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Cloning of mouse β-casein gene sequences

(Recombinant DNA; pBR322 cloning vehicle; messenger RNA purification; hybridizations)

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SUMMARY

Casein messenger RNAs (mRNAcsn) were purified from lactating mammary glands of BALB/c mice and used as a starting material for cloning of casein gene sequences. Double-stranded casein cDNA (ds-cDNAscn) was prepared and blunt-end ligated to *Hin*dIII-specific DNA linker molecules. After digestion with *Hin*dIII, the ds-DNAcsn was inserted into the *Hin*dIII site of plasmid pBR322, using T4 DNA ligase. *Escherichia coli* strain RH202 was transformed with the hybrid plasmids, and transformants were selected for resistance to ampicillin. Electrophoresis of *Hin*dIII-digested hybrid plasmid DNAs, followed by Southern transfer and hybridization to [^{32}P]cDNAcsn, revealed that one of the hybrid-plasmid-containing colonies, designated pCas51, contained a 400-bp insert which hybridized to the [^{32}P]cDNAcsn. Purification of the individual casein mRNAs (mRNAcsn α, β and γ) and solution hybridization of nick-translated insert DNA to each of these revealed that pCas51 contained sequences complementary primarily to mRNAcsn β.

Caseins constitute the major milk proteins and their synthesis is under hormonal control. The induction of the mRNAs for the three major caseins is the result of the synergistic stimulatory action of a glucocorticoid (hydrocortisone) and a polypeptide (prolactin) hormone (Banerjee, 1976). Accumulation of mRNAcsn as measured by hybridization to cDNAcsn requires the presence of both hormones (Ganguly et al., 1979; 1980; Mehta et al., 1980). We were interested, therefore, in determining the specific regulatory role of each of these two hormones at the transcriptional level of control of the casein gene. Accordingly, studies were undertaken to amplify the casein gene sequences by cloning in a bacterial plasmid, so that cloned DNA sequences could be hybridized to labeled nuclear transcripts synthesized in vitro, in conditions of DNA excess (McKnight and Palmiter, 1979). Since casein mRNAs are relatively abundant in mammary gland alveolar cells, we used purified casein mRNAs from lactating mammary glands of BALB/c mice as a starting material for the cloning experiments.

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Abbreviations: bp, base pairs; cDNAcsn, DNA complementary to casein messenger ribonucleic acid; csn, casein; ds-cDNAcsn, double-stranded cDNAcsn; dTT, dithiothreitol; kb, kilobase pairs; mRNAcsn, casein messenger ribonucleic acid; SH-agarose, sulfhydryl agarose; ss-cDNAcsn, single-stranded cDNAcsn; TCA, trichloroacetic acid.

Total mammary gland RNA was isolated by extraction with phenol-chloroform (Ganguly et al., 1979), mRNAcsn purified and used as the template for synthesis of a single-stranded cDNA, using AMV reverse transcriptase as previously described (Ganguly et al., 1979). Double-stranded DNA synthesis was performed using 25 units/ml *E. coli* DNA polymerase I and 20 μg/ml of ss-cDNAcsn, at 30°C for 60 min. Starting with about 25 μg of purified mRNA, we obtained 2 μg of ss-cDNAcsn and 1.5 μg of ds-cDNAcsn.

After S1 digestion, ds-cDNAcsn was electrophoresed on a 1.5% agarose gel in Peacock's buffer (Peacock and Dingman, 1967) at 150 V for 2 h. Molecules of ds-cDNAcsn ranging in size from 600 to 1 500 bp were cut out, and eluted. DNA "linker" sequences specific for the restriction endonuclease *Hin*dIII (C-C-A-A-G-C-T-T-G-G) were phosphorylated and labeled at the 5'-end using [γ-^{32}P]dATP (10 Ci/mmol), and the ^{32}P-labeled linker DNA was ligated to ds-cDNAcsn using T4 DNA ligase (BRL). The bacterial plasmid pBR322 and the ds-cDNAcsn were separately digested with *Hin*dIII and then ligated together with T4 DNA ligase in a 50-fold molar excess of plasmid DNA.

