cDNA-derived molecular characteristics and antibodies to a new centrosome-associated and G₂/M phase-prevalent protein

Karsten Rothbarth¹, Christian Petzelt², Xiang Lu¹,³, Ivan T. Todorov⁴, Gaby Joswig¹, Rainer Pepperkok⁵, Wilhelm Ansorge⁵ and Dieter Werner¹,*

1Division of Cellular Biochemistry, German Cancer Research Center, P.O. Box 10.1949, W-6900 Heidelberg, FRG
2Laboratoire International de Biologie Cellulaire Marine, F-85 350 Ile d’Yeu, France
3Present address: Institute of Biological Sciences, National Research Council of Canada, Ottawa, ON, K1A OR6, Canada
4Institute of Cell Biology and Morphology, Bulgarian Academy of Sciences, G. Bonchev Street B-25, 1113 Sofia, Bulgaria
5European Molecular Biology Laboratory (EMBL), P.O. Box 10.2209, W-6900 Heidelberg, FRG

*Author for correspondence

SUMMARY

Differential screening of a murine RNA-based cDNA library with cell cycle phase-specific transcripts released a cDNA clone (λCCD41) to a mRNA (1.349 kb) which, according to the mode of its detection, increases as expected during the cell cycle. The molecular characteristics of the protein (27 × 10³ M₀) encoded by this mRNA were deduced from the cDNA sequence and antibodies were prepared against the recombinant protein. Immunofluorescence studies performed with PtK₂ cells revealed that the amount of the antigen specified by the CCD41 sequence increases during the cell cycle out of proportion with the DNA content. In G₁ phase cells, the antigen is exclusively located at the site of the centrosome. During cell cycle progression the antigen becomes also detectable in perinuclear vesicles that increase in number and size, reaching a maximum in G₂ phase cells. The centrosomal location of the CCD41 antigen was investigated in relation to another centrosomal antigen, centrosomin A. Since the latter antigen is detected by a monoclonal antibody reacting specifically and permanently with the centrosomes in PtK₂ cells throughout the cell cycle it was possible to investigate the relative positions of the two proteins at the site of the centrosome and to add new information about the general architecture of the organelle and its changes during the cell cycle. While the centrosomin A antibody detects the pronounced cell cycle stage-dependent shape changes of the centrosome, the CCD41-encoded protein appears to be localized as a compact structure inside the centrosome. Its epitopes are exposed throughout the cell cycle except during a brief period immediately after the formation of the daughter centrosome.

Key words: cell cycle, cell cycle phase-specific transcripts, differential hybridization, cDNA, post-translational modification, fusion protein, PtK₂ cells, centrosome, CCD41, centrosomin A

INTRODUCTION

One approach to obtain biological probes for proteins involved in cellular structures or functions of interest is based on differential screening of cDNA libraries. This approach is designed to detect those mRNAs with altered cytoplasmic abundances during phenotypic changes or other biological events including cell cycle stages (Lu et al., 1990). Principally, there exist three different ways of regulation by which gene products actively involved in the cell cycle control could achieve their optimal levels of activities: (a) the gene is expressed at high level only during the time when its product is required for cell cycle progression; (b) the mRNA is translated only when the protein is needed for special cell cycle events; (c) the (inactive) gene product is post-translationally modified, resulting in an ‘active’ product when required. Of course, only those genes that belong to group (a) are detectable by the differential screening approach.

The majority of the known cell cycle phase-dependent and transcriptionally regulated genes are those required for DNA synthesis, coordinating DNA replication tightly with the cell cycle. The G₂/M transition is another decisive boundary marked by the activity of a family of genes, termed ‘cyclins’ (reviewed by Nurse, 1990). For example, human cyclin B has been reported to be regulated at the level of transcription because its mRNA increases in G₂ phase severalfold over the amount present in G₁ phase (Pines and Hunter, 1989). However, there must be other cell cycle genes belonging to group (a) that have not been discovered. Accordingly, we used the differential screening approach in order to detect new genes differentially expressed during different cell cycle stages.

