Journal of Cellular Biochemistry 68:309–327 (1998)

Oct-1 Enhances the In Vitro Replication of a Mammalian Autonomously Replicating DNA Sequence

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Abstract A 186-base pair fragment of *ors*8, a mammalian autonomously replicating DNA sequence isolated by extrusion of nascent monkey DNA in early S phase, has previously been identified as the minimal sequence required for replication function in vitro and in vivo. This 186-base pair fragment contains, among other sequence characteristics, an imperfect consensus binding site for the ubiquitous transcription factor Oct-1. We have investigated the role of Oct-1 protein in the in vitro replication of this mammalian origin. Depletion of the endogenous Oct-1 protein, by inclusion of an oligonucleotide comprising the Oct-1 binding site,inhibited the in vitro replication of p186 to approximately 15–20% of the control, whereas a mutated Oct-1 and a nonspecific oligonucleotide had no effect. Furthermore, immunodepletion of the Oct-1 protein from the HeLa cell extracts by addition of an anti-POU antibody to the in vitro replication reactioninhibited p186 replication to 25% of control levels. This inhibition of replication could be partially reversed to 50–65% of control levels, a two- to threefold increase, upon the addition of exogenous Oct-1 POU domain protein.

Site-directed mutagenesis of the octamer binding site in p186 resulted in a mutant clone, p186-MutOct, which abolished Oct-1 binding but was still able to replicate as efficiently as the wild-type p186. The results suggest that Oct-1 protein is an enhancing component in the in vitro replication of p186 but that its effect on replication is not caused through direct binding to the octamer motif. J. Cell. Biochem. 68:309–327, 1998.

Key words: in vitro replication; *ors*8; Oct-1 transcription factor; POU domain; mammalian autonomously replicating DNA sequence

The specific initiation of DNA replication is a crucial step in cell growth and reproduction. Eukaryotic origins of DNA replication consist of a core component, which is indispensable for origin function, and *cis*-acting auxiliary components, which enhance core origin function [DePamphilis, 1993]. These auxiliary components include binding sites for specific cellular transcription factors. Understanding how these transcription factors activate eukaryotic origins of DNA replication might aid in understanding the mechanisms that regulate the initiation of DNA replication.

Several mechanisms have been proposed to explain the direct role of transcription factors in activating the replication origins of eukaryotic viruses [DePamphilis, 1993]. In one model, transcription factors activate origins by initiating RNA primer synthesis at the initiation site of replication [Clayton, 1991]. Transcription factors may also stimulate replication by facilitating the assembly and activity of an initiation complex [Chiang et al., 1992; Mul and van der Vliet, 1992]. Evidence also exists in support of a model, wherein transcription factor binding may prevent repression of origin activity due to chromatin structure [reviewed in DePamphilis, 1993].

Several mammalian origins of DNA replication also contain putative auxiliary transcription factor binding sites. For example, the Chinese hamster DHFR origin has a consensus binding site for Oct-1 and AP-1 [DePamphilis, 1993], the hamster rhodopsin origin has AP-1 and Oct-1 sites, and CP-1/CAAT box [Gale et al., 1992], while the murine IgH intronic enhancer Eμ contains binding sites for Oct-1, 2, and 3 [Staudt and Lenardo, 1991]. Mammalian origin-enriched (*ors*) and autonomous replicat-

Contract grant sponsor: Medical Research Council of Canada; Contract grant number: MT7965; Contract grant sponsor: Cancer Research Society, Inc.; Contract grant sponsor: Le Défi Corporatif Canderel.

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Received 4 August 1997; Accepted 29 September 1997

ing sequences also contain various transcriptional regulatory elements, such as CTF/NF-1 and related sequences, CACCC, the β-globin transcriptional control sequence, as well as Oct-1 sequence [Rao et al., 1990; Landry and Zannis-Hadjopoulos, 1991; Wu et al., 1993]. Thus, the octamer consensus motif is present in the auxiliary regions of several viral and eukaryotic origins [Staudt and Lenardo, 1991; DePamphilis, 1993; Iguchi-Ariga et al., 1988]. The octamer sequence has also been reported to serve as a putative origin for cellular DNA replication, since enhanced replication of a transfected plasmid depended on the presence of an intact octamer binding site [Iguchi-Ariga et al., 1993].

The octamer consensus sequence, ATGC(A/T)AAT, is recognized by a group of proteins that have been identified at various developmental stages and in a variety of tissues and organs [reviewed in Schöler, 1991]. One of the octamer recognizing proteins is the 93 kDa, ubiquitously expressed transcription factor, Oct-1 [Singh et al., 1986; Staudt et al., 1986; Sturm and Herr, 1988]. It is a member of the POU family of transcription factors, which have been implicated in tissue-specific gene expression, cell differentiation and DNA replication [Pruijn et al., 1986; Rosenfield, 1991]. Oct-1 protein is composed of a transcription activation domain and a DNA binding domain, the POU domain. The POU domain consists of two subdomains: a 60-amino acid (aa) C-terminus domain, called the homeodomain, recognizing the 3′ half of the octamer sequence (TAAT), and a 75-aa N-terminus POU-specific domain, binding to the 5′ half of the octamer sequence (TATGCA) [Verrijzer et al., 1992]. The A/T overlap is recognized by both subdomains.

The Oct-1 POU domain is sufficient to stimulate adenovirus DNA replication, as demonstrated by deletion analyses [Verrijzer et al., 1990]. In vitro, Oct-1 enhances adenovirus replication three- to seven-fold, depending on the pTP-pol concentration and on a DNA-independent interaction between the pTP-pol complex and the POU homeodomain [Coenjaerts et al., 1994]. Furthermore, mutations in the Oct-1 POU specific recognition helix decreased the ability of the POU domain to enhance adenovirus replication [van Leeuwen et al., 1995]. Recently, it was shown that the Oct-1 POU domain stimulates replication by increasing the binding of the pre-terminal protein–DNA polymerase complex (pTP-pol) to the core origin and stabilizing the pre-initiation complex through a direct interaction between the pTP and the POU homeodomain [van Leeuwen et al., 1997]. Thus, the DNA binding domain of Oct-1 not only binds to the octamer motif, but it also interacts directly with a key component of the adenovirus replication machinery, the pTP protein.

In adenovirus, the Oct-1 binding site is important for optimal replication in vivo. However, if other compensating DNA sequences, such as the transcription factor binding sites SP1, ATF, and EBP-1, are present, deletion of the octamer sequence fails to produce a replication effect, indicating functional redundancy between different transcription factor binding sites at the left terminus of the adenovirus genome [Hatfield and Hearing, 1993]. Furthermore, bending at the octamer site, produced by Oct-1 binding, is not sufficient for replication enhancement [Verrijzer et al., 1991]. DNA bending may have an architectural role in the formation of an initiation complex by facilitating interactions between different proteins. Substitution of the Oct-1 binding site by an AP1 site does not stimulate replication in the presence of *fos-jun*, which are able to bend DNA in a similar fashion to Oct-1 [Coenjaerts et al., 1994; reviewed in van der Vliet, 1996]. Thus, protein-protein interactions between the POU DNA binding domain of Oct-1 and pTP are critical for enhancement of adenovirus replication.

Previously, we cloned origin-rich sequences (*ors*) isolated by extrusion of nascent DNA from replicating monkey (CV-1) cells at the onset of S phase [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985]. The *ors* are capable of transient autonomous replication in vivo, upon transfection in monkey (CV-1 and Cos-7) and human (HeLa) cells [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991] and in an in vitro replication system that uses HeLa cell extracts [Pearson et al., 1991]. Both in vivo and in vitro replication initiates within the *ors*, is semiconservative, bidirectional and sensitive to the action of aphidicolin [Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991, 1994a]. Among the functional *ors*, *ors*8 has been analyzed in detail both in vivo [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Zannis-Hadjopoulos et al., 1992] and in vitro [Pearson et al., 1991; Zannis-Hadjopoulos et

al., 1992]. In vitro replication of *ors*8 is semiconservative, as shown by the in vitro incorporation of bromodeoxyuridine (BrdUTP) and separation of BrdUTP-substituted DNA on both neutral and alkaline CsCl density gradients [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992]. Time course and electron microscopy mapping of the origin of replication on the *ors*8 plasmid showed that replication starts within the *ors* and proceeds bidirectionally [Pearson et al., 1991; 1994a]. Recently, we identified, by deletion analyses, a 186-base pair (bp) fragment of *ors*8 as the minimal sequence required for origin function both in vivo and in vitro [Todd et al., 1995]. This minimal replicating DNA sequence contains an imperfect 44-bp IR capable of extruding into a cruciform, several direct repeats, an imperfect consensus binding site for Oct-1, and several binding sites for the GATA family of transcription factors [Merika and Orkin, 1993; Pevny et al., 1991] (Fig. 1). A measurable sequence-directed DNA bent was also detected within the minimal origin of *ors*8 [Todd et al., 1995].

In order to determine the role of transcription factors on the replication activity of mammalian origin, we investigated the role of Oct-1 in the in vitro replication of p186, a pBR322-based plasmid containing the minimal *ors*8 autonomously replicating sequence [Todd et al., 1995].

In this study, we show that Oct-1 enhances the in vitro replication of p186. Addition of an Oct-1-specific double-stranded oligonucleotide in the in vitro reaction inhibited p186 replication by approximately 80–85%, whereas neither a nonspecific nor a mutated Oct-1 oligonucleotide produced any effect. Furthermore, immunodepletion of the Oct-1 protein by addition of an anti-POU antibody inhibited replication by 75–80%. In both instances, the inhibition was reversed to 50–65% of control levels when purified Oct-1 POU was added exogenously. Replication levels of a p186 mutant clone, however, which is unable to bind Oct-1, were similar to wild-type p186 levels, indicating that the enhancing replication effect of Oct-1 does not result from its direct binding to the octamer motif within the origin, but rather through an indirect mechanism, most likely, involving protein–protein interaction.

