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Identification of bacterial clones encoding bovine caseins by direct immunological screening of the cDNA library

(Recombinant DNA; nucleotide sequence; fused proteins; pBR322 plasmid vector; autoradiography; cow)

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SUMMARY

A sensitive immunoassay was used to identify recombinant plasmids carrying cDNA fragments of bovine caseins in the cDNA library from bovine mammary gland mRNA. Colonies grown on nitrocellulose filters were lysed in situ and proteins from the lysates were blotted onto CNBr-activated cellulose filter paper. Antigens covalently bound to CNBr-activated paper or bound to nitrocellulose filters were detected by reaction with antiserum to caseins, followed by ^{125}I-labelled *Staphylococcus aureus* protein A and autoradiography. Six clones were found positive among 5400 of the cDNA library: 3-Al, 3-B2, 3-B5, 3-H7, 2-A5 and 2-C9. The molecular weights of chimeric pre-β-lactamase : casein proteins synthesized in *Escherichia coli* were estimated by immunoblotting. Colony hybridization and nucleotide sequence analysis showed that clone 3-B5 contained a cDNA fragment of bovine κ-casein, clone 3-H7 contained a cDNA fragment of β-casein, while clones 2-A5 and 2-C9 carried cDNA fragments of α_{s1}-casein.

INTRODUCTION

The main function of the mammary gland is to synthesize and secrete a large amount of milk proteins. In bovine milk, the lactoproteins include six primary translation products: caseins α_{s1}, α_{s2}, β, and κ, and the whey proteins, α-lactalbumin and β-lactoglobulin (Fox and Mulvihill, 1982).

Recently, cDNA fragments coding for α_{s1}-and κ-casein were cloned in our laboratory (Kershulite et al., 1983; Gorodetsky et al., 1983). The principal method of clone identification was hybridization selection-translation followed by determination of the nucleotide sequence of the fragments.

The cloning of ds cDNA inserted into the plasmid pBR322 at the *Pst*I site by means of oligo(dC): oligo(dG) tailing often results in expression of a chimeric protein which consists of 183 β-lactamase a.a. residues and at least some protein antigenic

Abbreviations: a.a., amino acid; Ap, ampicillin; bp, base pairs; BSA, bovine serum albumin; buffer B,C, see MATERIALS AND METHODS, section **b**; cDNA, DNA complementary to mRNA; CNBr, cyanogen bromide; ds, double-stranded; PMSF, phenylmethyl sulphonylfluoride; ^{R} (superscript), resistance; ^{S} (superscript), sensitivity; SDS, sodium dodecyl sulfate; Tc, tetracycline; [ ], indicates plasmid-carrier state.

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determinants encoded by the cDNA (Villa-Komaroff et al., 1978). The appropriate clones may be identified by immunoscreening (Villa-Komaroff et al., 1978; Burrel et al., 1979). In the present study we employed this approach to identify *E. coli* clones whose plasmid DNA coded for bovine caseins.

MATERIALS AND METHODS

(a) Construction of recombinant plasmids and transformation

The poly(A)$^+$mRNA from bovine mammary gland was isolated as described earlier (Gorodetsky et al., 1984). The synthesis of ds cDNA was carried out essentially by the procedure of Land et al. (1981) as described by Kershulite et al. (1983). The ds cDNA and *Pst*I-digested pBR322 were tailed with about 20 nucleotides of oligo(dC) and oligo(dG), respectively (Roychoudhury et al., 1976). After annealing, the recombinant plasmids were used to transform *E. coli* JC5183 as described by Kushner (1978). The transformants were screened for the Tc^R, Ap^S phenotype. About 42% of the Tc^R Ap^S colonies (2300) showed a positive reaction with mammary gland-specific [^{32}P]cDNA.

