The EMBO Journal vol.10 no.5 pp.1225-1236, 1991

The maternally expressed *Drosophila* gene encoding the chromatin-binding protein BJ1 is a homolog of the vertebrate gene *Regulator of Chromatin Condensation, RCC1*

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Communicated by C.Nüsslein-Volhard

Using monoclonal antibodies I have identified a nuclear protein of *Drosophila*, BJ1 (M_r ~68 kd), and isolated its gene. Biochemical analysis demonstrates that the BJ1 protein is associated with nucleosomes and is released from chromatin by agents which intercalate into DNA, as previously shown for the high mobility group proteins (HMGs). On polytene chromosomes the protein is localized in all bands, with no preference for particular loci. Both the BJ1 protein and in particular the BJ1 mRNA are strongly expressed maternally. In early embryos all nuclei contain equal amounts of BJ1. During neuroblast formation, BJ1 mRNA becomes restricted to cells of the central nervous system, and higher protein levels are found in the nuclei of this tissue. In late embryonic stages, the mRNA almost completely disappears, but significant amounts of BJ1 protein persist until morphogenesis. The BJ1 gene encodes a 547 amino acid polypeptide featuring two different types of internal repeats. The sequence from amino acids 46 to 417 containing seven repeats of the first type has been highly conserved in evolution. 45% of the amino acids in this region are conserved in seven similar tandem repeats of the human gene *Regulator of Chromatin Condensation, RCC1*. The phenotype of a cell line carrying a mutation of *RCC1* suggested a main function for this gene in cell cycle control. A yeast gene, *SRM1/PRP20*, also contains these repeats and shows 30% amino acid identity to BJ1 in this region. Mutations in this gene perturb mRNA metabolism, disrupt nuclear structure and alter the signal transduction pathway for the mating pheromone. Complementation experiments argue for a common function of these genes in the different species. I propose that their gene products bind to the chromatin to establish or maintain a proper higher order structure as a prerequisite for a regulated gene expression. Disruption of this structure could cause both mis-expression and default repression of genes, which might explain the pleiotropic phenotypes of the mutants.

Key words: cell cycle control/chromatin structure/non-histone proteins/*RCC1*/SRM1/PRP20

Introduction

In nuclei of eukaryotic cells the DNA is complexed with proteins to form a compact structure, the chromatin. The basic unit of the chromatin is the nucleosome, which contains an octameric core of four different histones with ~150 bp of DNA wrapped around them. The nucleosomes can form higher order structures, resulting in variable degrees of chromatin condensation along the interphase chromosomes. It appears that not only the presence of specific transcription factors (activators or repressors), but also the degree of chromatin condensation may play a decisive role in the expression or repression of a gene (Weinstraub and Groudine, 1976; Wu *et al.*, 1979; reviewed by Felsenfeld and McGhee, 1986; Widom, 1989). Thus, inactivation of genes can occur when these are brought into the vicinity of highly condensed heterochromatin by chromosomal rearrangements (reviewed by Henikoff, 1990). In normal development particular states of expression can be stably maintained even after the disappearance of regulatory proteins that are required to establish expression or repression initially. It has been proposed that this imprinting of genetic activity occurs via the chromatin structure (Paro, 1990).

Many data have accumulated pointing to the role of both the histones and the non-histone proteins in determining the higher order chromatin structure of genomic regions. In particular, the presence of histones H1, $H1^0$, or H5, which bind to the linker regions between the nucleosomes, can induce assembly of more condensed structures and repression of genes (Schlissel and Brown, 1984; Sun *et al.*, 1989; Wolffe, 1989). Of the non-histone proteins influencing chromatin structure, the class of the moderately abundant high mobility group proteins (HMGs) has been studied in most detail. The term HMG proteins has been operationally defined according to biochemical properties that facilitate isolation of these proteins (reviewed in Johns, 1983). While HMG14 and HMG17 are preferentially associated with decondensed, transcriptionally active chromatin (Weisbrod *et al.*, 1980; Dorbic and Wittig, 1987), other HMGs such as the α-protein are thought to induce specific positioning of nucleosomes and higher order chromatin structures (Strauss and Varshavsky, 1984). However, the function of these proteins is far from being clear (Einck and Bustin, 1985). Presumably there exist additional proteins influencing chromatin structure which have not been isolated because of their lower abundance or because their biochemical properties differ from those of the HMGs.

Monoclonal antibodies have been successfully used to dissect the complexity of nuclear protein fractions and to analyze the functions of their individual components (Saumweber *et al.*, 1980. Dreyer *et al.*, 1981; Kuo *et al.*, 1982; Lacroix *et al.*, 1985; Garzino *et al.*, 1987). In *Drosophila*, this approach has allowed the identification of nuclear proteins involved in gene expression (Risau *et al.*, 1983; Frasch and Saumweber, 1989), in chromatin condensation (James and Elgin, 1986; Eissenberg *et al.*, 1990), and in structural functions of the nucleus (Fuchs *et al.*, 1983; Frasch *et al.*, 1988). In such an immunological screen, I obtained monoclonal antibodies against 39 different nuclear proteins (Frasch, 1985). The most frequently obtained class of antibodies recognized a 68 kd antigen with

moderate abundance in embryonic nuclei, called BJ1. Here, I describe the chromatin binding of BJ1, its expression pattern, and the cloning and sequencing of the BJ1 gene. The sequence of BJ1 turned out to be strongly conserved in evolution, and genes coding for related proteins are found from yeast to humans. A homologous gene in vertebrates, *Regulator of Chromatin Condensation (RCC1)*, was studied most extensively, and its main function appears to be in cell cycle control (reviewed in Nishimoto, 1988). In a hamster cell line with a temperature sensitive mutation for *RCC1*, premature initiation of mitosis occurs when the cells are shifted to the restrictive temperature during S phase (Ajiro *et al.*, 1983; H.Nishitani, M.Ohtsubo, K.Yamashita, H.Iida, J.Pines, H.Yasuda, Y.Shibata, T.Hunter and T.Nishimoto, submitted). However, temperature shifts in G_1 phase result in a different phenotype, and cause alterations in gene expression (Nishimoto *et al.*, 1981). In yeast, the phenotypes of mutations in a homologous gene, called *SRM1* (Clark and Sprague, 1989) or *PRP20* (Aebi *et al.*, 1990), are also compatible with the idea that the gene product is required for normal gene expression and chromatin structure. The properties of the *Drosophila* BJ1 protein provide important clues to the function(s) of these proteins in the nucleus and may explain the pleiotropic phenotypes observed in these mutants.

Results

Monoclonal antibodies against BJ1

Monoclonal antibodies were produced against protein fractions from *Drosophila* embryonic nuclei. Forty one independent hybridoma clones obtained from one particular protein fraction (see Materials and methods) secreted antibodies against nuclear proteins. On Western blots, 29 of these antibodies recognized an antigen with an electrophoretic mobility of 68 kd present in total nuclear proteins from embryos (Figure 1A, lane 3) and from *Drosophila* tissue culture cells (K_c cells, Figure 1A, lanes 4–7). Several lines of evidence suggested that the antibodies recognized at least four different epitopes in this 68 kd polypeptide, which I call BJ1. The four classes of antibodies were represented by the antibodies Bj10, Bj43, Bj59, and Bj70. These antibodies reacted with different peptides after partial digestion of BJ1 with V8 protease (data not shown; Cleveland *et al.*, 1977). BJ1/βGal fusion proteins of different length allowed the partial mapping of the binding regions of these antibodies within the BJ1 polypeptide. The antibodies also differed in terms of their crossreaction with proteins from other species (see below). Furthermore, with nuclear proteins from K_c cells they recognized minor bands in addition to the 68 kd band in four different patterns (Figure 1A).

Interestingly, the 60 kd band recognized by the Bj70 antibody (Figure 1A, lane 7) appears to be identical to the nuclear protein D1 described previously (Alfageme *et al.*, 1980; Levinger and Varshavsky, 1982a,b). The D1 protein is quantitatively extracted from nuclei with 5% perchloric acid (PCA), together with only few other proteins including histone H1 and A13 (Bassuk and Mayfield, 1982. Figure 1B, lanes 2 and 4). In a Western blot with the Bj70 antibody, a 60 kd band corresponding to D1 was stained with the PCA extract, whereas BJ1 (68 kd) remained in the insoluble fraction (Figure 1B, lanes 6 and 7). Thus, the nuclear proteins BJ1 and D1 of *Drosophila* are antigenically related.