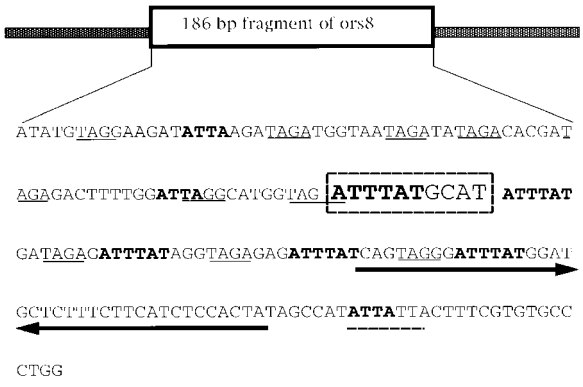


Fig. 1. Sequence characteristics of the 186-bp autonomously replicating DNA sequence of *ors*8. The imperfect Oct-1 binding site (boxed), the imperfect inverted repeat (head–head arrows), the direct repeat (dashed underline), and the repeated sequence motifs TAGA and TAGG (solid underline) and ATTA and ATTTAT (bold) are indicated. Adapted from Todd et al. [1995].

MATERIALS AND METHODS

Plasmids

Plasmid p186 consists of the *Nde*I–*Rsa*I subfragment of *ors*8 subcloned in the *Nru*I site of pBR322 [Todd et al., 1995]. It is capable of autonomous replication both in vivo and in vitro [Todd et al., 1995]. Plasmids p186 and pBluescript II KS- (used as a negative control) were propagated in *Escherichia coli* HB101 as previously described [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991]. Plasmid p186-MutOct consists of the p186 plasmid with a mutated Oct-1 binding site (see Site-Directed Mutagenesis below). It was propagated in *Epicurian coli* XL-1 Blue (Stratagene, La Jolla, CA). Plasmid DNAs were isolated using the QIAGEN-tip 500 columns according to the manufacturer's specifications (QIAGEN, Santa Clarita, CA).

The 186-bp fragment of *ors*8 and the 206-bp fragment of pBR322 (from nucleotide 860–1065) used in the competition bandshift assay were amplified by polymerase chain reaction (PCR) and purified by isotachophoresis, as previously described [Ruiz et al., 1995] or by using the QIAEX II gel extraction kit (QIAGEN).

Oligonucleotides

The Oct-1-specific oligonucleotide, 5′TGTCGAATGCAAATCACTAGAA3′, was purchased from Promega Corporation (Madison, WI, OCT1 Consensus Oligonucleotide, Cat. # E3241) as a double-stranded fragment and used in the in vitro replication, competition and bandshift assays. It is not homologous to the 186-bp fragment but contains a perfect Oct-1 binding site

that can readily compete for Oct-1 binding in the in vitro replication assays. A nonspecific double-stranded DNA fragment was prepared by annealing two oligonucleotides (5′TTCCGAATACCGCAAG3′, synthesized by Sheldon Biotechnology, Montreal, Canada), representing the region of pBR322 from nucleotides 838–854. The mutated Oct-1 oligonucleotide was prepared by annealing two single strands of a DNA fragment representing a 31-bp region of the 186-bp fragment of *ors*8 (synthesized by Sheldon Biotechnology, Montreal, Canada), in which the Oct-1 binding site was altered according to Kemler et al. [1989] in order to abolish Oct-1 binding. The sequence of the mutated Oct-1 oligonucleotide is 5′GGCATGGTAG**CGACCGTG**ATATTTATGATAG 3′ (the nucleotides in bold indicate the mutated Oct-1 binding site).

All the annealing reactions, including that of the Oct-1-specific oligonucleotide (Promega, Madison, WI), were performed using a 1:1 ratio of the two complementary single-stranded oligonucleotides in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA. The oligonucleotides were placed at 90°C for 2 min, and allowed to cool to room temperature. The efficiency of annealing was verified by 5′ end-labeling an aliquot (typically 10 ng) of the reaction mixture with 1 unit T4 polynucleotide kinase (New England Biolabs, Mississauga, Ontario, Canada) in the presence of 200 µCi [γ^{32}P]-ATP and was found to be approximately 80–90%.

Preparation of Cell Extracts

HeLa S3 nuclei and cytosol were purchased from Cellex Biosciences (Minneapolis, MN). The extracts were made as previously described [Pearson et al., 1991; Todd et al., 1995]. The protein concentrations of the nuclear and cytoplasmic extracts were 4.1 mg/ml and 6.4 mg/ml, respectively.

In Vitro Replication Assay

In vitro replication was carried out as previously described [Pearson et al., 1991], with slight modifications. Standard reactions included HeLa cytoplasmic (70 µg) and nuclear (18.5 µg) extracts, 2 mM ATP, 100 mM each CTP, GTP, UTP, dATP, and dGTP, 10 µCi of [α^{32}P]-dTTP and 10 µCi [α^{32}P]-dCTP, 0.2 units of pyruvate kinase (Boehringer Mannheim, Laval, Quebec, Canada), and equimolar amounts of either p186, p186-MutOct, or pBluescript (200 ng, 200 ng, and 130 ng, respectively).

The molar excess of the Oct-1-specific, mutated Oct-1, and nonspecific double-stranded oligonucleotides or of the anti-POU antibody (a gift from Dr. Peter C. van der Vliet, Utrecht University, The Netherlands) was in relation to the p186 input plasmid DNA. (That is to say, if 200 ng of p186 plasmid DNA (4549 bp) was used, then 0.97 ng of the Oct-1-specific oligonucleotide (22 bp) would represent equimolar amounts. If a 100-fold molar excess of the oligonucleotide was required, 97 ng would be used). The experiments were performed by pre-incubating the HeLa cell extracts with the oligonucleotides or the anti-Oct-1 POU antibody for 20 min on ice.

When the Oct-1 POU protein (a gift from Dr. Peter C. van der Vliet, Utrecht University, The Netherlands) or bovine serum albumin (BSA) (New England BioLabs, Mississauga, Ontario, Canada) was used, it was added to the reaction mixture containing the extracts and 250-fold molar excess of Oct-1 oligonucleotide or the extracts and the anti-Oct-1 POU that had been pre-incubated on ice for 20 min. The POU domain and BSA proteins were added to replace the depleted levels of endogenous Oct-1, and pre-incubated on ice for a further 20 min.

When aphidicolin (Boehringer-Mannheim) was used, it was added to a final concentration of 80 µM to the reactions containing the extracts and oligonucleotides that had been pre-incubated on ice; the reactions were subsequently pre-incubated at 30°C for 15 min.

Following the pretreatment steps, the DNA, nucleotides, pyruvate kinase and PEG solution (125 mM Hepes-KOH pH 7.8, 6 mM EGTA, 32 mM $MgCl_2$, 1.5 M ethylene glycol, 32% w/v polyethylene glycol) were added and the reactions were incubated at 30°C for 1 h. The reactions were terminated by the addition of 30 mM EDTA/1% SDS. The input DNA and the reaction products were purified as previously described [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994; Todd et al., 1995]. The reaction products were divided into three samples; one third was digested with 1 unit *Dpn*I (New England BioLabs) for 60 min at 37°C in the presence of 1× NEB buffer 4 (50 mM KCl, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT), 200 mM NaCl and 300 ng of pBluescript DNA, used as carrier DNA and indicator of *Dpn*I digestion. The *Dpn*I-digested (1/3) and

undigested (1/3) products were resolved on 1% agarose gel in 1× TAE buffer (16–20 h, 50–55 V). The gels were dried and exposed to Kodak BioMax MS film.

Quantification was performed, as previously described [Diaz-Perez et al., 1996], on *Dpn*I-digested products by densitometric measurements using a Phosphoimager analyzer (Fuji BAS 2000, Stamford, CT) or a Bio Image Densitometer (MillGen/Biosearch, Ann Arbor, MI); both methods yielded similar results. Quantification involved measuring the density of the bands corresponding to the relaxed circular (II) and linear (III) forms of the plasmid DNA. Quantification of the supercoiled (form I) band was not possible because of overlap with the *Dpn*I digestion products. These results were corrected for the amount of DNA recovered from the in vitro replication assay by quantitative analysis of the ethidium bromide picture of the gel. The amount of radioactive precursor incorporated into the DNA was expressed as a percentage of the control p186 in vitro reaction that did not contain any oligonucleotide or antibody and was considered to have 100% replication activity.

DNA Binding Assay

DNA binding assays were performed as previously described [Pearson et al., 1994b; Ruiz et al., 1995]. Reactions were performed in 20 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 µg poly(dI-dC) (Pharmacia, Baie d'Urfé, Québec, Canada), with 10 µg total HeLa cell extracts or 2.5 ng of the Oct-1 POU domain protein, and 1 ng ^{32}P-labeled Oct-1-specific or mutated oligonucleotide (6.8 fmol/reaction) or 0.1 ng of labeled 186-bp DNA (0.78 fmol/reaction), respectively, in final volume of 20 µl. When the anti-Oct-1 antibody (Trans Cruz Gel Supershift reagent, Santa Cruz Biotechnologies, Santa Cruz, CA, Cat. # sc-232) was used in the binding reaction to interfere with the binding of endogenous Oct-1 to the Oct-1-specific or mutated oligonucleotides, the extracts and the probe were pre-incubated at room temperature for 20 min, and then 1.5 µg of anti-Oct-1 antibody was added and incubated for a further 25 min at room temperature. When competition studies were done, cold DNA, either Oct-1 oligonucleotide and nonspecific 16-bp pBR322 fragment (nucleotide 838–854), or 186-bp fragment DNA and 206-bp pBR322 DNA fragment (nucleotide 860–1065), was included in the reaction in molar excess, as indicated in the figure legends. After a 30-min incubation on ice, loading dye (25% Ficoll, 25 mM EDTA, 0.2% BPB, 0.2% xylene cyanol) was added and the samples were subjected to electrophoresis on a 4% polyacrylamide gel in 1× TBE at 180 Volts for 1 h at room temperature. The gels were then dried and exposed for autoradiography.

