p9ka mRNA abundance in some normal rat tissues and in different rat mammary cell lines including another myoepithelial-like cell line, Rama 401 (10), to delineate more exactly the level of control of synthesis of p9ka protein during the conversion process.

MATERIALS AND METHODS

Reagents and Equipment

Where possible reagents were autoclaved prior to use and equipment coming into contact with RNA or DNA was sterilized by autoclaving or by treatment with 0.1% diethyl pyrocarbonate. Glassware used for nucleic acid isolation was siliconised.

Restriction Endonucleases

Restriction endonucleases were obtained from Boehringer Mannheim Corporation and conditions of digestion were those recommended by the supplier.

Cell Culture

Rat mammary cells were grown as previously described (3,15,16). L2

yolk-sac tumour cells (17,18) were a gift from Dr. Ulla Wewer (Institute of Pathological Anatomy, Copenhagen, Denmark). Conditions of culture for L2 cells were those described for Rama 25 cells (3), except that hormones were omitted from the culture medium.

Isolation, Fractionation and Translation of mRNA

Total cellular nucleic acid was extracted from cultured cells as described previously (16) and enriched for poly(A)-containing RNA by two passages through oligo(dT)-cellulose (19). Rat liver and rat uterus were frozen in liquid N_2, ground to a powder in a pestle and mortar and homogenised in 5-volumes of 0.1 M Tris-HCl pH 9.0, 0.5% sodium dodecyl sulphate, 5-volumes of 80% phenol in water. RNA was isolated as described previously (16). Poly(A)-containing RNA was isolated from lactating rat mammary glands using the method of Rosen et al. (20,21). In some experiments, RNA was isolated from tissues and cell lines by the method of Chirgwin et al. (22) using guanidinium isothiocyanate.

A low-molecular-weight fraction of cell-line mRNA, for use in cDNA synthesis and bacterial colony screening, was prepared by centrifuging 220 µg poly(A)-containing RNA through an 11 ml, 10-30% (w/v) sucrose gradient in 1 mM EDTA, 10 mM sodium acetate, 100 mM NaCl, pH 5.0, at 2°C for 16 hr at 150,000 g(av). The RNA was dissolved in 100 µl 1 mM EDTA pH 5.0 and heated to 70°C for 1 min prior to centrifugation. Gradients were collected from the top using an Isco model 185 gradient fractionator pumping at 0.75 ml/min. The top 1.2 ml of the gradient was discarded and the RNA from the next 2.7 ml, which contained p9ka mRNA, was recovered by ethanol precipitation in the presence of 0.2 M NaCl at -20°C for 48 hr, and centrifuging at 10,000 g(av) for 1 hr. After a single reprecipitation with ethanol from 200 mM Hepes-KOH pH 7.6, the integrity of the fractionated RNA was checked by translation in vitro with a nuclease-treated reticulocyte lysate, followed by analysis of the radioactive translation products using two-dimensional gel electrophoresis, as described previously (16).

Synthesis of Double-Stranded cDNA

20 µg of the low-molecular-weight fraction of Rama 29 cell mRNA from the sucrose gradient was denatured with 3 mM methylmercury hydroxide and incubated at 42°C for 1 hr in 100 µl 500 mM Tris-HCl, pH 8.3, 50 mM NaCl, 8 mM $MgCl_2$, 10 mM DTT, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, containing 10 µCi [^{3}H]dCTP (specific activity 21 Ci/mmol), 50 µg/ml oligo(dT) (12-18 nucleotides) and 500 units/ml AMV reverse transcriptase (supplied by Dr. J.W. Beard, Life Sciences Inc., U.S.A.). Reactions were stopped with 0.2% SDS, 8

mM EDTA, extracted with phenol (23) and subjected to chromatography on Sephadex G-50. mRNA was hydrolysed in 0.25 M NaOH at 70°C for 15 min and then neutralised. The length of the single-stranded cDNA was in the range 500-1100 bases when measured on alkaline agarose gels (24).

Second strand of cDNA was synthesised using reverse transcriptase (400 units/ml) at 46°C for 3 hr (25) and the double-stranded cDNA was treated with S_1 nuclease (which had previously been titrated with both double-stranded and single-stranded DNA), as described by Shenk et al. (26). 15-20 residues of dCMP were added to the 3' termini using terminal deoxynucleotidyl transferase in the presence of 2 mM $CoCl_2$ and using the cacodylate buffer system of Roychoudhury et al., (27).