E. coli strain RH202 (Adams et al., 1979) was transformed with the recombinant plasmid DNA. The cells were grown at 37°C for 90–120 min with good aeration to allow expression of the transformed genes before selection. Transformants were selected by plating onto T-agar supplemented with 40 μg/ml ampicillin. They were then scored for tetracycline sensitivity because the *Hin*dIII cloning site lies at the promoter end of the gene for resistance (Rodriguez et al., 1979; Widera et al., 1978), and some transformants with cloned genes might not express resistance. Purified preparations of plasmid DNA were obtained essentially as described (El-Gewely and Helling, 1980) and with yields of 2 to 5 mg of supercoiled DNA per litre culture. Recombinant plasmid DNA preparations, with and without digestion by appropriate restriction endonucleases, were electrophoresed through 1% agarose gels and the bands transferred to nitrocellulose filters by the procedure of Southern (1975) as modified by Thomas (1980). Of the colonies thus screened, one colony (pCas51) was found to contain an inserted sequence of about 400 bp (Fig. 1), though it still conferred tetracycline resistance. Hybridization of the gel bands to [^{32}P]-

Fig. 1. Hybridization of [^{32}P]cDNAcsn with recombinant plasmid pCas51. Left panel: Electrophoresis of pCas51 DNA either without digestion or after digestion with *Hin*dIII or *Eco*RI. Staining is with ethidium bromide. Right panel: Autoradiography of the gel bands shown in the left panel after Southern blotting and hybridization to [^{32}P]cDNA.

cDNAcsn showed that the inserted DNA segment hybridized to this probe (Fig. 1) and, therefore, contained a cloned segment of DNA corresponding to part of one of the murine casein genes.

The restriction endonuclease cleavage patterns of the complete pCas51 plasmid DNA and of the cloned fragment showed that *Alu*I, *Pst*I, and *Rsa*I each cut the cloned sequence at a single site (Fig. 2). The following enzymes did not cut the cloned DNA: *Ava*I, *Bam*HI, *Eco*RI, *Hae*III, *Hin*dIII, *Hin*fI, *Mbo*I, *Sal*I, *Taq*I.

Individual casein mRNAs were purified by electrophoresis of total oligo(dT)-bound mammary gland RNA on a 1.5% agarose preparative gel. Bands corresponding to each of the two 15 S casein mRNAs

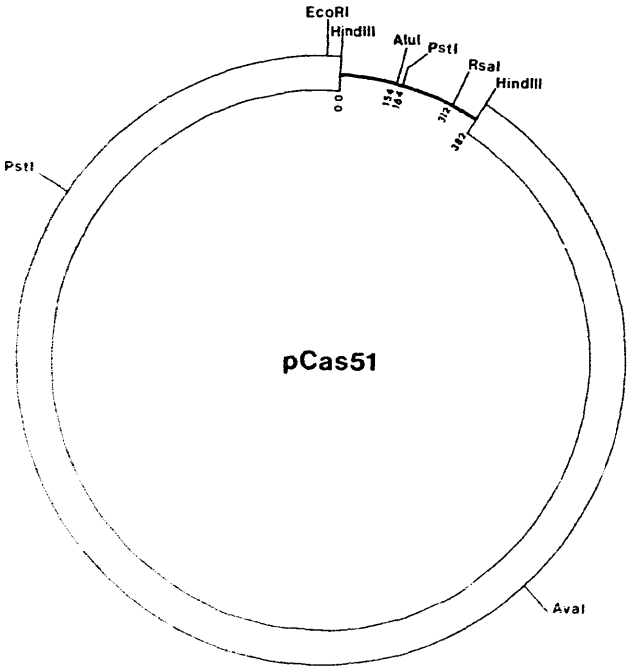

Fig. 2. Map of pCas51. The size of the plasmid (double line) is approx. 4.65 kb. The insert DNA (single line) has single restriction sites for *AluI*, *PstI* and *RsaI*. The numbers indicate the size of the fragments in base pairs.