Two classes of Bj antibodies also crossreacted with proteins from species that are very distantly related to

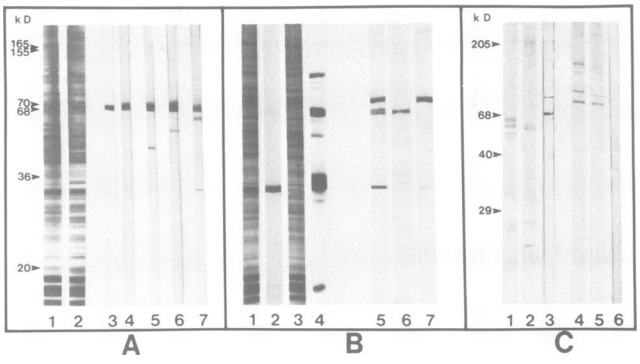

Fig. 1. Characterization of BJ1 and related proteins by Western blotting. **A.** Western analysis of *Drosophila* nuclear proteins with four different antibodies against BJ1. Lanes 1 and 2:total nuclear proteins from embyros or K_c cells, respectively, stained with Coomassie brilliant blue. Lane 3: Western blot of embryonic nuclear proteins, as in lane 1, with Bj43 antibody. Lanes 4–6: Western blot of K_c cell nuclear proteins, as in lane 2, with the antibodies Bj43 (lane 4), Bj10 (lane 5), Bj59 (lane 6) and Bj70 (lane 7) **B.** Extraction and Western analysis of D1 protein. Lanes 1–4: Coomassie stainings of total nuclear proteins form K_c cells (lane 1), 5% perchloric acid extract enriched for D1 (lane 2 with 3 μg of protein loaded, lane 4 with 30 μg), and residual nuclear proteins after PCA extraction (lane 3). Lanes 5–6: Western analysis of immobilized proteins as in lanes 1–3 with Bj70 antibody. **C.** Cross reaction of BJ1 antibodies with proteins from nuclei of embryonic chicken (lanes 1 and 4), with proteins from calf liver nuclei (lanes 2 and 5) and with proteins from total yeast cells (lanes 3 and 6). Lanes 1–3 were stained with Bj59 antibody, lanes 4–6 with Bj70 antibody.

Drosophila. On Western blots with nuclear proteins from embryonic chicken brains and calf liver, bands of 55–64 kd were stained with the Bj59 class, and two bands of ~80 kd and 90 kd with the Bj70 antibody class (Figure 1C, lanes 1, 2, 4 and 5). These antibodies exclusively stained the nuclei on tissue sections from chicken embryos (data not shown). On Western blots with total proteins from yeast, two bands were recognized by the Bj59 antibody class (69 kd and 84 kd), and a weak band by the Bj70 antibody class (40 kd). These results suggest that the epitopes recognized by these antibodies have been conserved in several nuclear proteins during evolution.

Chromatin-binding of BJ1

Since BJ1 was found to be antigenically related to the HMG-like protein D1, I examined if it shared any biochemical properties with HMG proteins. In particular, I tested whether BJ1 (and D1) is specifically released from nuclei upon alteration of the DNA conformation by intercalation. 'Elutive intercalation' has been used previously to release HMG-like proteins and other DNA-binding proteins from whole nuclei. The composition of the eluted protein fraction depended on the nature and concentration of the intercalating agent used, and on the ionic strength (Schröter *et al.*, 1985, 1987; Schulman *et al.*, 1987).

When nuclei from K_c cells were treated with 7.5 mM ethidium bromide and 50 mM NaCl, the eluted protein contained one major band with a mobility of 68 kd (Figure 2A, lane 3). This band corresponded to the BJ1 protein, as shown by western analysis (Figure 2B, lane 3) and by co-purification of the antigenic activity with the 68 kd band, when the extract was further fractionated on an FPLC MonoS column (data not shown). At a slightly higher ionic strength, 100 mM NaCl, BJ1 was eluted even more

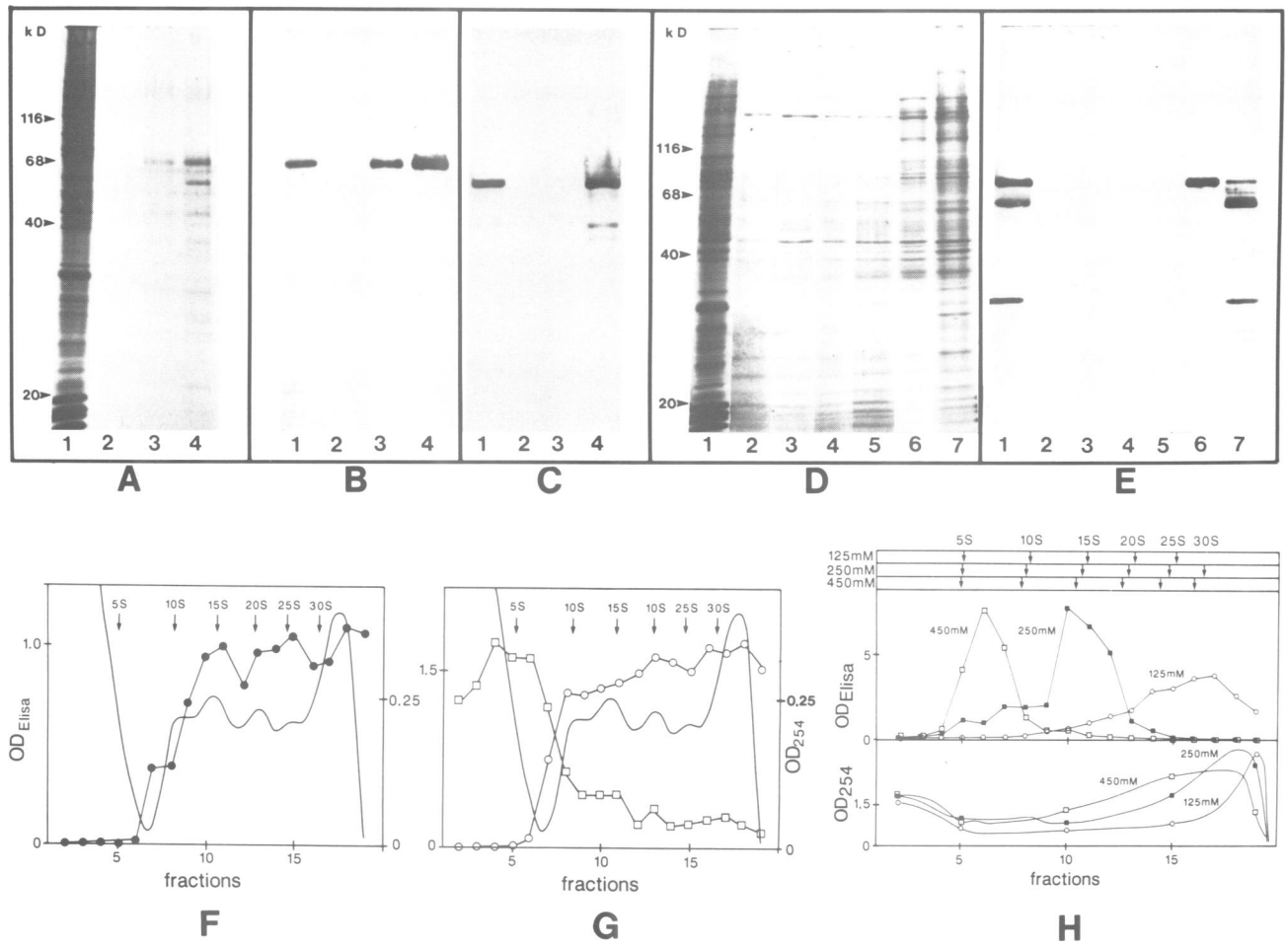

Fig. 2. Chromatin-binding and elution of BJ1. **A–C**. Intercalative elution of BJ1 and D1 from nuclei. **A**. Lanes 1–4: Coomassie staining of K_c nuclear proteins (lane 1), proteins eluted with buffer X2, 7.5 mM ethidium bromide (EtBr, lane 2), proteins eluted with buffer X2, 7.5 mM EtBr, 50 mM NaCl (lane 3), and proteins eluted with buffer X2, 7.5 mM EtBr, 100 mM NaCl (lane 4). **B**. Western analysis of immobilized proteins as in A, lanes 1–4, with Bj43 antibody (antiBJ1). **C**. Western analysis as in B, but with Bm10 antibody (antiD1, Frasch *et al.*, 1986). **D**. Salt extraction of BJ1 and D1 from nuclei. Lanes 1–7: Coomassie blue stained proteins from total K_c cell nuclei (lane 1), proteins successively extracted with buffer X2, 0 mM NaCl (lane 2), 50 mM NaCl (lane 3), 100 mM NaCl (lane 4), 250 mM NaCl (lane 5), 300 mM NaCl (lane 6), and 350 mM NaCl (lane 7). **E**. Western analysis of proteins as in D with Bj43 antibody (antiBJ1; upper bands at 68 kd) and, in a second step, with Bm10 antibody (antiD1; lower bands at 60 kd). **F–H**. Sedimentation profiles of antigens on chromatin gradients. Top fractions of the 10–30% sucrose gradient are to the left and bottom fractions are to the right. Chromatin separation was recorded measuring OD_{254} (ordinate scale right); the antigen distribution was analyzed by ELISA (left scale). **F**. Analysis of gradient fractions with Bj43 antibody (antiBJ1, ●), **G**. Analysis of gradient fractions with BX65 (anti-histone H2A, ○) with P11 (antiRNP, □). **H**. Sedimentation profiles of chromatin and BJ1 in sucrose gradients containing 125 mM NaCl (○), 250 mM NaCl (■), and 450 mM NaCl. (□). The S values were determined using a Fortran program written by R.Burberg.

efficiently by ethidium bromide (Figure 2A, B, lane 4). As judged by Western analysis, >70% of the BJ1 protein was eluted from the nuclei under these conditions. At 7.5 mM ethidium bromide and 100 mM NaCl, a second prominent band at 60 kd appeared in the eluate (Figure 2A, lane 4). Western analysis with a monoclonal antibody specific for D1 (Frasch *et al.*, 1986; Figure 2C, lane 4) and FPLC fractionation demonstrated that this band corresponded to the D1 protein. In the absence of ethidium bromide, neither BJ1 nor D1 were released under these ionic strengths (Figure 2D,E, lanes 3,4). BJ1 was only released, among many other proteins, at 300 mM NaCl, and D1 at 350 mM NaCl (Figure 2D,E, lanes 6 and 7). The specific elution of BJ1 upon treatment of nuclei with ethidium bromide, similar to that of the DNA-binding protein D1 (Levinger and Varshavsky, 1982a,b, Levinger, 1985), indicates that BJ1 is also a DNA-binding protein.

