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Single stranded nucleic acid binding structures on chicken lampbrush chromosomes

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SUMMARY

In chicken oocytes, proteins of the K/J family or their analogs, such as are known to be involved in mRNA processing in humans, are closely associated with nascent C-rich RNA transcripts on the loops of lampbrush chromosomes. Using labelled single stranded nucleotide probes and an antibody to protein K, these C-rich transcripts have been mapped to six different pairs of lampbrush loops situated on 3 macrochromosomes, the sex bivalent (ZW) and certain microchromosomes. Each of these loop pairs has a distinctive morphology. The observations represent cytological evidence of the connection between K-proteins and C-rich RNA transcripts. Another structure, the spaghetti marker of macrochromosome II, also preferentially binds C-rich homonucleotides. This spaghetti marker has a highly distinctive fine structural organization that is quite unlike that of lampbrush loops. Its proteins are not recognised by antibodies to protein K. Homonucleotide binding loops are recommended as potentially extremely valuable as markers on physical maps of chicken chromosomes.

Key words: lampbrush chromosome, K-protein, homonucleotide binding, immunofluorescence, chromosome mapping

INTRODUCTION

It is now well known that both snRNP and hnRNP complexes play important roles in the complicated and highly regulated processing of nuclear pre-mRNAs. Some steps of the pre-mRNA processing (e.g. splicing and polyadenylation) include a transcript sequence-specific recognition, and it seems likely that many other steps must also begin with a sequence-specific binding of RNA by proteins from snRNP and hnRNP complexes.

Proteins from hnRNP complexes have been shown to recognize single stranded (ss) nucleic acids, both ssDNA and ssRNA, and many of them specifically bind homonucleotide sequences (Patton et al., 1991; Datar et al., 1993; Dreyfuss et al., 1993). In particular, homonucleotide tracts are often present in upstream, non-coding, portions of genes. They have regulatory roles and are likely candidates as sites of recognition by regulatory RNPs and proteins. Indeed, several hnRNP proteins that play a role in transcription regulation are capable of recognizing single stranded nucleic acid homopolymers (Takimoto et al., 1993).

Several proteins from hnRNP complexes belong to the family of RNP consensus RNA-binding proteins. They contain one or two RNA-binding domains responsible for the specific interaction of the proteins with RNA, in particular with oligo-RNA homonucleotides (Ghetti et al., 1992; Gorlach et al., 1992; Dreyfuss et al., 1993). Some of these proteins are evolutionarily conserved (Datar et al., 1993).

Lampbrush chromosomes (LBC) are characterized by widespread RNA polymerase II transcription of various DNA sequences. Nascent RNA transcripts, complexed with proteins, make up the RNP matrix of lateral loops of LBCs and are abundant in nuclei of growing oocytes during the LBC stage (Callan, 1986). It is therefore not surprising that many snRNPs and proteins of hnRNP complexes involved in pre-mRNA processing are found in association with LBCs (DiMario et al., 1989).

In preparations of amphibian LBCs, snRNPs are present both in loops and in specific structures, snurposomes, several kinds of which have been found in association with chromosomes or free in the germinal vesicle (Gall, 1991, 1992). Also, several proteins of hnRNP complexes are localized in lateral loops of amphibian LBCs (Pinol-Roma et al., 1989; Soulard et al., 1993). Localization of snRNP or protein components of hnRNP complexes on bird LBCs have not yet been described.

We have recently described a peculiar object associated with the short arm of chicken LBC II, so called the spaghetti marker (SM) (Fig. 1). The SM consists of a loose bundle of 15-16 nm thick fibres. Cytochemical tests indicate that its fibres contain little, if any, nucleic acid and are largely proteinaceous in nature (Solovei et al., 1992). The SM fibres bear no resemblance to lateral loop RNP matrix when examined by transmisson or high resolution scanning electron microscopy (Solovei et al., 1992). The SM lies next to a chromomere to which it seems to be attached. The exact nature of the attachment is impossible to resolve.

When fluorescence in situ nucleic acid hybridization (FISH) with the plasmid pHuR93 (Moyzis et al., 1988) containing the human-derived telomeric DNA repeat was carried out in our laboratory on chicken LBCs, the SM was always strongly labelled. This was consistent with an earlier personal communication from Dr Nancy Hutchison in which she informed us that the structure that we later characterized as the SM labelled with pHuR93. Both Dr Hutchison and ourselves recognised that the binding of pHuR93 to the SM was most likely to involve the poly-(dC) x poly-(dG) tracts flanking the telomere repeat in the recombinant plasmid.

