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Effect of anti-cruciform DNA monoclonal antibodies on DNA replication

M.Zannis-Hadjopoulos, L.Frappier, M.Khoury and G.B.Price

McGill Cancer Centre, McGill University, 3655 Drummond St, Montreal, Quebec, H3G 1Y6 Canada

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To study the possible involvement of DNA cruciforms in the initiation of DNA replication, we used two monoclonal antibodies, 2D3 and 4B4, with anti-cruciform DNA specificity. Synchronized CV-1 cells were released into S phase for hourly intervals up to 6 h and permeabilized in the presence of monoclonal antibodies, under conditions that allow limited DNA replication. Exposure of the permeabilized cells to 2D3 or 4B4 resulted in a 2- to 6-fold enhancement of incorporation of labeled precursor nucleotide over the 6 h period. Approximately 50% of the enhanced synthesis was sensitive to aphidicolin, and the enhancing effect of 2D3 was abolished by absorption with immunobead anti-mouse immunoglobulin. Dot-blot hybridization analyses of DNA isolated from anti-cruciform antibody treatment groups showed a similar 2- to 11-fold increase in the relative copy number of low copy probes. In contrast, exposure of the permeabilized cells to a monoclonal antibody directed against Z-DNA and B-DNA had no significant effect on DNA synthesis. The results suggest that cruciforms are present in replicating DNA and that they are recognized and stabilized by the monoclonal antibodies.

Key words: antibodies/cruciform binding/mammalian DNA replication/replication origin

Introduction

Inverted repeat DNA (palindromic) sequences are widely distributed in the chromosomal DNA of many eukaryotes (Wilson and Thomas, 1973, 1974; Schmid *et al.*, 1975). They have the potential of intrastrand base pairing, which, in a negatively supercoiled molecule, can lead to the formation of cruciform structures interspersed among linear DNA sequences. It has been suggested that such structures may form under physiological conditions to serve as recognition signals for specific regulatory proteins of DNA replication or transcription. For example, inverted repeat sequences have been found at the operator and transcription termination regions in prokaryotes (Maniatis *et al.*, 1975; Rosenberg and Court, 1979) as well as at the replication origin region of many prokaryotes and mammalian viruses (Frisque, 1983; Stow and McMonagle, 1983; Zyskind *et al.*, 1983; Weller *et al.*, 1985; Lockshon and Galloway, 1986). They are also present in monkey DNA segments enriched for origins of DNA replication (Zannis-Hadjopoulos *et al.*, 1984, 1985; Frappier and Zannis-Hadjopoulos, 1987). Recently, palindromic sequences have been found in association with the amplified *myc* genes of human tumor cell lines and amplified *CAD* genes in drug-resistant BHK cell lines (Ford and Fried, 1986). These observations support the hypothesis that inverted repeat sequences may represent potential initiation sites for DNA replication (Bollum, 1975; Edenberg and Huberman, 1975; Hobom *et al.*, 1979; Meijer *et al.*, 1979; Tamm *et al.*, 1979) serving as the attachment site for initiator proteins (Hand, 1978). In support of this Collins *et al.* (1982) found that although there were no S1 nuclease sensitive sites in non-proliferating cells, the number of such sites increased when the cells moved from G_0 to G_1; the number of sites peaked at the onset of S phase. Cruciforms are sensitive to single-strand specific nucleases like S1 (Lilley, 1980; Panayotatos and Wells, 1981) by virtue of their single-strandedness at the tip of the stem-loops.

Although the existence of cruciforms *in vitro* has been demonstrated (Karrer and Gall, 1976), their existence *in vivo* has not been directly shown (Sinden *et al.*, 1983). In a recent report, Panayotatos and Fontaine (1987) present positive evidence for the presence of native cruciform DNA structures in bacteria *in vivo*. Because cruciform structures are energetically unfavorable, rapidly reverting to linear molecules, they are thought to form transiently *in vivo* as stable structures. It has been suggested that the action of cellular factors such as DNA helicases, binding proteins and other transcription and replication enzymes may affect the equilibrium and the rate of formation of cruciform structures *in vivo* (Mizuuchi *et al.*, 1982). In an effort to facilitate the detection of cruciforms *in vivo* we recently produced monoclonal antibodies with unique specificity to cruciform DNA structures (Frappier *et al.*, 1987). These antibodies recognize conformational determinants specific to DNA cruciforms and do not bind linear double-stranded (ds) DNA, linear single-stranded (ss) DNA, ssDNA containing a stem-loop (hair-pin) structure or tRNA. We report that the use of these anti-cruciform DNA antibodies in a permeabilized cell system capable of carrying out DNA replication, results in a 2- to 11-fold enhancement of DNA replication, as measured by (i) the incorporation of precursor nucleotide into acid-precipitable material, and (ii) the enhancement in copy number of low copy (unique) genetic elements.

Results

Cell synchrony

In order to facilitate our study, we decided to use cells synchronized at the G_1/S interphase, which have been reported to potentially contain transient but stable cruciforms that might function as signals for the initiation of replication (Collins *et al.*, 1982). CV-1 cells were blocked at the G_1/S boundary by serum starvation and aphidicolin (Zannis-Hadjopoulos *et al.*, 1984; Kaufmann *et al.*, 1985) and were then released into S phase at hourly intervals up to 6 h post-block, prior to being permeabilized and exposed to the anti-