Preparation and Homopolymer Tailing of pAT 153

Supercoiled pAT 153 (28) was propagated in E. Coli JA 221 (supplied by Dr. P. Meacock, Biotechnology Centre, University of Leicester) in Luria broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl pH 7.4) containing 10 µg/ml tetracycline. Plasmid DNA was prepared as described by Clewell & Helinski (29), after plasmid amplification with chloramphenicol, and purified by one-cycle of CsCl-gradient centrifugation (30). Supercoiled plasmid DNA was cut with restriction enzyme Pst 1, and an average of 13 residues of dGMP residues/3' end were added using terminal deoxynucleotidyl transferase (27). Tailed plasmid was annealed with tailed double-stranded cDNA at 350 fmole/ml in a buffer of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, for 2 hr at 44°C, and then slowly cooled to room temperature. 5 ng of the annealed DNA was used to transform E. Coli JA 221 cells made competent for DNA uptake by the method of Lederberg & Cohen (31) in 1 x SSC as described by Cohen et al. (32). Cells were spread onto nutrient agar plates containing 10 µg/ml tetracycline and incubated at 37°C overnight. Transformation frequencies with supercoiled pAT 153 were 0.65 - 6 x 10^6 colonies/µg DNA and with recombinant DNA 1-5 x 10^3/µg DNA. 80% of the colonies were ampicillin-sensitive.

Screening of Transformant Colonies

Tetracycline-resistant, ampicillin-sensitive colonies were screened by "colony hybridisation" (33,34) by incubating each 9 cm filter with 1 x 10^6 cpm of single-stranded cDNA preparations which had been radioactively labelled with [^{32}P]dCTP to a specific activity of 15 µCi/µg using reverse transcriptase as described above, but the incubation mixture included 83 µg/ml actinomycin D. Colonies were hybridised separately with cDNA of both Rama 25 cuboidal cell mRNA and Rama 29 elongated cell mRNA.

Prior to secondary screening, bacterial colonies containing recombinant plasmids were cultured in Luria broth containing tetracycline. Plasmid DNA was isolated from the bacteria by the NaOH-lysis procedure of Birnboim & Doly (35) as modified by Ish-Horowicz and Burke (36) and digested with pancreatic ribonuclease A that had been treated to remove residual DNAse activity (23). Twenty µg of denatured recombinant plasmid DNA preparations were spotted onto 3 mm circles of nitrocellulose (Schleicher & Schuell, BA85) and hybridised by the method of Parnes (37) to 19 µg of unfractionated Rama 29-cell poly(A)-containing RNA. Hybridised mRNA was eluted (37), translated in the reticulocyte lysate cell-free protein synthesising system (16) and the resulting [^{35}S]methionine-labelled translation products were analysed by two-dimensional gel electrophoresis (16, 38). Gels were impregnated with Autofluor (National Diagnostics) and subjected to autoradiography at -70°C (39). Large-scale isolation of recombinant plasmid DNA was carried out by scaling up the alkaline lysis procedure (35, 36).

Agarose Gel Electrophoresis and Filter Hybridisation

Plasmid preparations and restriction enzyme digests were analysed on 1% agarose (Seakem) gels which were run in the submerged mode in 13.3 mM Tris-acetate pH 7.7, 0.33 mM EDTA. Gels were post-stained in 5 µg/ml ethidium bromide. Bacteriophage λ DNA, digested with restriction enzyme Hind III, and bacteriophage ØX 174 DNA, digested with restriction enzyme Hae III, were used as molecular size markers.

RNA was subjected to electrophoresis in 1.1% agarose gels either containing 5 mM methylmercury hydroxide as described by Bailey & Davidson (40) or after denaturation of the RNA with glyoxal as described by McMaster & Carmichael (41,42).

RNA separated by electrophoresis was transferred to nitrocellulose as described by Thomas (43). When the gels were run in the presence of methylmercury hydroxide they were treated as described by Alwine et al. before transfer (44). Nitrocellulose filters were baked and hybridised as described previously (43) with 5 x 10^6 cpm of nick-translated DNA (specific activity 1 x 10^8 cpm/µg), washed twice in 2 x SSC, 0.1% SDS and then four times for 15 min each in 0.1 x SSC, 0.1% SDS at the temperatures indicated in the figure legends. In some experiments increasing amounts from 0.05 µg to 5 µg of poly(A)-containing RNA dissolved in 2 to 200 µl 10x SSC were spotted onto nitrocellulose filters in a Bio-Dot apparatus (Bio-Rad Laboratories). Filters were baked at 80°C for 2 hr, hybridised as above and washed in 0.1%

SSC, 0.1% SDS at 50°C. Radioactivity bound to each spot was determined by scintillation counting.

Recovery of DNA from Preparative Agarose Gels

Two hundred µg recombinant plasmid DNA was digested with restriction enzyme Pst 1 and the reaction was stopped with 0.2% SDS, 8 mM EDTA, extracted with phenol and precipitated with ethanol. The sample (1.85 ml) in 4% glycerol, 0.02% bromophenol blue was heated to 65°C for 5 min and loaded into a 3 mm x 18.8 cm slot cut to a depth of 3.3 mm in a 1% agarose gel. DNA was electrophoresed into a second slot containing electrophoresis buffer, and the DNA was recovered by ethanol precipitation.