(b) Immunoscreening

The immunoglobulin fraction of rabbit anti-bovine casein serum was isolated and used to prepare specific antibodies to casein by means of affinity chromatography on Sepharose 4B-immobilized casein. The screening procedure was essentially as described by Kemp and Cowman (1981). *E. coli* colonies were replicated onto a nitrocellulose filter which was then placed on an L-agar plate containing 20 μg Tc/ml and incubated overnight at 37°C. The colonies were lysed by placing the nitrocellulose filter on a piece of blotting paper saturated with 0.1 M $NaHCO_3$–1% Triton X-100–2 mg lysozyme per ml for 30 min in a closed dish with an atmosphere saturated with chloroform vapor. CNBr-activated filter paper was saturated with a binding buffer (0.1 M $NaHCO_3$–0.1% Triton X-100) and placed onto the nitrocellulose filter bearing the lysed colonies. The filter sandwich was wrapped in aluminium foil and left for at least 4 h to allow covalent binding of protein. Two filters were obtained as a result of this procedure, the CNBr-paper filter which contained proteins of the lysed colonies covalently bound to cellulose and the nitrocellulose filter with adsorbed proteins of the same lysed colonies.

Before exposure to antibodies the CNBr filters were washed with 1 M glycine–0.1 M $NaHCO_3$–0.5% Triton X-100–2% BSA and the nitrocellulose filters were incubated in 0.14 M NaCl–0.02 M Tris · HCl pH 7.5–3% BSA for 4 h. Both types of filters were then washed with buffer B (0.1 M NaCl–0.1% Triton X-100–0.005 M EDTA–0.02 M Tris · HCl pH 7.5–5 mg/ml BSA) and placed for 4 h into petri dishes containing 6 ml buffer B and 200 μg antibodies. Excess antibodies were removed by washing each filter at least four times with 20 ml of buffer C (0.1% SDS-1% Triton X-100–0.3 M LiCl–0.02 M Tris · HCl pH 7.5) and once more with 20 ml of buffer B. The filters were then incubated with 3 ml of a ^{125}I-labelled *S. aureus* protein A solution (3.3×10^5 cpm/ml) for 1 h, washed as above, blotted, dried at room temperature, and autoradiographed.

(c) Isolation of plasmid DNA

Plasmid DNA was isolated as described by Birnboim and Doly (1979). The DNA was further purified by CsCl density-gradient centrifugation.

(d) Colony hybridization

This was carried out according to the procedure of Grunstein and Hogness (1975).

(e) Hybridization selection-translation

Linearized plasmids containing cDNA inserts were immobilized on Millipore filters (type HA, 0.45 μm) and hybridized with total mammary gland poly(A)$^+$RNA as described by Maniatis et al. (1982). After elution, the RNA was translated in a cell-free reticulocyte system and immunoprecipitated with the specific antiserum and a 10% suspension of formalinized *S. aureus* cells. The immunoprecipitated proteins were solubilized and separated by electrophoresis in a 12% SDS-polyacrylamide gel (Laemmli, 1970).

(f) Gel electrophoresis of DNA fragments

The digestion of recombinant DNA with restriction enzymes was performed according to the instructions furnished by the supplier. Restriction fragments were separated on horizontal 1% agarose gels.

(g) Nick-translation

The nick-translation labelling of DNA probes was performed as described by Rigby et al. (1977).

(h) Analysis of recombinants by Western blotting

Colonies scored as positive in the immunoscreening procedure were grown overnight in 10 ml of L broth containing 15 μg Tc/ml. The cells were harvested by centrifugation and resuspended in 75 μl of 25% sucrose, 50 mM Tris · HCl pH 8.0, 2% Triton X-100, 5 mg/ml lysozyme. DNase I (final concentration 0.5 μg/ml) and PMSF (final concentration 0.5 mM) were then added and the cells incubated at room temperature for 2 h. The lysates were centrifuged at 20000 rev./min, 4°C for 40 min (Beckman JA-21 rotor). Proteins in the supernatant were separated electrophoretically in SDS-polyacrylamide gels and transferred to nitrocellulose filters as described by Towbin et al. (1979). After the transfer, the filters were incubated with antibodies and [^{125}I]protein A of *S. aureus* as described above in section **b**.