In this article we report the results of the incubation of chicken LBCs with DNA and RNA probes and their competitors which were carried out under conditions that precluded the true hybridization of nucleic acids. The binding of single stranded RNA and DNA homopolymers by the SM and by the RNP matrix of a range of distinctive loops on chicken LBCs is demonstrated and discussed.

MATERIALS AND METHODS

Chromosome isolation

Lampbrush chromosomes (LBCs) were manually isolated from sexually-mature chickens (*Gallus gallus domesticus*, Rhode Island Red cross) by standard techniques (Macgregor and Varley, 1988) modified for bird oocytes (Solovei et al., 1992, 1993). After complete dispersion of nuclear sap, LBCs were centrifuged onto the surface of a microscope slide, prefixed in 2% paraformaldehyde (5 minutes), fixed in 70% ethanol (1 hour) and then stored in 70% ethanol at 4°C.

Probes and competitors

Two types of labelled DNA probes were used for this study: (1) plasmid pHuR93 with an insertion (240 bp) of the human telomere repeat TTAGGG which was prepared by tailing the telomere repeat sequence with oligo-(dC) and ligating it to oligo-(dG)-tailed pBR322 (Moyzis et al., 1988). This plasmid was labelled using a nick-translation kit and Biotin-16-dUTP (Boeringer Mannheim). The plasmid was used after denaturation by heating at 75°C (15 minutes) as a single stranded probe or directly as a double stranded probe; in the latter case, after nick-translation, DNA fragments were end-filled using Klenow fragments; (2) oligo DNAs $(dC)_{30}$, $(dG)_{30}$, $(dA)_{30}$, all synthesized on a Applied Biosystems 394 DNA synthesizer and labelled with one molecule of Biotin on 5′-end.

The following homonucleotides were used as unlabelled competitors for the biotinylated pHuR93 and oligo-$(dC)_{30}$: (1) oligo-$(dC)_{25}$ and oligo-$(dG)_{25}$, both synthesized using an Applied Biosystems 394 DNA synthesizer, and (2) RNA homopolymers poly-(C), poly-(G), poly-(U), and poly-(A) (Pharmacia).

All probes were dissolved either in standard hybridization mixure (50% formamide, 2× SSC, 10% dextran sulfate) or in pure water. Plasmid pHuR93 was always used at a concentration of 50 ng/μl. Concentrations of synthesized oligonucleotides varied from 0.1 to 100 ng/μl. The concentration ratio between labelled sequence and competitor varied from 1/10 to 1/10,000.

Incubation conditions

Incubations of LBC preparations with the biotinylated probes were carried out under the conditions that preclude true nucleic acid hybridization. DNA of LBCs was not denatured to prevent DNA/DNA hybridization. To prevent DNA/RNA-transcript hybridization some slides with chromosomes were treated with ribonuclease A (100 μg/ml in 2× SSC, 1 hour at 37°C). 5 μl of probe was placed on a dry slide (with 4 sets of LBCs), covered by a coverslip, sealed with rubber cement and incubated on a slide warmer at 37°C or at room temperature for 2-12 hours. The competitors were either mixed directly with the labelled probe or the chromosomes were preincubated with a competitor prior to addition of the biotinylated probe. After incubation, slides were washed 3 times in 2× SSC at 37°C and 3 times in 2× SSC at room temperature. Labelled sequences bound to the chromosomes were detected with avidin DN conjugated with FITC (Vector Laboratories) without amplification steps. LBCs were counterstained with DAPI and propidium iodide (PI), air dried and mounted in antifade media (1% DABCO, 50% glycerol, 1× SSC).

Immunofluorescent staining

The monoclonal antibody 3C2 against human protein K (Matunis et al., 1992) was kindly donated by Dr G. Dreyfuss at the Howard Hughes Medical Institute Research Laboratories, USA. Lampbrush chromosomes were incubated in monoclonal antibody ascitic fluid at dilutions of 1:1,000 and 1:10,000. Detection was with FITC-conjugated goat anti-mouse F(ab′)2 (ICN ImmunoBiologicals) diluted 1:200.