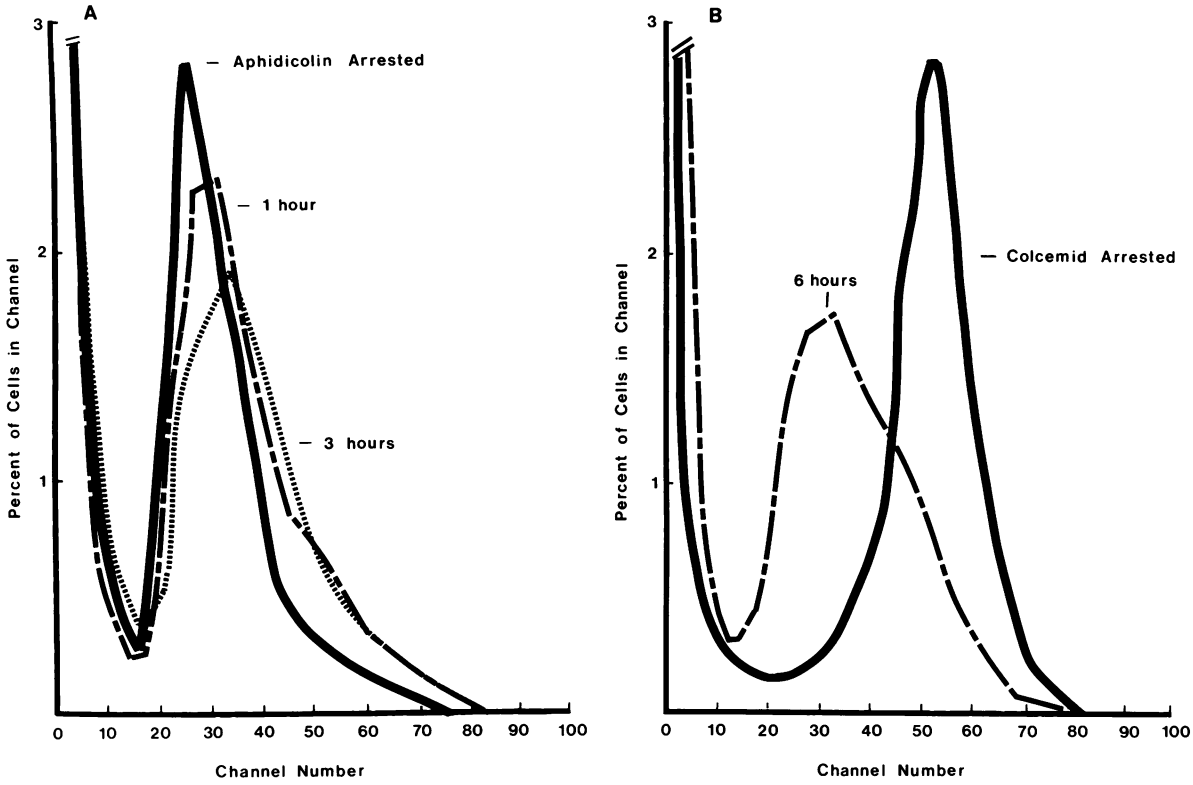

Fig. 1. Flow microfluorometric analysis of CV-1 cells blocked at G_1/S with aphidicolin and then released into S phase for intervals up to 6 h. (**A**) CV-1 cells were analysed after being blocked at the G_1/S boundary (2*n* DNA content) by simultaneous treatment with aphidicolin (2 μg/ml) and 20% FCS for 16 h, as well as at 1 h and 3 h after removal of the aphidicolin block. (**B**) Similarly, the cells were analyzed 6 h after removal of the aphidicolin, as well as after having being exposed to colcemid for 15 h (G_2 + M; 4*n* DNA content).

body. The degree of synchrony achieved by this method was monitored by flow cytometry (Figure 1). The results show that after 16 h in the presence of aphidicolin plus 20% serum, the vast majority of the viable cells were distributed in G_1 (Figure 1A) but started synthesizing DNA within 1 h after removal of the aphidicolin and continued for 3 h. By 6 h after removal of the drug the majority of the cells were still distributed in the early and mid-part of S phase with only a small fraction of cells in late S/G_2 phase (Figure 1B).

Effect of 2D3 and 4B4 mAb on DNA replication in permeabilized cells

The membranes of intact living cells are impermeable to antibodies. In order to bypass this limitation we took advantage of existing *in vitro* systems that utilize permeabilized cells and are capable of sustaining *bona fide* DNA replication with high fidelity, though at a capacity much reduced (Kristensen and Prydz, 1985; Burhans *et al.*, 1986). In these systems DNA replication has been shown to be semiconservative and, by and large, a continuation of replicons that had initiated *in vivo*, although some initiation of new replicons *in vitro* has been reported (Hand and Gautschi, 1979).

CV-1 cells that had been grown in the presence of [^{3}H]-thymidine for a period of 24–30 h for uniform labeling of the DNA were collected either at the G_1/S boundary or at hourly intervals up to 6 h after their release into S phase, and were permeabilized in buffer containing all the ribonucleotide and deoxyribonucleotide precursors necessary for *in vitro* DNA synthesis, with [α-^{32}P]dCTP as the labeled precursors, as described in Materials and methods. Prior to the addition of the permeabilizing buffer, the cells had been resuspended in media containing either the anti-cruciform mAbs, 2D3 or 4B4, or the control mAbs, P3 or BN18, respectively, to ensure the immediate exposure of the native chromatin to the antibody upon permeabilization. Following a 30 min incubation in ice, that allowed binding to occur between the antibody and cruciforms present on the chromatin, the DNA was replicated for 30 min at 37°C and the incorporation of [α-^{32}P]dCTP into acid-precipitable material was monitored (Figure 2). (It should be noted that 0 h represents a post-aphidicolin pulse at 37°C for 30 min; it, therefore, does not represent the base level, but rather it is indicative of the first 30 min in S.) The results, expressed as a ^{32}P : ^{3}H ratio to normalize for variation in cell numbers, show a 3- to 4-fold enhancement of [α-^{32}P]dCTP incorporation in the presence of 2D3 (Figure 2A) over that observed in the presence of P3; and a 4–5-fold enhancement in the presence of 4B4 (Figure 2B) over that observed in the presence of BN18. The incorporation profiles obtained in the presence of P3 and BN18 were identical to those obtained when the cells were resuspended in only ITS medium (−Ab) or PBS (no treatment). Figure 3 shows a plot of relative enhancement (stimulation of incorporation by 2D3 mAb over P3) at each time point, as an average of five different experiments (except for the 6 h time point which is an average of three experiments). As the error bars indicate, although there is variability from experiment to experiment, especially during the first hour of synthesis, there is on the average a reproducible stimulation of synthesis by 2D3 at each time point, in excess of 3-fold.

