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The gene for the hypothalamic peptide hormone oxytocin is highly expressed in the bovine corpus luteum: biosynthesis, structure and sequence analysis

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Expression of the vasopressin and oxytocin genes has been described so far only in the hypothalamus. We report here that at least the oxytocin gene is highly transcribed in the bovine corpus luteum during the mid-luteal phase of the oestrous cycle. Luteal cDNA sequence analysis as well as cell-free translation studies showed that the luteal mRNA is essentially similar to that in the hypothalamus, except that in the corpus luteum the poly(A) tail of this mRNA is shorter. When calculating the relative amounts per organ, the active corpus luteum produces ~250 times more oxytocin mRNA than a single hypothalamus.

Key words: cDNA sequence/neurophysin/oxytocin/vasopressin/corpus luteum

Introduction

The nonapeptide hormones vasopressin and oxytocin, are synthesized by the magnocellular neurones of the hypothalamus and transported axonally to the neurohypophysis where they are released into the blood circulation. Studies of the expression of the oxytocin and vasopressin genes have shown that they are synthesized as much longer polyproteins with their corresponding neurophysin carriers; the vasopressin, but not the oxytocin precursor, includes additionally a glycoprotein moiety at its C terminus (Land *et al.*, 1982,1983; Schmale *et al.*, 1983; Ivell and Richter, 1984; Ruppert *et al.*, 1984).

Vasopressin and oxytocin have also recently been immunologically identified in some steroid-producing tissues such as the ovarian corpus luteum, testis and adrenal gland (Wathes and Swann, 1982; Wathes *et al.*, 1983; Flint and Sheldrick, 1982; Nicholson *et al.*, 1984). However, it is not clear whether the two hormones found in these organs are taken up from the circulation to be released later on specific stimulation of the tissue, or whether they are synthesized locally. Nor is it known what functions these peptides may have in the peripheral organs.

Here we report our investigations of the bovine corpus luteum using a combination of DNA hybridization, cDNA cloning and *in vitro* translation, firstly to establish whether or not the hormones vasopressin and oxytocin are locally synthesized, and secondly to determine whether the synthesis proceeds *via* a mRNA and polyprotein precursor as in the hypothalamus, or whether, as has been demonstrated for other hormones, there are tissue-specific differences in transcription (Amara *et al.*, 1982; Kitamura *et al.*, 1983) and translation products (Herbert, 1981).

Results

Analysis of luteal mRNA

Because of the high homology between the oxytocin and vasopressin genes, especially in the central region corresponding to the neurophysin (Figure 1a; Land *et al.*, 1982,1983), specific probes can only be prepared from the 3′ regions of the cDNA molecules. Using these 3′ probes, poly(A)$^+$ RNA isolated from bovine corpora lutea of various sizes and appearance, and hence stages in the luteal cycle, were screened by dot-blot analysis for products of the nonapeptide genes (Figure 1b). Oxytocin mRNA was present in all corpora lutea, with the greatest amount in a corpus luteum (III, Figure 1b) which from size and external appearance corresponds to the peak of the luteotrophic phase. Vasopressin mRNA was detectable in this corpus luteum (III, Figure 1b) at a level three orders of magnitude lower than the corresponding oxytocin mRNA.

Luteal cDNA sequence

Southern analysis of genomic DNA prepared from calf cerebellum and digested with different restriction enzymes using a ^{32}P-labelled DNA probe specific for oxytocin, gives always only a single positively hybridizing DNA fragment (Ivell and Richter, unpublished). Thus, in the calf, oxytocin synthesis must result from the activity of only a single oxytocin gene. However, alternative splicing might yield an oxytocin mRNA which is not identical with that transcribed in the hypothalamus. Therefore poly(A)$^+$ RNA from corpus luteum was cloned into the plasmid pUC9 by the direct method of Heidecker and Messing (1983). Of ~300 galactosidase-negative, ampicillin-resistant colonies screened with the 3′ oxytocin-specific probe, four gave a positive signal. The plasmid with the longest insert was sequenced by the method of Maxam and Gilbert (1980) to give the sequence in Figure 2. The cloned cDNA does not contain the initiation site for translation, hence only four amino acids of the signal peptide sequence can be deduced. Otherwise the nucleotide sequence is identical to that reported for the hypothalamic cDNA (Land *et al.*, 1983) and the bovine oxytocin gene (Ruppert *et al.*, 1984).