Fluorescence microscopy

Labelled structures were visualized with a fluorescence microscope and imaged using a Bio-Rad MRC600 Confocal Scanning Laser Attachment to a Zeiss Axiovert microscope. Grey-scale images were false coloured using the ‘autumn look-up table’ prior to being hard copied via a Sony UP-3000P colour video printer.

RESULTS

Two types of structure on chicken LBCs bind biotinylated single stranded nucleic acid probes under incubation conditions that preclude nucleic acid hybridization. They are the SM on the short arm of the lampbrush chromosome II and several pairs of lateral loops with distinctive morphologies at specific sites on the chicken LBC complement.

The spaghetti marker (SM)

The SM gave a strong fluorescent signal after treatment with denatured pHuR93 plasmid under conditions that precluded the formation of nucleic acid hybrid molecules (Fig. 2A). In the same experiments, the terminal chromomeres and certain interstitial sites of LBCs, all of which are known to contain telmomeric DNA sequences and bind pHuR93 under the true hybridization conditions (Solovei et al., 1994, A. Rodionov, personal communication), were never labelled. Likewise, the short transcription units situated at the very ends of LBC chromatids that contain transcribed telomeric DNA sequences and bind pHuR93 under conditions of DNA to RNA-transcript hybridization were never labelled (Solovei et al., 1994). Taken together, these observations srongly support our claim that the incubation conditions used in our experiments did indeed preclude the true hybridisation of nucleic acids. Labelling of the SM was therefore most probably due to binding of some part of pHuR93 to SM proteins.

Plasmid pHuR93 was constructed from pBR322 and contains 240 bp of human telomere repeat (TTAGGG) flanked by at least 25 bp of (dC)x(dG) (Moyzis et al., 1988). Control experiments demonstrated that neither pure pBR322, nor PCR-generated pure telomere repeat were recognized by the SM. Unlabelled oligo-$(dC)_{25}$, used as a competitor during incubation with pHuR93 (1:1000), completely abolished the fluorescent signal on the SM, whilst oligo-$(dG)_{25}$ did not affect it (Fig. 2A). Binding of pHuR93 to the SM did not take place unless the plasmid DNA was denatured.

We conclude that the SM shows a preferential affinity for single-stranded poly-(C) nucleotide arrays and that the binding of poly-(C) to the SM is not of the kind that characterises the formation of nucleic acid hybrid molecules but results from binding of single stranded poly-(C) to SM proteins.

This conclusion was reinforced when we used biotinylated oligo-$(dC)_{30}$ as a probe in binding experiments with lampbrush chromosomes. The SM strongly bound biotinylated oligo-$(dC)_{30}$ under a variety of conditions, in standard nucleic acid hybridization mixture or in pure water, at 37°C or at room temperature. The fluorescent signal was easily observable in all these experiments down to a probe concentration of 1 ng per slide (1 ng/5ul).

When chromosomes were incubated with biotinylated oligo-$(dC)_{30}$ in the presence of a range of concentrations of poly C-RNA as a competitor, the SM was progressively less strongly labelled as the unlabelled competitor concentration was increased until, at a ratio of 1:1,000 labelled:unlabelled probe, the SM was no longer visibly labelled (Fig. 2B, Table 1). In the same way the binding of biotinylated oligo-$(dC)_{30}$ to the SM was suppressed by poly-(rG), although less strongly than it is by poly-(rC). Poly-(rU) had little or no effect on the binding of biotinylated oligo-(dC) (Table 1). Poly-(rA) had no effect at all. Therefore only poly-(rC) and poly-(rG) were able successfully to compete out the binding of oligo-$(dC)_{30}$ and could therefore be said to be specifically recognized by the SM. Neither biotinylated oligo-$(dG)_{30}$ nor biotinylated oligo-$(dA)_{30}$ were bound by the SM.