The majority of the enhanced incorporation (>50% at the 2 and 5 h peaks in the case of 2D3) was sensitive to the ac-

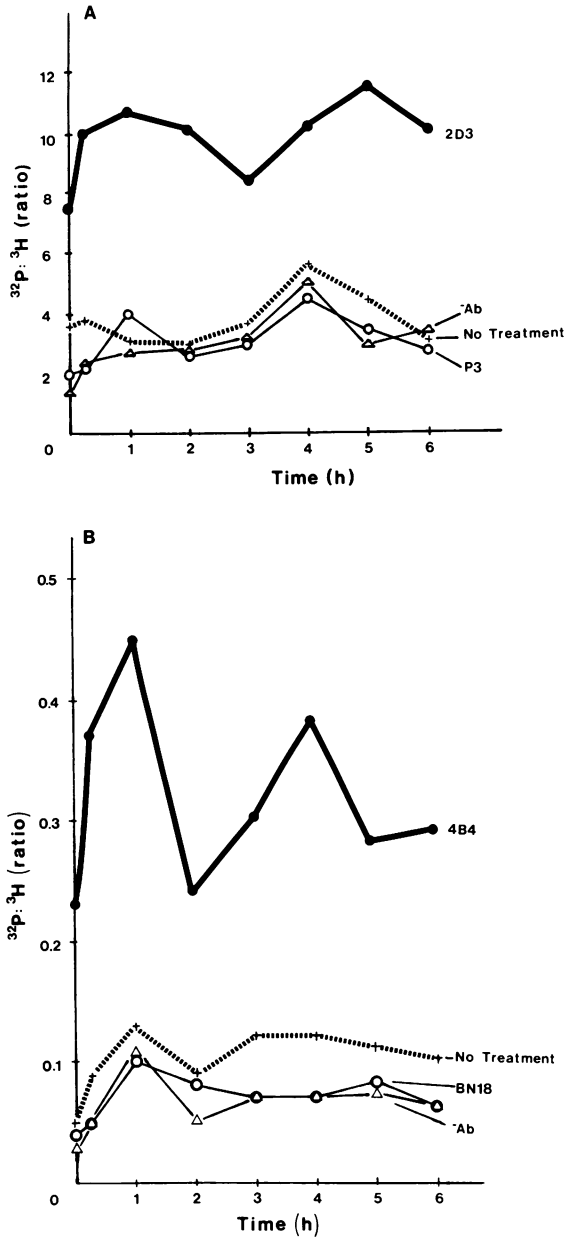

Fig. 2. Effect of anti-cruciform DNA mAbs on the incorporation of [α-^{32}P]dCTP into acid precipitable material of permeabilized cells. Cells were grown for 24–30 h in the presence of [^{3}H]thymidine (10 nCi/ml for 2A; 0.1 μCi/ml for 2B), then depleted of serum for 24 h and finally blocked at the G_1/S interphase by being treated with 20% FCS and aphidicolin, as in Figure 1. At the time intervals indicated following their release into S phase, samples were collected, resuspended in (**A**) 2D3 mAb (IgG$_1$; ●) or (**B**) 4B4 mAb (IgM; ●) and permeabilized in the presence of [α-^{32}P]dCTP. After incubating 30 min in ice, the DNA was replicated for 30 min at 37°C and then precipitated in 5% TCA, filtered through GF/C filters and counted in scintillation fluid. Parallel incubations were done in the presence of the control mAbs, P3 and BN18 (○), as well as in the presence of ITS medium with 2% FCS (△) and PBS (+). 0 h represents a post-aphidicolin pulse of 30 min at 37°C.

tion of aphidicolin (Figure 4) at a concentration of 8 μg/ml, indicating that most of the synthesis observed was due to the action of DNA polymerase α and/or δ (Hammond *et al.*, 1987). The variation in relative aphidicolin effects may reflect the differences in the level of antibody-induced enhancement. In general, greater enhancement by 2D3 is associated with a greater sensitivity to aphidicolin. It can

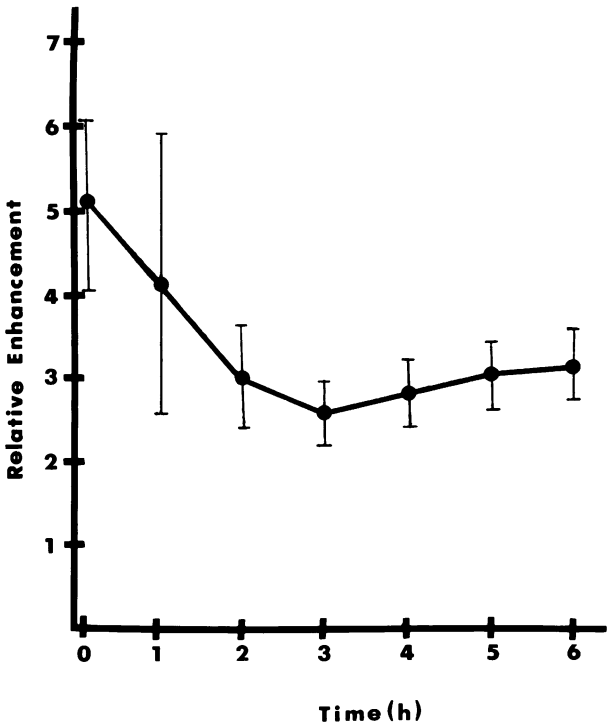

Fig. 3. Relative enhancement of incorporation of radioactivity by 2D3. Incorporation of [α-^{32}P]dCTP in the presence of 2D3 P3 mAbs was averaged for five different experiments, identical to that described in the legend of Figure 2, and the ratio of the two was plotted against time following the removal of aphidicolin (the 6 h time point represents an average of three experiments). Bars = 1 SD.

be noted that the majority of synthesis in permeabilized cells at the G_1/S boundary was relatively insensitive to the action of aphidicolin (8 μg/ml), while at later time points in S, synthesis became increasingly sensitive to the drug. The reverse was true in the presence of 2′,3′-dideoxythymidine triphosphate (ddTTP), a nucleotide analogue that inhibits polymerase β and γ but not α (Edenberg *et al.*, 1978), where maximal inhibition (50–60%) occurred within the first 15 min in S and decreased as cells progressed into S (data not shown). These results agree with those previously reported by others (Delfini *et al.*, 1985; Burhans *et al.*, 1986). The incorporation of precursor nucleotide into DNA either in the presence of P3 control antibody or in the complete absence of antibody (TTS medium or PBS) was also sensitive to this dose of aphidicolin (8 μg/ml), ranging from 22% inhibition at the G_1/S boundary to 50% at the later time points.