Immunodetection Assay

Western blots were performed as previously described [Pearson et al., 1994b; Ruiz et al., 1995]. Polyacrylamide gel electrophoresis (PAGE) was performed as previously described [Laemmli, 1970], using 500 ng bacterially expressed His6tagged Oct-1 POU domain (a gift from Dr. P. C. van der Vliet, Utrecht University, The Netherlands), 70 µg HeLa cell cytoplasmic extracts and 18.5 µg nuclear extracts (these amounts correspond to those used in the in vitro replication assays). Proteins were electroblotted to Immobilon-P membranes (Millipore) and probed with anti-POU rabbit polyclonal antibody (a gift from Dr. P.C. van der Vliet). The blots were developed using the ECL Detection system (Amersham, Oakville, Ontario, Canada) with the HRP-labeled antirabbit antibody. The membranes were exposed for autoradiography. Determination of the amount of Oct-1 protein in the HeLa nuclear extracts was performed by preparing a Western blot with different dilutions of the Oct-1 POU protein. Then, the films were scanned using a Bio Image Densitometer (Milligen/Biosearch) and a graph of peak area versus protein concentration was plotted. The concentration of the Oct-1 protein present in the HeLa nuclear extracts was determined from the standard curve.

Site-Directed Mutagenesis and Sequencing Reactions

Mutagenesis of the Oct-1 binding site in p186 was performed using the Chameleon Double-Stranded Site-Directed Mutagenesis kit (Stratagene, Cat. # 200509) with the following modifications. 0.25 pmol of p186 DNA, 25 pmol of *Sca*I/*Mlu*I unique site elimination selection primer (Pharmacia Biotech, Cat. # 27-1666-01) and 100 pmol of mutagenic primer, carrying the Oct-1 site mutation, were used. The mutagenic primer was synthesized, 5'-phosphorylated and purified by PAGE by Gibco BRL (Burlington, Ontario, Canada). The sequence of the 33-bp primer is 5'-PGGCATGGTAGCGACCGT-

GATATTTATGATAGAC 3'. This sequence is identical to the mutated oligonucleotide used in the in vitro replication assays, except for the additional two bases, AC, at the 3'. The primers were extended by incubating the annealed primers/template reaction mixture with a T7 DNA polymerase/T4 DNA ligase mixture at 37°C for 2.0 h. The reactions were digested with 20 units of *Sca*I (Stratagene) and the subsequent steps were performed as per manufacturer's specifications.

The potential mutant clones were screened by restriction endonuclease digestion with *Sca*I (Stratagene) and *Mlu*I (Gibco BRL). Clones that were not digested by *Sca*I, yet linearized by *Mlu*I, were sequenced using the T7 Sequencing kit from Pharmacia Biotech, as per manufacturer's instructions to "read short," with the following modifications. The sequencing reactions contained 1.0 µg miniprep DNA, 2.5 µM of primer H′ (from region 994–980 of pBR322), and 12.5 µCi [α^{35}S]-dATP. Then, 3.0 ml of the reaction mixture was denatured and loaded on an 8% sequencing gel. The gel was run at 1,700 V for 1.0 h. Subsequently, the gel was dried and exposed for autoradiography. The mutant clone, p186-MutOct, was then used in the in vitro replication reaction as described above.

RESULTS

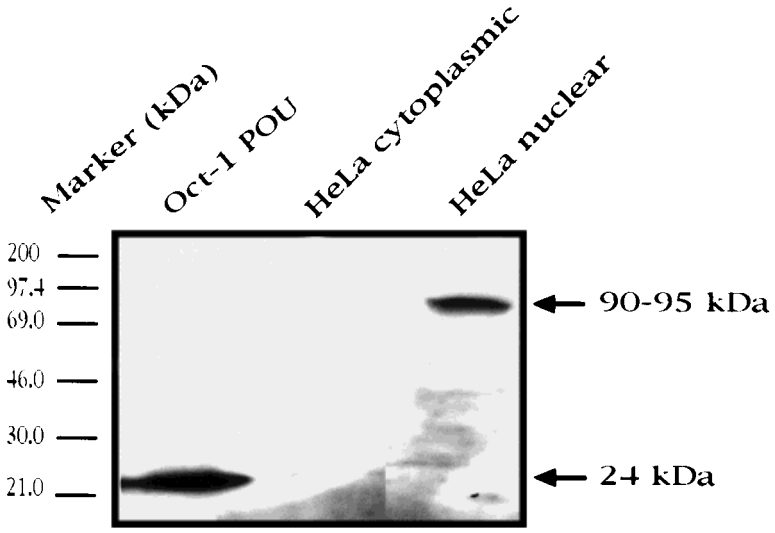
Endogenous Oct-1 Protein Binds the 186-bp Fragment of *ors*8

To verify that our preparation of HeLa cell extracts contained the ubiquitous Oct-1 protein, Western blot analysis of nuclear and cytoplasmic extracts was performed using a rabbit polyclonal antibody against the Oct-1 POU domain protein (Fig. 2A). The amount of nuclear and cytoplasmic extracts in these analyses corresponded to the amount used in a single in vitro replication assay, 18.5 µg and 70 µg, respectively. The 24-kDa Oct-1 POU domain protein was used as a positive control. The HeLa nuclear extracts contained the Oct-1 protein (approximately 93 kDa), in agreement with Oct-1 being a nuclear protein (Fig. 2A). By densitometric analyses, we determined that 18.5 µg protein from HeLa nuclear extracts contained approximately 100 ng of Oct-1 protein. Upon longer exposures, very low levels of Oct-1 protein were also detected in the cytoplasmic extracts, suggesting some protein leakage into the cytoplasm during the preparation of the extracts (data not shown).

To determine whether the endogenous Oct-1 protein was able to bind to the imperfect octamer motif within the 186-bp fragment (Fig. 1), bandshift and competition bandshift assays were performed in which labeled 186-bp DNA fragment was reacted with protein from total HeLa cell extracts. We have previously shown that, when labeled 186-bp fragment DNA was used in a bandshift with total HeLa cell extracts, three major protein–DNA complexes were formed [Ruiz et al., 1995; and Fig. 2B, lane 2). Complexes 1 and 2 were due to specific binding, since they were competed when cold 186-bp fragment DNA was used as a specific competitor, whereas complex 3 was shown to be due to nonspecific binding, as it was abolished in the presence of increasing molar amounts of a 206-bp fragment of pBR322, used as nonspecific competitor [Ruiz et al., 1995]. Here, we show that complex 1 is specifically competed by a 10-fold molar excess of the cold Oct-1-specific oligonucleotide (Fig. 2B, lane 3). By 50-fold excess of cold competitor, the complex completely disappeared (Fig. 2B, lane 4). Furthermore, complex 1 is supershifted when the anti-POU antibody is included in the binding reaction (data not shown). This suggests that this complex represents a specific interaction between the 186-bp fragment and the endogenous Oct-1 protein. Complex 2 was also competed in the presence of much higher amounts, 250-fold molar excess, of the Oct-1 specific oligonucleotide. As we have shown previously, complex 2 is formed by the interaction of the 186-bp fragment with the *ors* binding activity protein (OBA) [Ruiz et al., 1995]. The competition of complex 2 by the Oct-1-specific oligonucleotide

Fig. 2. **a:** Western blot analysis. 500 ng of purified, bacterially expressed His6tagged, Oct-1 POU domain protein, 70 µg protein from HeLa cytoplasmic and 18.5 µg protein from HeLa nuclear extracts were loaded on a 8% PAGE. The proteins were electrotransferred to Immobilon-P membranes for 1 h at 100 V in transfer buffer containing 20% methanol. The blot was probed with anti-POU polyclonal antibody and developed as described in Material and Methods. The 24-kDa POU domain protein and the 90- to 95-kDa Oct-1 protein are indicated. **b:** Competition bandshift assay showing the binding specificity of the endogenous Oct-1 protein to the 186-bp fragment of *ors*8. Reactions contained 1 ng of labeled 186-bp fragment DNA and 10 µg protein from total HeLa cell extracts (+NC, **lane 2**) in the presence of increasing molar fold excess amounts of the cold Oct-1 oligonucleotide (**lanes 3–5**), as indicated. The unbound 186-bp fragment DNA, the three major protein complexes (1, 2, 3), and the 186-bp secondary structure (*) are indicated.

a.


Marker (kDa)
Oct-1 POU
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HeLa nuclear
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69.0
46.0
30.0
21.0
90-95 kDa
24 kDa

b.

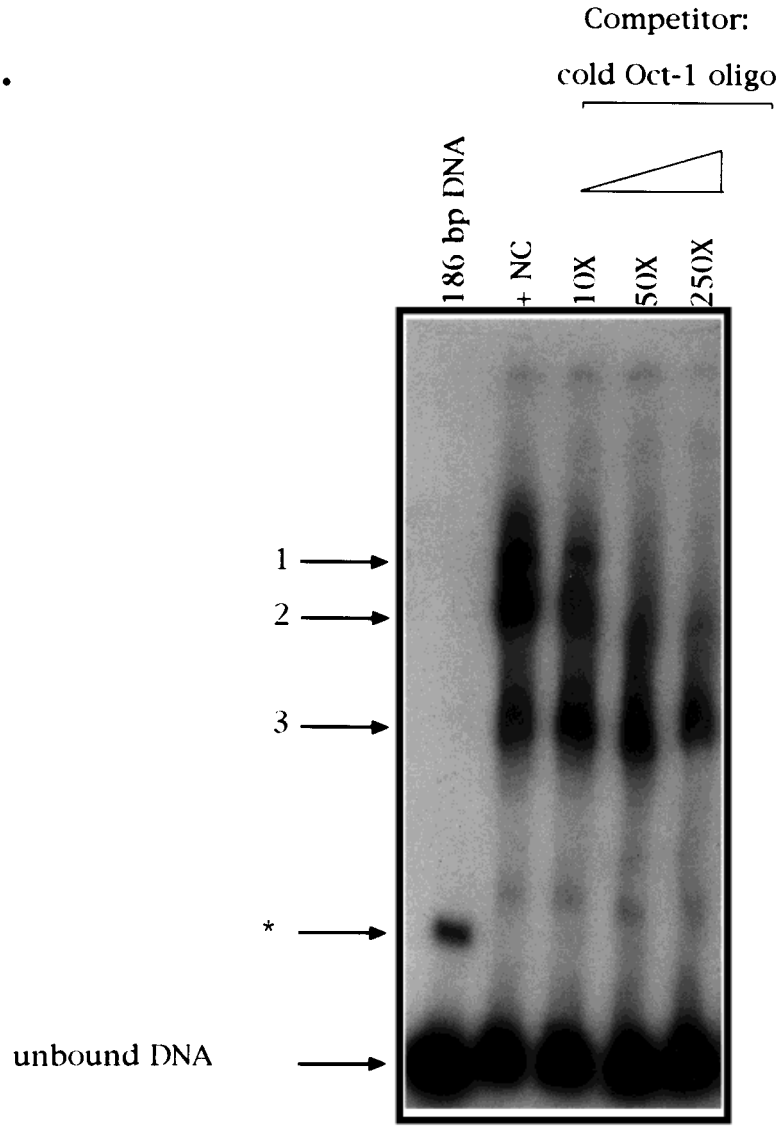
Competitor:
cold Oct-1 oligo
186 bp DNA
+ NC
10X
50X
250X
1
2
3
*
unbound DNA

Figure 2

might be due to an interaction of OBA with Oct-1. Finally, complex 3 was not competed even in the presence of 250-fold molar excess of cold competitor, suggesting that Oct-1 binding is not involved in its formation.