We realized that the success of poly-(rG) as a competitor in our experiments could have been a consequence of annealing with the biotinylated probe oligo-$(dC)_{30}$ rather than binding to SM proteins. Accordingly, in subsequent experiments competitor oligonucleotides were applied before the incubation with labelled oligo-$(dC)_{30}$. Chromosomes were preincubated for 2 hours at 37°C with unlabelled oligo-$(dC)_{25}$, oligo-$(dG)_{25}$, poly-(rC), or poly-(rG), washed in 2× SSC (3× 3 minutes), and then incubated for 2 hours at 37°C with biotinylated oligo-$(dC)_{30}$. The ratios of labelled probe concentration to the concentration of competitors used for preincubation were 1:1,000. For poly-(rG), the ratio of 1:10,000 was also tried. Poly-(rC) completely abolished labelling of the SM, signifying that the SM proteins have a greater affinity for single stranded RN homopolymers than they have for single stranded DN homopolymers. Unlabelled poly-(rG) and oligo-$(dC)_{25}$ also suppressed labelling of the SM by oligo-$(dC)_{30}$ detectably but less effectively than poly-(rC) (Table 2).

We conclude that the SM is a structure on chicken lampbrush chromosomes that contains proteins that preferentially bind single stranded poly-(C) RNA and oligo-(C) DNA.

LBC lateral loops

After incubation of chicken LBCs with biotinylated oligo-$(dC)_{30}$ under conditions excluding hybridization with nucleic acids, several binding sites were found in addition to the SM (Fig. 1). These sites include: (1) the pair of marker loops PBL11 on bivalent I (Fig. 3A); (2) a pair of very small loops near the terminal chromomere on the long arm of bivalent II;

