Volume 15 Number 3 1987 Nucleic Acids Research

A monoclonal antibody to triplex DNA binds to eucaryotic chromosomes

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Received September 26, 1986; Revised and Accepted January 9, 1987

ABSTRACT

A monoclonal antibody (Jel 318) was produced by immunizing mice with poly[d(Tm^5C)].poly[d(GA)].poly[d($m^{45}CT$) which forms a stable triplex at neutral pH. Jel 318 did not bind to calf thymus DNA or other non pyrimidine.purine DNAs such as poly[d(TG)].poly[d(CA)]. In addition the antibody did not recognize pyrimidine.purine DNAs containing m^6A (e.g. poly[d(TC)].poly[d(Gm^6A)]) which cannot form a triplex since the methyl group blocks Hoogsteen base-pairing. The binding of Jel 318 to chromosomes was assessed by immunofluorescent microscopy of mouse myeloma cells which had been fixed in methanol/acetic acid. An antibody specific for duplex DNA (Jel 239) served as a control. The fluorescence due to Jel 318 was much weaker than that of Jel 239 but binding to metaphase chromosomes and interphase nuclei was observed. The staining by Jel 318 was unaffected by addition of *E. coli* DNA but it was obliterated in the presence of triplex. Since an acid pH favours triplex formation, nuclei were also prepared from mouse melanoma cells by fixation in cold acetone. Again Jel 318 showed weak but consistent staining of the nuclei. Therefore it seems likely that triplexes are an inherent feature of the structure of eucaryotic DNA.

INTRODUCTION

Unlike bacteria, eucaryotes contain large amounts of non-random sequences (1,2). Many of these are strictly repeating pyrimidine.purine stretches containing all pyrimidines on one strand and all purines on the other. For example, the 5′-flanking region of one gene of the mouse MHC region contains three regions of oligo[d(TC)].oligo[d(GA)] of lengths greater than 50 base-pairs (3). Several other dramatic examples of these unusual sequences have been reported recently (4,5,6). Because these sequences frequently occur in the 5′-flanking regions of genes, there exists the possibility that they are involved in some sort of control function. This idea has received considerable support because *in vivo* and *in vitro* these sequences are hypersensitive to the action of single-strand specific nucleases (7,8). Two theories have been put forward to explain these results.

The first suggests that pyrimidine.purine sequences adopt an unusual

duplex conformation (9,10). On the basis of susceptibility of oligo[d(TC)].oligo[d(GA)] to alkylating agents Pulleyblank has proposed the adoption of an `S´ helix which contains alternating Watson-Crick (A-T) base-pairs with Hoogsteen bonded (G-C) base-pairs (9). The `S´ helix certainly rationalizes some features of the chemical and nuclease sensitivity of these sequences but not all. More recently a heteronomous structure has also been proposed (10).

The second theory proposes that pyrimidine.purine DNAs can dismutate to form a triplex together with a single-stranded purine (4,5). Triplexes are favoured by negative supercoiling and a low pH (11). These conditions not only favour chemical sensitivity and nuclease attack but also the formation of a single-stranded purine strand is consistent with some of the patterns of nuclease digestion (4,5). Of course, there is no good reason why both `S´ helices and triplexes might not exist simultaneously under some conditions in the same genome (5). We became interested in the idea that these sequences might potentially form triplexes because of our observation that pyrimidine.purine DNAs containing m^5C form stable triplexes at neutral pH (12). Because poly[d(Tm^5C)].poly[d(GA)].poly[d($\overset{+5}{m}CT$)] forms a stable triplex at pH 7, this also suggested an approach to the question of whether triplexes have a biological role.

Although this triplex is only poorly immunogenic a monoclonal antibody (Jel 318) specific for this structure has been prepared. In this paper, we demonstrate by immunofluorescent microscopy that Jel 318 stains metaphase and interphase chromosomes. Of course, any critical evaluation of this work is dependent on the specificity of the antibody. This problem has been solved in part by preparing analogues of poly[d(TC)].poly[d(GA)] containing m^6A. Pyrimidine.purine DNAs containing m^6A cannot form triplexes because the extra methyl group interfers with Hoogsteen base-pairing (Figure 1). Jel 318 does not bind to these DNAs.