Specific Binding of Endogenous Oct-1 Protein Found in HeLa Extracts to an Oligonucleotide Containing the Oct-1 Binding Site

A bandshift assay was used to show the presence of binding activity for the Oct-1-specific double-stranded oligonucleotide in total HeLa cell extracts (Fig. 3A). Three major specific protein-DNA complexes were formed (Fig. 3A, lane 2, Complex A, B, C). The binding specificity of the oligonucleotide in each of the complexes was tested by competition bandshift assays (Fig. 3A), with increasing molar excess amounts (5- to 150-fold) of cold Oct-1 oligonucleotide (lanes 3–6), used as specific competitor, or cold pBR322 oligonucleotide (lanes 7–10), used as nonspecific competitor. At 5- and 25-fold molar excess of the specific competitor, only the binding of complex A was competed for (lanes 3 and 4), while at 50- and 150-fold molar excess, all three complexes were specifically competed by the cold Oct-1 oligonucleotide (lanes 5 and 6). In contrast, none of the complexes was competed

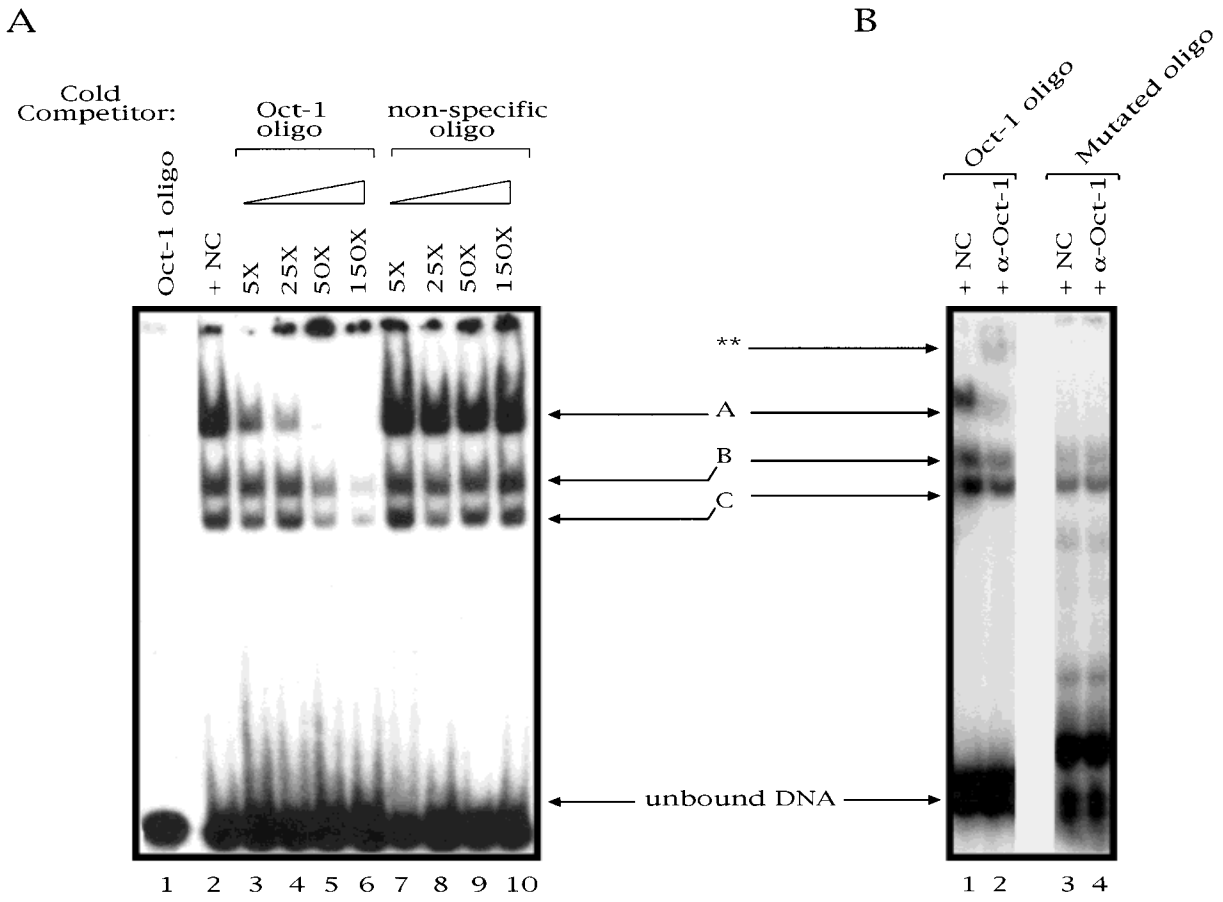

Fig. 3. **A:** Competition bandshift assay showing binding specificity of the Oct-1 oligonucleotide to the HeLa cell endogenous Oct-1. Reactions contained 1 ng labeled Oct-1 oligonucleotide and 10 mg protein from total HeLa cell extracts (+NC, **lane 2**) in the presence of increasing molar excess amounts of cold Oct-1 oligonucleotide (**lanes 3–6**), added as specific competitor, or cold 16-bp pBR322 DNA fragment (nucleotides 838–854; **lanes 7–10**), added as nonspecific competitor, as indicated. The free Oct-1 oligonucleotide DNA (unbound DNA) and the three major complexes (A, B, C) are indicated. **B:** Interference of Oct-1 binding to the 186-bp fragment by addition of the anti-Oct-1 antibody (α-Oct-1). Reactions were performed by preincubating the extracts and the labeled oligonucleotide [Oct-1-specific (**lane 1**) or mutated (**lane 3**)] at room temperature for 20 min prior to the addition of 1.5 μg of the anti-Oct-1 antibody (**lanes 2, 4,** respectively). Upon addition of the antibody, the reaction was incubated for a further 25 min at room temperature. The reactions were loaded on a 4% PAGE gel and ran at 180 V for 1 h. The three major complexes (A, B, C), the Oct-1 supershifted complex (**), and the unbound Oct-1-specific or mutated Oct-1 oligonucleotides are indicated.

by the nonspecific competitor (lanes 7–10). Hence, all three complexes represent a specific interaction of the extracts with the Oct-1-specific oligonucleotide.

To demonstrate which of the complexes were the result of Oct-1 binding, an anti-Oct-1 antibody was included in the binding reaction. Addition of the antibody supershifted complex A (**), but had no major effect on the two faster migrating complexes B and C (Fig. 3B, lane 2). To show that complex A was indeed the result of the endogenous Oct-1 protein binding to the Oct-1-specific oligonucleotide, an oligonucleotide with a mutated Oct-1 binding site was also used in a bandshift reaction with total HeLa cell extracts (Fig. 3B, lane 3). Complex A, which results from Oct-1 binding, did not form, while the two protein DNA complexes corresponding to complexes B and C did (Fig. 3B, lane 3). Addition of the anti-Oct-1 antibody did not supershift either of these complexes (Fig. 3B, lane 4). These results show that the Oct-1-specific oligonucleotide specifically binds the endogenous Oct-1 protein, whereas the mutated oligonucleotide does not, and confirms that the retarded complex A is due to the binding of Oct-1. Complexes B and C are the result of the Oct-1 oligonucleotide binding to unidentified proteins whose binding was not competed even in the presence of cold oligonucleotide corresponding to the SP1, AP1, and AP2 binding sites (Promega, personal communication). These two specific complexes are not the result of Oct-1 binding since they also form in the presence of the mutated Oct-1 oligonucleotide and they are not recognized by the Oct-1 antibody.

Addition of the Oct-1-Specific Oligonucleotide Results in Inhibition of Replication

In vitro replication assays of the p186 plasmid were performed as described previously [Todd et al., 1995], except that increasing molar excess amounts, relative to the amount of the input p186 DNA, of the Oct-1-specific, mutated Oct-1, and nonspecific oligonucleotides were also included in the reaction, in order to study the effect of removal of the endogenous Oct-1 protein on the in vitro replication of p186. The extracts and the oligonucleotides were preincubated on ice for 20 min, to allow binding of the oligonucleotide to the endogenous Oct-1 protein present in the extracts. The products of the in vitro replication reactions included open circular (II), linear (III), and supercoiled (I) forms of the plasmid DNAs (Fig. 4A). Lower levels of form I DNA were recovered after the in vitro replication reaction, by comparison to those of form II and form III DNA, in agreement with other studies [Decker et al., 1986; Pearson et al., 1991].The ladder of bands between the relaxed circular and supercoiled plasmid DNAs in the gel of undigested samples (Fig. 4A, -*Dpn*I) corresponds to topoisomeric molecules [Pearson et al., 1991]. DNA forms that migrate slower than the open circular form II represent catenated dimers and replicative intermediates [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994].