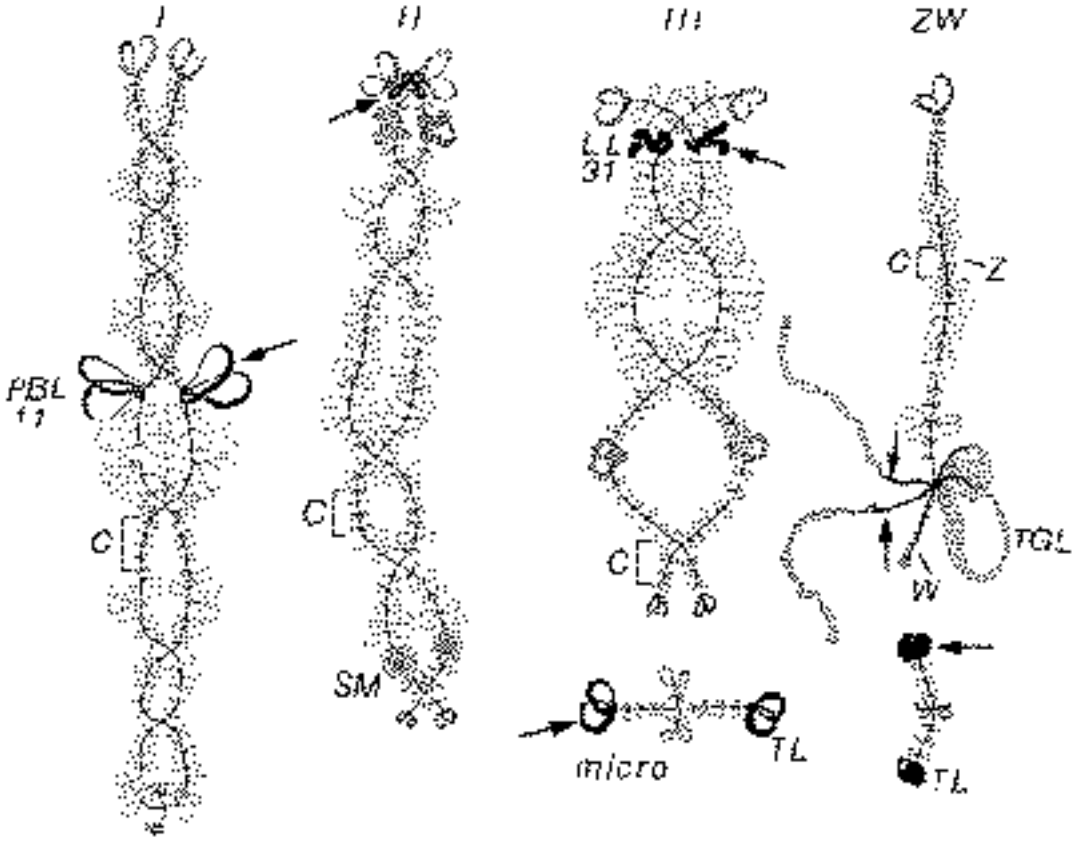

Fig. 1. Schematic drawing of the first three chicken lampbrush macrobivalents (numbers *I, II* and *III)*, the sex bivalent (*ZW)* and some microchromosomes (*micro)* showing the set of loops (coloured black and indicated by arrows) that bind poly(C) single stranded RNA and antibodies against human K protein. *PBL 11*, loops at the proximal border of the 'bald' region 1 of chromosome 1; *LL 31*, lumpy loops 1 on chromosome 3; *TGL*, telomere giant loops of the ZW bivalent; *TL*, telomere loops of the microchromosomes; *C*, centromere region. *SM* indicates the spaghetti marker on the second bivalent which binds poly(C) single stranded RNA and DNA but does not bind 3C2 antibodies. All loops and chromosome regions, with the exception of the Spaghetti Marker, are named according to the nomenclature introduced by Chelysheva et al. (1990)

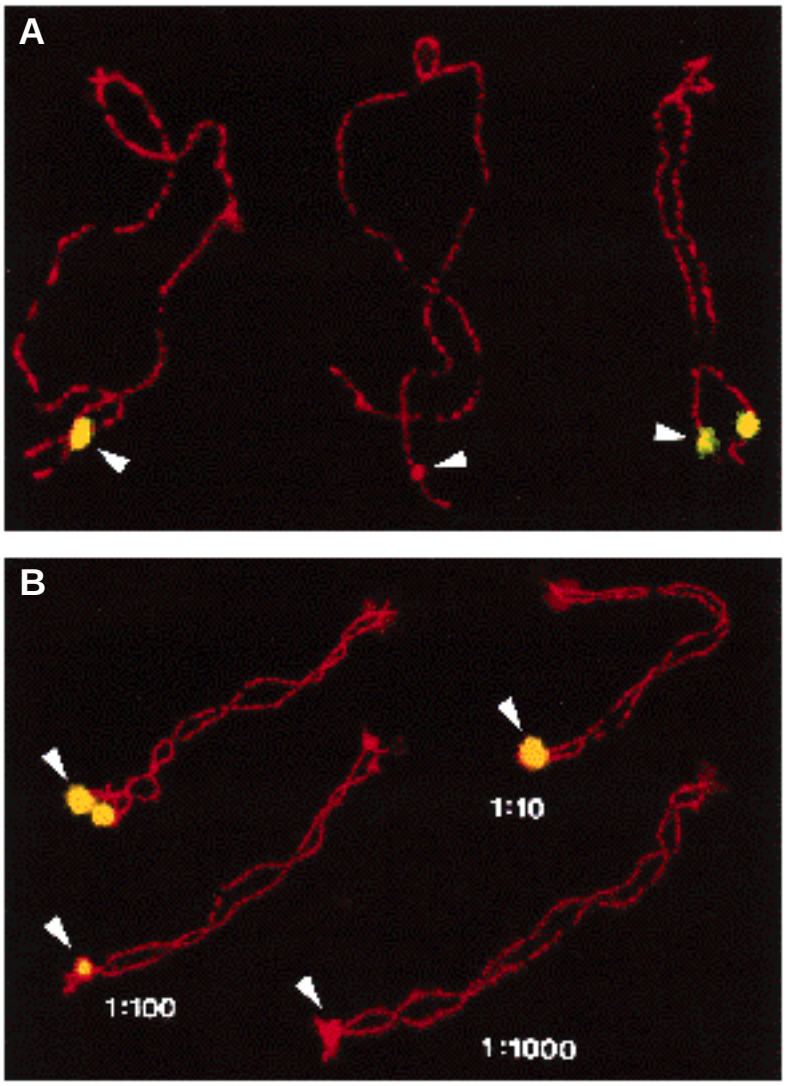

Fig. 2. Labelling of SM (arrowheads) on account of binding of biotinylated pHuR93 or biotinylated oligo-(C)DNA alone or in competition with other unlabelled homonucleotide polymers. (A) Bivalent II after incubation with denatured biotinylated pHuR93 (left) and with the same denatured labelled plasmid probe plus unlabelled oligo-$(dC)_{25}$ (middle) or oligo-$(dG)_{25}$ (right), respectively, as competitors. (B) Labelling of SM with biotinylated oligo-$(dC)_{30}$ after preincubation with non-biotinylated poly-(rC) as a competitor. The concentration ratios labelled probe:competitor are indicated in each case. A bivalent II incubated with pure biotinylated oligo-(dC), with no unlabelled competitor present, is shown at the top left.