In a different assay, I tested whether BJ1 was associated with nucleosomes. After RNase A treatment of K_c nuclei and limited digestion with micrococcal nuclease, soluble chromatin was extracted under low salt conditions and size-fractionated on a sucrose gradient. The sedimentation profile of nucleosomes and BJ1 antigen were compared. The gradient fractions were tested for BJ1 by a solid phase immunoassay (ELISA), and the distribution of the nucleosomes was monitored through their DNA absorbance. Figure 2F shows that the nucleosomes were separated from each other up to the 4-mers, followed by a faster sedimenting peak containing longer nucleosomal chains. The profile of BJ1 antigen closely paralleled that of the nucleosomes (Figure 2F) and was similar to the sedimentation profile of histone H2A (Figure 2G). The fraction with the mono-nucleosomes, however, contained less BJ1. In contrast to BJ1, the RNA-associated protein P11 remained in the top fractions of the gradient which contained the RNA fragments (Figure 2G). The extraction of BJ1 at low salt conditions (<300 mM NaCl) was dependent on the treatment of the nuclei with micrococcal nuclease (Figure 2E). These results strongly

suggest that the BJ1 protein is associated with nucleosomal chromatin.

BJ1 is released from nucleosomes by increased ionic strengths. For the experiment shown in Figure 2H, chromatin of larger nucleosomal chain length was separated on sucrose gradients containing different concentrations of NaCl. At 125 mM NaCl, BJ1 was found in the fractions containing the bulk of the chromatin. As expected from the

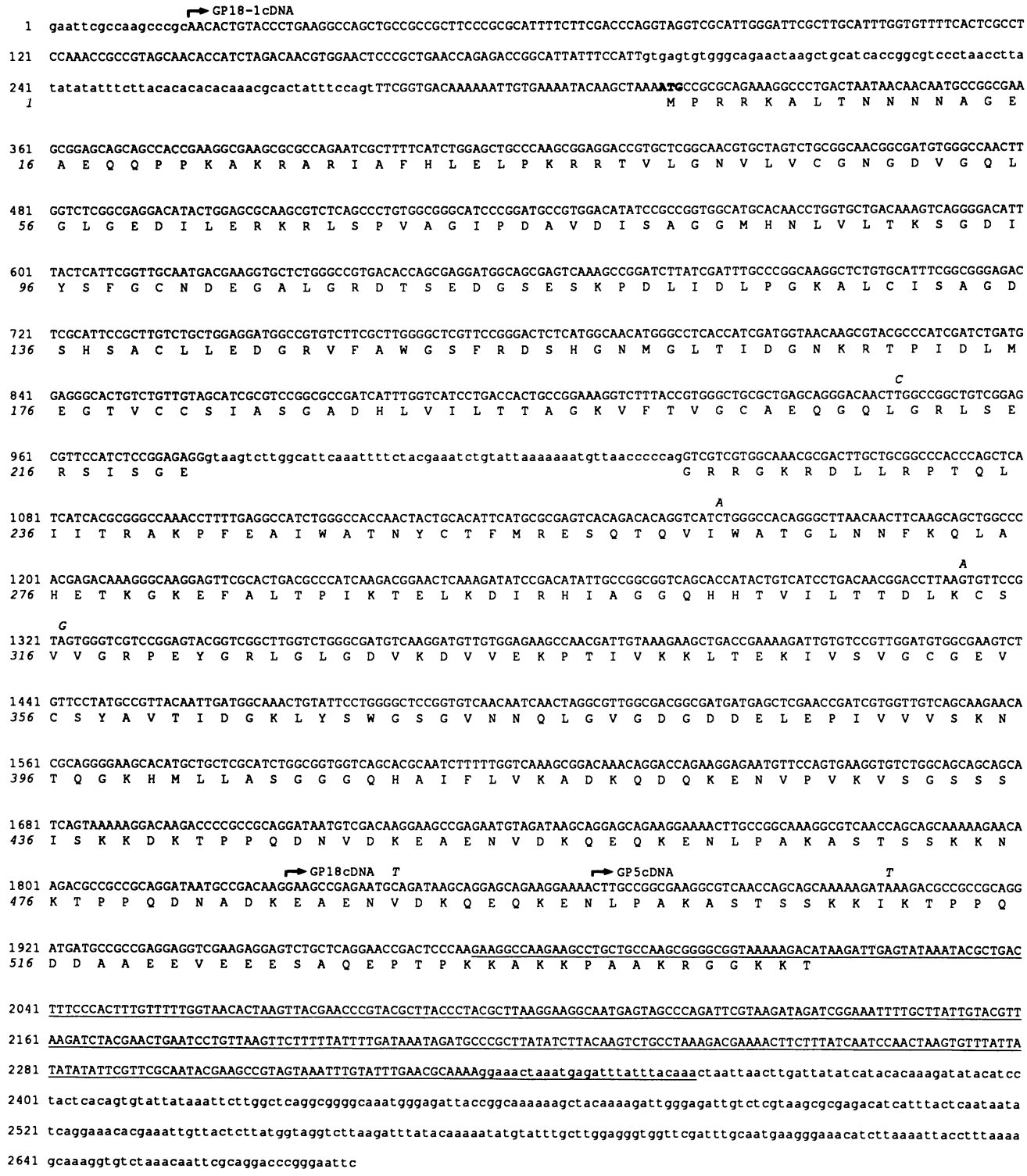

Fig. 3. Genomic and cDNA sequences of BJ1. The nucleotide sequence of a 2.7 kb genomic *Eco*RI fragment is shown. Sequences also present in the GP18-1 cDNA (2172 bp) are written with capital letters. Above the genomic sequence, several polymorphic nucleotide exchanges in the GP18-1 cDNA sequence are shown in italics. All of them are in the third positions of the codons. The conceptual translation of the GP18-1 open reading frame is shown below the nucleotide sequence. The arrows indicate the 5′ ends of cDNAs mentioned in the results section. The underlined sequence (382 bp) shows strong similarity to a sequence of similar length in a genomic λ phage (GP18.2, map position 10B on the X-chromosome; data not shown), which was also isolated under high stringency conditions with GP18 cDNA as a probe. The homology did not extend outside this sequence and there is no evidence for it being expressed.

results with intact nuclei, BJ1 was released from the chromatin at 450 mM NaCl and sedimented with a sedimentation coefficient (SW_{20}) of ~7S in the top fractions. 250 mM NaCl also released BJ1 from the nucleosomes. This concentration is slightly lower than the one required for its release from intact nuclei (Figure 2E,H). Interestingly, after release at 250 mM NaCl, BJ1 sediments with a higher velocity than at 450 mM NaCl (SW_{20} ~15S). From this result I conclude that BJ1 is bound to chromatin as a protein–protein complex that is released at increased ionic strength and falls apart at an even higher salt concentration. Presently I do not have any information about the composition of this complex.

Isolation and sequencing of the BJ1 gene

cDNAs carrying parts of the BJ1 open reading frame were isolated from a λgt11 expression library made from 0–2 h embryonic mRNA (U.Rosenberg, unpublished). A mixture of the antibodies Bj43, Bj46, Bj59 and Bj70, recognizing different epitopes, was used for this screen. Two of the isolated λ phages, GP18 and GP4, encoded fusion proteins recognized by all four monoclonal antibodies. Since BJ1 was the only common polypeptide recognized by all four antibodies, these clones were expected to contain BJ1 cDNA sequences. One λ phage, GP5, gave a signal with the antibody Bj46 only. Subsequent analysis showed that all three inserts corresponded to the 3′ end of the BJ1 transcript. The GP18 insert was used to isolate longer cDNAs and genomic clones. The 2.2 kb insert of the longest cDNA, GP18-1, closely matched the length (2.2 kb) of the BJ1 transcript, as determined by Northern analysis (Figure 5A). The entire GP18-1 cDNA sequence was contained on a 2.7 kb *Eco*RI fragment of genomic DNA (derived from the phage clone GP18.8). The 2.7 kb of genomic DNA and the cDNAs GP5, GP18 and GP18-1 were sequenced (Figure 3). Comparison of the GP18-1 cDNA sequence with the genomic sequence revealed the presence of two small introns (87 bp and 59 bp). The sequence of the GP18-1 cDNA starts close to the *Eco*RI site of the genomic sequence. A canonical polyadenylation signal, AATAAA, was not found, but the poly(A) of the cDNA is preceded by AT-rich sequences. A long open reading frame extends from nucleotide 213 to nt 1853 of the cDNA. The nucleotide sequence TAAA preceding the first ATG closely matches the consensus sequence for *Drosophila* translation start sites (Cavener, 1987). The ORF of the cDNA encodes a polypeptide of 547 amino acids. The calculated molecular mass of 58.85 kd is lower than the electrophoretic mobility of BJ1 (68 kd). This difference might be due to its high content of charged amino acids (36 Asp, 39 Glu, 24 Arg, 54 Lys).