In order to test further whether the observed enhancement of precursor incorporation into DNA was a specific effect of the anti-cruciform monoclonal antibodies, we also tested for comparison, using the same assays, the effect of anti-dsDNA monoclonal antibody, HB2, which reacts specifically with Z-DNA and B-DNA (Monier *et al.*, 1984). The results (Table I) show that the incorporation in the presence of HB2 was similar to that obtained in the presence of the control antibodies BN18 and P3, and in the complete absence of antibody (−Ab) from the reaction mixture.

The biological activity of 2D3 was associated with immunoglobulin. Adsorption of 2D3 onto immunobead rabbit anti-mouse immunoglobulin and subsequent removal of the beads by centrifugation, was done prior to the addition

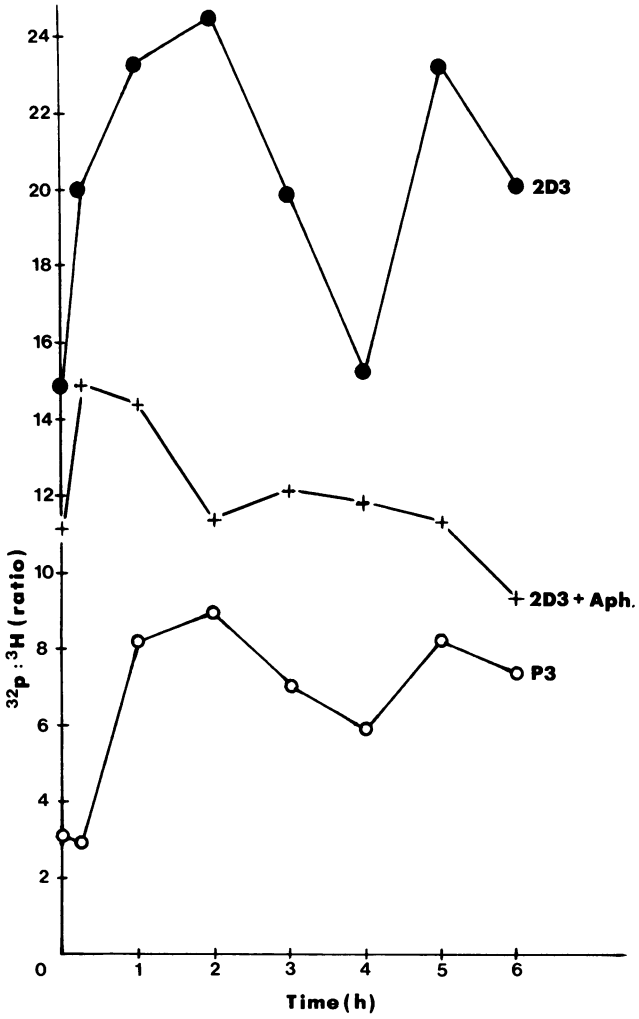

Fig. 4. Effect of aphidicolin on the enhancement by 2D3 mAb of incorporation of [α-^{32}P]dCTP. The cells were treated as described in Figure 2 except that an additional set of cells was included (+) which contained aphidicolin (8 μg/ml) in its permeabilization buffer.

Table I. Comparison of the effect of various antibody treatments on the incorporation[a] of [α-^{32}P]dCTP into acid precipitable material in permeabilized[b] CV-1 cells

Time in S phase (h)[c]	mAb				−Ab
	2D3	P3	HB2[d]	BN18	
0	21.8 (3.9)[e]	5.6	3.6	4.7	4.1
2	17.6 (3.1)	5.6	4.8	8.4	6.5
4	18.1 (2.3)	7.8	6.1	8.4	5.9

[a]Incorporation is expressed as ^{32}P : ^{3}H ratio (see text for explanation).
[b]The cells were synchronized in G_1/S by treatment with aphidicolin, then released into S phase by removal of the drug. Treatment with the mAb, permeabilization and DNA replication are described in Materials and methods.
[c]Time past removal of aphidicolin.
[d]Monoclonal antibody with reactivity against Z-DNA and B-DNA.
[e]Numbers in brackets indicate (−)-fold enhancement of incorporation in the presence of anti-cruciform mAb, 2D3, relative to its control antibody, P3.

of the depleted supernatant culture fluid onto the cells. This removal of immunoglobulin eliminated the enhancing effect of 2D3 and decreased the level of [α-^{32}P]dCTP incorporation to the level observed in the presence of P3, ITS medium or PBS (data not shown).