(i) Nucleotide sequencing

Sequencing was performed according to the method of Maxam and Gilbert (1980) after recloning the cDNA fragments into pUR222 (Rütter et al., 1981).

RESULTS

(a) Immunoscreening of recombinant clones coding for caseins

A population of 5400 colonies carrying recombinant plasmids were screened for the production of casein antigenic determinants, and a few gave intense spots on the autoradiograms with anti-casein antibodies and [^{125}I]protein A. The clones were confirmed to be immunoreactive by repeated screening of a small population, and designated 3-A1, 3-B2, 3-B5 and 3-H7 (Fig. 1). It is noteworthy that the colonies produced a noticeable background reaction on nitrocellulose, and a simultaneous analysis on CNBr-activated cellulose allowed exclusion of such artifacts. The immunological specificity of the reaction was confirmed by adding unlabelled com-

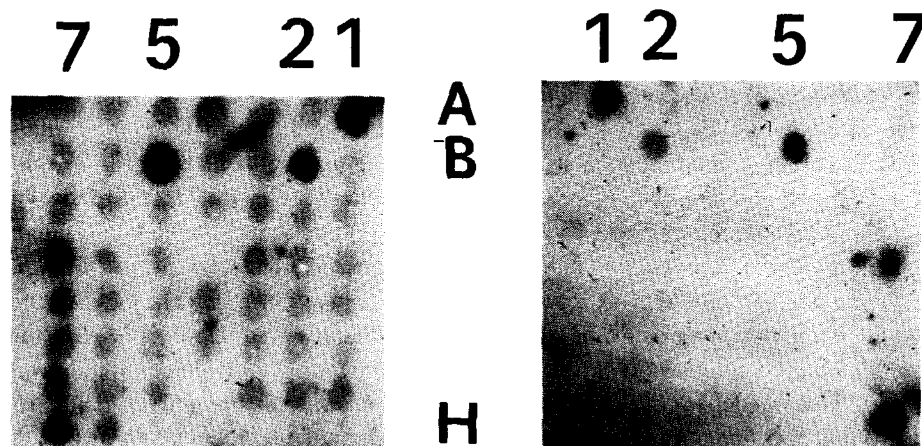

Fig. 1. Immunoscreening of bacterial clones carrying cow mammary gland cDNA sequences. Following lysis, colonies were immobilized on nitrocellulose (left) or CNBr-activated cellulose (right) filters. The filters where then treated with antibodies to casein and ^{125}I-labelled protein A. Columns are labelled 1–7 and rows A–H. All colonies of this minilibrary were given the prefix 3, e.g. 3-A1, 3-H7, etc. Clones 3-A1, 3-B2, 3-B5 and S-H7 produced casein antigenic determinants.