In this paper we describe the characterization of a cDNA
MATERIALS AND METHODS

Recloning and sequencing of the λCCD41 insert

The insert of the recombinant phage λCCD41 contains an internal EcoRI recognition sequence. In order to reclone the complete insert in the EcoRI-digested and alkaline phosphatase-treated pBluescript vector (Stratagene), amplified phage DNA was only partially digested with EcoRI. Inserts released from the phage arms, but not cleaved at the internal EcoRI site, were extracted from low melting point agarose gels and ligated with the EcoRI and alkaline phosphatase-treated pBluescript vector. The insert of the resulting plasmid pCCD41 was sequenced according to Sanger et al. (1977) by means of the DNA sequencing kit from Pharmacia. The insert was sequenced in both directions using commercially available M13 as well as custom-made primers and reverse primers.

Production of antigenic fusion protein and preparation of antibodies

An expression vector was constructed which could be induced to express a recombinant β-galactosidase/CCD41 fusion protein. For this purpose, the insert of the plasmid pCCD41 was released by HincII / EcoRI. The recessive EcoRI end was made blunt and this fragment was inserted into the Smal site of the prokaryotic expression vector pEX2 (Boehringer). The orientation of the CCD41 insert in the expression plasmid pEX-CCD41 was determined by the size of BamHI restriction fragments. The fusion protein was expressed in Escherichia coli pop2136 (Boehringer), where it was deposited in inclusion bodies consisting essentially of the fusion protein (not shown).

Pre-immune serum was taken from rabbits. This was followed by four successive subcutaneous injections of 1 mg of protein contained in inclusion bodies at intervals of three weeks. The antigenic fusion protein was emulsified in Freund’s complete adjuvant (first injection) or Freund’s incomplete adjuvant in successive injections. Immune serum was collected two weeks after the last injection. The serum was pre-adsorbed by treatment with an acetone powder prepared according to Harlow and Lane (1988) from Ehrlich ascites tumor cells grown in vivo. The serum was pre-adsorbed by treatment with an acetone powder prepared according to Harlow and Lane (1988) from Ehrlich ascites tumor cells grown in vivo.

In vitro translation of pCCD41 in vitro transcripts and in vitro post-translational modification

The plasmid pCCD41 was linearized with SspI and transcribed in vitro from the T3 promoter of the vector. The transcripts were either used to stimulate reticulocyte lysates (BRL) without processing fractions and reticulocyte lysates supplemented with different amounts of microsomes from canine pancreas (Boehringer) designed to induce co-translational processing of proteins (Blobel and Dobberstein, 1975; Kurjan and Herskowitz, 1982; Rothblatt and Meyer, 1986). Assay conditions were as recommended by the manufacturer of the lysate and the microsomal fraction. The translation assays were not treated with proteases before gel electrophoresis on 12% (w/v) SDS-polyacrylamide gels.

Cell culture and synchronization procedures

PtK2 cells were maintained in MEM-medium supplemented with 5% fetal calf serum, non-essential amino acids, and 30 mM HEPES, pH 7.2, in a 5% CO2 atmosphere at 36°C. To obtain a high level of synchronously developing cells, semi-confluent cultures were shaken off, the resulting mitotic cells in the supernatant were centrifuged (1000 g, 5 min) and resuspended in fresh medium. Such a procedure gave better than 80% initial synchrony. The cells were then seeded onto coverslips and processed for immunofluorescence at various time intervals.

Immunofluorescence

Cells were first washed once in PBS (37°C). For double immunofluorescence detection of centrosomin A and CCD41 cells were permeabilized with buffer A for 5 min. For double immunofluorescence detection of CCD41 and microtubules they were treated for the same time with buffer B. Such different treatments are necessary because permeabilization of the cells with buffer A destroys a substantial part of labile microtubules whereas the staining of the centrosome remains unaffected in this buffer. Treatment with buffer A has been chosen because permeabilization results in a much reduced background staining. Buffer A: 0.1 M PIPES; 2 mM EGTA, 1 mM MgCl2, 0.1 % Triton X-100, pH 7.2; buffer B: same, but without Triton. In both cases the pH value was adjusted with KOH. Thereafter, the cells were washed with buffer B and fixed in cold ethanol (~5°C) for 7 min. After two washes with buffer B the cells were incubated with 1% BSA in PBS for 10 min to reduce unspecific staining, which was followed by incubation for 45 min at 37°C in sequence with the antibodies. CCD41 antibody: rabbit-derived polyclonal antibody, working dilution was 1:500 in 1% BSA, PBS. Centrosomin A antibody (Joswig and Petzelt, 1990; Joswig et al., 1991): affinity-purified mouse monoclonal antibody, working dilution was 1 µg/ml of 1% BSA, PBS. α-Tubulin antibody (Sigma): mouse monoclonal antibody against α-tubulin, working dilution 1:1000 in 1% BSA, PBS. After several washes with PBS the cells were
Single cell fluorometry and microscopy