Comparison of hypothalamic and luteal mRNA

The poly(A)$^+$ RNA from corpus luteum was also hybridized to probes derived from different parts of the cDNA (Land *et al.*, 1982,1983) using the Northern blot technique (Figure 3). Luteal oxytocin mRNA migrated as a single band of ~630 bases (Figure 3, lane 4), somewhat smaller than oxytocin mRNA of hypothalamic origin (~690 bases; Figure 3, lane 3), and was identified using DNA probes derived from the 5′ end of the hypothalamic cDNA (Figure 3, lane 10), the central region (Figure 3, lane 8) and the 3′ end (Figure 3, lane 4). These fragments correspond approximately to exons A, B and C, respectively, of the recently sequenced bovine gene

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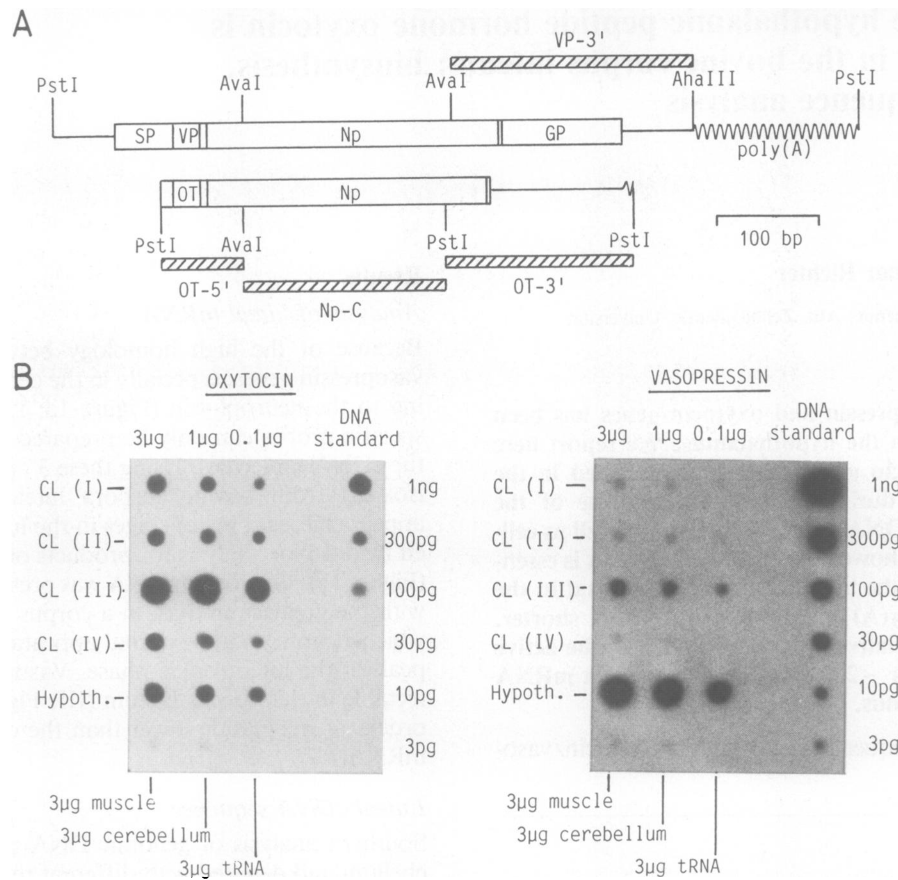

Fig. 1. (A) Scheme illustrating the derivation of specific DNA probes from the bovine hypothalamic cDNAs for the vasopressin-neurophysin II (upper) and oxytocin-neurophysin I (lower) precursors. **(B)** Dot-blot analysis of poly(A)$^+$ RNA from different bovine corpora lutea and hypothalamus. **Left:** hybridized with 3′ oxytocin-specific probe (OT-3′); **right:** hybridized with the 3′ vasopressin-specific probe (VP-3′). As calibration standards, increasing quantities of OT-3′ subcloned in pUC8 **(left)** or the entire vasopressin-neurophysin II cDNA cloned in pBR322 **(right)** were spotted as indicated. In controls (not shown) no cross-reactivity between the two probes could be detected.