Recovery of RNA from Agarose Gels and Translation by a Reticulocyte Lysate

5.7 µg Rama 29-cell poly(A)-containing RNA was subjected to electrophoresis on a methylmercury hydroxide-containing 1.1% low-melting agarose gel (40). One-mm transverse slices of the track were cut from the gel, melted briefly at 70°C and cooled to 37°C. Two-µl samples of the molten agarose were incubated in a reticulocyte lysate system for cell-free protein synthesis for 2 hr at 37°C as described by Brandt & Hackett (45). Translation products were analysed by two-dimensional polyacrylamide gel electrophoresis and radioactivity which was incorporated into the p9ka spot on the gel was quantitated as described previously (15).

Determination of Poly(A) Content of mRNA Preparations

Poly(A)-containing RNA (50-150 ng) was hybridised with 40 pmoles 3H-polyuridylic acid (specific activity 500 mCi/mmol of nucleoside residue) in 10 µl of 400 mM NaCl, 10 mM Tris-HCl, pH 7.5 at 37°C for 1 hr. Reaction mixtures were cooled to 30°C and adjusted to a volume of 50 µl containing 360 mM NaCl, 4.5 mM $ZnCl_2$, 150 mM sodium acetate buffer pH 4.5 and 10 units of S_1 nuclease and the mixtures were incubated for 10 min. Undigested RNA was precipitated onto glass fibre filters with 5% trichloroacetic acid containing 1% sodium pyrophosphate. After washing with 5% trichloroacetic acid, ethanol and ether, counts per minute trapped by the filters were determined by scintillation counting. Calibration curves over the range 2-10 ng poly(A) were obtained by replacing poly(A)-containing RNA with polyadenylic acid which had been bound and eluted from an oligo(dT)-cellulose column. The poly(A) content of mRNA preparations ranged from 21 to 62 ng/µg RNA.

Radioactive Labelling of Double-Stranded DNA

Double-stranded DNA was labelled with deoxycytidine 5'-[α-^{32}P] triphosphate to a specific activity of 1 x 10^8 cpm/µg by nick translation (46).

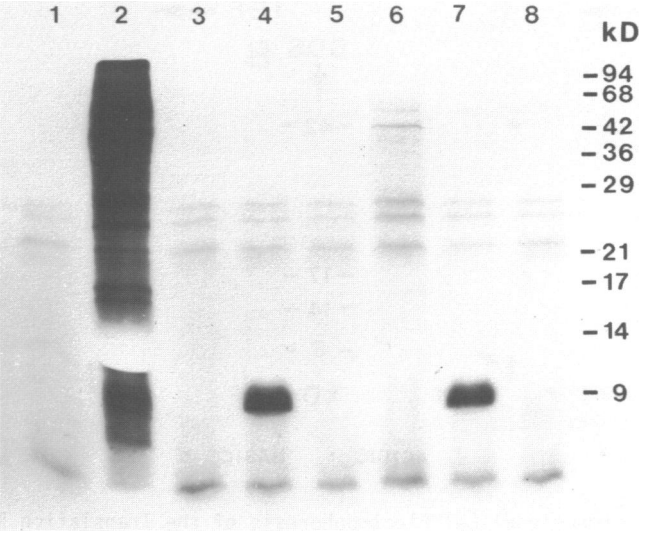

FIGURE 1 Translation of mRNA Selected by Binding to Plasmid DNA Isolated from Individual Recombinants. 20 µg denatured plasmid DNA from 5 separate recombinants or native pAT 153 DNA were bound to individual nitrocellulose filters. Filters were incubated with 38 µg poly(A)-containing RNA isolated from Rama 29 elongated cells. mRNA released from the hybrids was translated in the reticulocyte lysate and the polypeptides synthesised were subjected to polyacrylamide gel electrophoresis. The [^{35}S]methionine-labelled translation products were detected by autoradiography after exposure to photographic film for 3 days. Track 1, endogenous pattern of protein synthesis by the lysate; Track 2, Rama 29 cell mRNA total translation products; Track 3, translation products produced by any mRNA selected by pAT 153 DNA; Tracks 4-8, translation products produced by mRNA bound to 5 individual recombinant plasmids. Molecular weight markers in kilodaltons.

Biohazard Procedures

Recombinant DNA work was carried out under conditions of Good Microbiological Practice as laid down by the U.K. Genetic Manipulation Advisory Group. All manipulations with methylmercury hydroxide were carried out in a fume cupboard.