The GP18 cDNA starts with codon 486, and GP5 with codon 499. Therefore the antibodies Bj43, Bj59, and Bj70 appear to bind to epitopes in the region between amino acids 486 and 499, and Bj46 (Bj10 class) binds somewhere between amino acids 499 and 547 (Figure 3, Figure 4A).

The BJ1 gene maps to 64F on the left arm of the third chromosome.

Structure of the BJ1 protein and sequence homologies

The primary structure of the BJ1 polypeptide is shown schematically in Figure 4A. Internal sequence comparison showed that a large portion of the BJ1 protein consists of homologous, internally repeated domains. Seven tandem copies of the first type of repeat (repeat lengths of ~50 amino acids) are found in the region between amino acids

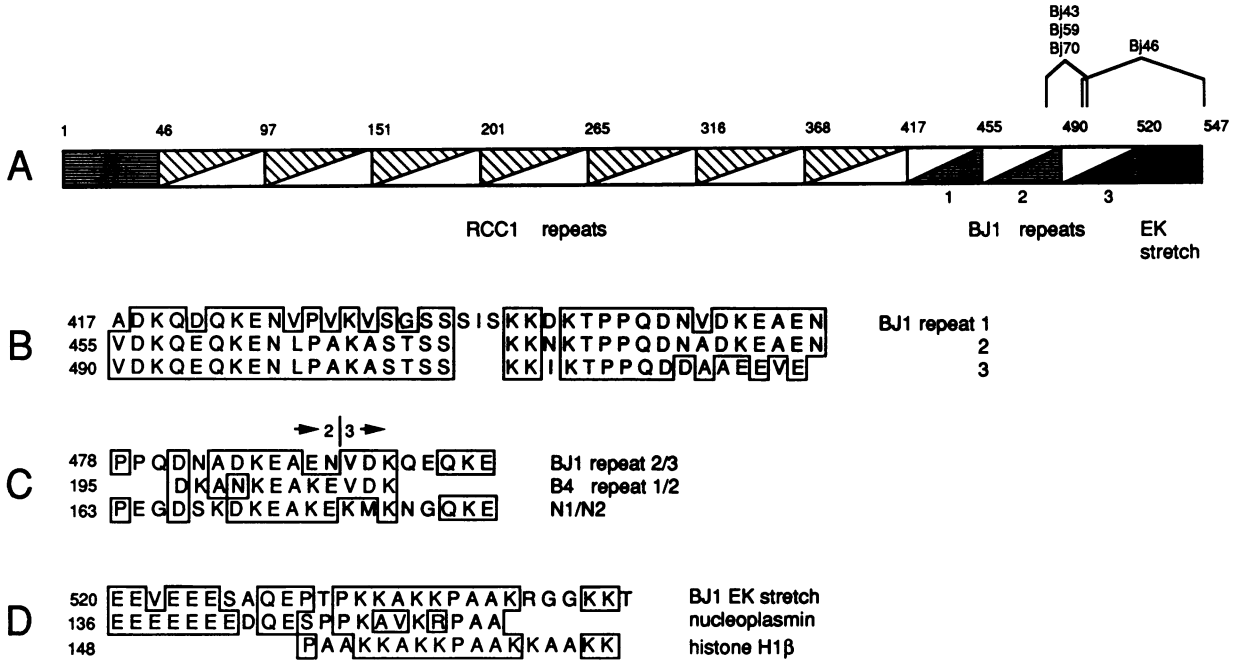

Fig. 4. Primary structure and sequence comparisons of the BJ1 protein sequence. **A.** Schematic diagram of the predicted BJ1 protein. The seven repeats with weak internal homology (amino acids 46–417) are strongly homologous to similar repeats of the vertebrate *RCC1* genes. Three repeats of a different type (amino acids 417–520) are highly homologous to each other. The C-terminal EK-stretch is glutamine- and lysine-rich. The brackets indicate binding regions of different monoclonal antibodies. Because of the strong sequence similarities of the BJ1 repeats, the antibodies may have multiple binding sites on each protein molecule. **B.** Sequence comparison of the BJ1 repeats. Amino acids present in two or all three repeats are boxed. **C** and **D.** Comparison of sequences from BJ1 with sequences from nuclear proteins of other species, B4, N1/N2, and nucleoplasmin are from *Xenopus laevis* (Smith *et al.*, 1988; Kleinschmidt *et al.*, 1986; Dingwall *et al.*, 1987). Histone H1β is from *Strongylocentrotus purpuratus* (Lai and Childs, 1988).

46 and 417. Most interestingly, this region displays a high sequence homology to the gene *RCC1 (Regulator of Chromatin Condensation)* from humans, hamster and *Xenopus* (Ohtsubo *et al.*, 1987; Uchida *et al.*, 1990; Nishitani *et al.*, 1990). A sequence comparison between the homologous regions of BJ1 and RCC1 from these species is shown in Figure 1 of the accompanying publication (Ohtsubo *et al.*, 1991). While the homology of these repeats of BJ1 among each other is rather low, 45% of the amino acids in this region have been conserved between *Drosophila* and humans. In yeast, a gene homologous to BJ1 called *SRM1* (Clark and Sprague, 1989) or *PRP20* (Vijayraghavan *et al.*, 1989; Aebi *et al.*, 1990) has been characterized. The amino acid identity between *Drosophila* and yeast in the region of the seven repeats ('RCC1 repeats') is 30% (Figure 1, Ohtsubo *et al.*, 1991). Thus, the sequence of the RCC1 repeat region has been highly conserved in evolution. Each of these proteins has an N-terminal domain of 40−50 amino acids. These domains lack sequence similarities but in all cases have a high content of charged amino acids.

Unlike the yeast and vertebrate homologues, BJ1 has a C-terminal extension of 130 amino acids. Immediately following the RCC1 repeats, BJ1 contains three additional repeats of a second type ('BJ1 repeats', repeat length ~35 amino acids, Figure 4A). These repeats are highly homologous to each other (Figure 4B), but are not related to the RCC1 repeats. A nuclear protein from *Xenopus*, B4, contains similar, but shorter repeats in its C-terminal part (Figure 4C; Smith *et al.*, 1988). A second nuclear protein from *Xenopus*, the histone-binding protein N1/N2, also contains a stretch of 21 amino acids with a similar sequence. C-terminal to the BJ1 repeats there is a cluster of glutamic acids, followed by a lysine-rich sequence ('EK-stretch', Figure 4A). This part of BJ1 has some similarities to sequences of nucleoplasmin from *Xenopus*, and to H1 histones (Figure 4D; Dingwall *et al.*, 1987; Lai and Childs, 1988).

Expression and subcellular localization of BJ1

Figure 5 shows a Northern and Western analysis of the abundance of the BJ1 mRNA and protein throughout the *Drosophila* life cycle. A strong signal from the BJ1 2.2 kb mRNA was seen with 0−2 h embryonic RNA (Figure 5A). Since there is no transcription at this stage, this mRNA must be provided to the embryo maternally. A signal of the same intensity was obtained with 2−4 h old embryos. In later stages of embryonic development the abundance of the BJ1 mRNA decreased drastically. In late embryos and in all following stages of development, only a faint signal was detected. Only adult females contain a level of BJ1 mRNA similar to that seen in early embryos. This is probably due to transcription of BJ1 in the ovaries (see below).

In contrast to the mRNA, only a small amount of BJ1 protein was detected in 0−2 h old embryos (Figure 5B). The level of BJ1 protein increased up until 8−12 h of embryonic development and slowly decreased again in later stages of embryonic and larval development. A stronger signal was obtained with young pupae and adult females. The differences in the patterns of BJ1 mRNA and protein can be explained by translational control and a higher stability of the protein as compared with that of the mRNA.