Inclusion of increasing amounts of the Oct-1-specific oligonucleotide decreased the total incorporation of label in p186 (Fig. 4A, lanes 6–10), whereas the nonspecific oligonucleotide (Fig. 4A, lanes 2–5) and the mutated Oct-1 oligonucleotide (data not shown) had no effect. In addition to incorporation that is due to replication, some is due to repair [Pearson et al., 1991]. To differentiate between incorporation due to repair or replication, the in vitro replication products were digested with the restriction endonuclease *Dpn*I [Peden et al., 1980]. Using the *Dpn*I resistance assay, we have previously demonstrated that the in vitro replication of *ors*8 is semiconservative [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992], as it is in vivo [Frappier and Zannis-Hadjopoulos, 1987].

*Dpn*I digestion of the samples confirmed that the Oct-1-specific oligonucleotide inhibited the replication of p186 (Fig. 4B, lanes 6–10), in contrast to the samples in which the nonspecific oligonucleotide (Fig. 4B, lanes 2–5) or the mutated Oct-1 oligonucleotide (data not shown) were included. Quantification of the *Dpn*I-resistant bands corresponding to forms II and III was performed (as described under Materials and Methods) and the percentage of DNA replication relative to the positive control (Fig. 4B, lane 1), p186, was plotted (Fig. 4C). A decrease in DNA replication was not observed at the 50-fold molar excess level of Oct-1-specific oligonucleotide, probably because this amount was not sufficient to bind all the endogenous Oct-1 present in the extracts. However, the relative percent of DNA replication decreased to approximately 37% in the presence of competitive 100-fold molar excess of Oct-1-specific oligonucleotide, and down to 12–25% with 250-molar fold of the same oligonucleotide (Fig. 4C, black bars). This level of replication remained

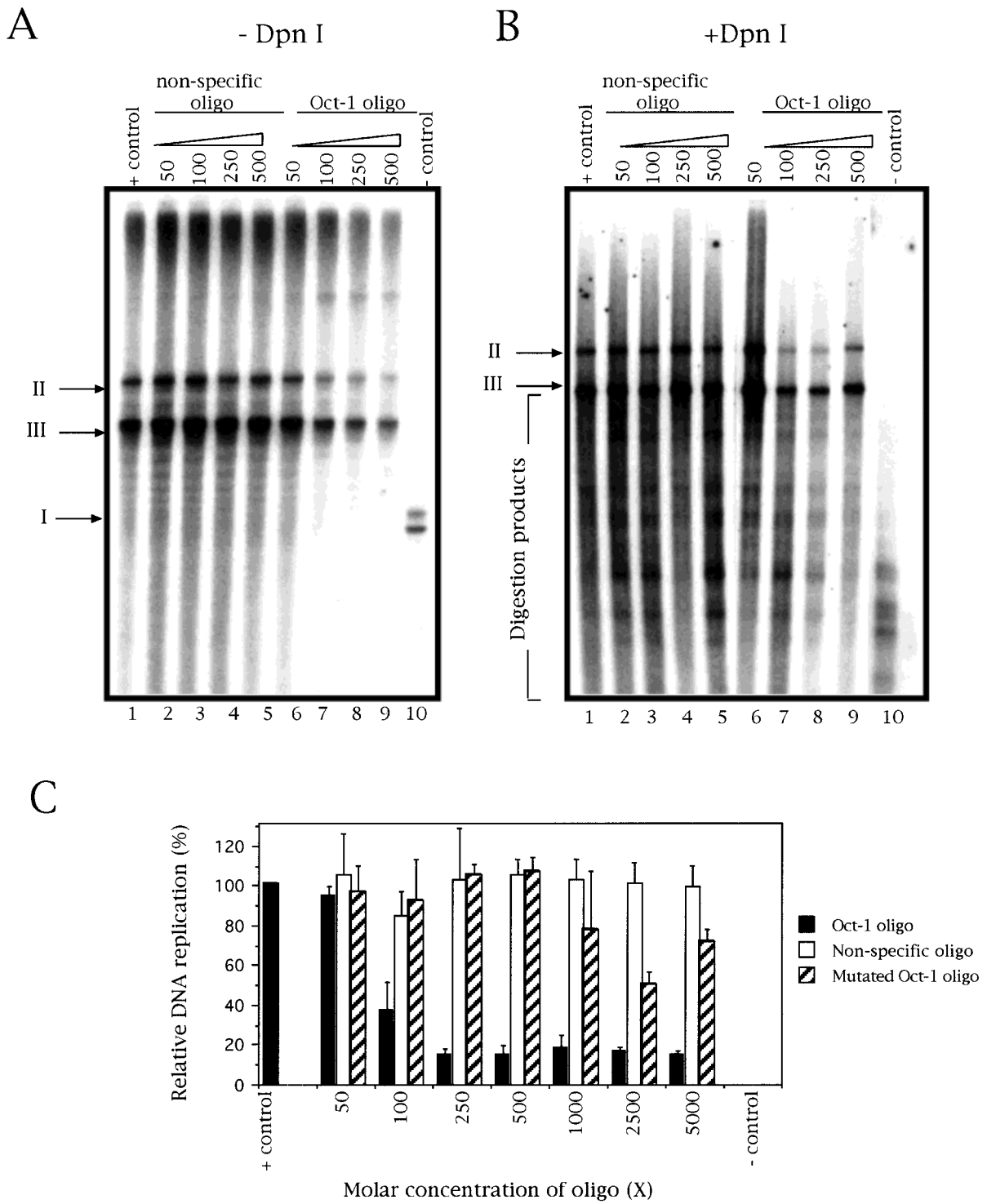

Fig. 4. In vitro replication assay showing the inhibitory action of the Oct-1 oligonucleotide. HeLa cell extracts were incubated with increasing amounts of Oct-1-specific oligonucleotide or nonspecific oligonucleotide of pBR322 on ice for 20 min. Then, p186 DNA and the remaining components of the in vitro reaction were added and the reactions were incubated at 30°C for 1 h, as described under Materials and Methods. The negative control corresponds to the pBluescript plasmid. The DNA products were purified and concentrated. One third of the reaction was left untreated (*Dpn*I) and a third was digested with 1 unit of *Dpn*I (+*Dpn*I), as described under Materials and Methods. The samples were electrophoresed on a 1% agarose gel in 1× TAE buffer at 50–55 V for 16–20 h. The gels were dried and exposed for autoradiography. **A:** Typical autoradiograph of undigested in vitro products is shown. The open circular (II), linear (III), and supercoiled (I) forms of the replicated DNA are indicated. **B:** *Dpn*I digestion of the samples in part A. Forms II and III, and the *Dpn*I digestion products are indicated. **C:** Bar graph showing the relative DNA replication of p186 with increasing amounts of Oct-1-specific (black bars), nonspecific (white bars), or mutated Oct-1 (striped bars) oligonucleotides. The positive control corresponds to the p186 and the negative control to the pBluescript plasmid in the absence of any oligonucleotide (black bars). Quantifications were done on the *Dpn*I-digested samples as described under Materials and Methods. Data are expressed as a percentage of the control p186 in vitro reaction lacking any oligonucleotide. Each bar represents two to seven experiments; 1 SD.

constant even when the specific oligonucleotide was increased to a molar excess of up to 5,000-fold (Fig. 4C, black bars). No inhibition of replication was observed when the nonspecific oligonucleotide (white bars) or the mutated Oct-1 oligonucleotide (striped bars) were included in the in vitro replication reaction of p186 up to levels of 500- to 1,000-fold molar excess (Fig. 4C). Above 1,000-fold molar excess, some nonspecific effects were eventually detected for the mutated Oct-1 oligonucleotide (striped bars), although the nonspecific oligonucleotide still exhibited no effect on the level of DNA replication (white bars). Similar results were obtained when the extracts and oligonucleotides were pre-incubated at 30°C, rather than on ice for 20 min (data not shown). The data suggest that, depletion/competition of the Oct-1 protein, by the addition of >250-fold molar excess of Oct-1-specific oligonucleotide, inhibited DNA replication to 18% of the control.

In Vitro Nucleotide Incorporation in the Presence of the Oct-1 Oligonucleotide Is Sensitive to Aphidicolin

The incorporation of precursor nucleotide into p186 plasmid DNA during its replication in vitro in the presence of the Oct-1 oligonucleotide was sensitive to 80 μM aphidicolin (Fig. 5). Aphidicolin, a tetracyclic diterpenoid, is an inhibitor of the eukaryotic replicative polymerases α and δ [Lee et al., 1985], while the nonreplicative, eukaryotic DNA polymerases β and γ, are resistant to it. Therefore, the results suggest that it is the replicative DNA polymerases that are largely responsible for the in vitro incorporation of precursor nucleotide, observed in the presence of both the Oct-1-specific and nonspecific oligonucleotides. Incorporation attributable to repair synthesis in the presence of the oligonucleotides was constant, approximately 15–30%, as determined by quantification of the in vitro products following correction for the amount of DNA loaded onto the gel (data not shown). These data are in agreement with previously reported results [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994].