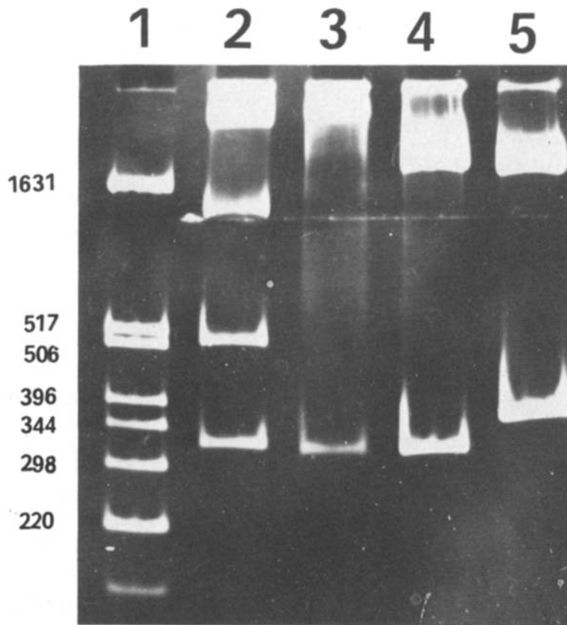

Fig. 2. Size determination of cDNA fragments of clone 3-B5 (lane 3), 3-H7 (lane 4) and 3-B2 (lane 5). Electrophoresis was in a 6% polyacrylamide gel following *Pst*I digestion of the plasmid DNA. The M_r markers used were *Hinf*I fragments of pBR322 (lane 1) and *Pst*I fragments of clone 1-A3 plasmid DNA (lane 2). Fragment sizes (in bp) are shown at the left.

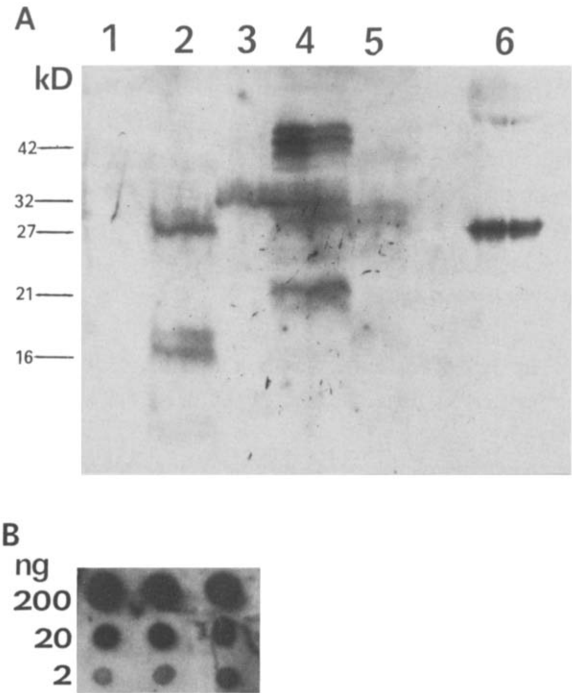

Fig. 3. Immunoassay of caseins. (Panel A) Immunoblotting of bacterial lysate proteins. Shown are the control plasmid pBR322 (lane 1), clone 3-A1 (lane 2), clone 3-B2 (lane 4), clone 3-H7 (lane 5) and 50 ng β-casein (lane 6). The proteins were separated by electrophoresis in a 15% polyacrylamide gel, transferred to a nitrocellulose filter and treated with antibodies to casein and [^{125}I] protein A. kD = kDal. (Panel B) Calibration of interaction of casein with antibodies against it and [^{125}I] protein A. The quantity of casein immobilized on the filter (in ng) is shown at the left.

petitive casein to the reaction mixture. The sizes of the cDNA inserts in plasmids 3-A1, 3-B2, 3-B5 and 3-H7 were about 300, 350 and 330 bp, respectively (Fig. 2).

Cell extracts of *E. coli* JC5183 carrying recombinant or vector plasmids were analyzed by Western blotting to determine the sizes of the chimeric pre-β-lactamase: casein proteins (Fig. 3). The pre-β-lactamase residue had an M_r of 20000. *E. coli* cells containing the recombinant plasmids gave a complex antigen pattern as revealed by anti-casein antibodies and radioiodinated protein A. The major protein antigen species of 32 kDal in the 3-B2 extract was absent from extracts of *E. coli* [pBR322]. Clones 3-A1 and 3-H7 each produced four polypeptides 27, 17, 16 and 12 kDal, reacting with casein antibodies. Clone 3-B5 showed three polypeptide groups of 40–42, 32–38, and 19–21 kDal. The smaller polypeptides seem to have arisen from processing of the largest antigenic protein.