MATERIALS AND METHODS

Nucleic Acids: m^6dATP was synthesized by methylation of dATP via the Dimroth rearrangement (13). Synthetic polymers were prepared as described previously (12,14). Poly[d(TC)].poly[d(Gm^6A)] was synthesized by incorporating m^6dATP in place of dATP. By adding both m^6dATP and dATP in varying ratios to the reaction cocktail, the proportion of m^6A in the final polymer could be adjusted (13). The ratio of incorporation was estimated from the Tm (14) since Engel and von Hippel have shown that the depression

in Tm is proportional to the incorporation of m^6A (13). The triplex poly[d(Tm^5C)].poly[d(GA)].poly[d($\overset{+5}{m}CT$)] was prepared from the parent duplex by treatment with S_1 endonuclease. The DNA (6 A260 units in 1 ml) in 100 mM Na acetate buffer pH 5.0 was incubated at 37°C for 1 hour to ensure complete triplex formation (12). Then $ZnCl_2$ was added to a concentration of 5 μM together with 25 units of S_1 endonuclease. The incubation at 37°C was continued (for about 3 hours) until the pH 5 ethidium bromide fluorescence assay showed that half of the polypurine strands had been digested (11,15). The sample was then treated with 50 μg/ml proteinase K and purified by gel exclusion chromatography in 10 mM Na acetate pH 5.0.

Monoclonal antibody, Jel 318: After immunizing C57/Black mice three times with 50 μg of triplex complexed to 50 μg of methylated BSA hybridomas were prepared by fusions with MOPC 315.43 as described previously (16,17). Hybridoma supernatants were screened for the presence of antibody against poly[d(Tm^5C)].poly[d(GA)].poly[d($\overset{+5}{m}CT$)] by a solid phase radio immune assay (SPRIA) (16,17). After ten fusions and the screening of approximately 20,000 hybridomas, one (Jel 318) which showed strong binding to the triplex was successfully cloned. This compares to a success rate of about one positive hybridoma for 100 tested for many proteins (unpublished observations). Clearly the triplex is not highly immunogenic. Antibody Jel 318 was prepared in large quantities from ascites fluid by purification of the IgG with gel exclusion chromatography as described previously (17). The preparation of Jel 239 which is specific for duplex DNA has been reported recently (18).

Solid Phase Radio Immune Assays: SPRIA and competitive SPRIA were performed in PBS buffer (pH 7.2) with a 1 in 10 dilution of the hybridoma supernatant (16,17).

Immunofluorescent Microscopy: The mouse myeloma cell line, MOPC 315.43 was used for the preparation of fixed metaphase chromosomes. Logarithmically growing cells (50 mls) were treated with 0.025 μg/ml colcemid for 1 hour before harvesting the cells by centrifugation. They were then resuspended in 0.075% KCl for 15 minutes, recentrifuged and treated three times for half an hour with 3:1 (v/v) methanol/acetic acid. After the final centrifugation the nuclei were resuspended in 1 ml of 3:1 (v/v) methanol/acetic acid and several drops were released from about 60 cms onto microscopic slides. The slides were dried overnight at 50% humidity.

A mouse melanoma cell line (B16F10), a gift of Dr. R.C. Warrington, was attached to slides directly by placing sterilized microscope slides in

petri dishes of actively dividing cells. They were then fixed by the cold acetone technique and dried at 20°C (19).

After fixing, both types of cells were treated with a solution of the monoclonal antibodies in PBS for 1 hr. Jel 318 IgG from ascites was used at a hundredfold dilution while Jel 239 from the hybridoma supernatant was diluted two fold. For competitive experiments, DNA (either triplex or duplex E. coli DNA) was added together with the antibodies. The slides were then washed individually with PBS before incubating the slides with a solution of fluorescein-conjugated goat anti-mouse IgG (10 μg/ml from Sigma). After 1 hour the slides were again washed and a drop of PBS containing 0.1% p-phenylenediamine and 10% glycerol was added to prevent

Table 1
Solid-Phase Radioimmune Assay Results for Jel 318 Binding to Various DNAs[a]

Nucleic Acid	Percentage of Maximum Binding
Poly[d(Tm^{5}C)].poly[d(GA)].poly[d(m^{+5}CT)]	100
Poly[d(Tm^{5}C)].poly[d(GA)]	83
Poly[d(TC)].poly[d(GA)]	53
Poly[d(TTm^{5}C)].poly[d(GAA)]	39
Poly(dA).poly(dT)	34
Poly(dA).poly(dT).poly(rU)	33
Poly(dT).poly(dA).poly(dT)	19
Poly(dG).poly(dm^{5}C)	<5
Poly[d(Tm^{5}Cm^{5}C)].poly[d(GGA)]	<5
Poly[d(TG)].poly[d(CA)]	<5
Poly[d(TC)].poly[d(Gm^{6}A)]	<5
Calf Thymus DNA	<5
Heat-denatured Calf Thymus DNA	<5
Poly[d(Gm^{5}C)].poly[d(Gm^{5}C)]	<5
Blank Well	<5