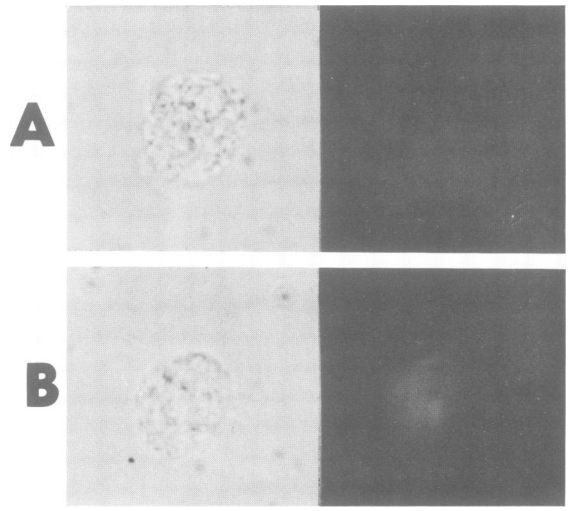

Fig. 5. Demonstration of the localization of anti-cruciform DNA, mAb and 2D3 in nuclei of permeabilized CV-1 cells. (**A**) Representative labeled nuclei, washed and stained with fluorescein-conjugated goat anti-mouse IgG 30 min after exposure to (**A**) P3 or (**B**) 2D3. Left panel is phase contrast and right panel is fluorescence image. Magnification is 787×.

In five out of eight experiments, a biphasic incorporation profile was obtained, with the two peaks occurring at 1−2 and 5−6 h, respectively, into the S phase (Figures 2 and 4). In the other three experiments a single peak was observed at either 3 or 4 h. These results may reflect a temporal activation of early- and mid-S replicating clusters of replicons and the observed variations in time may be due to variations in the cell synchrony from experiment to experiment.

Electrophoretic analysis and autoradiography of the DNA synthesized in the presence of the four mAbs in the permeabilized cells, performed both on non-denaturing agarose and denaturing polyacrylamide gels, showed [α-^{32}P]dCTP incorporated predominantly into DNA chains of $\leq$1000 bp in length, the majority being between 400 and 700 bp (data not shown). This is in agreement with previously published data using the same *in vitro* synthesizing system (Burhans *et al.*, 1986).

Detection of 2D3 and 4B4 mAbs in the nuclei of permeabilized cells

In order to confirm that entry of mAbs into the cell nucleus was achieved in our system, samples of cells that were permeabilized and treated with 2D3 and P3 as described above were allowed to react with fluorescein-conjugated goat anti-mouse IgG (Figure 5). With this indirect immunofluorescence method, 2D3 was detectable in the reacted nuclei (Figure 5B) while P3 was not (Figure 5A). Equivalent results have been obtained with 4B4 and BN18 using a fluorescein-conjugated anti-mouse IgM (data not shown).

Dot-blot hybridization analysis of the DNA synthesized in the permeabilized cells

In order to explain the apparent enhancement of [α-^{32}P]-dCTP incorporation into DNA in the presence of 2D3 antibody by comparison to the control antibody, P3, we isolated the *in vitro* synthesized DNA from the two mAb treatments

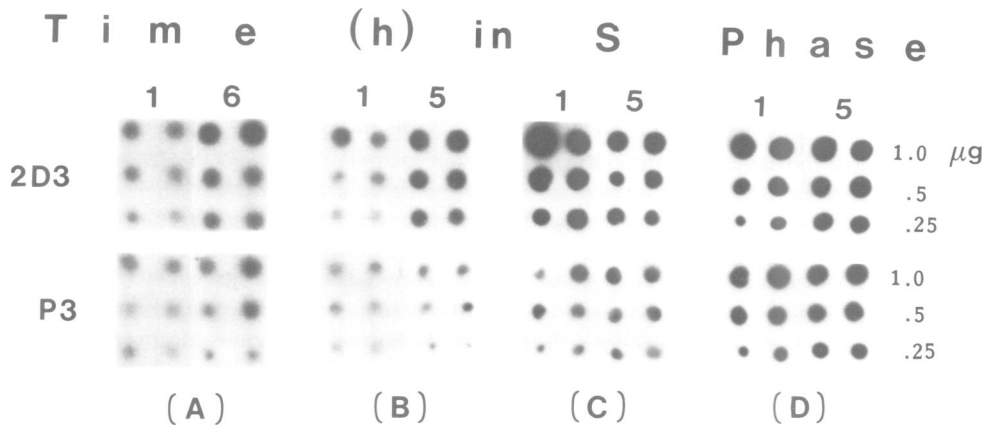

Fig. 6. Effect of 2D3 mAb on DNA synthesis of (**A**) *ors 8*, (**B**) *DHFR*, (**C**) c-*myc* and (**D**) c-*fos* gene sequences in permeabilized CV-1 cells, measured by dot-blot hybridization. Gene probes were the inserts of plasmids pBR32/*ors 8*, pSV2-*DHFR*, pHSR-1 and pc-*fos* (human)-1, respectively.

and dot-blotted it for hybridization analysis with probes, whose copy number in the monkey genome is known and whose synthesis is known to take place in the early part of S phase (Figure 6). The amount of hybridizable radioactivity of each probe was quantitated for each time point by densitometry, as a measure of relative copy number in that particular DNA, present after each mAb treatment. The results (Table II) show that when we use a low-copy probe (*ors 8*; five copies per haploid CV-1 genome) (Zannis-Hadjopoulos *et al.*, 1984), we observe a 3- to 6-fold increase in its copy number in the DNA that was synthesized *in vitro*, at 1–6 h into the S phase in the presence of 2D3 (average magnitude of [α-^{32}P]dCTP incorporation enhancement, 2-fold) over that synthesized in the presence of P3 (Figure 6A). *Ors 8* is a 483 bp long clone of CV-1 DNA, obtained by a procedure that enriches for sequences that contain origins of replication (Zannis-Hadjopoulos *et al.*, 1985) and, as we showed recently, it can initiate autonomous replication in monkey and human cells (Frappier and Zannis-Hadjopoulos, 1987). It contains an imperfect 27 bp inverted repeat at its center (Zannis-Hadjopoulos *et al.*, in preparation). Similar results were obtained using a cDNA clone of the single-copy mouse *DHFR* gene (Chang *et al.*, 1978) as probe (Figure 6B), but this time a 2- to 5-fold amplification of copy number was observed in the DNA that was synthesized *in vitro* at 1–5 h into the S phase, in the presence of 2D3; the magnitude of this enhancement was also similar to that observed for incorporation (see also Figures 2 and 3). Replication of mammalian DNA sequences containing the *DHFR* gene occurs immediately on entry of the cells into the S phase but has also been reported to occur throughout the first 5 h of S (Heintz and Hamlin, 1982). Similarly, we probed the *in vitro* synthesized DNA with c-*myc* and c-*fos*, two cellular proto-oncogenes that are thought to play a role in the regulation of cell proliferation. An enhancement of 11-fold or higher, much greater than that observed for *ors 8* and DHFR, was observed for c-*myc* (Figure 6C and Table II) in the presence of 2D3 and during the first hour of synthesis; however, synthesis of c-*fos* in the presence of either 2D3 or P3 was quantitatively the same (Figure 6D). Finally, we observed no difference in the copy number of highly reiterated sequences such as *Alu* family (Jelinek *et al.*, 1980) or alpha satellite (McCutchan *et al.*, 1982) when we used them as probes (data not shown).