The expression and localization of BJ1 mRNA and protein were studied in more detail by *in situ* hybridizations and

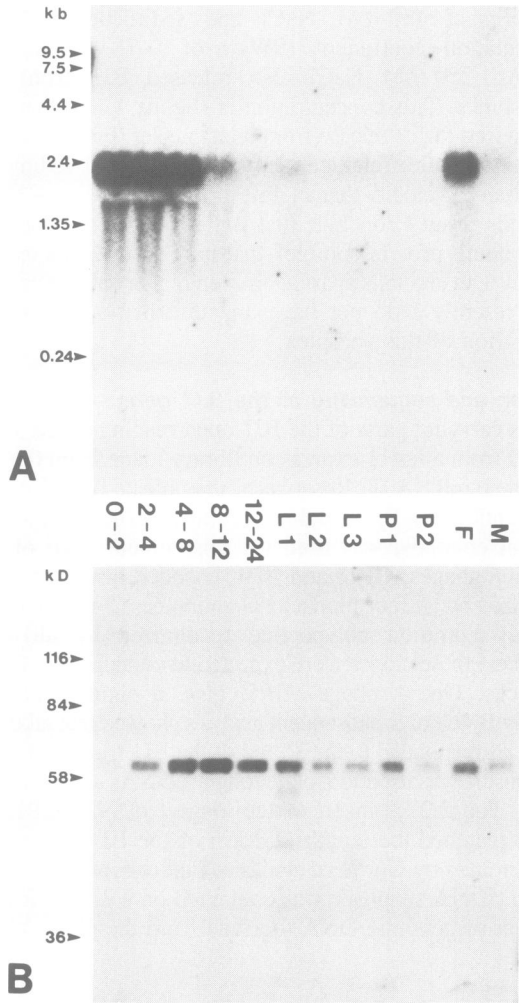

Fig. 5. Developmental profiles of BJ1 mRNA and BJ1 protein accumulation. **A.** Northern analysis of BJ1 mRNA accumulation. 5 μg of total RNA from different stages were loaded in each lane. A random-primed 1.3 kb *Sac*I−*Xba*I fragment from the 5′ part of the GP18-1 cDNA detected a 2.2 kb RNA. **B.** Western analysis of the BJ1 protein accumulation. 50 μg of total proteins from different stages were loaded in each lane and tested with the Bj43 antibody. All samples were collected at 22°C. Abbreviations: 0−2 to 12−24: embryonic stages in hours after egg deposition; L1,L2,L3: first, second and third larval instars; P1, P2: early and late pupal stages; F: adult females; M: adult males.

antibody stainings of ovaries and embryos. Figure 6A−C shows the expression of BJ1 mRNA during follicle development. In *Drosophila*, the oocyte develops from a cyst containing 16 interconnected germ line cells. Fifteen of these cells develop into nurse cells which provide the 16th cell, the prospective oocyte, with RNA and protein. The surrounding follicle cells are of somatic origin.

In contrast to most other maternal transcripts, the BJ1 mRNA is strongly enriched in the prospective oocyte of young follicles, and only low levels are detected in the nurse cells. Figure 6A shows a germarium with two cysts. The BJ1 transcript appears to be localized to two of the 16 cells of each cyst. From stage 1 to 9 of follicle development (King *et al.*, 1956), BJ1 mRNA is mainly found in the future oocyte (Figure 6B,C). At stage 10, when the oocyte grows by taking up yolk, strong expression starts in the nurse cells

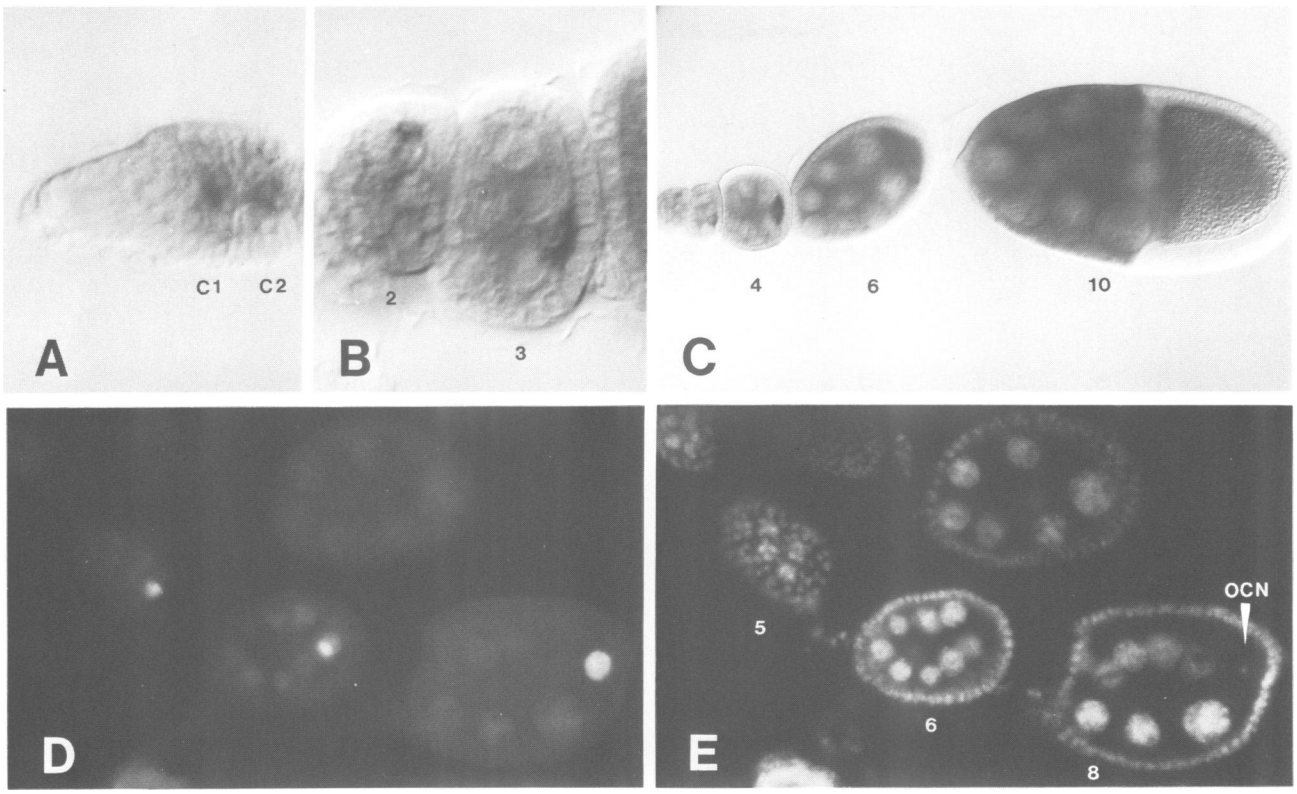

Fig. 6. BJ1 expression during oogenesis. **A–C**. Detection of the BJ1 mRNA in follicles of different stages by whole mount *in situ* hybridization with a digoxigenin-labeled cDNA probe. **A**. Germarium representing the earliest stages of oogenesis with two cysts (C1, C2). BJ1 mRNA is mainly detected in one or two out of the 16 cells of each cyst. **B**. Follicles of stages 2 and 3. BJ1 mRNA accumulates most strongly in the prospective oocytes. **C**. Same ovariole as in B, with more advanced stages of follicle development. In stages 4 and 6, high levels of BJ1 mRNA are found in the oocytes, and only low levels in the nurse cells. In stage 10, the nurse cells also accumulate high levels of BJ1 transcript. **D**. Detection of the BJ1 protein by immunofluorescence on frozen sections of follicles (stages 5, 6 and 8). BJ1 protein accumulates strongly in the oocyte nuclei. The nurse cell nuclei are weakly stained. **E**. DNA staining with Hoechst dye of the section shown in D. OCN: oocyte nucleus.

(Figure 6C). At later stages this mRNA is transported into the oocyte and in early embryos it is equally distributed. No transcript was detected in follicle cells (Figure 6A–C).

The BJ1 protein is also strongly enriched in the oocytes of follicles from early stages onwards. BJ1 is exclusively localized in the nuclei (Figure 6D). In germinal vesicles, BJ1 is detected in the nucleoplasm and even higher levels are seen on the condensed chromatin. Nurse cell nuclei contain less BJ1, and in follicle cell nuclei the protein was not detected (Figure 5D). The presence of BJ1 in very early oocytes suggests that, in addition to its role as a maternal store, the protein has a function in the oocyte nucleus itself.

In embryos undergoing the first cleavages, BJ1 protein is concentrated in the nuclei and is probably also present at a lower level in the cytoplasm (Figure 7A). From blastoderm onwards BJ1 protein is exclusively nuclear during interphase (Figure 7B). In elongated germ band embryos (stage 10), BJ1 mRNA disappears first from the ectoderm and then also from the mesoderm. High levels of BJ1 mRNA persist only in neuroblasts, and later in ganglion cells derived from those, until stage 13 (Figure 7C,E). Also for the BJ1 protein, higher levels are detected in nuclei of the neuroblasts and the cells of the CNS (Figure 7D,F). In contrast to the mRNA, significant levels of BJ1 protein persist in all the tissues until the end of embryogenesis.

During mitosis, the BJ1 protein leaves the nuclei and becomes evenly distributed throughout the cells. This was best seen in fluorescent antibody stainings of embryos post-gastrulation, where defined patches of cells divide simultaneously (Foe, 1989). In these mitotic domains the whole cells were stained (Figure 8A, arrow; Figure 8C), whereas the neighboring, non-dividing cells showed nuclear staining. High magnification views of cells progressing through the cell cycle are shown in Figure 8D–H (Bj59 antibody stainings) and I–M (DNA stainings). In interphase nuclei, BJ1 has a similar distribution to that of the chromatin, but is not enriched in the centromeric regions that are brightly stained with Hoechst dye (D, I). From early prophase to early telophase, granular BJ1 staining is seen throughout the cells (E,J; F,K) and BJ1 is not associated with the chromosomes. During telophase BJ1 reaccumulates in the nuclei (G,L). In Figure 7H, mitotic cells were stained with the antibody Bj70, which crossreacts with the nuclear protein D1 on Western blots (see above). In histological stainings, a composite signal derived from both BJ1 and D1 was also seen with this antibody (Figure 8H). In contrast to BJ1, D1 remains bound to the chromosomes during mitosis, being most concentrated at the centromeres (H,M, arrows; Figure 2 in Frasch *et al.*, 1986).