[a] The results are expressed as percentage of maximum binding after substraction of the background. The maximum cpm was about 3,000 with a background of 200.

oxidation of the fluorescein under U.V. irradiation (20). Finally, the slide was sealed with a coverslip surrounded by Permaseal (Fischer).

The slides were viewed in a Zeiss photomicroscope II with epifluorescence attachment. Photographs were taken with Ilford XP1 400 with a x100 oil immersion objective lens.

RESULTS

Initially the specificity of Jel 318 was investigated by SPRIA, the results of which are shown in Table I. Jel 318 bound best to poly[d(Tm^{5}C)].poly[d(GA)].poly[d($^{+5}$mCT)] but there was also good binding to several other duplex DNAs. However, all of these are pyrimidine.purine DNAs and they can potentially form triplexes. Indeed at pH 7.2 at which pH the SPRIA is performed both poly[d(Tm^{5}C)].poly[d(GA)] and poly[d(TTm^{5}C)].poly[d(GGA)] can dismutate to form triplexes (12). Moreover, it has been accepted for some time that poly[d(TC)].poly[d(GA)] forms a minor proportion of multi-stranded structures at neutral pH ever since the observation that different preparations of this DNA have different bouyant densities (21). A small amount of triplex may also be present in preparations of poly(dA).poly(dT). It should be noted that the 53% binding of poly[d(TC)].poly[d(GA)] does not imply that this DNA contains 50% triplex under these conditions--the SPRIA is only a qualitative binding assay as has been noted previously (16,22). Of more

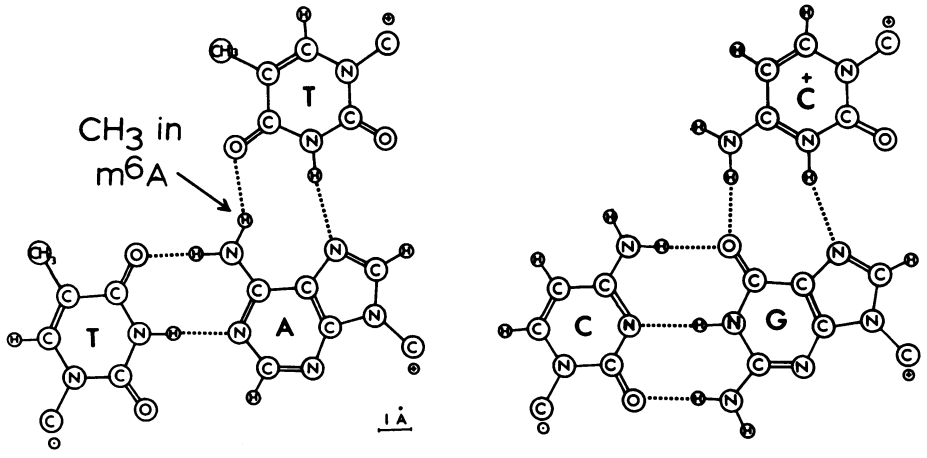

Figure 1. Structures of the base triads T-A-T and C-G-C$^+$ involved in triplex formation. Notice that the presence of m^{6}A interferes with Hoogsteen base-pairing.

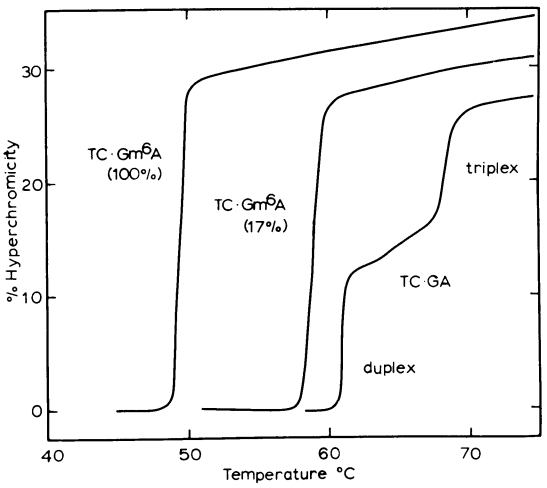

Figure 2. Thermal denaturation profiles for poly[d(TC)].poly[d(GA)] poly[d(TC)].poly[d(Gm^6A)] and poly[d(TC)].poly[d(Gm^6A)] (17%) (i.e. containing 17% m^6A in place of A) measured in 5 mM NaCl, 0.01 mM EDTA and 10 mM HEPES pH 7.0.