The system for single cell fluorometry and quantification of DNA contents will be described in detail elsewhere (Pepperkok et al., unpublished). Briefly, PtK2 cells were immunostained with the CCD41 antibody and their nuclei with Hoechst dye 33258. The images were recorded and digitized with an inverted microscope (Zeiss) equipped with appropriate optical filters and a SIT low light level video camera coupled to a DVS Realtimeboard (Hamahatsu), which allows real-time enhancement of the images by recursive filtering, background subtraction or pseudocolour representation. For off-line improvement of the images and quantitation of fluorescence an IBAS Image Processing System (Zeiss) was used. Images of the staining with antibody and with Hoechst dye were loaded into two separate image buffers of the IBAS system. Both images were corrected for background fluorescence using the IBAS function BLOWGR (IBAS reference manual). Examples of such images are shown in Fig. 2 (below). Cell nuclei were detected and localized automatically in the image for Hoechst dye staining by a segmentation procedure using a dynamic threshold. For each image pixel the mean grey value of its neighbourhood region was calculated using an average low-pass Filter (IBAS reference manual). This mean grey value plus a constant offset determined the local threshold value. Image pixels with grey values below the threshold were recorded as background, while those with grey values above the threshold were identified as objects, e.g. cell nuclei. The amount of Hoechst dye per nucleus (integrated optical density) was calculated by multiplication of the average intensity with the area of cell nuclei, which reflects the relative amount of DNA per nucleus (Crissman et al., 1985). Antibody-stained dots were recorded by visual inspection of the corresponding image.

Other immunostained preparations were viewed using the AXIOVERT 405 (Zeiss) with a x100 Plan-Neofluar. Photographs were taken using either Kodak Tri-X 400 or Ektachrome 400 film.

Biocomputing

The program package HUSAR (Heidelberg unix sequence analysis resources), supplied by S. Suhai, German Cancer Research Center, Heidelberg, FRG, was used for inspection of nucleotide and amino acid sequences. Especially, the fast search program FASTA of Pearson and Lipman (1988) was applied to search for similar sequences in nucleotide databases (EMBL release 12/91 and Genbank release 9/91) and in protein data bases (PIR, release 9/91, SwissProt, release 9/91). Putative post-translational modification sites were detected by means of the comparison tables of A. Bairoch, Medical Biochemistry Department, University of Geneva, Switzerland. Signal sequence analysis was performed according to Heijne (1986).

RESULTS

Origin and characteristics of the recombinant cDNA \( \lambda \text{CCD41} \)

The cDNA clone \( \lambda \text{CCD41} \) was detected in a murine RNA-based \( \lambda \text{gt10} \) cDNA library by differential screening with cell cycle phase-specific probes whose preparation and significance has been described previously in detail (Lu et al., 1987; Lu and Werner, 1988; Lu et al., 1990). Briefly, cDNA prepared from RNA of Ehrlich ascites cells grown in vivo and separated into fractions of phase-synchronous cells by centrifugal elutriation (Lu et al., 1987) was cloned into the in vitro transcription vector pBluescript (Lu and Werner, 1988). In vitro transcriptions of such cell cycle phase-specific cDNA libraries were used to screen the \( \lambda \text{gt10} \) library (Lu et al., 1990). The \( \lambda \text{CCD41} \) plaque was selected because it showed a significantly stronger hybridization signal with the G2/M phase-specific transcripts than with the S phase-specific transcripts. Only weak hybridization was observed with the radio-labelled probe prepared in vitro transcription of the G1 phase-specific cDNA library (Lu et al., 1990). These results and appropriate controls with first-strand cDNA probes published previously (Lu et al., 1990) indicate that the \( \lambda \text{CCD41} \) insert reflects a transcript whose copy number per Ehrlich ascites tumor cell is low after mitosis (G1-phase) but increases during the cell cycle progression, reaching its maximum in G2/M phase.