RESULTS

Construction of cDNA Library and Screening for mRNA Sequences

1090 ampicillin-sensitive bacterial colonies were screened by "colony hybridisation" with single-stranded cDNA copies of the low-molecular-weight fraction of cell line mRNA preparations from both cuboidal Rama 25 cells and elongated Rama 29 cells in turn. Colonies showing a positive hybridisation

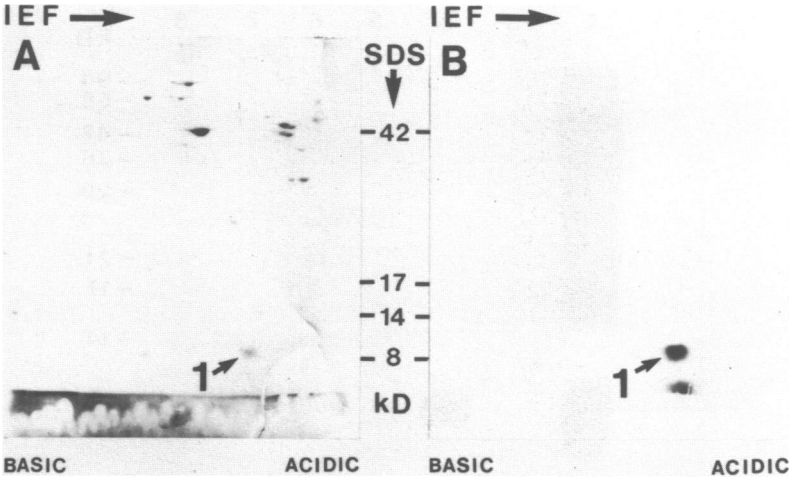

FIGURE 2 Two-dimensional Gel Electrophoresis of the Translation Products of mRNA Selected by Binding to Plasmid 1 Recombinant. 15 µl of the translation reaction shown in Fig. 1, track 4, was subjected to two-dimensional gel electrophoresis in the presence of 600 µg unlabelled Rama 29 cell protein. Both the gel, stained with coomassie blue (A), and the autoradiograph of A showing [^{35}S]methionine-labelled translation products (B) are shown. Polypeptide 1 is p9ka. IEF = isoelectric focussing; molecular weights are in kilodaltons.

signal with the cDNA from Rama 29 elongated cells and a weaker signal with the cDNA from Rama 25 cuboidal epithelial cells were subsequently screened in batches of 10 by hybrid-selected translation using unfractionated mRNA from Rama 29 elongated cells. Positive batches were identified by looking for a 9,000 molecular weight [^{35}S]methionine-labelled translation product which co-migrated with non-radioactive p9ka when subjected to two-dimensional gel electrophoresis. Individual colonies of one positive batch of 10 clones were examined separately by one-dimensional SDS gel electrophoresis (Fig. 1). Two positive recombinant plasmids from this batch of bacterial clones (clones 1 & 4) contained a 400 base-pair insert in the Pst 1 site of pAT 153, with a single Eco R1 site within the inserted DNA. Both plasmids 1 and 4 hybridised to mRNA which translated to give a product that co-migrated exactly with p9ka during two-dimensional gel electrophoresis (Fig. 2). Recombinant plasmids positive for p9ka mRNA showed an additional radioactive spot, which migrated on two-dimensional gels just behind the dye front when 12½% acrylamide SDS gels were used (Fig. 2). This radioactive spot and the radioactivity which co-migrated with p9ka were not detectable in the endogeneous polypepetide translation products of the nuclease-treated reticulocyte lysate. Since