importance is the observation that Jel 318 does not bind to calf thymus DNA or poly[d(TG)].poly[d(CA)] which cannot form triplexes under any conditions. There is also no measurable binding to poly[d(Tm^5m^5C)].poly[d(GGA)] or poly(dG).poly(dm^5C) suggesting that Jel 318 has a sequence preference for triplexes containing a high proportion of T-A-T triads.

The lack of binding to poly[d(TC)].poly[d(Gm^6A)] is also significant since this DNA cannot form a triplex due to the interference of the methyl group with Hoogsteen base-pairing (Figure 1). The parent polymer readily forms a triplex upon heating at pH 7.0 as demonstrated previously (12) and shown in the thermal denaturation profile of Figure 2. On the other hand, even a polymer containing 17% m^6A (poly[d(TC)].poly[d(Gm^6A)] (17%)) will not form a triplex at pH 7.0 (Figure 2). Does this partially methylated polymer bind Jel 318?

Competition binding experiments are shown in Figure 3. The amount of competitor required to reach 50% inhibition of binding can be used to estimate relative binding constants. As expected for an antibody which only binds triplex, Jel 318 binds to poly[d(Tm^5C)].poly[d(GA)]. poly[d($m^{+5}CT$)] about five times better than to poly[d(Tm^5C)].poly[d(GA)] which contains equal amounts of both strands but which partially dismutates

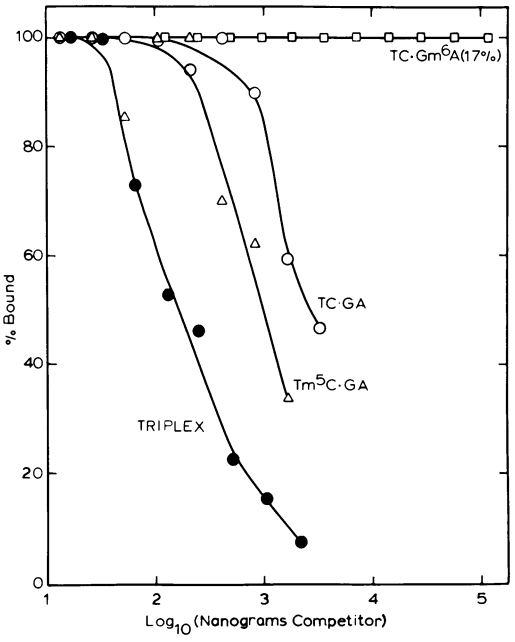

Figure 3. Competition binding experiments for the hybdridoma supernatant of Jel 318. The plates were coated with triplex.
● = Triplex-poly[d(Tm^{5}C)].poly[d(GA)].poly[d(m^{+5}CT)]
Δ = Poly[d(Tm^{5}C)].poly[d(GA)], O = Poly[d(TC)].poly[d(GA)]$_6$ and
□ = Poly[d(TC)].poly[d(Gm^{6}A)] (17%) (i.e. containing 17% m^{6}A in place of A).

to a triplex (12). The binding to poly[d(TC)].poly[d(GA)] is about 20 fold less, while there is no measurable binding to poly[d(TC)].poly[d(Gm^{6}A)] (17%).

Could the lack of binding be due to the extra methyl group which occurrs, on average, every 12 base-pairs? If it is assumed that one arm of the IgG occludes 6 base-pairs and that the other arm binds independently (due to flexibility of the hinge region) then the theory of overlapping binding sites (23) can be used to estimate that five out of eleven binding sites will still be available to the IgG even in the presence of an obstructive methyl group. In other words for a duplex binding IgG the binding constant to duplex poly [d(TC)].poly[d(Gm^{6}A)] (17%) would be expected to be (at the most) about two fold lower than to duplex poly[d(TC)].poly[d(GA)]. As shown in Figure 3 the ratio of binding constants is at least 500 fold. Thus this data is not consistent with antibody recognition of duplex DNA and the level of binding to