Addition of Purified Oct-1 POU Partially Reverses the Inhibition of Replication

To determine whether the inhibition of replication observed in the presence of the Oct-1-specific oligonucleotide was due to the depletion of the Oct-1 protein, purified Oct-1 POU

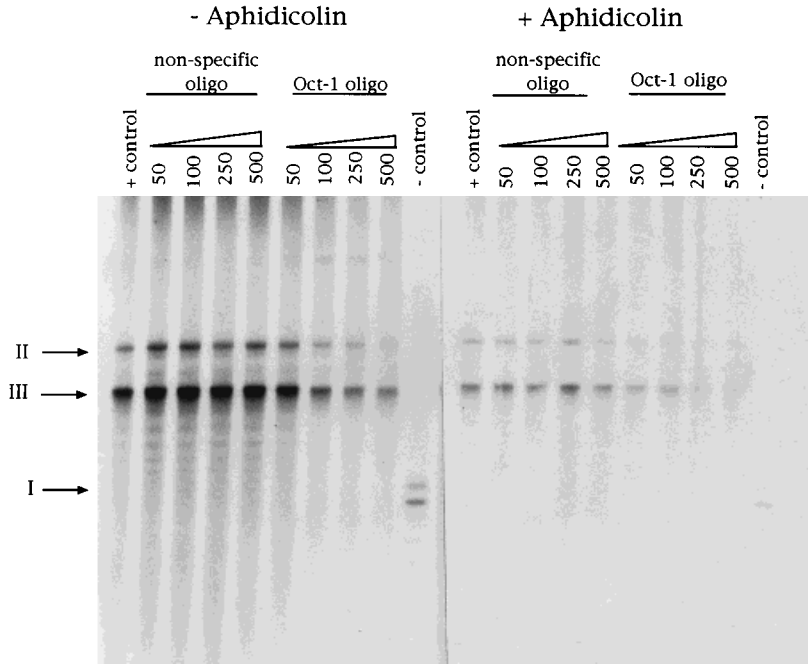

Fig. 5. In vitro replication assay in the presence of aphidicolin. Reactions were performed by pre-incubating the extracts with the indicated molar excess amounts of the Oct-1-specific or nonspecific as described in Figure. 4. Aphidicolin was added to a final concentration of 80 μM and the reactions were incubated at 30°C for 15 min. The DNA products were purified and concentrated. The samples were electrophoresed on 1% agarose in 1× TAE buffer at 50–55 V for 16–20 h. Gels were dried and exposed for autoradiography. Forms I, II, and III are indicated. + and - control correspond to p186 and pBluescript, respectively, in the absence of any oligonucleotide.

domain protein was added back to the inhibited reaction. First, the binding of the Oct-1 POU domain to the imperfect octamer site present in 186-bp fragment was verified by a bandshift assay (Fig. 6A, lane 2; Fig. 6B, lane 1). The specificity of the binding was tested in a competition bandshift assay in which either cold 186-bp fragment DNA, used as a specific competitor (Fig. 6A, lanes 3–7), or a 206-bp pBR322 fragment, used as nonspecific competitor, was added (Fig. 6B, lanes 2–6). The 186-bp fragment–Oct-1 POU complex (**) was specifically competed with 50- to 500-fold molar excess of cold, specific competitor, whereas competition was not observed with increasing amounts (up to 500-fold molar excess amounts) of cold, nonspecific competitor. The slower migrating band seen in all the lanes (indicated by * in Fig. 6A,B) results from the formation of a secondary structure induced during the purification of the 186-bp fragment [Ruiz et al., 1995]. This complex is not competed for in the presence of increasing amounts of the cold competitor, further confirming that the observed binding/competition with the POU-186-bp fragment is specific.

The purified Oct-1 POU domain was then added to the Oct-1 oligonucleotide-inhibited in vitro replication reaction of p186 in order to restore normal replication levels. The in vitro replication of p186 was first inhibited by preincubating the extracts with 250-fold molar excess of Oct-1-specific oligonucleotide, resulting in the depletion of the endogenous Oct-1 protein (approximately 100 ng, as deduced from Fig. 2A) and inhibition of replication to 18% (Fig. 4C). Then, increasing amounts of the Oct-1 POU domain or BSA protein (used as a control) were added to the reaction mixture and incubated for a further 20 min on ice before carrying out the in vitro reaction. The results indicate that the addition of 1.5 ng of POU domain protein increased replication only marginally (increase of 5%), while the addition of 80 ng and 160 ng of the POU domain protein restored replication to nearly 50% of control levels (Fig. 6C, black bars). Consequently, when recombinant Oct-1 POU domain protein was added at amounts approximately replacing the oligonucleotidedepleted Oct-1 levels, a twofold restoration in replication was achieved. Addition of the same amounts of BSA did not result in any increase in replication (Fig. 6C, striped bars). Also, when purified POU domain protein was added to the in vitro replication reaction of p186, in the absense of any oligonucleotide, no significant effect on DNA synthesis was observed (data not shown), suggesting that the mere addition of the POU protein does not result in stimulation of replication and that there exists a threshold level of Oct-1 in the extracts. The results suggest that the increase in replication obtained with the addition of purified Oct-1 POU was due to the restoration of the depleted Oct-1 protein (Fig. 6C).

Specific Immunodepletion of Oct-1 Protein Inhibits the In Vitro Replication of p186

The inability of the purified POU domain protein to fully restore replication to control levels (Fig. 6C) may be explained by several possibilities (discussed below). One of them is that the Oct-1-specific oligonucleotide is depleting not only the Oct-1 protein but also other unidentified protein(s)/factors (see Fig. 3A, complex B or C), whose depletion may contribute in part to the observed inhibition of replication. To determine the contribution, if any, of these putative Oct-1 oligonucleotide binding protein(s) on the in vitro replication of p186, we immunodepleted the HeLa cell extracts of Oct-1 protein using an anti-POU antibody (a gift from Dr. van der Vliet). This antibody recognizes a single protein from total HeLa cell extracts, as determined by Western blot analysis (Fig. 2A) and is specific for the Oct-1 protein (Fig. 3B, lane 2).

Addition of a 50-fold molar excess of the antibody relative to the endogenous Oct-1 protein inhibited replication to approximately 28% of the control (Fig. 7, anti-POU), while at 150- and 250-fold molar excess the antibody did not inhibit replication further (data not shown). Thus, the maximum level of inhibition of replication observed upon immunodepletion of the extracts corresponds to that which is obtained upon depletion of the Oct-1 protein by inclusion of the Oct-1-specific oligonucleotide (Fig. 4A–C). Addition of nonspecific polyclonal antibodies, such as antigoat or antimouse IgG, at greater concentrations (70- and 140-fold) than that of the anti-POU antibody did not affect the in vitro replication of p186 (data not shown).

To determine whether the inhibition of replication was due to the specific immunodepletion of the Oct-1 protein, purified POU domain was, once again, added exogenously. Addition of a 50-fold molar excess of the POU domain restored replication to approximately 65% of con-

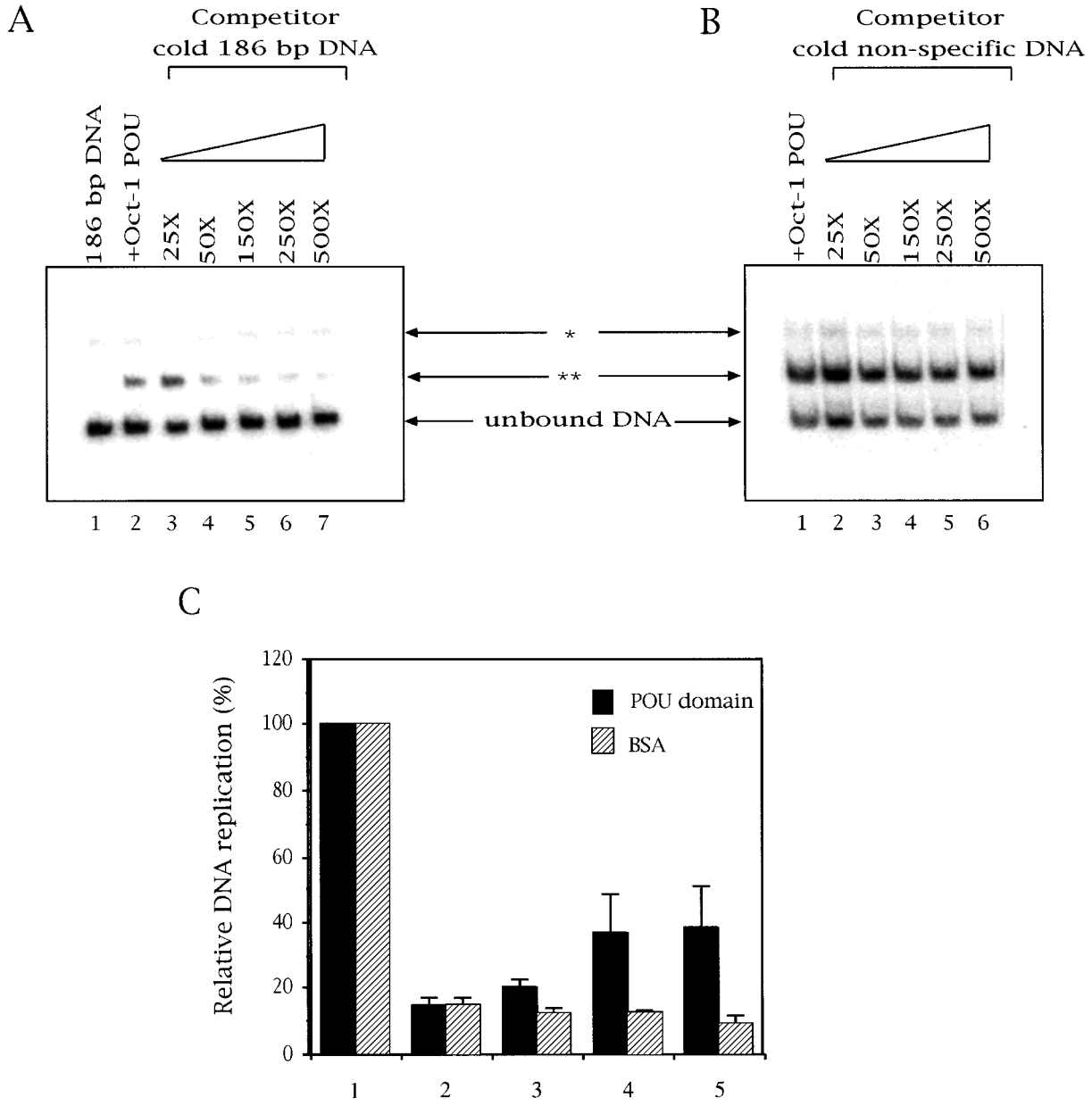

Fig. 6. **A:** Competition bandshift assay showing specificity of binding of the Oct-1 POU domain protein to the 186-bp fragment. Reactions contained 0.1 ng of ^{32}P-labeled 186-bp fragment, 2.5 ng Oct-1 POU protein and increasing molar excess amounts of cold 186-bp fragment DNA, as indicated. The free 186-bp DNA(unbound DNA), the protein DNA complex (**), and the 186-bp secondary structure (*) are indicated. **B:** As in A, except that increasing molar excess amounts of a cold nonspecific competitor from nucleotides 860–1065 of pBR322 was added to the binding reaction. **C:** In vitro replication data showing the reversal of replication inhibition with the addition of exogenous Oct-1 POU. In vitro replication was inhibited by pre-incubating the HeLa extracts with 250-fold molar excess of Oct-1 oligonucleotide (relative to the input p186 DNA) for 20 min on ice. Then, different amounts of the Oct-1 POU domain protein or BSA protein were added to replace the amount of Oct-1- specific oligonucleotide depleted Oct-1 and further incubated on ice for 20 min. The reactions were performed as described under Material and Methods. The data are expressed as a percentage of replication relative to a control reaction lacking any oligonucleotide or exogenous Oct-1 POU protein (**lane 1**). Black bars, addition of purified POU domain protein (**lane 2,** 0 ng; **lane 3**, 1.5 ng; **lane 4**, 80 ng; **lane 5**, 160 ng); striped bars, addition of equivalent amounts of BSA. Bars represent results obtained from two different experiments; 1 SD.

trol levels (Fig. 7, anti-POU + Oct-1 POU). Addition of higher amounts of the purified POU protein did not result in a greater increase in replication (data not shown).