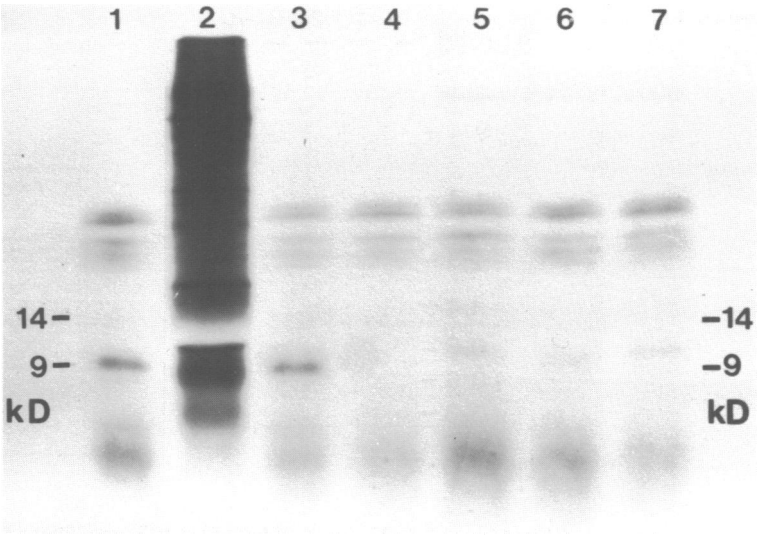

FIGURE 3 Gel Electrophoresis of Translation Products of mRNA from Different Cell Lines after Hybridisation to Filter-Bound Plasmid 1 Recombinant DNA. 6 µg mRNA from cell lines were hybridised to 5 µg filter-bound plasmid 1 DNA (Tracks 1, 3, 6), or to filter-bound pAT 153 DNA (Track 7), or a blank filter (Track 4). Tracks, 1, 4, and 7, Rama 29 myoepithelial-like cell mRNA; Track 3, Rama 401 myoepithelial-like cell mRNA; Track 6, Rama 25 epithelial cell mRNA. Tracks 1, 3, 4, 6, 7, mRNA selection; Tracks 2 and 5 are non-selected translation of Rama 29-cell mRNA (Track 2) and endogenous translation products (Track 5).

exactly the same results were obtained when either plasmids 1 or 4 were used, purified insert DNA from plasmid 1 was used in the experiments described below. When the cDNA library to the low-molecular-weight mRNA was screened with the insert from recombinant plasmid 1 by colony hybridisation, 3% of the total number of colonies were positive; this corresponds to the abundance of p9ka translatable mRNA in the low-molecular-weight RNA fraction from Rama 29 elongated cells.

Detection of p9ka mRNA in Cell Lines by Hybrid-Selected Translation

To characterize more fully the specificity of plasmid 1 DNA, hybrid-selected translation experiments were carried out using poly(A)-containing RNA from several different rat mammary cell lines. Plasmid 1 DNA hybridised to mRNA, which could be subsequently translated into a 9kd polypeptide, from Rama 401 myoepithelial-like cells (10) (Fig. 3) and from rat mammary fibroblast cells (not shown). Both cell lines contain p9ka protein. This 9kd translation product showed exact co-migration with p9ka protein on two-

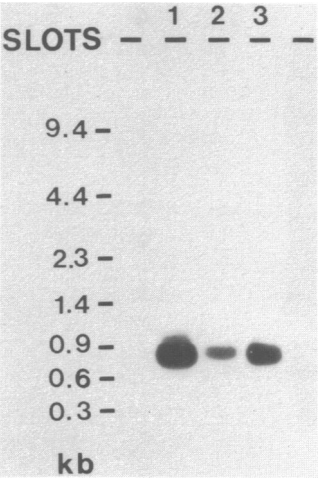

FIGURE 4 Northern Hybridisation of Cell-Line mRNA with a Probe Prepared from the 400 Base-Pair Recombinant cDNA. Poly(A)-containing RNA preparations, isolated from cell lines, were subjected to agarose gel electrophoresis with methylmercury hydroxide, blotted onto nitrocellulose filters and hybridised as described under Materials and Methods. Filters were washed at 50°C in 0.1 x SSC, 0.1% SDS and radioactive hybrids visualised by autoradiography. Track 1, 5 µg mRNA from Rama 29 myoepithelial-like cells; track 2, 5 µg mRNA from Rama 25 epithelial cells; track 3, 6 µg mRNA from Rama 401 myoepithelial-like cells. Molecular sizes are given in kilobases.

dimensional gels (results not shown). The relative intensities of the p9ka radioactive products of hybrid-selected translation of these cell lines reflected the relative intensities of p9ka when the mRNA preparations were translated in the reticulocyte lysate without prior hybridisation selection. When any mRNA from cuboidal epithelial Rama 25 cells, that was bound to plasmid DNA on filters, was translated in the cell-free protein synthesising system, there was no detectable synthesis of p9ka (Fig. 3). Similarly when any mRNA from Rama 29 cells that bound to the parent pAT 153 DNA was translated, no polypeptide corresponding to p9ka was detected (Fig. 3).

Sizing of pp9ka mRNA

When Poly(A)-containing RNA from elongated Rama 29 cells was denatured with glyoxal or methylmercury hydroxide and subjected to agarose gel electrophoresis, blotted onto nitrocellulose filters and probed with radioactive plasmid 1 insert DNA, a single radioactive band of hybridisation was obtained which corresponded to RNA of length 780 bases (Fig. 4). It is unlikely that this 780-base mRNA arises from breakdown of a larger molecule during isolation of RNA with phenol, since the same hybridisation pattern is