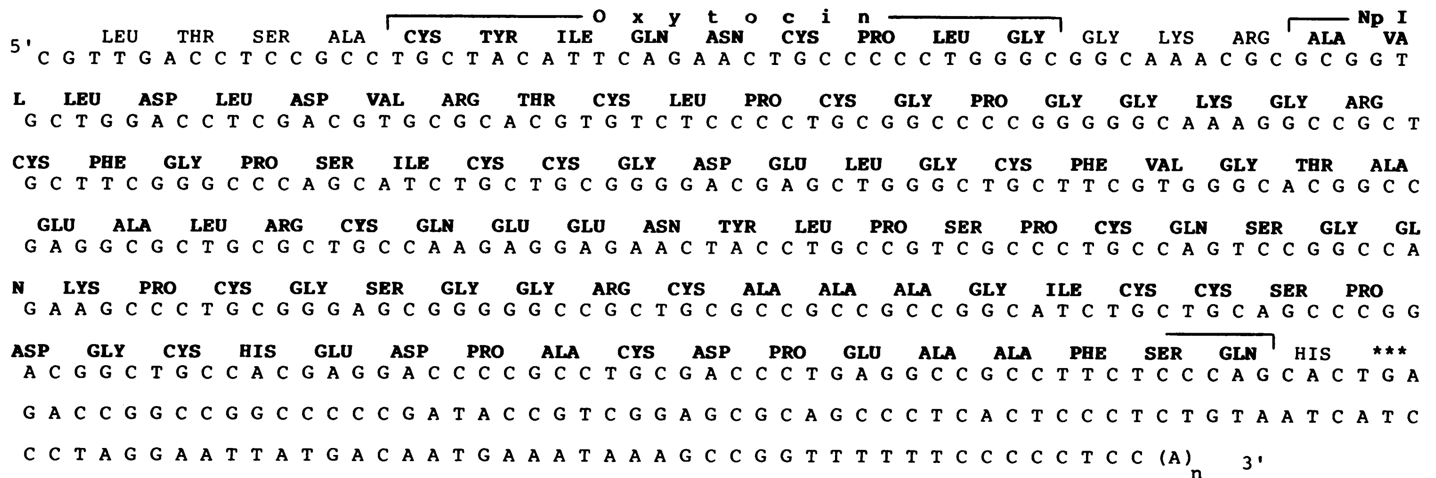

Fig. 2. cDNA and amino acid sequence corresponding to the bovine luteal mRNA for the oxytocin precursor. The 5′ end is truncated in the region of the signal peptide. Enclosed in boxes are the hormone oxytocin and the neurophysin I polypeptide.

(Ruppert *et al.*, 1984). With the high stringency of the hybridization conditions employed, this also shows that the luteal oxytocin mRNA is similar to that of hypothalamic origin and is not a product either of a second gene or alternative splicing. This confirms the sequencing data from the cloned cDNA. The vasopressin mRNA detected by dot blots was below the limit of detection for the Northern blot procedure (Figure 3, lane 2).

The difference in length of the hypothalamic and luteal mRNAs may be a consequence of differential 3′ polyadenylation. To substantiate this, incubation of the poly(A)-rich RNA with oligo(dT) and RNase H, which only cleaves double-stranded or heteroduplex RNA, reduced the size of both hypothalamic and luteal oxytocin mRNA to a similar size of ~590 bases (Figure 2, lanes 13 and 14). Thus the tissue-specific size heterogeneity of the oxytocin mRNA is a func-