(3) a pair of marker lumpy loops LL31 on the long arm of bivalent III (Fig. 3C); (4) the 'thin' ends of the telomere giant loops on the chiasmate ends of the Z and W sex chromosomes; (5) the telomere loops on the short arms of some microchromosomes (Fig. 3E). In all these cases binding of labelled oligo-$(dC)_{30}$ was RNase-stable and was abolished by competing with unlabelled poly-(rC). As with the SM, labelling of these loops with oligo-$(dC)_{30}$ was suppressed by the unlabelled competitor poly-(rG), but not by oligo-$(dG)_{25}$, oligo-$(dA)_{25}$, poly-(rA) nor poly-(rU).

Table 1. Labelling of SM by biotinylated oligo-(dC) in presence of competitor RNAs

Probes and competitors	Probe/competitor ratio	Brightness of SM
Biotinylated oligo-$(dC)_{30}$/no competiton		+++
Biotinylated oligo-$(dC)_{30}$/poly-(rC)	1/10	++
	1/100	+
	1/1,000	–
Biotinylated oligo-$(dC)_{30}$/poly-(rG)	1/1,000	–
Biotinylated oligo-$(dC)_{30}$/poly-(rU)	1/1,000	+++

Experiments with antibodies

Monoclonal antibody 3C2, against human protein K, used in dilutions of 1:1,000 or 1:10,000, gave very strong positive reactions with all 5 kinds of oligo-(dC)-absorbing loops (Fig. 3B,D,F) but no reaction whatsoever with the SM (Fig. 1). The

Table 2. Labelling of SM by biotinylated oligo-(dC) after preincubation with competitor nucleic acids

Competitor nucleic acid for preincubation		Brightness of SM
Oligo-$(dC)_{25}$	(1:1,000)	+
Oligo-$(dG)_{25}$	(1:1,000)	+++
Poly-(rC)	(1:1,000)	–
Poly-(rG)	(1:1,000)	±
	(1:10,000)	–

nuclei of erythrocytes (which were deliberately placed alongside chromosome preparations) also gave a positive immunoreaction which appeared as a few separate spots. This is consistent with the data of Matunis et al. (1992) who showed positive immunoreaction to hnRNP K in interphase nuclei of HeLa cells using the same antibodies.