Verification of the G2/M phase prevalence of the protein encoded by the recombinant phage \( \lambda \text{CCD41} \)

Since the expression of a protein is not necessarily correlated to its mRNA level, experiments were designed to verify the overproportional increase of CCD41 mRNA-encoded protein during the cell cycle. For this purpose, the insert of the recombinant phage \( \lambda \text{CCD41} \) was recloned in the prokaryotic expression vector pEX2 resulting in the plasmid pEX-CCD41. E.coli pop2136 cells were transfected and induced to produce the recombinant fusion protein which was used to prepare antibodies directed against the protein specified by the CCD41 insert.

Using immunofluorescence techniques, it was found that the antigen is not species specific. For example, the antigen could be serologically detected in very different systems including sea urchin sperm (Fig. 1) and in PtK2 cells (Figs 2-7).

With respect to the expected cell cycle phase-specific distribution of the antigen, it was found that PtK2 cell cultures were composed of subpopulations of cells differing in their antibody-dependent immunofluorescence patterns which could be correlated with their DNA contents (Fig. 2, Table 1). Single cell fluorometry revealed that cells containing a single antibody-stained dot belong to the group of cells with G1 phase-specific DNA contents. A second group of cells was identified with multiple bright fluorescent dots in the cytoplasm close to the nucleus. Cells containing more than five of such dots showed the typical G2 phase DNA content. A third fraction of cells could be grouped with an intermediate DNA content, apparently S phase cells. Cells belonging to the latter fraction exhibited 2-3 antibody-stained dots per cell. The cells in mitotic stages showed the brightest antibody-specific fluorescence, indicating that the antigen apparently reaches its highest levels during the G2/M phase, which correlates well with the increased levels.
of the CCD41 sequence in G₂/M phase cDNA libraries (Lu et al., 1990).

**Localization of the CCD41-encoded antigen in PtK₂ cells**

Immunofluorescence techniques revealed that the antigen encoded by the CCD41 sequence is associated with two types of organelles. It could be localized in perinuclear vesicles that increase in number during the cell cycle (Fig. 2, Table 1) and it forms an integral part of the centrosome (Figs 1, 3-7).

**Identification of the centrosome in PtK₂ cells**

The centrosomes of PtK₂ cells can be accurately identified and followed throughout the cell cycle by their immunoreactivity with the established monoclonal antibody to centrosomin A (Joswig and Petzelt, 1990; Joswig et al., 1991). Accordingly, new centrosomal components, e.g. the CCD41-encoded antigen, can be identified by the co-localization with centrosomin A. Moreover, the application of the monoclonal antibody to centrosomin A together with the polyclonal antibody to the CCD41-encoded protein allows us to reveal the relative positions of the two antigens in the centrosome. Thus, by means of the two antibodies it became possible to investigate whether the two proteins occupy the same part of the centrosomal structure and whether the daughter centrosome shows the same composition as the mother centrosome.

**Localization of the CCD41 antigen during the cell cycle**

The cell cycle of PtK₂ cells lasts for about 22 hours. Their centrosomes show a growth cycle which can be visualized with the antibody to centrosomin A (Joswig and Petzelt, 1990). During the cell cycle progression the centrosome becomes enlarged and changes its shape. For example, ring-like structures with appendices appear, and immediately

---

**Fig. 1.** Sperm of the sea urchin *Paracentrotus lividus.* (A) Stained with CCD41 antibody; besides a weak staining of the sperm membrane the centrosome is clearly marked (arrow). (B) Stained with tubulin antibody. (C) Sperm nucleus stained with Hoechst H 33342. Bar, 10 µm.

**Fig. 2.** Out of proportion increase in the CCD41-encoded protein during the cell cycle progression of PtK₂ cells. (A) Hoechst 33258-stained cells. (B) Same window as shown above but cells in CCD41-specific staining. Single cell photometry shows that cells containing only one single CCD41-specific dot (a) have a DNA content typical of G₁ cells. Cells containing more than five CCD41-specific dots show a DNA content typical of G₂ phase cells (b). A summary of the data obtained by investigation of 69 cells is given in Table 1.