Table II. Relative enhancement of DNA synthesis by 2D3 mAb in permeabilized cells[a] determined by specific probes

Probe	Time in S phase (h)[b]		
	1	5	6
ors 8	3.1[c]	–	5.5
DHFR	2.1	4.2	–
c-*myc*	11.2	3.2	–
c-*fos*	0.8	1.0	–

[a]Incorporation is expressed as ^{32}P : ^{3}H ratio (see text for explanation).
[b]Time past removal of aphidicolin.
[c]The relative enhancement is shown as a ratio of radioactive probe hybridized to DNA isolated from 2D3-treated cells to that isolated from P3-treated (control) cells. Radioactivity was quantitated by densitometry scanning of the dots in the 0.25 μg row, shown in Figure 5.

Discussion

Regions of inverted repeats, which are widely distributed in DNA, have the potential of adopting a cruciform configuration (Platt, 1955). It has long been postulated that cruciforms may form transiently *in vivo*, to act as special regulatory signals on the DNA, for the initiation of DNA replication and serve as the attachment sites for the initiator proteins (Zannis-Hadjopoulos *et al.*, 1984; Lockshon and Galloway, 1986). The ability to form a stem-loop (cruciform) structure is known to be essential for function of the origin of replication on the plasmid ColE1, where mutations that disrupt folding result in replication failure (Masukata and Tomizawa, 1984). In this case a multiple stem-loop structure is necessary for the association of a primer-precursor RNA with the DNA template prior to generation of the primer by RNase H cleavage (Masukata and Tomizawa, 1984, 1986); control of the conformation of the primer-precursor controls plasmid replication (Masukata and Tomizawa, 1986). The ability to assume this configuration is conserved among different ColE1 type origins despite considerable divergence in their primary structure (Lacatena and Cesareni, 1981; Selzer *et al.*, 1983).

The potential for secondary structural interactions is also a characteristic of the region surrounding the light (L) and heavy (H) strand replication origins (O_L and O_H) of mitochondrial DNA (Clayton, 1982; Pepe *et al.*, 1983; Wong

et al., 1983). In the case of L strand initiation of DNA synthesis, the stem-loop structure found at the origin is essential for initiation *in vitro* in a human mitochondrial system (Hixon *et al.*, 1986).

Although the cruciform conformation is energetically unfavored in a relaxed DNA molecule, negative supercoiling favors it (Hsieh and Wang, 1975); studies using single-strand specific nucleases and electron microscopy have provided evidence for the existence of cruciforms in supercoiled DNA (Lilley, 1980; Panayotatos and Wells, 1981). Furthermore, it was recently reported that a dominant factor in the kinetics of cruciform extrusion is the DNA sequence that flanks the inverted repeat, with the sequence of the inverted repeat itself having little or no influence (Sullivan and Lilley, 1986). The flanking sequence with the dominant influence on cruciform kinetics is very AT-rich, acts in *cis* independent of polarity, and it can have an effect over distances of at least 100 bp.

Despite the evidence provided by the nuclease S1 digestion experiments (Collins *et al.*, 1982; Panayotatos and Wells, 1981) that argues in favor of the existence of cruciforms, their existence *in vivo* has not been directly demontrated (Sinden *et al.*, 1983). Panayotatos and Fontaine (1987) have recently reported the presence of a native cruciform structure in *E.coli*.

Recently we developed two monoclonal antibodies, 2D3 and 4B4, with specificity to cruciform DNA (Frappier *et al.*, 1987). The binding site for both antibodies has been mapped to the four-way (elbow-like) junction at the base of the cruciform step (Frappier *et al.*, submitted). Operating on the assumption that if cruciforms existed *in vivo*, even transiently, they could be stabilized by a suitable and specific protein, we studied the effect of our monoclonal antibodies on the DNA replication of synchronized CV-1 cells at various stages in the S phase, using a permeabilized cell system. Using this technique, we show that the monoclonal antibodies directed against cruciform DNA effectively enhance the incorporation of labeled precursor nucleotide into acid precipitable material by 2- to 6-fold (Figures 2−4), while the same effect is not observed in the presence of the control antibodies. Approximately half of the enhanced synthesis is due to the action of DNA polymerases α and/or δ, as it is sensitive to aphidicolin. The enhancing effect is abolished by absorbing the antibody with immunobead rabbit anti-mouse immunoglobulin prior to using it in the permeabilized cells. Dot-blot hybridization analysis of the DNA produced by the enhanced synthesis showed an enhancement of the same magnitude or greater in the copy number of early replicating DNAs, such as *ors 8* (five copies per haploid CV-1 genome), and *DHFR* and c-*myc* (single copy per haploid CV-1 genome), that were used as probes (Figure 6 and Table I). Amplification of c-*myc* was particularly striking and may be reflective of the proximity of the replication origin to the gene, which has been recently localized within a 2.2 kb restriction fragment containing the 5′-flanking region of the first c-*myc* exon (Duncan and Leffak, 1987). In contrast, dot-blot hybridizations using c-*fos* as probe revealed no enhancement in the copy number of this gene, suggesting that it either resides distal from the cruciform or is replicated at a period other than early S. Furthermore, for comparison, anti-dsDNA (Z-DNA and B-DNA) monoclonal antibody (Monier *et al.*, 1984) failed to significantly affect DNA synthesis.