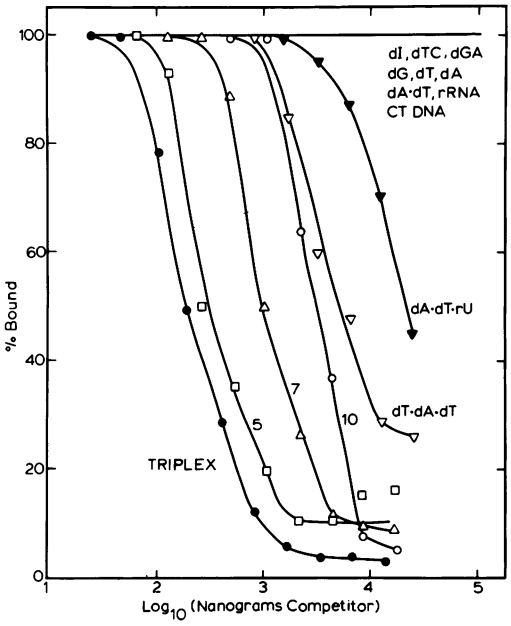

Figure 4. Competition binding experiments for hybridoma supernatant of Jel 318. The plates were coated with triplex.
● = Triplex-poly[d(Tm^{5}C)].poly[d(GA)].poly[d(m^{5}C$^+$T)]
□,△,○ = pH 5, pH 7, or pH 10-treated poly[d(Tm^{5}C)].poly[d(GA)]
▽ = poly(dT).poly(dA).poly(dT)
▼ = poly(dA).poly(dT).poly(rU)
No competition was observed with poly(dI), poly[d(TC)], poly[d(GA)], poly(dG), poly(dT), poly(dA), poly(dA).poly(dT), ribosomal RNA or calf thymus DNA.

poly[d(TC)].poly[d(GA)] is probably due to the presence of about 5% triplex in this polymer.

Since formation of the triplex is pH dependent the effect of pH on antibody binding was also determined (Figure 4). Poly[d(Tm^{5}C).poly[d(GA)] was treated at pH 5, 7 or 10 for 48 hrs at 0°C. The pH was returned to pH 7 by the addition of 150 mM HEPES buffer and then competition experiments were performed immediately. Because the duplex-triplex transformation shows considerable hysteresis, the DNA is expected to retain some "memory" of the pH at which it was incubated (12). As shown in figure 4, pH 5-treated poly[d(Tm^{5}C).poly[d(GA)] competes slightly less well than triplex poly[d(Tm^{5}C)].poly[d(GA).poly[d(m^{5}C$^+$T)] but better than pH 7-treated duplex which in turn is a more effective competitor than pH 10-treated duplex. If