BJ1 protein was also detected in nuclei of larval tissues, including the salivary glands. Thus it was possible to analyze the distribution of BJ1 on polytene chromosomes. Figure 8N shows that BJ1 is found in all bands, the more condensed regions of the chromosomes. Developmental puffs, heat-

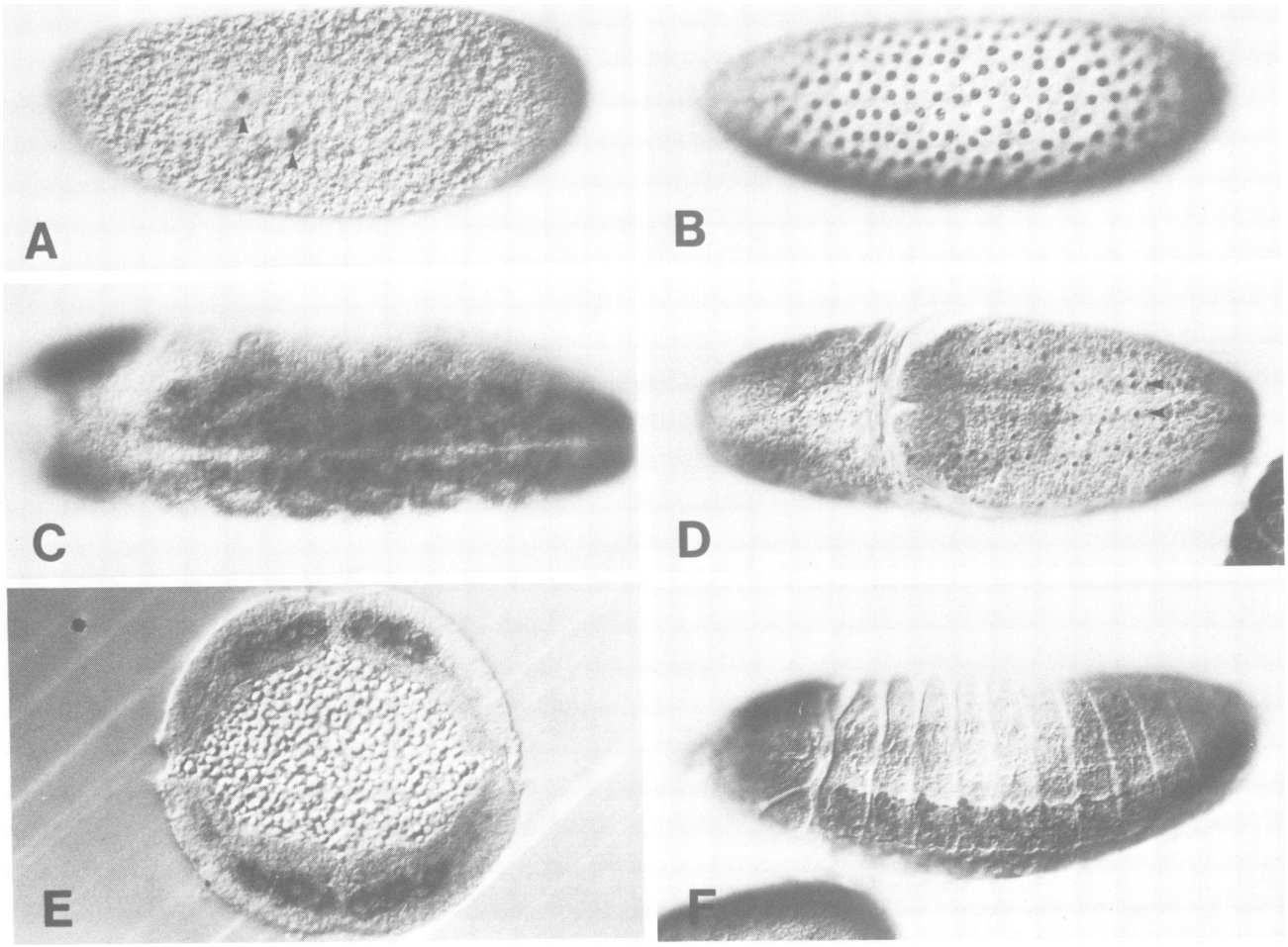

Fig. 7. Expression and localization of BJ1 in embryos. **A**. Whole mount staining of an early cleavage stage embryo with Bj59 antibodies. The nuclei are more strongly stained (arrow heads). **B**. Embryo in syncytial blastoderm stage, stained as in A. BJ1 protein is only detected in the nuclei. **C**. Whole mount *in situ* hybridization of a stage 10 embryo with a BJ1 cDNA probe, Ventral view, anterior is to the left. BJ1 mRNA is mainly detected in the neuroblasts. **D**. Stage 10 embryo, antibody staining as in A; dorsal view, anterior is to the left. The nuclei of neuroblasts are preferentially labeled (arrow heads). **E**. *In situ* hybridization of a stage 10 embryo as in C; cross section, showing BJ1 mRNA accumulation in the neuroblasts. **F**. Stage 13 embryo, antibody staining as in A; anterior is to the left, ventral is down. Nuclei in the CNS are more strongly labeled than in ectodermal cells.

shock puffs and interbands are negative. This staining is very similar to the one obtained with DNA dyes (Figure 8H). A 69 kd antigen with an identical distribution described by Fleischmann *et al.* (1987) may also correspond to BJ1.

Discussion

In *Drosophila* embryos and in cultured embryonic cells, BJ1 is a relatively abundant nuclear protein. I estimate that the amount of BJ1 protein in K_c cell nuclei is 2−5% of that of histone H1. Thus, the abundance of BJ1 is significantly higher than that of proteins which are known or presumed to act as transcription factors (Parker and Topol, 1984; Heberlein *et al.*, 1985; Wu *et al.*, 1987; Krause *et al.*, 1988; Perkins *et al.*, 1988). The level of BJ1 in nuclei is more comparable with that of high mobility group (HMG) proteins from other species and with that of the HMG-like protein D1 of *Drosophila* (Johns, 1983; Alfageme *et al.*, 1980; Bassuk and Mayfield, 1982). BJ1 shares several other properties with HMG proteins. The protein is extracted from nuclei at 300 mM NaCl, a salt concentration similar to that required for the release of HMGs (350 mM NaCl). BJ1 is bound to nucleosomes and is eluted from nuclei upon treatment with agents that intercalate into DNA, as has been shown for several HMG proteins (Schröter *et al.*, 1985; Schulman *et al.*, 1987). HMGs typically contain ~40% of acidic and basic amino acids. and the BJ1 protein also has a high content of such residues (31%). However, BJ1 does not meet all requirements for an HMG protein because of its rather high electrophoretic mobility (68 kd) and its insolubility in perchloric acid. In contrast to D1, it does not contain any sequence motifs that are typical of HMGs (Ashley *et al.*, 1989; Jantzen *et al.*, 1990; Schulman *et al.*, 1991). Although one class of monoclonal antibodies reacted with both BJ1 and D1, I did not detect any obvious sequence similarities between the two proteins. Perhaps these antibodies recognize a non-contiguous epitope with a similar structure on both proteins.

A special feature of the BJ1 primary sequence is its modular structure. The central 90% of the sequence is built