These results suggest that cruciform or cruciform-like structures (i.e. naturally occurring structures which present the antigenic determinant of our cruciform immunogens) do form in the DNA *in vivo* and that they are being recognized and stabilized by the monoclonal antibodies. Antibody stabilization of alternate DNA structures such as Z-DNA has been previously shown by other investigators (Lafer *et al.*, 1981). *Ors 8* was isolated as one of the sequences enriched for origins of replication that are activated early in S (Zannis-Hadjopoulos *et al.*, 1985) and we have subsequently shown that it functions as an origin of replication in mammalian cells and that it contains an inverted repeat (Frappier and Zannis-Hadjopoulos, 1987). The enhancement in the copy number of *ors 8*, induced by monoclonal antibodies to DNA cruciforms, suggests that cruciforms or cruciform-like structures exist at origins of DNA replication. Furthermore, the increase in copy number in the presence of 2D3 antibody for *ors 8*, *DHFR* and c-*myc* sequences suggests that the stabilization of the cruciform may result in multiple initiations at a single site. These putative multiple initiations are consistent with the times at which these sequences are replicated and active. This interpretation would also account for the enhanced DNA synthesis by 2D3 that we found to be insensitive to the action of aphidicolin, and which may represent synthesis mediated by the action of DNA primase, the enzyme implicated in the priming of initiation of DNA synthesis at the origin of replication (Kornberg, 1980). Eukaryotic DNA primases appear, in general, to be insensitive to the action of aphidicolin, which has been shown to allow initiation as well as a limited amount of DNA synthesis to take place in the immediate area of the origin of SV40 (Dinter-Gottlieb and Kaufmann, 1982) and of the amplified *DHFR* domain of CHOC 400 cells (Burhans *et al.*, 1986).

The steric factors involved in the binding of the antibodies to the cruciform are obviously not inhibitory to the activity at the origin of replication. It is possible that the actual synthesis begins distal to the binding site or that the antibody substitutes for the cellular factor(s) that normally recognize(s) these structures. Unlike a putative cellular factor, the antibody binds the DNA essentially irreversibly, thereby stabilizing the cruciform or cruciform-like structure and allowing multiple initiations to occur. We have no evidence that binding of 2D3 and 4B4 to DNA alters its sensitivity to DNase I digestion, as DNA molecules containing stable cruciforms (Frappier *et al.*, 1987) were found to be naturally resistant to digestion by DNase I at the site of binding, and gave the same DNase I banding pattern, both when bound and unbound by 2D3 and 4B4 (data not shown).

To test the possibility that our mAbs might stimulate DNA synthesis at replication forks rather than (or in addition to) cruciforms, we examined the ability of 2D3 mAb to stimulate the replication of SV40 DNA in SV40-infected CV-1 cells. We found that the presence of the antibody neither stimulated nor inhibited viral DNA replication, as assessed by Southern blot analyses of Hirt supernatants (data not shown) (Hirt, 1967). Thus, this result makes it unlikely that the enhancement of cellular DNA synthesis that we observed is due to the interaction of the antibodies with replication forks. Since we were unable to observe an effect on SV40 replication, *in vivo*, we also used 2D3 in a cell-free system similar to that developed by Decker *et al.* (1986) for the replication of SV40. Again, 2D3 had no effect on SV40 replication *in*

vitro (data not shown). Thus, 2D3 seemingly also does not bind to the SV40 origin of replication, which contains a large palindromic sequence that could potentially form a cruciform structure; either T antigen, the viral initiator protein, is blocking the site to which the antibody would normally bind or a cruciform does not form at the SV40 origin of replication. Experiments to clarify these alternative possibilities are currently in progress. It should be noted that the palindrome at the SV40 ori is ~90% GC and, therefore, it may not be kinetically possible to extrude in the cruciform configuration.

Current models of eukaryotic replicon activation are based on the assumption that different classes of replication origins are successively activated throughout the S phase (Hamlin, 1978; Rivin and Fangman, 1980; Fangman *et al.*, 1983). The results presented in this paper indicate a biphasic activation of origins during the first 6 h of DNA synthesis that follow the G_1/S block by aphidicolin. As we had pointed out previously (Zannis-Hadjopoulos *et al.*, 1984), transiently forming cruciform structures would be ideally placed at replication origin sites, since such sites must be activated only once per cell cycle in a normal cell. Temporal regulation of cruciform activation could potentially be achieved through the interaction of specific recognition (initiator) proteins with inverted repeat sequences, as they become accessible on the chromatin, and may result in the formation and transient stabilization of the cruciforms. Once replication has been initiated, the same cruciform would normally not become accessible again, and therefore it would not reform until the next cell cycle.

Materials and methods

Cells and media

CV-1 monkey cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS). The hybridoma cell lines were maintained in alpha medium with hypoxanthine, thymidine and 20% FCS. For harvesting the mAbs the hybridomas were grown in DMEM containing insulin, transferrin, selenium and 2% FCS, as described in Frappier *et al.* (1987).

Antibodies

The two anti-cruciform mAbs, 2D3 and 4B4, have been recently described (Frappier *et al.*, 1987). 2D3 mAb is isotype IgG_1 and 4B4 mAb is isotype IgM. They are secreted by hybridoma lines that were generated by the fusion of immunized mouse spleen cells to the mouse myeloma line, P3-X63Ag8. The mAb being secreted by this myeloma line, P3, an IgG_1, was used as control for 2D3; the control for 4B4 was an unreactive IgM mAb to rat myeloid cells, BN18 (courtesy of Dr A.K.Sullivan, McGill Cancer Centre, Montreal, Canada). An IgM mAb (mouse) to dsDNA (HB2; BIOSOFT), with reactivity to Z-DNA and B-DNA, but not with ssDNA, poly dA−poly dT, poly d(AT), poly dT and RNA (Monier *et al.*, 1984), was used as an anti-DNA control antibody.