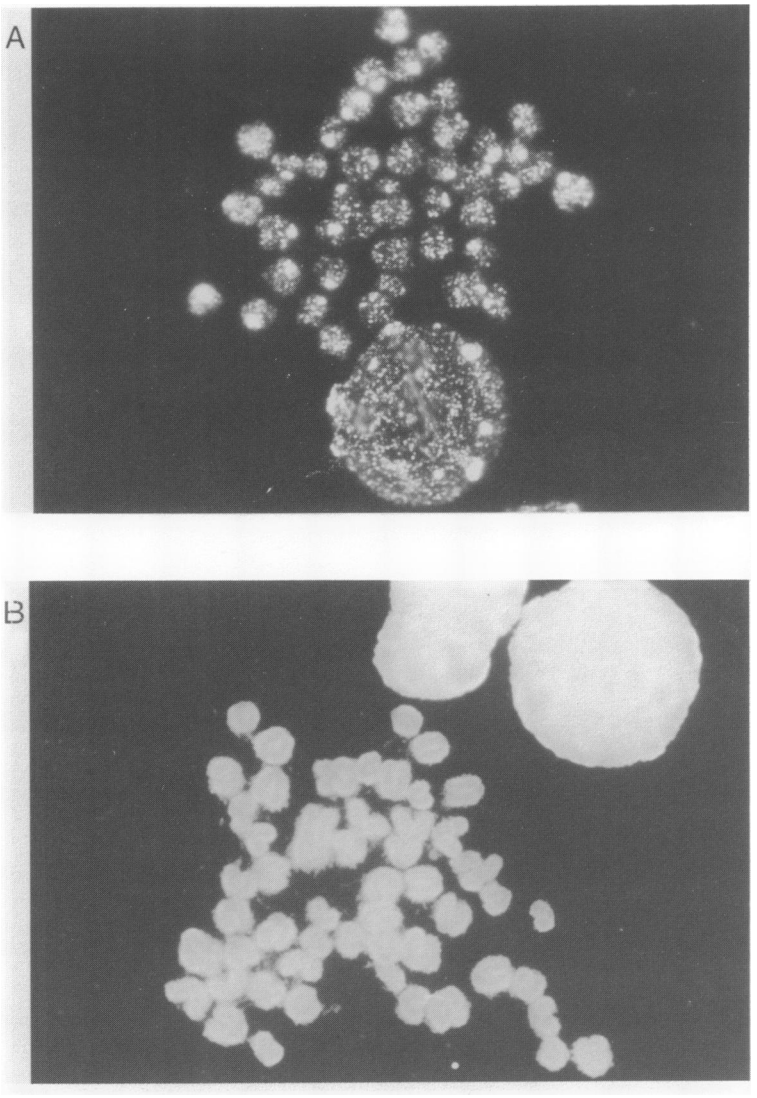

Figure 5. Immunofluorescent microscopy of metaphase chromosomes and interphase nuclei of mouse myeloma cells fixed in methanol/acetic acid (a) Jel 318, (b) Jel 239.

Jel 318 was duplex specific than exactly the opposite result would have been expected.

Figure 4 also demonstrates that the triplexes poly(dT).poly(dA).poly(dT) and poly(dA).poly(dT).poly(rU) bind to Jel 318

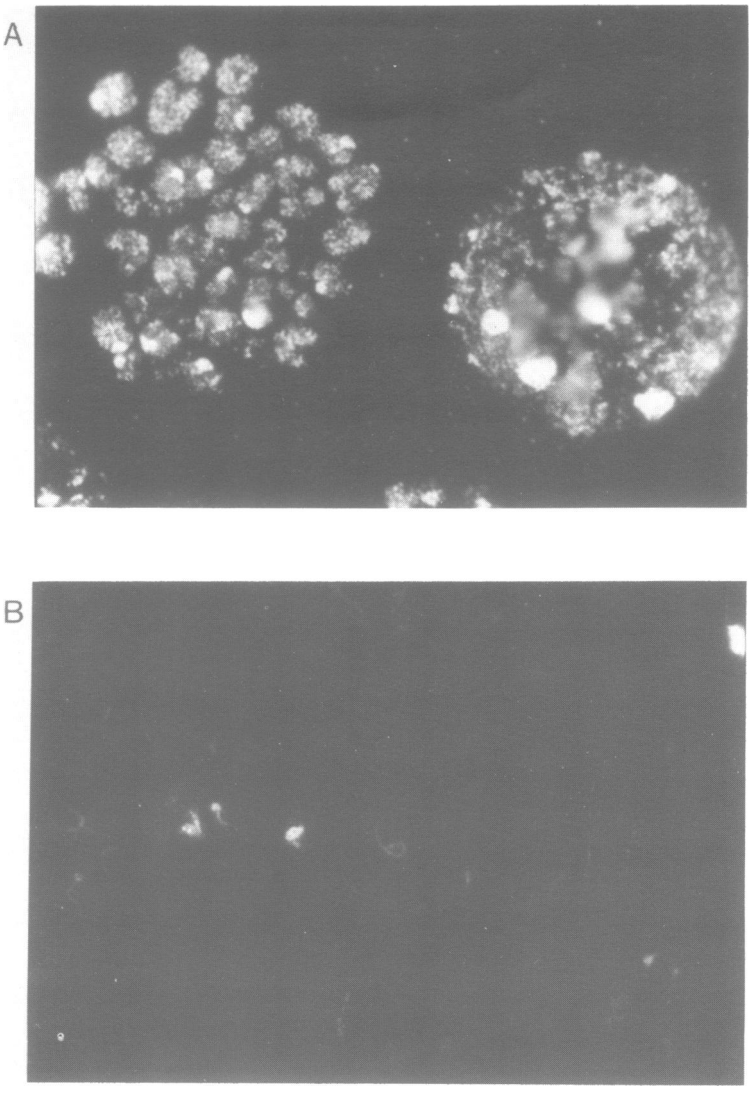

Figure 6. Immunofluorescent microscopy with Jel 318 of metaphase chromosomes and interphase nuclei of mouse myeloma cells fixed in methanol/acetic acid (a) In the presence of 25 μg/ml E. coli DNA (b) In the presence of 25 μg/ml triplex.