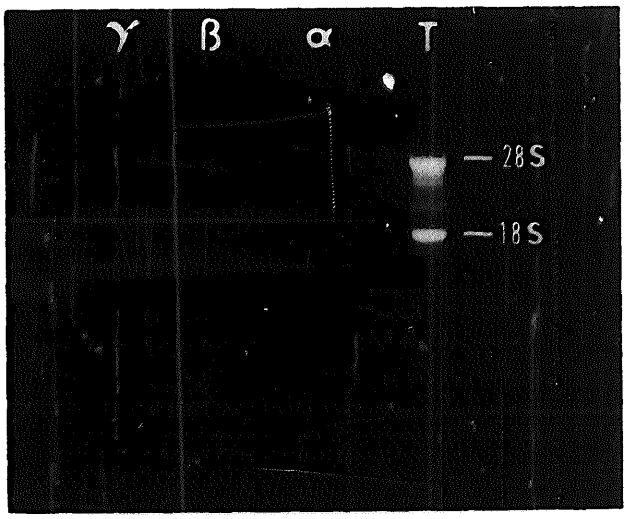

Fig. 3. Electrophoresis of purified casein mRNAs on 1.5% agarose gels. Oligo(dT)-bound RNA from 7–12 days lactating mice was first electrophoresed on a preparative agarose gel. 50 μg of RNA was applied to each of twelve lanes after heat denaturation at 70°C for 10 min. The bands were visualized by staining with ethidium bromide. Bands corresponding to the two 15 S casein mRNAs (mRNAcsn α and β) and the 12 S casein RNA (mRNAcsn γ) were excised from the gel and the RNA was eluted. Aliquots of each purified RNA were again heat denatured and re-electrophoresed on analytical agarose gels. 10 μg of total RNA (T) has been electrophoresed on an adjacent lane (extreme right) to indicate the positions of the 28 S and 18 S ribosomal RNAs. An RNA band of molecular weight close to that of the mRNAcsn γ could not be eliminated from the preparation.

(mRNAcsn α and mRNAcsn β) and the 12 S casein mRNA (mRNAcsn γ) were excised separately and eluted. Fig. 3 shows that each purified RNA migrates as a discrete band in analytical agarose gel electrophoresis.

pCas51 insert was nick-translated as described by Maniatis et al. (1975), yielding the ^{32}P-specific activity of 5×10^7 to 1×10^8 cpm/μg. Solution hybridizations of such ^{32}P-labeled DNA to purified mRNAcsn α, β, and γ were done using modifications of the procedure described by Bishop (1972). Table I shows that the cloned sequence is complementary primarily to mRNAcsn β. This result was confirmed by hybridization of each RNA with the insert DNA and subsequent translation in a rabbit reticulocyte lysate cell-free system followed by immunoprecipitation with mouse casein antibody. Only the synthesis of casein from mRNAcsn β was completely inhibited by the hybridization (data not shown).

TABLE I

Hybridization of purified casein mRNAs to [^{32}P]pCas51 insert DNA

Sample	Time of hybridization (h)	DNA hybridized [a] (cpm)	% Hybridization [b]
mRNAcsn α	12	296	2.43
	24	343	1.68
mRNAcsn β	12	1490	83.10
	24	1594	86.21
mRNAcsn γ	12	272	0.81
	24	335	1.14
Wheat germ tRNA	12	237	0
	24	316	0
pCas51 insert DNA	12	260	–
(reannealing)	24	318	–

[a] 3000 cpm of double-stranded pCas51 insert DNA was used per reaction. The DNA was denatured by heating at 100°C for 5 min and quick chilling. immediately before adding to the reaction mixture. Hybridized cpm were determined by S1 nuclease digestion and subsequent TCA precipitation.

[b] Percent hybridization values were obtained after correcting for the reannealing of the double-stranded DNA and for the fact that only one strand hybridizes to its complementary RNA.

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