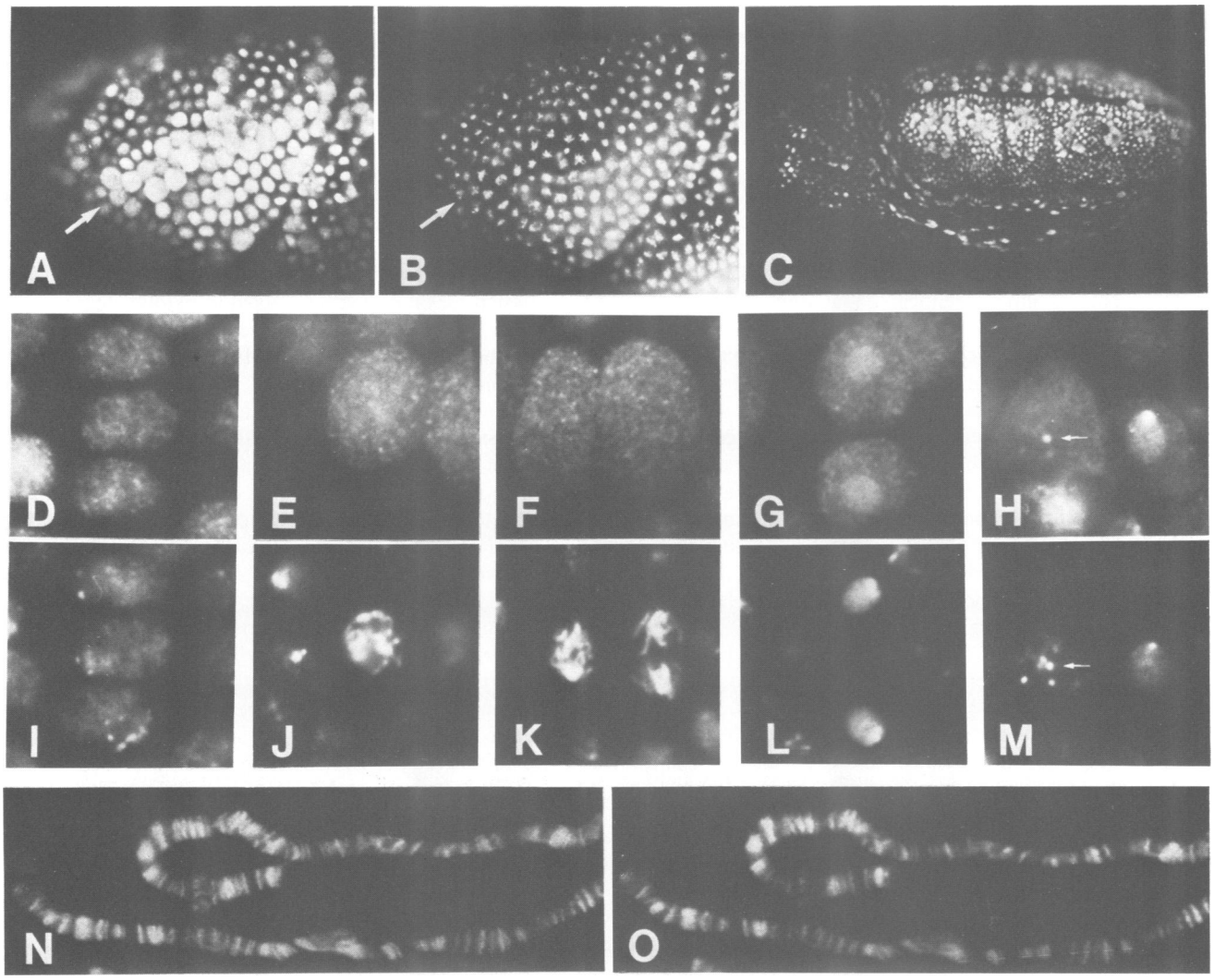

Fig. 8. Subcellular distribution of BJ1 protein during the cell cycle and its localization on polytene chromosomes. Indirect immunofluorescence with Bj59 antibodies (A–G), Bj70 antibodies (H), and Bj86 antibodies (Bj43 class; N). **A** Antibody staining and **B**. DNA staining of ectodermal cells in the head region of a stage 9 embryo. In the domains of mitotically active cells (cycle 14; arrows), BJ1 protein is distributed throughout the cells. **C**. Stage 10 embryo. The whole cells are stained in segmentally repeated, mitotic domains (cycle 15). **D–H**, Antibody stainings and **I–M**, DNA-stainings of embryonic cells during cycle 14. **D, I**. Interphase. BJ1 is strictly nuclear. **E, J**. Prophase. BJ1 is found in granules throughout the cells. **F, K**. Anaphase. BJ1 is cytoplasmic and not associated with the chromosomes. **G, L**. Telophase. BJ1 redistributes to the daughter nuclei. **H, M**. Prophase and telophase. Bj70 antibody detects BJ1 protein in the cytoplasm and D1 protein at the chromocenters (arrows). **N**. BJ1 antibody staining of the bands on polytene chromosomes from third instar larvae. **O**. DNA staining of the chromosomes from N.

from repeated units of ~30–60 amino acids each. According to their sequence, they fall into two classes: seven N-terminal repeats with low internal homology and a different series of three C-terminal repeats, which are highly homologous to one another. It is likely that the protein has evolved from two ancestral sequences by duplication events, with the C-terminal repeats ('BJ1 repeats') being of more recent origin. The proteins from vertebrates and yeast that are homologous to the seven N-terminal repeats of BJ1 lack the 'BJ1 repeats' (Ohtsubo *et al.*, 1987, 1991; Clark and Sprague, 1989). However, monoclonal antibodies binding to the BJ1 C-terminus react with other proteins from these species, and motifs with similar sequences have been found in nuclear proteins from *Xenopus*, particularly in the histone H1-like protein B4 and the histone-binding proteins N1/N2 and nucleoplasmin. Although these sequences do not correspond to any of the sequences known to interact with histones, the glutamine-rich stretch near the C-terminus of BJ1 is a good candidate for a histone-binding region (Kleinschmidt and Seiter, 1988; Erard *et al.*, 1988). The function of the repeated structure is not yet clear. The yeast nuclear protein $nuc2^+$ also contains 10 internal repeats and it has been proposed that these repeats form 'snap helices', which associate with each other intra- and intermolecularly (Hirano *et al.*, 1990; Sikorsky *et al.*, 1990).The DNA-binding activity of $nuc2^+$ resides in a domain outside the repeats. Obviously, a protein with such a dual function would be ideally suited to induce or stabilize higher order structures of chromatin. Presently, it is not known whether the repeated domains in BJ1 have a secondary structure similar to that proposed from $nuc2^+$ and related proteins. However, the sedimentation behavior of BJ1 released from chromatin at

250 mM NaCl argues for the occurrence of protein−protein interactions. It is also not known if BJ1 binds to DNA directly or through other proteins. The specific release of BJ1 upon alteration of the DNA and chromatin structure induced by intercalation argues for a direct binding. With 7.5 mM ethidium bromide and 50 mM NaCl, no other major polypeptide is released that could mediate BJ1 binding to DNA. If BJ1 is a DNA-binding protein, it must have a low sequence specificity, since its distribution on polytene chromosomes does not reveal any preference for particular sites or loci. In fact, the BJ1 pattern on polytene chromosomes is almost identical to that seen for histone H1 (Frasch, 1985).

The sequence homology of BJ1 to the gene products of *RCC1* from vertebrates and *SRM1/PRP20* from yeast covering 70% of the BJ1 polypeptide is highly significant and argues for a conserved function of these genes. The *RCC1* protein, which has been studied most extensively, shares many other features with BJ1. Like BJ1, it is a nuclear protein that diffuses into the cytoplasm during mitosis (Ohtsubo *et al.*, 1989). The RCC1 protein is also extracted from nuclei at 300 mM NaCl (Ohtsubo *et al.*, 1989) and a protein likely to be identical to RCC1 is released from nuclei by intercalating agents (Bischoff *et al.*, 1990). There is evidence for a DNA-binding affinity of RCC1 (Ohtsubo *et al.*, 1989). The patterns obtained with antibodies against the *Xenopus RCC1* gene product (Nishitani *et al.*, 1990) on Western blots with *Drosophila* extracts and in histological embryo stainings were identical to those obtained with the BJ1 monoclonal antibodies (unpublished results). Most importantly, the complementation of an *RCC1* mutation in a mammalian cell line with the *Drosophila* BJ1 gene, and also with the yeast gene, strongly argues for the idea that these genes have equivalent functions in different species (Ohtsubo *et al.*, 1991). The functions of these genes are not yet fully understood in any of the systems, vertebrates, yeast or *Drosophila*. Since the genes have been studied in each of these systems from a different perspective, we can now combine this information to get a more complete image of the cellular functions of their products.

Most information on the vertebrate *RCC1* gene was obtained from the analysis of a hamster cell line, tsBN2, carrying a temperature-sensitive mutation in this gene (reviewed by Nishimoto, 1988). The phenotype of this cell line at restrictive temperature suggested a function of RCC1 in the control of the cell cycle. In G_2 phase of wild type cells, a cascade of events involving newly synthesized proteins and post-translational modifications results in the activation of the M-phase- (or maturation-) promoting factor (MPF) that triggers entry into mitosis. The active component of the MPF complex is a protein kinase ($p34^{cdc2}$), which is also present, but inactive, during S phase (reviewed by Nurse, 1990). An unknown mechanism prevents activation of MPF before completion of DNA replication (Dasso and Newport, 1990). This mechanisms appears to be disrupted in the tsBN2 cells at restrictive temperature. The loss of RCC1 function in these cells after a temperature shift during S phase results in a premature activation of MPF and thus entry into mitosis (Nishitani *et al.*, 1991). This activation requires synthesis of new proteins, and probably also transcription of mRNA(s) (Nishimoto *et al.*, 1981; Nishitani *et al.*, 1991). Therefore in wild type, until completion of replication during S phase, RCC1 seems to inhibit the expression of one, or several, gene products which are involved in MPF activation.