although the interaction is several orders of magnitude weaker than to poly[d(Tm^5C)].poly[d(GA)].poly[d(m^5C^+T)]. It is also noticeable that neither poly(dT) nor poly(dA).poly(dT) show competition. Again this is

good evidence for the triplex specificity of Jel 318. Finally, no competition was observed with poly(dI), poly(dG), poly[d(GA)], poly[d(TC)], poly(dA), ribosomal RNA or duplex calf thymus DNA. Therefore, it was concluded that Jel 318 was specific for the triplex with which the mice were originally immunized. There is also some binding to triplexes of other sequences but there is no measureable binding to duplex DNA.

Metaphase chromosomes and interphase nuclei stained with Jel 318 are shown in Figure 5. As a control Jel 239 which is specific for duplex DNA was used concurrently. It can be seen that the fluorescence due to Jel 318 was considerably less than that due to Jel 239 and this difference was observed consistently with many different preparations. Although the staining with Jel 239 is relatively uniform that given by Jel 318 is not. In particular the chromosomes have a speckled appearance and many of the centromeres are more brightly fluorescent. We are currently attempting to quantitate the relative intensities given by Jel 318 and Jel 239 as well as use polytene and the chromosomes of other species to investigate staining patterns.

Figure 6 shows the binding of Jel 318 to nuclei and metaphase chromosomes in the presence of 25 μg/ml of E. coli DNA or triplex. E. coli DNA has little effect on the staining by Jel 318 whereas it is completely obliterated by the same concentration of triplex. We believe that the bright spots which appear in the presence of triplex are due to antibody-triplex complexes which precipitate and stick to the slides. This experiment again provides evidence that the antibody is triplex specific and will not bind to duplex DNA. On the other hand the staining of Jel 239 could be removed in the presence of E. coli DNA but not by triplex (Data not shown).

Since fixation of nuclei and chromosomes in methanol/acetic acid is expected to favour triplex formation because of the low pH, cells were also fixed in cold acetone. The staining of melanoma nuclei by Jel 318 and Jel 239 is shown in Figure 7. Clearly the intensity due to Jel 239 is much greater than that of Jel 318 but the nuclei of the melanoma cells are still visible.

DISCUSSION

Two major problems can arise in studies with immunofluorescent microscopy. The first relates to the specificity of the antibodies while the second involves artefacts which may occur during preparation of the