Cell synchronization

CV-1 cells were plated at 5×10^5 per 100 mm plate in the presence of 0.1 μCi/ml or 10 nCi/ml [^{3}H]thymidine (1 mCi/ml; 66 Ci/mmol; ICN) and were synchronized at the G_1/S boundary by serum starvation and aphidicolin as previously described (Zannis-Hadjopoulos *et al.*, 1984). (Aphidicolin was kindly provided by M.Suffness, National Institutes of Health, Bethesda, MD, USA.) Progression through S phase was monitored by removing the medium containing aphidicolin, washing the plates twice with phosphate buffered saline (PBS) and re-incubating the cultures in fresh media at 37°C for up to 6 h. Samples were taken at hourly intervals for analysis by flow cytometry, as previously described (Kaufmann *et al.*, 1985) and for permeabilization.

DNA replication in permeabilized cells

We used the method described by Burhans *et al.* (1986), with the appropriate modifications to accommodate the use of the mAbs. In brief, the synchronized cells were collected either at the G_1/S interphase or at hourly intervals following the removal of aphidicolin by scraping the cells off the plates and centrifugation. The cell pellets were immediately put in ice and resuspended in 1 ml of ice-cold hybridoma culture supernatant containing the appropriate mAb. [The mAb reactivities were all determined to be equivalent to the culture supernatants used in our previous work (Frappier *et al.*, 1987); the immunoglobulin concentration of both 2D3 and 4B4 is ~5−10 μg/ml; HB2 was used in concentrations demonstrated to be equivalent to the DNA-binding activity of 2D3, as assessed by gel retardation assays that we have previously described (Frappier *et al.*, 1987).] One volume of ice-cold 2× replication buffer [100 mM Hepes, pH 7.8, 200 μg heat inactivated, nuclease-free BSA; 2 mM DTT; 0.2 mM each dGTP, dTTP and dATP; 0.4 mM each CTP, GTP, UTP; 8 mM ATP; 30% glycerol; 20 mM $MgCl_2$; 2 μl [α-^{32}P]dCTP (3000 Ci/mmol; 10 mCi/ml; ICN)] containing 0.4% Nonidet P-40 (NP-40) and 2 mM PMSF was immediately added to the cell−mAb suspension (final mAb dilution was 50%). In some experiments, aphidicolin (final concentration of 8 μg/ml) or 2′,3′-dideoxythymidine triphosphate (ddTTP; final concentration of 50 μM) were included in the replication buffer (see Results). The mixture was incubated in ice for 30 min to allow the mAb to enter the nucleus and bind to cruciform DNA; the mixture was then transferred to 37°C for another 30 min to allow DNA replication to occur. The replication reaction was terminated either by the addition of 50% trichloroacetic acid (TCA) to a final concentration of 5%, followed by incubation in ice for 1 h, collection of the acid precipitable material on GF/C filters, extensive washing with 5% TCA, drying and scintillation counting; or by the addition of an equal volume of 2× lysis buffer (2.4% SDS, 400 mM NaCl, 80 mM EDTA, 200 mM Tris−HCl, pH 7.9) and proteinase K to 200 g/ml for subsequent extraction of high mol. wt DNA (Zannis-Hadjopoulos *et al.*, 1984).

Immunofluorescence procedure

Permeabilized cells from log-phase cultures of CV-1, exposed to 2D3 and P3 antibody as described above, were washed twice with cold PBS containing 1% FCS. The cells were then exposed to 1 mg RNase in 0.5 ml wash medium for 30 min at room temperature before a third and final wash. A 0.1 ml volume of a 1/10 dilution of fluorescein-conjugated $F(ab')_2$ fragment of goat anti-murine IgG (Cappel Laboratories, Cochranville, PA, USA) was added to the pellet. After 30 min at 4°C it was washed three times and the pellet resuspended in 0.1 ml PBS and 1% FCS. (It should be noted that after permeabilization using NP-40 the cellular preparation consists mainly of nuclei with large amounts of attached cytoplasm. In the process of the extensive washing required to prepare the cells for immunofluorescence the adherent cytoplasm is removed leaving only the nuclei.) The nuclei were placed on microscope slides and viewed with a Leitz Orthoplan fluorescence microscope.

Sizing of the in vitro replicated DNA

The DNA that was synthesized in permeabilized cells in the presence or absence of 2D3 mAb was extracted and concentrated by ethanol precipitation, as described above. Samples (2×10^5 c.p.m.) were resuspended in 5 μl of denaturing dye mix [80% v/v formamide, 10 mM NaOH, 1 mM EDTA, 0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue (Maxam and Gilbert, 1980)], denatured by boiling for 10 min and quick-chilled in ice. They were then loaded and electrophoresed onto a 4% polyacrylamide sequencing gel (Maxam and Gilbert, 1980). End-labeled λ *Hin*dIII DNA was treated in the same way and was co-electrophoresed as size markers.

Dot-blot hybridization of in vitro replicated DNA

DNA was synthesized in permeabilized cells in the presence of 2D3 or P3 mAb as described above except that, instead of [α-^{32}P]dCTP, cold dCTP was used at a final concentration of 0.1 mM. The isolated DNA was dot-blotted onto Genescreen Plus membranes (NEN) at concentrations of 0.25, 0.5 and 1 μg per dot, in duplicate. Dot-blotting, prehybridization and hybridization conditions were as described previously (Kaufmann *et al.*, 1985). The inserts of pBR/*ors 8* (Zannis-Hadjopoulos *et al.*, 1985), pSV2/*DHFR* (Chang *et al.*, 1978), pHSR-1 (c-*myc*; ATCC), pc-*fos*(human)-1 (*c-fos*; ATCC), Blur 8 (human Alu; ATCC) (Rubin *et al.*, 1980), and pCa1004 (a gift from Dr M.Singer, NCI, Bethesda, MD, USA), a clone of α-satellite DNA, were nick-translated to high specific activity (Rigby *et al.*, 1977) and used as hybridization probes.

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