The developmental expression of BJ1 would be consistent with a function in mitotic regulation. The embryo is provided with a large amount of maternal BJ1 mRNA and also with some maternal BJ1 protein. It is conceivable that these maternal products are necessary to allow the rapid nuclear divisions which occur in early embryos prior to zygotic gene expression. After cell divisions have ceased in the epidermis, BJ1 mRNA is only found in the nervous system, where cells continue to divide. This pattern of expression is similar to that found for other genes involved in mitotic control, such as the cyclins (Lehner and O'Farrell, 1990. Whitfield *et al.*, 1990) and *string* (Edgar and O'Farrell, 1989). However, unlike the cyclins and the *string* product, the BJ1 protein appears to be very stable and a significant amount of it seems to persist until larval or pupal stages. BJ1 protein is also found, at a lower level, in post-mitotic cells that are arrested in G_1, e.g. in the late embryonic ectoderm, or in polytene cells of the salivary gland. One can assume that, in these cells, BJ1 has a function different from regulation of cell cycle events. Similarly, tsBN2 cells mutant for *RCC1* display a phenotype which is not due to cell cycle defects, when they are shifted to restricted temperatures during G_1 phase (Nishitani *et al.*, 1991). Rather, these shifts lead to drastic alterations in gene expression (Nishimoto *et al.*, 1981). The *prp20* mutation in the *RCC1*-like gene from yeast also results in altered mRNA metabolism, and in perturbations of chromatin structure. The steady state levels of several transcripts decrease, while new transcripts seem to be expressed from cryptic promoters (Aebi *et al.*, 1990). *SRM1*, a (probably hypomorphic) allele of *PRP20*, was originally isolated as a suppressor that restored mating of pheromone-receptorless mutants (Clark and Sprague, 1989). This phenotype may be due to alterations in gene expression as well. Such alterations could include over-expression of components of the signal transduction pathway, or under-expression of inhibitors, resulting in constitutive signal transduction.

Thus, effects on gene expression appear to be a common theme in the phenotypes of *RCC1/SRM1/PRP20* mutations. As I have pointed out, the properties of BJ1 do not suggest that it acts as a transcription factor which binds to specific regulatory sequences of target genes. Rather, most data point to a role of BJ1 in the establishment or maintenance of a proper higher order structure of the chromatin. I suggest that BJ1, and the analogous genes in vertebrates and yeast, influence or stabilize chromatin structure in a way that allows correct regulation and expression of genes during interphase and S phase, including those genes controlling activation of MPF. The coupling of MPF activation to the completion of DNA replication could act through a separate mechanism, which only requires the function of BJ1/RCC1/PRP20 as a prerequisite. However, it is also possible that the higher order structure of chromatin and replication are more intimately connected to each other. The silencing of mating type loci in yeast, where the establishment of a particular chromatin structure depends on a replication event, could be a precedent for such a mechanism (Miller and Nasmyth, 1984; Kayne *et al.*, 1988; Johnson *et al.*, 1990). The identification and analysis of proteins associated with BJ1/RCC1/PRP20 (Figure 2; Ohtsubo *et al.*, 1989; Bischoff *et al.*, 1990) together with genetic and molecular studies in

Drosophila and yeast should help to further clarify the relationship between chromatin structure, gene expression and cell cycle control.

Materials and methods

Isolation of nuclei

10 g portions of 1–15 h old embryos were dechorionated and homogenized in 100 ml of embryo buffer (60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 1 mM DTE, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.1% Triton X-100, 15 mM Tris pH 6.4). The suspension was filtered through a nylon mesh and layered onto step gradients of 3 ml 2.4 M sucrose and 7 ml 1.3 M sucrose in embryo buffer. After centrifugation for 15 min at 6000 r.p.m. in a Sorvall HB-4 rotor, the pellets on top of the 2.4 M sucrose cushion were homogenized and diluted in 150 ml of embryo buffer. The nuclei were recentrifuged for 90 min at 25000 r.p.m. in a SW27 rotor on step gradients of 1 ml 2.4 M sucrose and 10 ml 2.2 M sucrose in embryo buffer. The nuclei were collected from the top phase of the 2.4 M sucrose cushion.

Nuclei from K_c cells were isolated as described previously (Risau *et al.*, 1983).

Generation of monoclonal antibodies against nuclear proteins

The preparation of fractions of nuclear proteins used for immunization has been described (Frasch and Saumweber, 1989). The Bj antibodies were obtained with a fraction of proteins that were released from chromatin at 450 mM NaCl and that were contained in the flow through of a QAE Sephadex column at 5 M urea, 150 mM NaCl, 0.5 mM DTE, 10 mM Tris pH 8.3 (Augenlicht and Baserga, 1973). Bj10, Bj46, Bj59 and Bj70 are IgG_1, Bj43 is IgG_3, and Bj86 is IgG_{2a}.

Preparation and fractionation of soluble chromatin

The digestion of nuclei from K_c cells with micrococcal nuclease and RNase A, the fractionation of the extracted chromatin on sucrose gradients and the analysis of the gradient fractions were done as described previously (Frasch and Saumweber, 1989).

Protein extraction by 'elutive intercalation'

This procedure was performed according to Schröter *et al.* (1985). 4.5×10^8 nuclei from K_c cells were resuspended in 3 ml of X2 buffer (1 mM Tris pH 7.0, 0.2 mM EDTA), 7.5 mM ethidium bromide, and NaCl at concentrations of 0, 50 or 100 mM. Extraction was for 45 min at 0°C with shaking. After centrifugation (10 min, 4000 *g*), the extracted proteins were precipitated with 20% TCA, washed with ethanol, and boiled in SDS gel loading buffer. The extract recovered from 5×10^7 nuclei (Coomassie staining) or 1×10^7 nuclei (Western blots) was loaded in each lane.

Gel electrophoresis and Western blots

Electrophoresis on SDS–polyacrylamide gels and Western blots were done as described (Frasch and Saumweber, 1989). The following proteins were used as molecular weight standards: trypsin inhibitor (soybean, 20.1 kd), carbonic anhydrase (29 kd), P11 antigen (Risau *et al.*, 1983; 36 kd), alcohol dehydrogenase (subunit, 39.8 kd), BSA (68 kd), S5 antigen (Risau *et al.*, 1983; 70 kd), β-galactosidase (*E.coli*, 116 kd), RNA polymerase (*E.coli*, 155 kd and 165 kd) and myosin (205 kd).

Isolation of cDNA clones

cDNA clones were isolated from a λgt11 expression library as described by Young and Davis (1983). The library was made from 0–2 h embryonic RNA with a size-selection for insert lengths in the 500 bp range (U.Rosenberg, unpublished). 240 000 phages were plated with a density of 14 000 phages per plate (11 × 11 cm). After IPTG induction, a mixture of the monoclonal antibodies Bj43, Bj46, Bj59 and Bj70, binding to different epitopes of the BJ1 protein, was used for filter incubation, followed by a secondary, alkaline phosphatase conjugated antibody. After the staining reaction, signals were obtained from 23 phages, 12 of which were further purified. Three of these could be detected by all four antibodies, two were only detected by Bj46, four were only detected by Bj59, and nine of them showed cross-hybridization of their inserts.

cDNAs with longer inserts were isolated from a pNB40-plasmid library prepared from 4–8 h embryonic RNA (Brown and Kafatos, 1988).

Sequencing

cDNAs and genomic fragments were subcloned into pBluescript KS+ and SK+ (Stratagene). Deletions were created with appropriate restriction enzymes and the sequencing reactions were carried out with M13 primers or with primers complementary to insert sequences. Sequencing was performed by the dideoxynucleotide sequencing method (Sanger *et al.*, 1977) using single stranded templates and a modified form of T7 polymerase (Sequenase, USB).

Northern analysis

Northern blots were done as described previously (Dohrmann *et al.*, 1990), but using total RNA rather than poly(A)$^+$ RNA.

In situ hybridization

In situ hybridizations to whole embryos and ovaries were carried out according to the procedure of Tautz and Pfeifle (1989). Digoxigenin conjugated dUTP and anti-digoxygenin antibodies were from Boehringer Mannheim. Hybridization probes were prepared according to the protocol of Feinberg and Vogelstein (1984), using a molar ratio of dTTP:DigdUTP of 2:1. For sections (10 μm), the embryos were embedded in Araldite after staining (Leptin and Grunewald, 1990).

Antibody stainings

For antibody stainings of whole embryos (Dequin *et al.*, 1984) and ovaries, fixation was carried out for 20 min in a buffer containing 45 mM KCl, 15 mM NaCl, 13 mM $MgCl_2$, 10 mM K-phosphate pH 6.8, 4% formaldehyde and 15% (v/v) of a saturated, aqueous solution of picric acid. When picric acid was not included, the cytoplasmic BJ1 protein was not retained. Frozen sections were made after the staining (Dequin *et al.*, 1984). Hoechst 33258 was used for DNA staining at 1 μg/ml (in PBS). For HRP stainings, the VECTASTAIN detection kit (Vector Laboratories) was used with diaminobenzidine as a substrate.

Fixation, squashing and staining of polytene chromosomes was done as described by Saumweber *et al.* (1980). Photographs were taken with Kodak 2415 technical pan film and developed with developer HC110 (Nomarski optics) or D19 (fluorescence).

Acknowledgements

I gratefully acknowledge the contributions of Raymond Dequin at the beginning of the project and would like to thank Harry Saumweber for his advice. I appreciate the expert technical assistance of Monika Wild and Meike Müller. I would like to thank Friedrich Bonhoeffer and Janni Nüsslein-Volhard for their support and encouragement. I also thank Takeharu Nishimoto for communicating unpublished results, and Christian Lehner and Daniel St Johnston for critically reading the manuscript.

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Received on January 30, 1991