Mutation of the Oct-1 Binding Site Does Not Affect the In Vitro Replication of p186

Since the endogenous Oct-1 is able to bind specifically to the 186-bp fragment and since its depletion, either by addition of the Oct-1-specific oligonucleotide or by addition of the anti-POU antibody, inhibited p186 replication, we investigated whether Oct-1 enhanced p186 replication by a mechanism that involved its direct binding to the octamer motif. We therefore mutated the Oct-1 binding site in p186 (see Materials and Methods) from TAAATACG to GCTGGCAC. Figure 8a shows the sequencing reactions of one of the resulting mutant clones, p186-MutOct, and wild-type p186, with the mutated or wild-type Oct-1 binding sites indicated in boldface. A comparison of the ability of these two plasmids to undergo in vitro replication showed that p186-MutOct replicated in vitro with the same efficiency as wild-type p186 (Fig. 8b). Quantitation of the *Dpn*I-resistant bands corresponding to forms II and III showed that the mutant plasmid replicated with an average efficiency of 99% compared to 100% for p186. Moreover, competition-depletion (using the Oct-1 oligonucleotide) and reconstitution (adding the POU domain) assays with p186-MutOct as template yielded results similar to those obtained with p186 (data not shown). Finally, to rule out the possibility that Oct-1 was able to bind elsewhere within the mutated 186-bp fragment, a bandshift assay was performed using purified POU domain protein and the mutated 186-bp fragment. No binding was detected by these assays (data not shown).

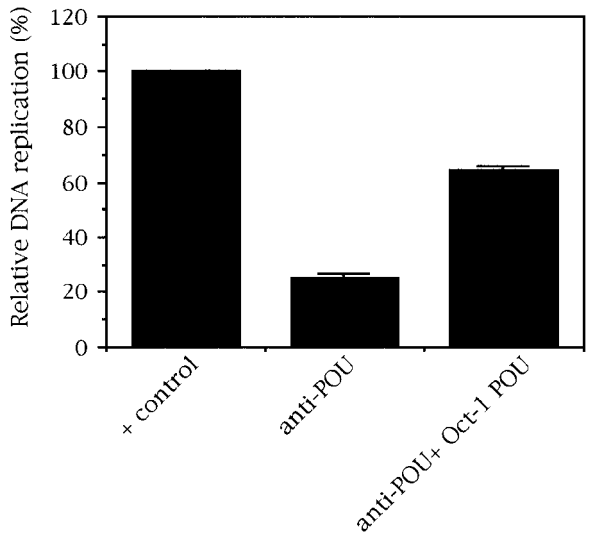

Fig. 7. In vitro replication of p186 showing the effect of anti-POU antibody. In vitro replication was inhibited by preincubating a 50-fold molar excess of anti-POU antibody (relative to the endogenous Oct-1) on ice for 20 min. Then, a 50-fold molar excess of purified POU domain protein (relative to the amount of anti-POU antibody) was added exogenously and further incubated on ice for 20 min. The reactions were performed as described under Materials and Methods. Data are expressed as a percentage of replication relative to a control reaction with no antibody or exogenous Oct-1 POU protein. Bars are results from two different experiments.

DISCUSSION

We have previously shown that *ors*8 (483 bp) can function as an origin of DNA replication in autonomously replicating plasmids in vivo [Frappier and Zannis-Hadjopoulos, 1987] and in a mammalian cell-free replication system [Pearson et al., 1991; 1994a]. We have also shown that a minimal origin lies within an internal fragment of 186-bp, which is also capable of autonomous replication in vivo and in vitro [Todd et al., 1995]. This minimal replicating DNA fragment of *ors*8 contains an imperfect 44-bp IR, several direct repeats, and an imperfect consensus binding site for Oct-1 (Fig. 1).

Here, we present evidence that Oct-1 protein, a cellular transcription factor, is an enhancing component in the in vitro replication of p186, but it seemingly does not exert its effect through direct binding to the octamer motif in the origin. We confirmed the presence of Oct-1 protein in the HeLa cell extracts that were used for the in vitro replication and determined that the HeLa nuclear extracts contained approximately 100 ng of Oct-1 protein (Fig. 2A). We also confirmed the potential for specific binding of this endogenous Oct-1 protein to the minimal origin of *ors*8 (Complex 1, Fig. 2B). Furthermore, we demonstrated, by competitive bandshift and supershift assays, specific binding of the endogenous Oct-1 protein to a double-stranded oligonucleotide containing the Oct-1 binding site (Complex A, Fig. 3A, B). Complex A was the result of Oct-1 binding, since it was (1) competed by increasing amounts of Oct-1-specific oligonucleotide; (2) supershifted by the anti-Oct-1 antibody; and, finally, (3) not formed with the mutated Oct-1 oligonucleotide. Complexes

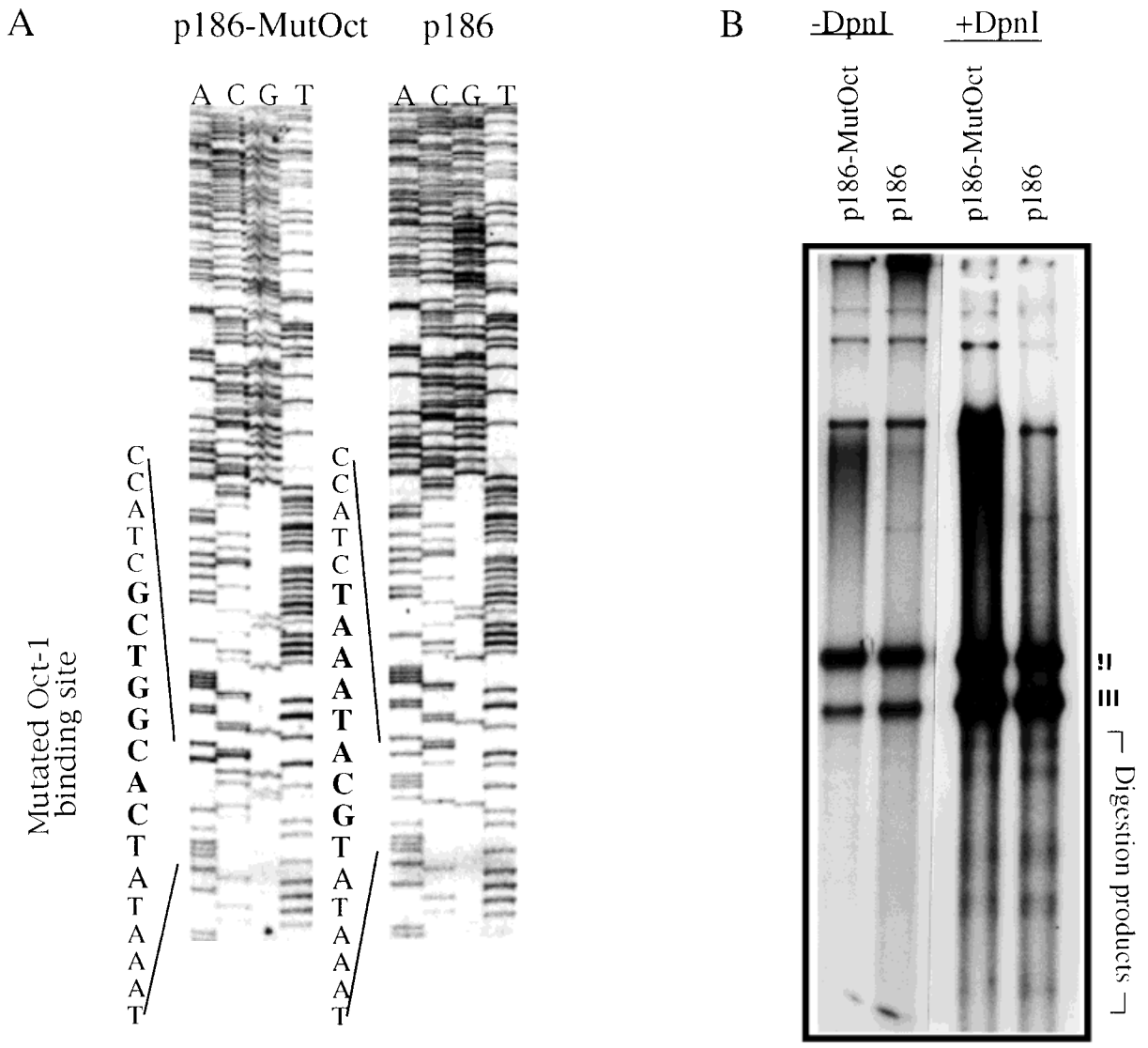

Fig. 8. Effect of a mutated Oct-1 binding site on the in vitro replication of p186. Site-directed mutagenesis was employed to alter the Oct-1 binding site in the p186, as described under Materials and Methods. Potential clones were first screened by restriction endonuclease digestion with *Sca*I and *MIu*I and then sequenced. **A:** Autoradiograph of an 8% sequencing gel with the mutated Oct-1 binding site in p186-MutOct and the wild-type octamer site of p186 indicated in bold. **B:** Comparison of the replication efficiency of the mutant and wild-type plasmids. In vitro replication was performed using 200 ng of either p186 or p186-MutOct template DNA. Autoradiograph of the undigested in vitro products (**lanes 1, 2**) and the *Dpn*I-digested products (**lanes 3, 4**).