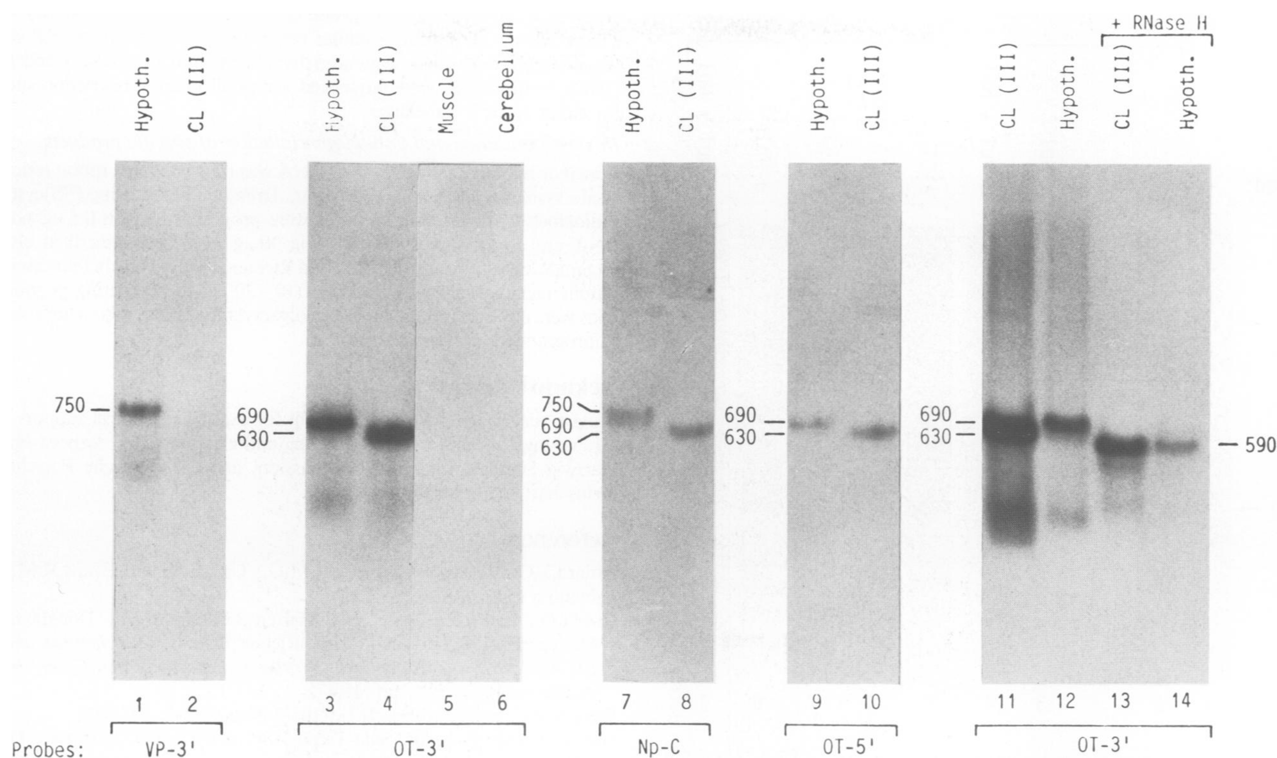

Fig. 3. Blot hybridization analysis of poly(A)$^+$ RNA separated by denaturing agarose gel electrophoresis. **Lanes 1–10:** 3 μg of poly(A)$^+$ RNA from the tissues as indicated were hybridized with different [^{32}P]nick-translated DNA probes as described in Figure 1A. Hybridization with DNA fragment Np-C which is 100% homologous between both vasopressin and oxytocin genes reveals a double band for the hypothalamus corresponding to the vasopressin and oxytocin mRNAs, but only a single band in the corpus luteum. **Lanes 13** and **14** show the results of treatment with oligo(dT) and RNase H, where the differently sized mRNAs are both reduced in size to 590 bases, implying the loss of 100 (A) residues from the hypothalamic message and 40 (A) residues from the luteal mRNA. **Lanes 11** and **12** are controls where RNase H was omitted.

tion of the extent of 3′ polyadenylation. Heterogeneity in the 3′-untranslated region of mRNA has been reported for the pro-opiomelanocortin mRNA (Civelli *et al.*, 1983) and may also relate to the finding of alternative polyadenylation signals (Heidecker and Messing, 1983) as in the rat oxytocin gene (Ivell and Richter, 1984). It would support the view that the 3′-untranslated region may be important for the regulation of gene expression (Heidecker and Messing, 1983).

In vitro translation of luteal mRNA

To show that the identified mRNA could indeed programme the anticipated oxytocin precursor, luteal poly(A)$^+$ RNA was translated *in vitro* in a rabbit reticulocyte lysate system (Figure 4). Antibodies raised against the oxytocin-associated neurophysin I (NpI) specifically selected a polypeptide of mol. wt. 16 500 (Figure 4, lane 3) migrating identically with the similar product derived from hypothalamic mRNA (Figure 4, lane 8; Schmale and Richter, 1981). The mol. wt. 16 500 luteal product also reacted specifically with oxytocin antisera (Figure 4, lane 5), identifying it, therefore, as the common precursor to oxytocin and neurophysin I (Schmale and Richter, 1981). This precursor is one of the major polypeptides visible in an autoradiogram of total translation products (Figure 4, lane 2 arrowed). No products were found which reacted either with anti-vasopressin (Figure 4, lane 7) or with anti-neurophysin II (not shown).