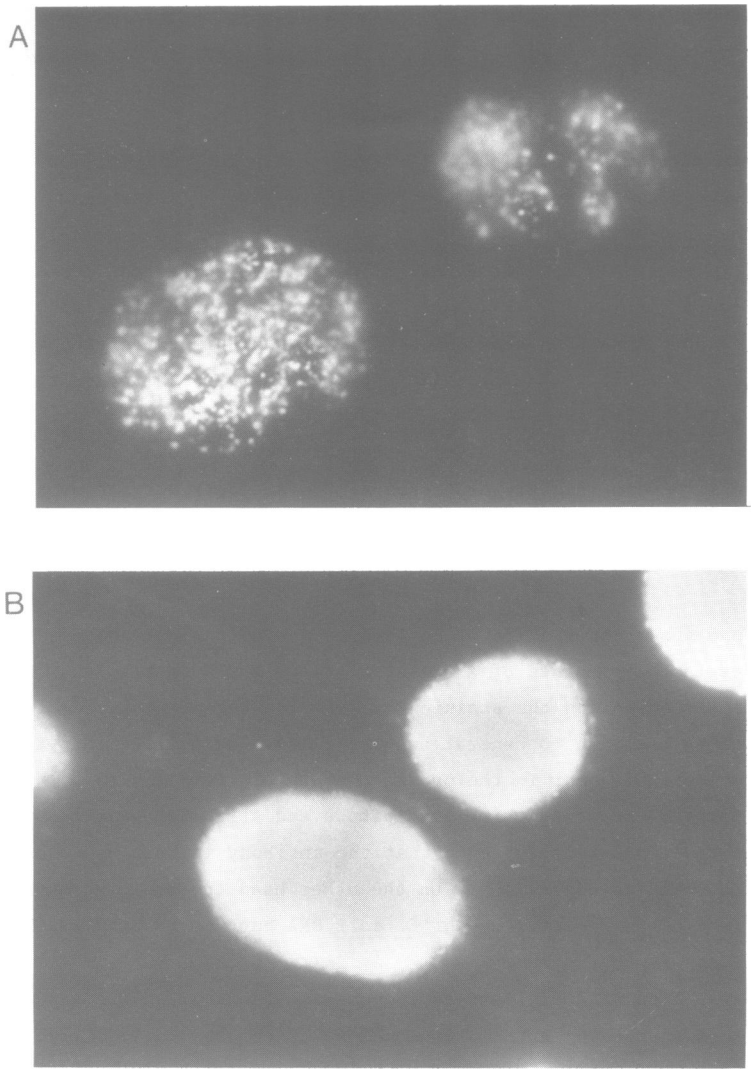

Figure 7. Immunofluorescent microscopy of mouse melanoma cells fixed in cold acetone (a) Jel 318 (b) Jel 239.

chromosomes. Similar problems have arisen with the `Z´ DNA story.

`Z´ DNA is highly immunogenic and therefore a battery of specific reagents have been prepared both as monoclonal antibodies and as sera from rabbits (22,24,25). Results from many different laboratories were in agreement that these reagents did not bind to the usual `B´ DNA

conformation. In the present case, the triplex poly[d(Tm^5C)].poly[d(GA)].poly[d($\overset{+5}{m}CT$)] is only weakly immunogenic so that rabbit antisera may be difficult to prepare and the preparation of monoclonal antibodies is an arduous task. It may be sometime before other antibodies with specificities similar to that of Jel 318 are available for comparative studies. Moreover the 'B' to 'Z' transition is well-defined and indeed some brominated polymers are locked into the 'Z' conformation (26). On the other hand, triplexes can only be locked into place by low pH and they are formed from two separate duplexes by dismutation (11). Thus one of the original duplexes is still present and it is virtually impossible to ensure that 100% conversion has occurred. Studies on the specificity of triplex binding antibodies such as Jel 318 therefore are inherently more complex. Without the availability of pyrimidine.purine DNAs containing m^6A it would have been difficult to define the specificity of Jel 318. As it is the competition experiments of Figure 3 suggest that the binding constant to the triplex is at least four order of magnitude greater than to duplex DNA. This degree of discrimination is not difficult to understand if one considers that the structural features of a triplex (27) are at least as different from 'B' DNA as those of 'Z' DNA (28). Binding of Jel 318 to DNA.DNA.RNA triplexes seems unlikely in view of the equally good staining of metaphase chromosomes and interphase nuclei. The latter are expected to contain far more RNA than metaphase chromosomes. Finally, although the competition experiments of Figures 3 and 4 are all consistent with a triplex specificity for the antibody, we are not able to rule out absolutely the possibility that the antibody is binding to an, as yet, unknown conformation or structure of nucleic acid. Even if this is the case, this unknown conformation or structure is not uniform because the staining of Jel 318 reveals a speckled pattern.

The second problem with immunofluorescent microscopy can arise during the preparation of the chromosmes. This is well illustrated by the studies of Hill and Stollar on the binding of 'Z' DNA antibodies to polytene chromosomes (29). They found that unfixed chromosomes showed no staining and staining only became apparent after treatment with methanol/acetic acid. In other words, 'Z' DNA was being formed only after treating the chromosomes at low pH. Since triplexes are favoured at low pH the same artefact is possible in the present studies with mouse myeloma cells fixed in methanol/acetic acid. However mouse melanoma cells fixed in cold acetone also showed good staining and therefore a low pH is not required to

observe the binding of Jel 318. Consequently it seems likely that triplexes are an inherent feature of the structure of eucaryotic DNA.

At present it is not possible to define what function these triplexes might be performing but two ideas come to mind. The first involves a structural role; that is the formation of triplexes holds the loops of DNA in place in the eucaryotic nucleoid (30,31). The second possibility is that triplexes have a regulatory function in gene expression. Triplex formation in the 5'-flanking region could either switch the genes 'ON' or 'OFF' as has been discussed previously (12). In any event the presence of multi-stranded structures in eucaryotic chromosomes must now be taken seriously.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. R.C. Warrington for advice and encouragement. Special thanks are due to Dr. A.R. Morgan for stimulating this line of investigation. This work was supported by MRC Canada by grants to J.S.L. R.P.B. is the holder of an MRC studentship.

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