---

**Table 1. Summary of the single cell fluorometry data**

<table>
<thead>
<tr>
<th>Number of CCD41-specific dots per cell</th>
<th>1</th>
<th>2-5</th>
<th>&gt;5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA content (arbitrary units; mean values)</td>
<td>154</td>
<td>205</td>
<td>293</td>
</tr>
<tr>
<td>Standard deviations</td>
<td>±50</td>
<td>±50</td>
<td>±125</td>
</tr>
<tr>
<td>Number of cells in the different groups</td>
<td>32</td>
<td>17</td>
<td>20</td>
</tr>
</tbody>
</table>

Hoechst 33258 and CCD41 antibody-stained PtK₂ cells of the kind shown in Fig. 2 (n=69) were investigated with respect to their relative DNA contents by single cell fluorometry. In addition, the CCD41-specific dots were counted and registered. Cells showing mitotic figures are not included in this table. The analysis of variance (F-test) confirmed that the differences in the DNA contents of the cells grouped according the number of CCD41 antibody-specific dots per cell are highly significant (>99%).
before the breakdown of the nuclear envelope, the centrosomes are divided into the two daughter centrosomes that migrate to the mitotic poles (Fig. 3A, C, E). Treatment of PtK2 cells with the antibody to the CCD41 antigen shows that this protein is another integral part of the centrosome where it is present essentially throughout the cell cycle (Fig. 3B, D, F). The antibody to the CCD41 antigen does, however, not reveal the growth cycle of the centrosome in the same clear way as can be observed with the antibody to centrosomin A. The structure occupied by the CCD41 protein remains smaller and more compact throughout the cycle than that marked by the antibody directed against centrosomin A. Are the two proteins superimposed in the centrosome? A comparison of the structures visualized by application of the two antibodies shows that the two proteins occupy different regions of the centrosome with partial overlapping of centrosomal regions stained with both antibodies. The CCD41 protein is located as a compact
body closer to the center of the centrosome than centrosomin A, which occupies a relatively larger space with filaments extending around the organelle (Fig. 4).

Localization of the CCD41 antigen during mitosis

During mitosis, at least until metaphase, the centrosome appears in almost all cells as a compact body which can be visualized by the appearance of its decoration with the antibody to centrosomin A (Joswig and Petzelt, 1990). During the same period the CCD41-specific labelling is as well located in a dense structure at the mitotic poles (Fig. 5). However, the structure detectable with the antibody to the CCD41-encoded protein does not show any shape changes of this dense structure during all mitotic phases (not shown). Although centrosomin A and the protein specified by the CCD41 sequence are located close together at the site of the centrosome, a profound and important difference is seen when the separating centrosomes are examined with respect to their compositions. From the beginning of the separation process the two centrosomes can be permanently visualized with the centrosomin A antibody. In contrast, the staining with the CCD41-specific antibody remains restricted to the mother organelle. No CCD41 is detectable within the newly formed daughter centrosome at this stage (Fig. 6). Only when the two centrosomes have reached the future mitotic poles, does the daughter centrosome also become stained with the CCD41 antibody, and no further difference in CCD41-specific immunofluorescence intensities of the two centrosomes can be observed. Therefore, for the first time, a definite albeit brief period of centrosome ‘maturation’ is demonstrated during which the two organelles are not alike.

Localization of the CCD41 antigen in non-centrosomal compartments

In addition to its centrosomal localization, the CCD41 protein shows a pronounced periodic appearance in the cytoplasm. In early G1 phase cells the CCD41-specific staining is virtually absent in all cellular areas other than the centrosome. After a few hours, additional staining appears associated with small vesicle-like structures around the nuclear envelope. These vesicles become abundant in G2 phase (Fig. 2), showing occasionally large lacunae of presumably fused vesicles. Their localization suggests a preference for the vicinity of the nuclear envelope. Perinuclear vesicles of this type have been suggested as being involved in sorting of proteins and in directing them to their proper intracellular destination (Darnell et al., 1986). In mitotic cells, after the dissolution of the nuclear envelope, the cell is filled with a vesicular staining but still the two centrosomes are the most prominently stained structures. No preferential reaction of the CCD41-specific antibody with the mitotic spindle could be observed (Fig. 5). The cell in mitosis remains positive for the CCD41 antibody until late telophase, even at the time when the two daughter nuclei have already re-formed. However, as soon as cytokinesis is terminated, the major CCD41-specific staining of cytoplasmic structures disappears, only the centrosome continues to react with the antibody to the CCD41 antigen.