(b) Hybridization selection-translation

Since we did not have monospecific antisera to individual bovine caseins at our disposal, the specificity of the nucleotide sequences (α_{s1}, β, α_{s2} or $\varkappa$) contained in our clones was assayed by the following three methods: hybrid selection-translation, colony hybridization to specific molecular probes in situ, and nucleotide sequence analysis. The first of the assays employed the plasmid DNA to select for specific mRNA by means of hybridization, followed by in vitro translation, immunoprecipitation, and M_r determination of the polypeptides. The translation products obtained from our clones are shown in Fig. 4. Lane 6 corresponds to proteins encoded by total unfractionated mRNA after immunoprecipitation with anti-casein antiserum. The DNA of

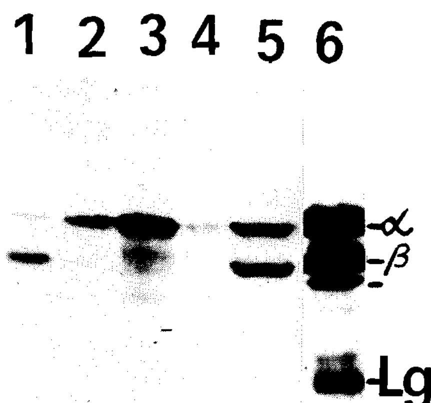

Fig. 4. Hybridization selection-translation. Translation products of total mammary gland mRNA following immunoprecipitation with antibodies to total milk proteins were used as a control (lane 6). ^{35}S-labelled translation products of mRNA selected by hybridization were immunoprecipitated and separated in a 15% polyacrylamide gel. Total mRNA was hybridized with plasmid DNA of clones 3-B2 (lane 1), 2-C9 (lane 2), 2-A5 (lane 3), 2-H11 (lane 4), 2-A2 (lane 5). Shown at the right are the main translation products of the mammary gland mRNA: α_{s1}-casein (α), β-casein (β), $\varkappa$-casein (below β) and β-lactoglobulin (Lg).

clones 3-A1 and 3-H7 failed to select a translationally active mRNA under our conditions. The mRNA hybridized with clone 3-B2 DNA, on the other hand, and produced a peptide reactive with anti-casein antibodies and having an M_r close to that of the β-casein precursor. Our assumption was that clone 3-B2 contained a fragment of β-casein or some related gene.

(c) Colony hybridization

The next stage consisted of a more accurate identification of casein-expressing clones by means of colony hybridization in situ with ^{32}P-labelled probes, the cloned cDNA fragment coding for α_{s1} casein from a.a. 12 through 176 and from a.a. 107 through 199, and the $\varkappa$-casein fragment from a.a. 70 through 148 (Kershulite et al., 1983; Gorodetsky et al., 1983). The fragments were excised from the respective plasmids by *Pst*I for the α_{s1}-casein probes and by *Hin*dIII + *Pst*I for the $\varkappa$-casein probe. The resulting fragments were isolated by means of electrophoresis in a 6% polyacrylamide gel, followed by extraction from the gel and nick-translation labelling with [α-^{32}P]dCTP to the specific radioactivity of 4–6 × 10^7 cpm/μg. The labelled probes were used for colony hybridization. The response to the left or right portion of the α_{s1}-casein coding sequence was negative, while clone 3-B5 did react with the $\varkappa$-casein probe (Fig. 5).

We then tried to determine whether the other expressing clones contained cDNA inserts representing overlapping sequences of a single casein or different caseins. To answer this question, we excised