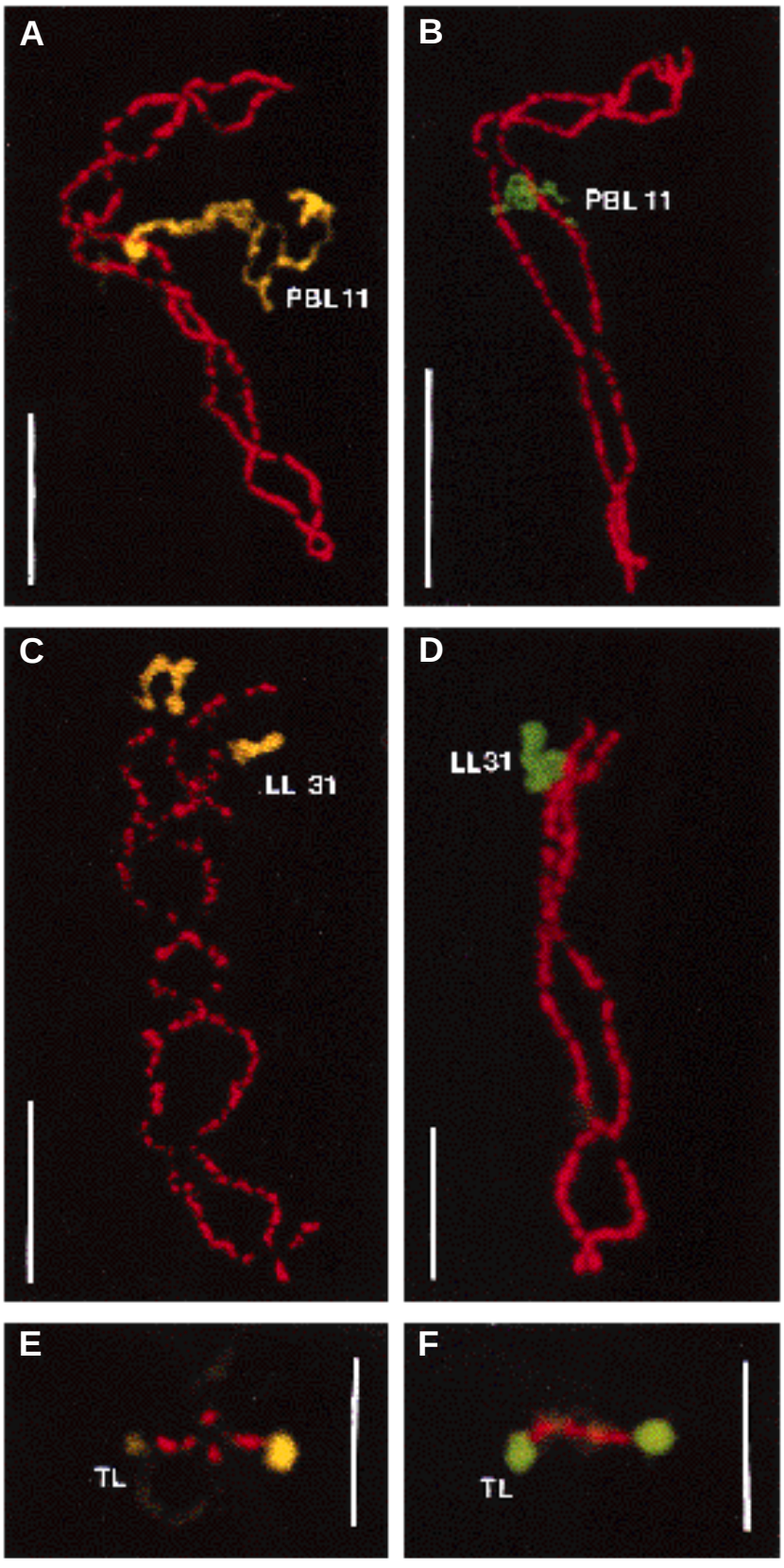

Fig. 3. Labelling of loops on lampbrush bivalent I (A and B), bivalent III (C and D) and a microbivalent (E and F) on account of binding of biotinylated oligo-$(dC)_{30}$ (A,C and E) and immunostaining with antibodies against K protein (B,D and F). Bars: (A-C), 20 μm; (D-F), 10 μm.

DISCUSSION

Our data indicate that two types of proteins that preferentially bind poly-(C) nucleic acids are present on chicken LBCs. One of the protein types forms part of the RNP matrix of a specific set of loops. It is immunologically similar to protein K/J from human (Matunis et al., 1993) and *Xenopus* (Siomi et al., 1993), and is most likely nothing more than the chicken equivalent of protein K/J. Proteins K/J bind poly-(C) nucleic acids and they are known to be amongst the major pre-mRNA binding proteins involved in mRNA processing (Matunis et al., 1992; Siomi et al., 1993; Burd and Dreyfuss 1994).

The human protein K sequence-specifically binds to the C-rich region of proto-oncogene c-myc and therefore might be involved in regulation of its transcription (Takimoto et al., 1993). This protein also possesses a high affinity for poly-(C) RNA (Matunis et al., 1993), which makes it a likely candidate for involvement in the processing of RNA transcripts. These and other lines of evidence about the functional significance of K protein have a purely biochemical basis.

Our results again support the conclusion that proteins K/J bind specifically to C-rich RNA but they put the matter on a more cytological level by showing that in chicken oocytes, proteins K/J or their analogs are closely associated with the nascent RNA transcripts on the loops of lampbrush chromosomes. Futhermore, using labelled single stranded nucleotide probes and antibodies has enabled us to map directly a well defined set of chromosome regions where these C-rich transcripts are situated, so providing an approach to localization of the sequences (genes) that specify such transcripts.

The long, branching fibres of the SM, with their smooth outlines and uniform dimensions, are structurally very different from the RNP matrix of loops (Solovei et al., 1992). RNase treatment does not affect the appearance of the SM, from which we have concluded that RNA at least is not a significant component of its structure (Solovei et al., 1992). The SM is not recognized by the antibody to hnRNP K/J. For the moment, we can only conclude that its fibres consist largely of a different protein that preferentially binds C-rich RNA. The true nature of this extraordinary structure remains a mystery.

Homonucleotide absorbing loops are potentially extremely valuable as convenient markers on the cytological maps of chicken LBCs. They could be especially useful for chicken gene mapping by FISH.

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