CCD41-encoded protein and the microtubular cytoskeleton

A fraction of polyploid giant cells is frequently found in PtK2 cell populations. Cells belonging to this fraction are permanently arrested in their cell cycle. Although the cell cycle stage of such cells cannot be determined precisely, they show, in addition to the CCD41-specific centrosome decoration, multiple vesicular structures marked by the CCD41-specific antibody around the nucleus (Fig. 7). In this case, the centrosome was identified by the convergence of the microtubules at this site. Again, no apparent relationship can be observed between the localization of the vesicle-like structures containing CCD41 protein and the distribution of the microtubules.
Sequence data
In order to obtain sequence data, the λCCD41 insert was recloned in the pBluescript vector (pCCD41) and sequenced.

Sequence characteristics of the pCCD41 cDNA
Fig. 8 shows the nucleotide sequence of pCCD41. It reveals that the insert of the plasmid pCCD41 reflects a complete cDNA. The ATG start-codon is preceded by a 5′ non-translated region of 88 nucleotides with two in-frame stop codons. The coding region has an open reading frame which encodes 244 amino acids. The stop codon (TAA) at the 3′ end of the coding sequence is followed by an additional 475 nucleotides of untranslated cDNA. The poly(A) tail is preceded by sequences that reflect putative polyadenylation signals (ATAAA, ATTAAA). Neither the complete nucleotide sequence nor longer sections of the sequence were found in the most recent data base releases, which indicates that the cloned sequence specifies a protein whose cDNA has not yet been isolated and sequenced before. Only the nucleotide sequence coding for the initial 18 amino acid residues showed a high degree of identity with the initial sections of several cDNAs which code for hydrophobic or membrane proteins (not shown).

Amino acid sequence characteristics of the polypeptide encoded by pCCD41
The nucleotide sequence of the open reading frame of the pCCD41 recombinant codes for a $27.14 \times 10^3 M_r$ precursor polypeptide. The N-terminal section of the deduced polypeptide exhibits a sequence motif that is typical for secretory or transmembrane proteins (Perlman and Halvorson, 1983). The three domains characteristic for a transmembrane leader peptide are found in this section. The short N-terminal domain is four amino acid residues in length and ends with a basic residue (K). This domain is followed by the hydrophobic core (amino acid positions 5–13) and the third domain with the putative signal sequence cleavage site begins with the helix-breaking residue (P). Based on the rules and probability tables of Heijne (1986),
the most likely cleavage site in this signal sequence is to be expected between amino acid positions 17 (A) and 18 (F). Consequently, the mature polypeptide has an expected $M_r$ of $25.3 \times 10^3$. Since the protein shows no ER-retention signal (Pelham, 1988), the possibility cannot be excluded that a portion of this protein is secreted. However, the immunofluorescence experiments (Figs 1-7) show that at least a significant portion of the protein is retained in cells.

PEST residues

Another sequence feature of the polypeptide chain is of interest with respect to the rapid degradation of the cellular protein during M/G1 transition. Regions that are flanked by basic residues and rich in proline (P), glutaminic acid (E), serine (S) and threonine (T) are considered to indicate short half-lives, or stage-dependent conditional short half-lives, of cellular proteins (Rogers et al., 1986). A region of this type is found in the CCD41-encoded polypeptide between amino acid positions 103 (K) and 131 (H). The portion of PEST residues in this region amounts to 56% while the preceding and succeeding windows of equal lengths contain only 33% and 10% PEST residues, respectively.

Post-translational modification predictions for CCD41

The amino acid sequence of the polypeptide encoded by pCCD41 was investigated with respect to putative functional sites and with respect to consensus motifs for post-translational modifications. No motifs were found that indicate the enzymic activity of the protein. However, a number of sites were detected that reflect potential sites for modification by phosphorylation. Protein kinase C exhibits a preference for the phosphorylation of serine or threonine residues that reside close to a C-terminal basic residue (Woodget et al., 1986; Kishimoto et al., 1985). Motifs meeting this prediction are located at amino acid positions 145 (SFR), 167 (TLK), 207 (TGR) and 237 (SGR). Other sites of the protein meet the consensus pattern for casein kinase 2 (S or T)-(2X)-(D or E) (Pinna, 1990). Such patterns can be found at amino acid positions 89 (TQFE), 177 (TRYE) and 188 (TCVE). Finally, the protein comprises strings that reflect potential asparagine glycosylation sites. The consensus sequence for asparagine glycosylation is (N)-(X, but not P)-(S or T)-(X, but not P) (Marshall, 1972; Bause, 1983; Gavel and Heijne, 1990). Asparagine residues that meet these conditions are found at amino acid positions 72 (NVTI), 139 (NGSA), 165 (NFTL) and 175 (NRTR).