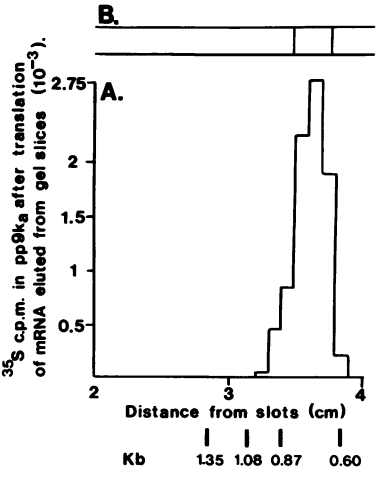

FIGURE 5 (A) Translation of Gel-Fractionated RNA in a Reticulocyte Lysate Cell-Free System of Protein Synthesis. Poly(A)-containing RNA from Rama 29 cells was subjected to agarose gel electrophoresis in the presence of methylmercury hydroxide. RNA was eluted from 1 mm slices of the gel and translated in a reticulocyte lysate as described under Materials and Methods. The [^{35}S]methionine-labelled translation products were subjected to two-dimensional gel electrophoresis and radioactivity incorporated into p9ka was determined. Molecular sizes in kilobases, were obtained by co-migration of marker nucleic acids as described in Materials and Methods. (B) the position of the 0.78 kb band of hybridisation is shown diagramatically.

obtained with RNA prepared using the guanidine isothiocyanate method of Chirgwin et al. (22). The presence of translatable mRNA for p9ka in the 780-base region of the gel was confirmed by subjecting Rama 29-cell poly(A)-containing RNA to electrophoresis on a methylmercury hydroxide-containing gel, eluting the RNA from 1 mm slices taken from the 780 base region and translating the mRNA in the reticulocyte lysate. Analysis of the resulting translation products by two-dimensional gel electrophoresis, confirmed the presence of p9ka mRNA in the fractions corresponding to the 780-base region of the original RNA gel (Fig. 5).

Quantitation of p9ka mRNA in RNA Preparations from Cell Lines and Tissues

The results of the Northern blotting experiments in Fig. 4 also showed that hybridisable p9ka mRNA was found in RNA isolated from the myoepithelial-like cell line derived from normal cuboidal epithelial cells, Rama 401 (10). With equal RNA loadings in the tracks of the agarose gel, the relative amount of radioactivity in the DNA/mRNA hybrids reflected the abundance of p9ka protein detected in the myoepithelial-like cells by two-dimensional gel

Table 1 Quantitation of p9ka mRNA by Filter Hybridisation (Dot-Blot)

Source of RNA		1st Experiment		2nd Experiment	
		Gradient* of slope ±SE (cpm/µg RNA)	Relative† Hybridisation	Gradient* of slope ±SE (cpm/µg RNA)	Relative† Hybridisation
Epithelial Cells (Rama 25)	1	948 ± 82	1.0	492 ± 11	1.0
	2	1296 ± 204	1.15	887 ± 24	1.5
	3	764 ± 88	0.99	N.D.	N.D.
Elongated Cells (Rama 29)	1	4908 ± 304	7.4	4578 ± 119	13.2
	2	5212 ± 373	6.5	5872 ± 147	14.0
Elongated Cells (Rama 401)		3393 ± 206	5.6	N.D.	N.D.
		3rd Experiment			
Epithelial Cells		266 ± 15	1.0		
Rat Uterus		167 ± 12	0.73	269 ± 82	0.64
Rat Liver	1	117 ± 11	0.31	117 ± 20	0.17
	2	179 ± 11	1.45	336 ± 67	1.48
Lactating Mammary Gland	1	40 ± 9	0.2	75 ± 29	0.2
	2	35 ± 14	0.12	48 ± 9	0.09
Ribosomal RNA		N.D.	N.D.	0.28 ± 19	6×10^{-4}
Transfer RNA		N.D.	N.D.	11 ± 29	0.022
L2 Yolk Sac Tumour Cells		N.D.	N.D.	116 ± 15	0.166

Dot-Blot hybridisations were carried out as described under Materials and Methods. Spots were cut from the filters and radioactivity determined by scintillation counting. The best-fit straight line of ^{32}P-bound/dot against µg RNA applied was calculated by linear regression (47). The extent of hybridisation is shown by the gradient of this line (*) ± standard error of the slope (47). Relative hybridisation (†) is the gradient expressed relative to that of one of the preparations of epithelial cell line mRNA (Rama 25) with a correction made for variations in the poly(A) content of different mRNA preparations. The values for ribosomal RNA and transfer RNA are not corrected for poly(A) content. Rows labelled 1, 2, or 3 refer to separate RNA preparations. N.D. = not determined.

electrophoresis. Some faint high molecular-weight RNA bands that hybridised to the 400 base-pair insert were visible, but only when autoradiographs were over-exposed. The hybridisation signal of the 780-base band was not decreased when filters were both prehybridised and hybridised in the presence