Discussion

The experiments reported here show that the oxytocin gene is actively transcribed in the bovine corpus luteum. This explains fluctuation of serum oxytocin paralleling the luteal cycle (Schams *et al.*, 1983) as well as higher nonapeptide concentrations in the ovarian vein than in arterial blood (Flint and Sheldrick, 1982). The relative levels of mRNA in corpus luteum for the two nonapeptides correspond well with the levels reported for oxytocin and vasopressin in corpus luteum (Wathes *et al.*, 1983). If the quantitative data are converted to relative amounts per organ, then the active corpus luteum (III, Figure 1b) altogether produces some 250 times more oxytocin mRNA than a single hypothalamus.

The significantly lower yields from corpora lutea at other stages of the luteal cycle indicate that luteal oxytocin production is phasic and may, as suggested by Wathes and Swann (1982), be linked to the prostaglandin F2α-induced luteolysis which begins to override progesterone production at this stage of the cycle. The very high cyclic levels of oxytocin synthesis by the corpus luteum call into question the relative importance of hypothalamic oxytocin production in peripheral reproductive functions dependent upon circulating oxytocin.

Materials and methods

Preparation and analysis of mRNA

Differently sized bovine corpora lutea (I, 2.0 g; II, 3.7 g; III, 5.4 g; IV, 7.7 g), hypothalami, muscle and cerebellum were collected from a slaughterhouse within 45 min of death and frozen in liquid nitrogen. RNA was prepared for all tissues by CsCl centrifugation of the phenol-extracted total nucleic acids (Hoffman *et al.*, 1983) and enriched for poly(A)$^+$ RNA by oligo(dT)-cellulose chromatography.