Evidence for glycosylation of CCD41

The consensus patterns for potential glycosylation sites detectable in the amino acid sequence of CCD41 are of special interest because the cellular protein shows a higher molecular mass than the polypeptide encoded by the pCCD41 cDNA. Western blots probed with the CCD41 antibody result in a specific signal indicating a protein that is as large as $50 \times 10^3 M_r$ (Fig. 9). Interestingly, this mature cellular protein is mainly detected in the ‘nuclear’ fraction, which seems to conflict with the cellular distribution detected by immunofluorescence techniques (Figs 2-7). However, it is well known that many cytoplasmic components, including centrosomes (Mitchison and Kirschner, 1984).
Fig. 7
remain rather firmly attached to nuclei and become thereby co-isolated with nuclei. Since CCD41 is predominantly detected in centrosomes and in perinuclear vesicles (Figs 2-7), it is not surprising that the majority of this antigen is co-isolated with nuclei. The apparent size of the cellular protein is best explained by a post-translational modification through glycosylation. This interpretation is supported by the experimental data shown in Fig. 10. This figure shows that in vitro translation of the cDNA transcripts results in a polypeptide of the expected size ($27.14 \times 10^3 \text{ M}_r$). Addition of microsomal fractions containing signal peptide cleavage factors and other post-translational modification factors including glycosylation activities (Blobel and Dobberstein, 1975; Kurjan and Herskowitz, 1982; Rothblatt and Meyer, 1986) could not reduce the size of the translation product. In contrast, the translation product increased in the presence of the co-translational processing fraction, which is most likely due to the potential of these fractions to induce in vitro glycosylation. Although the $M_r$ of the in vitro glycosylated product is smaller than that of the cellular protein found in Ehrlich ascites cells, it is conceivable to conclude that glycosylation is the post-translational modification event which explains the difference in size between the pCCD41-encoded polypeptide and the cellular protein. Consequently, it is likely that CCD41 is a cellular glycoprotein.

**DISCUSSION**

Theoretically, the differential hybridization approach is a useful method of identifying genes expressed differentially during dynamic biological processes. However, limited amounts of stage-specific mRNA and standardization of stage-specific mRNA probes are major problems with this technique. Previously, we proposed that the construction of stage-specific cDNA libraries in in vitro transcription vectors and the use of stage-specific in vitro transcripts, instead of first-strand cDNA, could eventually overcome these problems (Lu et al., 1990). By application of this method we detected a recombinant clone whose distribution in cell cycle stage-specific cDNA libraries appeared to be of special interest (Lu et al., 1990). From the distribution of the CCD41 sequence in phase-specific cDNA libraries it could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in $G_2/M$ phase and rare in $G_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific cDNA libraries could be suggested that this cDNA reflects a mRNA encoding a translation product that is prevalent in G$_2$/M phase and rare in G$_1$ phase. Since protein levels are not necessarily correlated with their mRNA levels it was of interest to verify the phase-prevalent distribution of the translation product at the cellular level. Our results show that this could be achieved by recombinant DNA techniques. The protein encoded by the CCD41 sequence could be expressed in *E. coli* and antibodies directed against the recombinant protein enabled us to verify that the level of the cellular protein specified by the CCD41 sequence increases out of proportion during the cell cycle progression of, e.g., PtK$_2$ cells. This result suggests that the different levels of the CCD41 sequence in phase-specific
Fig. 10. In vitro translation of pCCD41 cDNA in vitro transcripts in the absence and in the presence of microsomes from canine pancreas. Samples were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. The figure shows a contact print of the autoradiography of the dried gel. M, 14C-labelled marker proteins; (a) in vitro translation assay without addition of microsomes; (b)-(d) in vitro translation assays with increasing amounts of microsomes. The proteins were analysed on a 10% (w/v) SDS-polyacrylamide gel.

replace stage-specific first-strand mRNA populations in differential hybridization experiments and that significant clones can be obtained by this procedure.