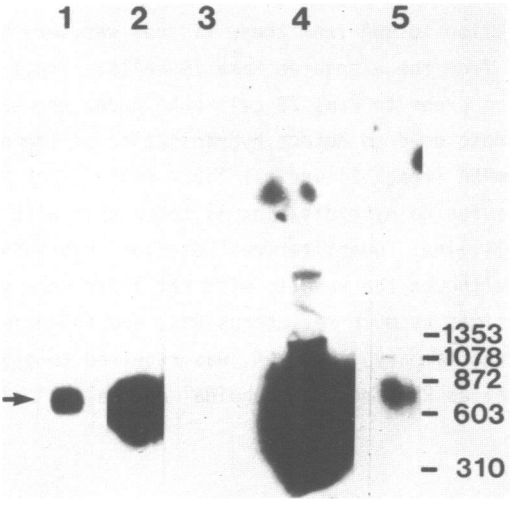

FIGURE 6 Northern Hybridisation of p9ka cDNA Probe to mRNA Isolated from Normal Rat Tissues and Cell Lines. 10 µg poly(A)-containing RNA, isolated from lactating rat mammary gland (Track 1), rat liver (Track 2), L2 yolk sac tumour cell line (Track 3), Rama 29 myoepithelial-like cell line (Track 4), and 5 µg poly(A)-containing RNA from rat uterus (Track 5), were subjected to agarose gel electrophoresis after glyoxal denaturation and transferred onto nitro- cellulose filters. The filters were incubated with 5 x 10^6 cpm ^{32}P-labelled probe (specific activity 2 x 10^8 cpm/µg), washed at 60°C in 0.1 x SSC, 0.1% SDS and subjected to autoradiography for 116 hr (Tracks 1-4) or 45 hr (Track 5). Molecular sizes are indicated in bases on the right of the tracks. The arrow points to the 780 base band that hybridises to the radioactive probe.

of a 1000-fold excess of poly(A), oligo(dT), oligo d(G) or oligo d(C) (data not shown).

Fig. 4 shows that the p9ka mRNA was also detected in poly(A)-containing RNA preparations from cuboidal epithelial Rama 25 cells, however, the signal strength was apparently less than in the case of the elongated cell lines. The relative amounts of p9ka mRNA in RNA preparations from cuboidal epithelial and elongated myoepithelial-like cells were determined following "Dot-Blot" filter hybridisations and the results, which take into account variations in the amount of poly(A)-containing RNA in the preparations, are shown in Table 1. There was a five to fourteen-fold increase in p9ka mRNA when poly(A)-containing RNA from elongated myoepithelial-like cells (Rama 29 and Rama 401) was compared with similar RNA preparations from cuboidal epithelial cells (Rama 25).

Fig. 6 shows that the 400 base-pair probe also detected a 780-base mRNA

sequence in normal rat mammary gland, rat liver and rat uterus. However the amount of hybridisation to RNA from these tissues was very much reduced when compared with that from the elongated Rama 29 cells. Track 4 of Fig. 6 shows hybridisation of the probe to Rama 29 cell mRNA under the same conditions and exposure-time as those used to detect hybridisation of the probe to normal rat mammary gland mRNA (track 1) and rat liver mRNA (track 2). Track 3 shows that at these exposures no hybridisation is detectable with RNA from the L2 yolk sac tumour cell line. Quantitative "Dot-Blot" hybridisations (Table 1) demonstrated that although the results with rat liver were variable, approximately 1.5 times as much rat uterus RNA, and approximately 5-10 times as much lactating rat mammary gland RNA, was required to give the same hybridisation signal as RNA from the cuboidal epithelial cell line Rama 25.

DISCUSSION

A 400 base-pair cloned cDNA has been shown to be complementary to part of the translatable mRNA coding for a 9kd acidic polypeptide (p9ka) found in cultured fibroblasts and myoepithelial-like cells. This mRNA is about 780 bases in length as determined on two denaturing gel systems using Northern blotting techniques and by translation of the mRNA eluted from agarose gels. This mRNA is longer than the 270 bases necessary to code for a 9kd polypeptide. The presence of an additional radioactive spot, which migrates near the dye front on two-dimensional gels, when this hybridised mRNA is subsequently translated into protein, raises the possibility that this 780-base p9Ka mRNA also encodes at least one other polypeptide smaller than p9ka. This additional polypeptide is also seen in the translation products of mRNA eluted from two other separate recombinant plasmids which also bind p9ka mRNA. mRNA molecules which code for more than one polypeptide have been reported to be present in some eukaryotic cells (48). However, the possibility that this smaller molecular weight radioactive spot may arise by some other mechanism (for example, by proteolytic degradation of p9ka in the reticulocyte lysate) cannot be ruled out at present.