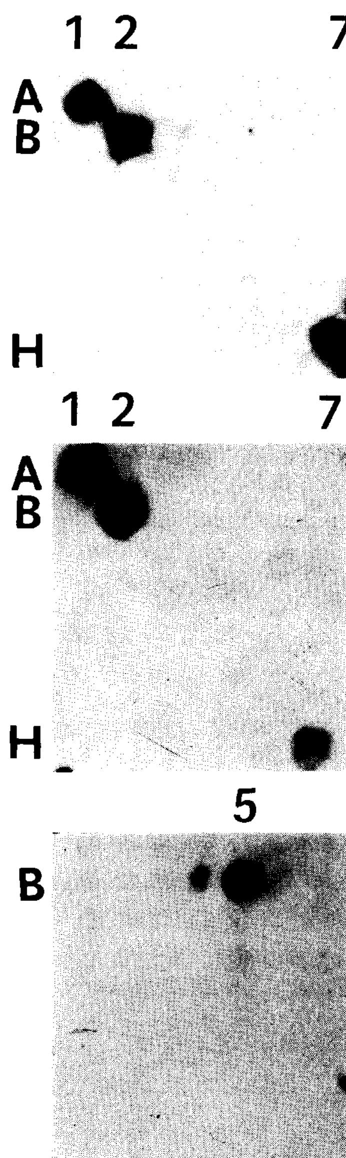

Fig. 5. Hybridization of cDNA minilibrary (arrangement the same as in Fig. 1) with ^{32}P-labelled probes: a 300-bp *Pst*I fragment of clone 3-H7 plasmid DNA (upper panel), a 350-bp *Pst*I fragment of clone 3-B2 plasmid DNA (center panel), and 250-bp *Hin*dIII/*Pst*I fragment of clone pKcas $\varkappa$-2 cDNA coding for $\varkappa$-casein (bottom panel).

PstI fragments from cDNA clones 3-B2 and 3-H7 and ^{32}P-labelled them by nick-translation. Three clones 3-A1, 3-B2 and 3-H7 produced hybridization signals with the labelled probes, the signal being the strongest for homologous and somewhat weaker for heterologous hybridization (Fig. 5). This suggested that clones 3-A1, 3-B2 and 3-H7 contained somewhat similar sequences.

(d) Partial nucleotide sequence of clone 3-H7

The nucleotide sequence of a clone 3-H7 subfragment is shown in Fig. 6. A comparison of it with the known amino acid sequences of bovine caseins (Ribadeau-Dumas et al., 1972) unequivocally indicated that the clone represented a portion of the β-casein gene. The clone received the name pKcasβ-7 and was used as a probe to search for clones with larger fragments of β-casein cDNA. Such clones with inserts of approx. 700 bp were indeed found in the mammary gland cDNA clone library.

An analysis of a partial nucleotide sequence of clone 3-B2 revealed certain zones of homology with the pKcasβ-7 sequence. However, the a.a. sequences predicted on the basis of this nucleotide sequence did not correspond to any of the known bovine caseins. This issue requires further investigation.

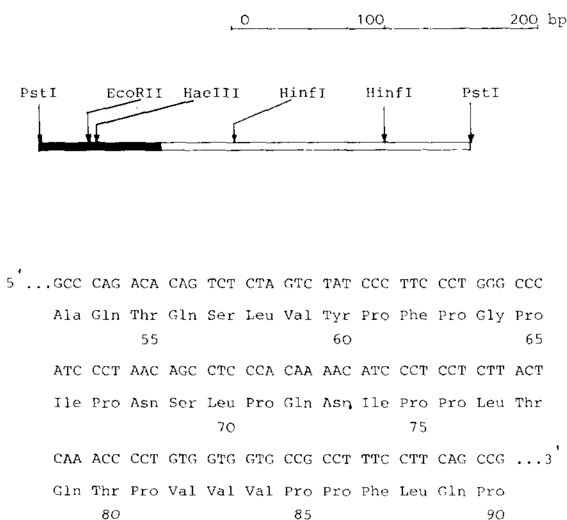

Fig. 6. Restriction map and a partial nucleotide sequence of the cDNA insert of clone 3-H7 (see RESULTS, section **a**). The nucleotide sequence is represented by a black bar and codes for the bovine β-casein region from a.a. 53 through a.a. 90; this clone was designated pKcasβ-7 (see RESULTS, section **d**).