B and C were also competed by increasing amounts of the Oct-1-specific oligonucleotide, but not by the nonspecific competitor, suggesting that they are the result of specific binding. However, these complexes were shown to be independent of Oct-1, since they also formed with the mutated Oct-1 oligonucleotide and were not recognized by the antibody against Oct-1. Therefore, these complexes do not result because of Oct-1 binding.

Addition of the Oct-1-specific oligonucleotide did not affect repair-type synthesis during the in vitro replication assays, since the background level of aphidicolin-resistant DNA repair (15–30%) incorporation remained constant regardless of the molar excess amount of oligonucleotide used and could be attributed to some amount of nicking in the input DNA (Fig. 5). Treatment with aphidicolin inhibited incorporation significantly (70–85%) when compared to

reactions without aphidicolin, confirming that the in vitro nucleotide incorporation is largely due to the replicative DNA polymerases α/δ.

Addition of >250-fold molar excess amounts of the Oct-1-specific oligonucleotide to the in vitro reaction, resulted in the depletion of the endogenous Oct-1 protein and inhibited replication of p186 by 80–85% compared to the control reaction in which p186 was replicated in the absence of this oligonucleotide (Fig. 4B, C); in contrast, the use of a mutated Oct-1 oligonucleotide (up to 1,000-fold excess) or a nonspecific oligonucleotide (up to 5,000-fold excess) produced no effect (Fig. 4C). The partial (20–40%) inhibition observed at high concentrations (>1,000-fold molar excess) of the mutated oligonucleotide may be caused by the removal of other, unidentified factors that bind to the mutated oligonucleotide (see Fig. 3B, lane 3). Although the Oct-1-specific oligonucleotide may also bind to these factors, the inhibition observed upon addition of this oligonucleotide (see Fig. 4C) is much higher and occurs at lower oligonucleotide concentrations. Furthermore, depletion of these unidentified factors does not seem to affect replication, since addition of the anti-POU antibody (Fig. 7), which only recognizes Oct-1 (see Fig. 2A), yielded similar results to those obtained with the Oct-1-specific oligonucleotide.The maximum level of inhibition by the Oct-1-specific oligonucleotide was 85%, even in the presence of 5,000-fold excess, suggesting that some basal level of replication does occur in the absence of Oct-1. On the other hand, this low level of replication may, in part, be due to residual activity of the oligonucleotide-bound Oct-1 protein, which is not removed in its bound state during the actual in vitro replication reaction. Nevertheless, the Oct-1-specific oligonucleotide inhibits replication, on the average, by 85%, by comparison to the mutated Oct-1 or nonspecific oligonucleotides, and the activity of the oligonucleotide-bound Oct-1, if any, is low.

We demonstrated that the inhibition of replication caused by the addition of the Oct-1-specific oligonucleotide was due, in major part, to depletion of Oct-1, since addition of the Oct-1 POU domain to the inhibited reactions restored replication, up to 50% of control levels (Fig. 6C). The amount of recombinant Oct-1 POU domain protein necessary to obtain a twofold restoration of replication correlates with the levels of endogenous Oct-1 protein that were depleted (see Results). In the adenovirus reconstituted DNA replication system, 5 binding units of POU domain (1 binding unit represents 1.7 ng POU domain) were used to stimulate DNA replication with 50 ng pTP-pol, 1 μg DNA binding protein (DBP) and 50 ng adenovirus-TP DNA [Coenjaerts and van der Vliet, 1995]. In our system, four times as much input DNA (200 ng) was used, thus requiring 34 ng POU domain. However, since we use cell extracts rather than a reconstituted system, it is not surprising that more protein is required. We also showed that immunodepletion of Oct-1 from the HeLa cell extracts using the anti-POU antibody could maximally inhibit p186 replication to approximately 25% of control levels (Fig. 7) and that the inhibition of replication was reversed, to 65% of control levels when recombinant POU domain protein was added exogenously (Fig. 7). Finally, although Oct-1 is able to bind to the imperfect wild-type octamer motif in p186 (Figs. 3A,B and 6A,B), it does not seemingly exert its effect on replication through direct binding, since mutation of this site yielded a clone with wild-type replication efficiency.

The inability of the POU domain to completely reverse the inhibition in the in vitro replication of p186 indicates that it is not sufficient by itself to fully enhance p186 replication. This is in contrast to adenovirus replication where, the Oct-1 POU domain and the DNA binding domain of nuclear factor I (NFI) suffice for enhancement of replication of the viral DNA [Gounari et al., 1990; Verrijzer et al., 1990]. The POU domain stimulates replication through a direct interaction with the pTP during the preinitiation step [Coenjaerts et al., 1994; van Leeuwen et al., 1997]. Through this protein-protein interaction, the POU domain stabilizes the preinitiation complex, and together with NFI, position the complex to the core origin, leading to increased binding of the pTP-pol to its target site. This enhanced binding of the pTP-pol correlates very well with the increased levels of stimulation on replication [van Leeuwen et al., 1997]. In adenovirus, the four initiator proteins, pTP-pol, NFI and Oct-1, can form a stable complex even in the absence of DNA [van Leeuwen et al., 1997]. Direction of the pTP-pol complex to its target site is also dependent on the specificity of the intact POU domain; mutation of the POU domain resulted in a decrease in adenovirus DNA replication [van Leeuwen et al., 1995]. One explanation for the lack of full restoration of p186 replication in vitro might be

that the POU domain protein may not function effectively in other protein interactions that may be required to execute that reaction in higher eukaryotes. In the adenovirus in vitro DNA replication system, the POU domain is only interacting with virus-encoded proteins and not with any cellular proteins. Furthermore, unlike the adenovirus system, the transcription activation domain, in addition to the binding domain of Oct-1, may also be required for the full activation of replication of p186. For example, the transcription activation domain could be required for enhancement if the mechanism by which stimulation occurs involves distortions to the origin structure by the transcription factor, thus facilitating the assembly of a pre-initiation complex [van der Vliet, 1996]. In addition, the flexible nature of the transcription activation domain may increase the potential for different, specific interactions with replication proteins [van der Vliet, 1996].

Significant conformational changes (e.g., bending) in the DNA must occur to promote all the necessary protein–protein interactions and destabilize the origin, thus facilitating the initial unwinding step of replication. Such bending is achieved by several ways in adenovirus. First, the protein-free origin is intrinsically bent [Zorbas et al., 1989]. Second, binding of NFI enhances the intrinsic bent [Zorbas et al., 1989]. Finally, the POU domain is also capable of bending its recognition sequence to some degree [Klemm et al., 1994]. Therefore, although binding of pTP is essential, it has been postulated that it may not be the only role of the adenovirus POU domain. However, the in vitro replication efficiency of a virus with a mutated octamer sequence has not been examined thus far. What has been looked at is the ability of other transcription factors to compensate for a deleted octamer sequence in vivo. When other DNA sequences, such as SP1, ATF, and EBP1 sites, are present at the left terminus of the adenovirus genome, deletion of the octamer sequence does not inhibit replication, indicating functional redundancy between different transcription factor binding sites [Hatfield and Hearing, 1993]. In those studies, depletion of the Oct-1 protein was not investigated. However, when the Oct-1 binding site was substituted by an AP1 site, to which *fos–jun* can bind and bend DNA similarly to Oct-1, replication was not stimulated [Coenjarts et al., 1994], again indicating the importance of protein–protein interaction between the POU DNA binding domain of Oct-1 and the pTP protein of adenovirus.

The data presented here suggest that the specific depletion of Oct-1 inhibits replication, although mutation of its binding site does not produce any effect (Fig. 8b). Consequently, a direct role of Oct-1 in unwinding the minimal origin of *ors*8 at its binding site is unlikely, even though it has the potential to bind to this site (Fig. 2B). As discussed earlier, a more plausible function for this transcription factor would be its involvement in protein–protein interactions, as is its main role in adenovirus [van Leeuwen et al., 1997]. Thus, Oct-1 may be interacting with other replication factor(s), which would also become depleted during the in vitro competition assays.

A growing number of origins in multicellular organisms are being shown to contain binding sites for specific cellular transcription factors, in addition to binding sites for the replication initiator proteins. Several reports suggest that not only are transcriptional regulatory elements a common feature of viral, yeast, and lower eukaryotic origins of DNA replication, but they also modulate the initiation of replication by binding to their recognition sites in the origin [reviewed in Boulikas, 1996; DePamphilis, 1993]. However, the significance of these sites is still unclear in higher eukaryotic origins since mutational analyses have not been performed and their contribution remains uncertain. It is tempting to assume that the transcription factor binding sites in eukaryotes act as enhancers of replication in a similar mechanism as in yeast and viral systems [DePamphilis, 1988; Heintz, 1992; van der Vliet, 1991]. Using p186, which contains the minimal origin of *ors*8, the data presented here indicate that depletion of the Oct-1 transcription factor inhibits the replication of p186, although the actual octamer binding site is not essential for in vitro replication. Therefore, the involvement of Oct-1 in p186 DNA replication most likely occurs through protein–protein interactions with the replication machinery. The POU domain proteins have been postulated to be regulators of viral replication through their involvement in interaction with viral proteins [van Leeuwen et al., 1997]. For instance, a direct interaction has been reported between Oct-1 and the Herpes simplex transactivator protein [O'Hare et al., 1988], between Oct-6 and the JC papovavirus T

antigen [Renner et al., 1994] and between Oct-1 and adenovirus pTP [van Leeuwen et al., 1997].

A better understanding of the relationship among transcription factors, transcription factor binding, and the initiation of DNA replication will require the examination of several different eukaryotic origins in order to eventually lead to an understanding of the regulation of this process.

ACKNOWLEDGMENTS

We thank Dr. Peter C. van der Vliet (Utrecht University, The Netherlands) for generously providing the Oct-1 POU domain and the polyclonal POU antibody. This research was supported by grant MT7965 from the Medical Research Council (MRC) of Canada (M.Z.H.) and the Cancer Research Society (G.B.P.). D.M. is the recipient of studentships from the Cancer Research Society and Le Défi Corporatif Canderel; M.T.R. is the recipient of a studentship from the Cancer Research Society.

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