For dot-blot analysis, RNA or DNA, in the quantities indicated, was de-

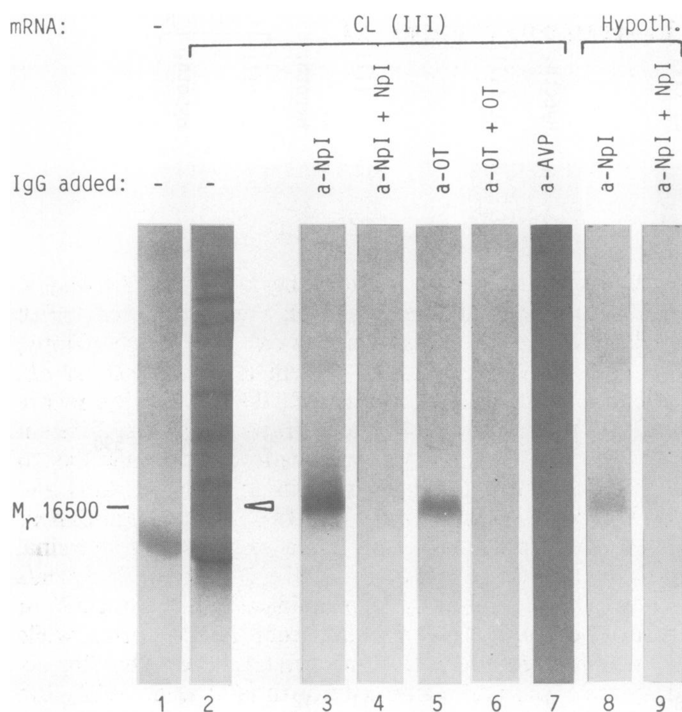

Fig. 4. *In vitro* translation of luteal mRNA and immunoprecipitation of the oxytocin-neurophysin I common precursor. **Lanes 1** and **2:** 2 μl of total translation products without **(lane 1)** or with **(lane 2)** added luteal mRNA. **Lanes 3** and **4:** immunospecific polypeptides selected by anti-NpI (UCB Bioproducts, Brussels) in the absence **(lane 3)**, or presence **(lane 4)** of 10 μg unlabelled NpI. **Lanes 5** and **6:** the NpI-immunoreactive polypeptides from 4 x 50 μl translation reactions were eluted from an unfluorographed polyacrylamide gel (Ivell and Richter, 1982) and reacted with 30 μg anti-oxytocin (Ferring, Kiel, FRG) in the absence **(lane 5)** or presence **(lane 6)** of 30 μg unlabelled oxytocin. **Lane 7:** as **lane 3** except using anti-vasopressin IgG. **Lanes 8** and **9:** as **lanes 3** and **4**, except that the translations were programmed by hypothalamic mRNA.

natured by heating at 60°C for 15 min followed by 15 min on ice, in 10 μl of a solution containing 6 x SSC, 7.5% formaldehyde and calf thymus tRNA to give a total nucleic acid concentration of 0.3 μg/μl. The nucleic acid was then spotted onto nitrocellulose membrane filters as indicated. The filters were dried *in vacuo* at 80°C, pre-hybridized in a solution containing 50% formamide, 5 mM sodium phosphate, pH 7.5, 0.9 M NaCl, 5 mM EDTA, 0.1% SDS, 1 x Denhardt's and 0.5 mg/ml denatured herring sperm DNA, at 42°C overnight, and then hybridized for 48 h at 42°C in a similar solution containing [^{32}P]nick-translated DNA probe as indicated, at 10^6 c.p.m./ml and specific activity $>10^8$ c.p.m./μg. Filters were washed twice in 50% formamide, 5 mM sodium phosphate, pH 7.5, 0.9 mM NaCl and 0.1% SDS, at 42°C and 50°C (20 min each), and once in 0.1 x SSC, 0.1% SDS at 50°C for 20 min, before exposing to X-ray film.

To determine the size of the specific mRNA, RNA samples were denatured in 10 mM $HgCH_3OH$ and run on vertical 1.3% agarose gels in borate buffer also containing 10 mM $HgCH_3OH$ (Gal *et al.*, 1983). Gels were rinsed as in Gal *et al.* (1983) and blotted onto nitrocellulose membranes which were dried and hybridized to the indicated probes as above. To estimate the length of the poly(A) tail 5 μg poly(A)$^+$ RNA was incubated with 0.5 μg oligo(dT) in 20 μl H_2O at 21°C for 10 min, then for a further 10 min at 21°C after addition of 5 μl 250 mM KCl. 25 μl of a buffer containing 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM KCl, 0.2 mM dithiothreitol and 10% sucrose were then added together with 2.5 units of RNase H (BRL, Neu Isenburg,FRG) and the reaction mix incubated at 37°C for 1 h. RNA was then precipitated with 2.5 vol of ethanol at −20°C overnight and electrophoresed as above. Controls were run where the RNase H was omitted.

Preparation and cloning of cDNA from luteal poly(A)$^+$ RNA

2 μg of poly(A)$^+$ RNA from the corpus luteum where the dot-blots indicated maximal oxytocin transcriptional activity, were cloned into oligo(dT)-tailed pUC9 by the direct method of Heidecker and Messing (1983). Resultant recombinant plasmids were transformed into *E. coli* JM 103. Ampicillin-resistant colonies were screened by *in situ* colony hybridization using the ^{32}P-labelled, 3′ oxytocin-specific probe (OT-3′). Of the four positively hybridizing colonies, all indicated similar restriction maps, therefore that with the longest insert was then sequenced by the method of Maxam and Gilbert (1980); both strands were sequenced across all internal restriction sites used for either 5′ or 3′ labelling.

In vitro translation and immunoprecipitation of specific products

Luteal or hypothalamic poly(A)$^+$ RNA was translated in a rabbit reticulocyte lysate system (New England Nuclear, Dreieich, FRG) using [^{35}S]cysteine as radiolabel. 25 μl reaction mixtures were programmed with 0.5 μg poly(A)$^+$ RNA and immunoprecipitated using 30 μg of IgG as described elsewhere (Schmale and Richter, 1981; Ivell and Richter, 1982). Control immunoprecipitations included unlabelled antigen (10−30 μg) as competing peptide. Proteins were electrophoresed on 15% polyacrylamide/SDS gels, which were then fluorographed and exposed to film.

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