Several characteristics of the protein identified by this approach are remarkable: its greatly increased transcription and translation during the G2 phase, and its continuous localization at the centrosome throughout the cell cycle, except for a very brief phase when the daughter centrosome is formed. The latter quality suggests that the protein is involved in a typical function of the centrosome whose central role in cell cycle progression (Maniotis and Schliwa, 1991) and the control of the three-dimensional structure of the cytoplasm has been well established (reviewed by Mazia, 1987; Bornens, 1991). However, if the CCD41-encoded protein is only involved in a centrosomal function, why do we find such an intense extra-centrosomal accumulation during the G2/M phase in the cytoplasm of PtK2 cells? It could be argued that the CCD41 protein is somehow related to other proteins whose levels increase greatly during the cell cycle and which are known to activate periodically protein kinases (Murray and Kirschner, 1989; Nurse, 1990). Proteins with these characteristics have been termed cyclins. However, at present we have found no indication of a structural relationship between the CCD41 protein and the cyclins. The CCD41 protein contains, like the cyclins, a PEST motif (Rogers et al., 1986), which is considered to be responsible for a stage-dependent half-life. However, no further reasons for a closer structural relationship with known G2/M regulators could be detected on the sequence level. Further studies are designed which should reveal whether CCD41 is functionally related to G2/M phase regulators; for example, whether the CCD41 protein is able to form complexes with protein kinases.

The available data add some valuable information to the general architecture of the centrosome. It appears from our results as well as from those of others (Mazia, 1987) that the centrosome is a complex structure composed of several different proteins. It undergoes shape changes during the cell cycle, probably reflecting its control function in the cell. However, not all proteins that are part of this structure exhibit the same shape changes of the organelle, which points to the existence of core and surface proteins. Since the antibody against centrosomin A indicates the shape changes more clearly than the antibody to CCD41 it is suggested that centrosomin A belongs to the centrosomal surface proteins instead. In contrast, CCD41 seems to reflect a more dense core component. In addition, the antibody to CCD41 points to the partial non-identity of the two centrosomes just after the formation of the daughter centrosome.

It should be noted that several other proteins with known or unknown functions have been identified which are either components of the centrosome or are at least centrosome-associated. γ-Tubulin has been shown to be present at the centrosome in a broad range of eukaryotes (Aspergillus nidulans: Oakley et al., 1990; Schizosaccharomyces pombe: Horio et al., 1991; Drosophila: Zheng et al., 1991; Xenopus and mammalian cells: Stearns et al., 1991) and its role in nucleating microtubules is plausible (Oakley and Oakley, 1989; reviewed by Oakley, 1992). For example, it has been shown that microinjected antibodies to γ-tubulin bind to native centrosomes at all stages of the cell cycle and interfere with the regrowth of microtubules after artificial depolymerization (Joshi et al., 1992). Cyclin B has been convincingly demonstrated within the astral microtubules in Drosophila embryos (Maldonado-Codina and Glover, 1992) as well as in a variety of other cell types. However, a clear distinction should be made between components accumulating in the vicinity of the centrosome, such as cyclin B (Maldonado-Codina and Glover, 1992) or Ca2+-transport vesicles (Petzelt and Hafner, 1986), and those components located in the centrosome, such as γ-tubulin, various antigens to which antibodies can be found in the sera of patients with autoimmune diseases, centrosomin A (Joswig and Petzelt, 1990), and CCD41 (this paper), respectively. Whereas the former antigens may reflect characteristics of the cytoplasmic environment necessary for the function of the centrosome, the latter, as part of the structure itself, may provide more direct information on the various centrosomal functions.

So far, a detailed functional analysis at the genetic level has been performed only with one centrosomal component, γ-tubulin (Oakley et al., 1990; Oakley and Oakley, 1989; reviewed by Oakley, 1992). The antibodies and the genetic probes to the two new centrosomal proteins, centrosomin A and CCD41, now provide the required tools for the functional analysis of other intrinsic centrosomal proteins.

This work was supported by grants of the Deutsche Forschungsgemeinschaft (Werner, We 589/2-2) and by the European Com-
munities (Petzelt, Contract SC1*CT91.0640). One of us (I.T.) is grateful for a fellowship of the German Cancer Research Center.

The nucleotide sequence of full-length CCD41 cDNA published here has been deposited at the EMBL sequence data bank (1986).

REFERENCES


(Received 16 April 1992 - Accepted 23 September 1992)