When the rat low-molecular-weight cDNA library is screened with plasmid 1 insert DNA, additional bacterial colonies are found to be positive; however, none contain inserted sequences longer than 400 base pairs. When these inserts are mapped around the single Eco R1 site, the results suggest that each of these is complementary to the same region of the mRNA. That this region corresponds to the 3' end of the mRNA, is suggested by preliminary nucleotide sequence data, and the observations that the original plasmid cDNA will neither arrest translation of p9ka mRNA, nor protect the

mRNA coding sequence from S_1 nuclease digestion.

The proportion of p9ka mRNA, assayed by filter hybridisation, is 5 to 14 fold higher in poly(A)-containing RNA from elongated myoepithelial-like cell lines (Rama 29 and Rama 401) than with poly(A)-containing RNA from an epithelial stem cell line (Rama 25) (Table 1). This observation is consistent with a previous result, obtained by cell-free translation of isolated mRNA, that the increased synthesis of p9ka by the elongated cells depends upon an increased amount of translatable mRNA (16). The increased proportion of p9ka mRNA in elongated myoepithelial-like cells when compared with that in the epithelial stem cell line (Rama 25) may arise in several ways. It may result from increased transcription of the nuclear gene, from increased processing of nuclear pre-mRNA, from increased transport of mRNA into the cytoplasm, or by an increase in the half life of p9ka mRNA within the elongated cells compared to the epithelial cells. The results in the present paper probably rule out a simple control mechanism in which translatable p9ka mRNA in the myoepithelial-like cells arises by increased processing and transport of a precursor molecule present in both the epithelial and the myoepithelial-like elongated cells, since total nuclear and cytoplasmic poly(A)-containing RNA was used in the hybridisations. However, the results do not distinguish between a control mechanism based on changes in mRNA transcription or one based on altered mRNA half life in the two cell lines. Since it has been shown previously that newly-converted myoepithelial-like cells derived from Rama 25 epithelial cell cultures contain the level of p9ka characteristic of the elongated cell lines (16), a change in control at transcription or a change in half life of p9ka mRNA may occur during the formation of the myoepithelial-like cells in culture.

The question arises as to whether the increase in the proportion of hybridisable p9ka mRNA in the Rama 29 cells compared with that in the epithelial Rama 25 cells can account quantitatively for the increased proportion of p9ka protein that is detected by two-dimensional gel electrophoresis of extracts of Rama 29 cells. Such estimates depend on the accurate determination of small amounts of radioactivity incorporated into p9ka by the epithelial cells. Rama 25 epithelial cells incorporate about 0.03% of the total TCA-insoluble [^{35}S]methionine cpm into p9ka (the average of 19 separate determinations); the proportion of incorporation into p9ka by Rama 29 elongated cells (0.24%) is about 8-fold greater. This corresponds to the 5 to 14-fold increase in abundance of mRNA which hybridises to the p9ka probe, and suggests that the increase in p9ka in Rama 29 cells occurs with a

similar increase in the abundance of p9ka mRNA. This interpretation is strengthened by experiments which suggest that there is a four-fold increase in the proportion of incorporation into the p9ka translation product by Rama 29 cell mRNA in the reticulocyte lysate when compared with the incorporation resulting from translation of Rama 25 cell mRNA.

p9ka has previously only been identified in cultured cells, particularly those derived from the rat mammary gland (15,16); however, the same-sized mRNA for this protein has now been detected in RNA preparations from normal lactating rat mammary gland, rat liver and rat uterus. Quantitative "Dot-Blot" hybridisations show that uterus contains the 780-base mRNA in approximately half the abundance of that found in the cuboidal rat mammary epithelial cell line while in liver the abundance may be greater. These levels of p9ka mRNA in the normal rat tissues may be a reflection of a low level of its expression in all the cells of the tissue, of its expression in a few cells at a particular stage of the cell cycle (49,50) or p9ka mRNA may be present in abundance in a cell type which constitutes a minor population of cells within these normal tissues.

In apparent contrast to the liver and the uterus, lactating rat mammary gland RNA, which contains the 780-base p9ka mRNA, as detected by Northern hybridisation experiments, gives a signal in "Dot-Blot" experiments corresponding to a 5-10 times lower level of p9ka mRNA than in the cultured mammary epithelial cells. In part this may be due to the predominance of milk protein synthesis in the lactating mammary gland. p9ka mRNA is also abundant in the elongated cell line, Rama 401, which has been previously described as a mammary myoepithelial cell (10). In the intact mammary gland the content of myoepithelial cells is unlikely to exceed 5-10% of the total number of cells (Rudland, P.S. unpublished observation), thus yielding a theoretical abundance of at least 10-20 times lower than that anticipated from isolated myoepithelial cells; the abundance of p9ka mRNA in lactating mammary gland is 20-40 times lower than that of the myoepithelial cell line Rama 401. This is consistent with the possibility that if p9ka is confined to a minor population of cells within the normal mammary gland this population may be the myoepithelial cells.

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