(e) A search for clones expressing α_{s1}-casein

Direct immunoscreening of the mammary gland cDNA library failed to reveal clones producing α_{s1}-casein. This could be due to instability of the chimeric protein containing a α_{s1}-casein fragment, so that the immunoscreening signal was fainter than for β- or κ-casein. We therefore investigated a minilibrary of clones enriched with casein cDNA. The minilibrary was selected from clones that showed positive hybridization with 16S poly(A)$^+$mRNA of the mammary gland. Earlier we demonstrated that the α_{s1}-casein mRNA was in that poly(A)$^+$mRNA fraction. The same minilibrary was also screened by hybridization with the ^{32}P-labelled α_{s1}-casein probe coding for a.a. 107 through 199 (Fig. 7). Among clones showing hybridization with the α_{s1}-casein probe, 2-A5 and 2-C9 did produce antigenic determinants of α_{s1}-casein (Fig. 7, left). Hybridization selection-translation showed also that clone 2-A5 DNA hybridized with α_{s1}-casein mRNA (Fig. 4). The cDNA insert sizes were 800 and 990 bp for 2-C9 and 2-A5, respectively. As was shown by restriction analysis and partial nucleotide sequencing, clone 2-A5 contained an almost complete sequence of α_{s1}-casein cDNA without a small 3′-terminal portion.

DISCUSSION

The immunochemical assay of antigenic determinants is an effective method of screening bacterial clones for gene products encoded by foreign DNA fragments: it allows detection of incompletely translated protein products as well as proteins that cannot be easily assayed by other methods. A number of studies on various modifications of the immunological clone screening in situ have been reported in the last few years, employing immobilized antibodies (or fragments thereof) reacting with proteins of lysed colonies (Villa-Komaroff et al., 1978; Burrel et al., 1979; Erlich et al., 1978), or colony proteins immobilized after lysis and interacting with specific antibodies (Kemp and Cowman, 1981; Whittle et al., 1982; Helfman et al., 1983). The sensitivity of the reaction can be markedly improved by using *S. aureus* [^{125}I]protein A, which interacts with the

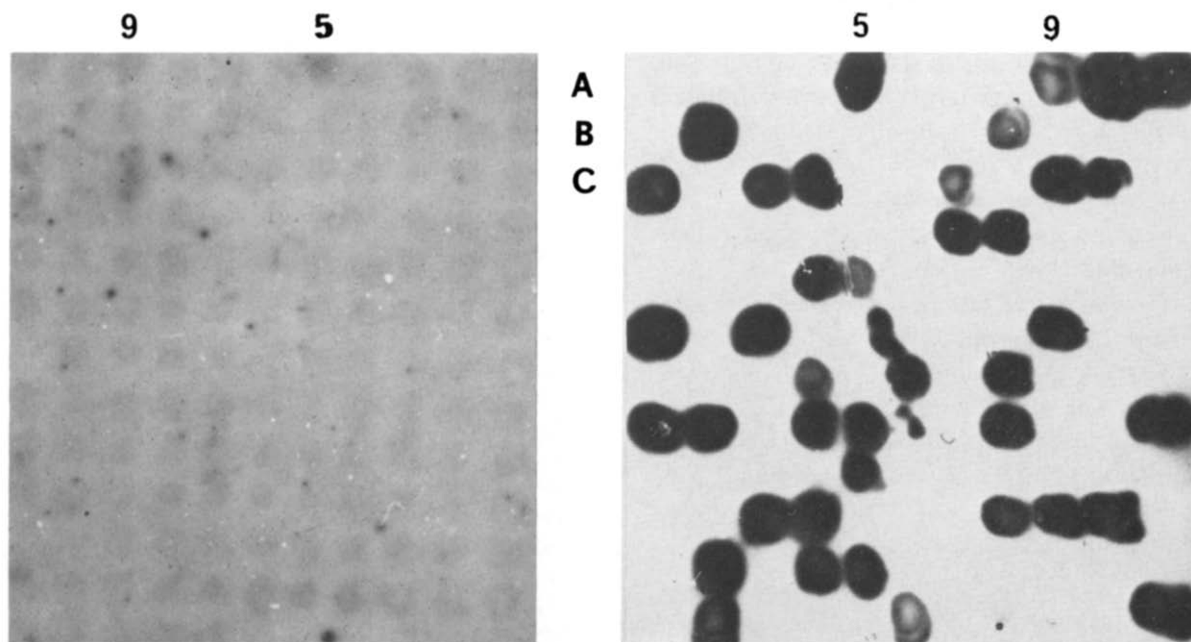

Fig. 7. Immunoscreening of the mammary gland cDNA minilibrary, selected by hybridization with 16S poly(A)$^+$ mRNA, for expression of casein antigenic determinants. Columns are labelled 1–11 and rows A–M. All colonies of this minilibrary were given the prefix 2, e.g. 2-A1, 2-A5, etc. Clones 2-A5 and 2-C9 produced casein antigenic determinants (left). Hybridization of the same minilibrary with a molecular probe for α_{s1}-casein: ^{32}P-labelled 750-bp *Pst*I fragment of clone pKcas α-17 coding for α_{s1}-casein from a.a. 107 through a.a. 199 (right panel). Left filter was treated with antibodies to casein and ^{125}I-labelled protein A.

Fc region of the antibody (Kemp and Cowman, 1981; Erlich et al., 1978).

We tested various modifications of immunoscreening and found the combined procedure employing both nitrocellulose and CNBr-activated cellulose to immobilize lysate proteins to be the most efficient (Kemp and Cowman, 1981; Helfman et al., 1983). The method combines high effectiveness of protein immobilization on nitrocellulose and low background values for proteins covalently bound to activated cellulose. We also preferred to immobilize antigens rather than antibodies since that allowed an efficient use of the indirect method with highly labelled protein A. Clones found by means of immunoscreening can be used to construct molecular probes for detection of large nontranslated inserts. We used this approach for screening clones carrying large β-casein cDNA inserts. Work on determining the complete nucleotide sequence of β-casein cDNA is now in progress.

We observed a good correlation between the cDNA insert sizes and the sizes of casein fragments in the chimeric proteins of β-casein clones and clone 3-B2. The correlation did not hold, however, for clone 3-B5, which codes for ϰ-casein. The origin of the 40-kDal polypeptides that reacted with antibodies remains unclear. The cDNA insert of this clone (300 bp) can encode a protein of only 10 kDal; thus the β-lactamase fusion protein should correspond to about 30 kDal. It is possible that the cloned cDNA insert did not contain any terminators and the chimeric protein was a read-through product into the C-end of β-lactamase.

It also remains unclear which protein is encoded by the cDNA of clone 3-B2. This could be some minor casein of cow's milk. It should also be noted that so far not all milk or mammary-gland proteins are known; a few years ago, for example, the nucleotide sequence of a novel whey protein, WAP, was determined (Hennighausen et al., 1982).

Some more convenient immunoscreening versions have become available recently. Thus, Rüther and Müller-Hill (1983) proposed a series of expression plasmids suitable for the screening of specific cDNAs in a clone library. Their method revealed clones expressing mouse β-casein antigenic determinants.

For a cDNA clone to be detectable by immuno-

screening the direction and frame of β-lactamase and cDNA transcription should coincide, the cDNA-encoded protein fragment should contain the antigenic determinant, and the fused protein should have certain stability in *E. coli*. It is no wonder, therefore, that only 6 clones expressing antigenic determinants of different caseins have been found among 5400 clones of the cDNA library. Non-expressing clones of the library had inserts ranging from 250 to 1000 bp. As nucleotide sequence and restriction analyses have shown, they coded for different mRNA regions: 5′-terminal, central and 3′-terminal. The complete α_{s1}-casein cDNA molecule was assembled from two partially overlapping clones (Kapelinskaya, T.V., personal communication), while the β-casein molecule was constructed from three clones of the cDNA library.

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