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The N-terminal domain of human DNA ligase I contains the nuclear localization signal and directs the enzyme to sites of DNA replication

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DNA replication in mammalian cells occurs in discrete nuclear foci called 'replication factories'. Here we show that DNA ligase I, the main DNA ligase activity in proliferating cells, associates with the factories during S phase but displays a diffuse nucleoplasmic distribution in non-S phase nuclei. Immunolocalization analysis of both chloramphenicol acetyltransferase (CAT)–DNA ligase I fusion proteins and epitope tagged DNA ligase I mutants allowed the identification of a 13 amino acid functional nuclear localization signal (NLS) located in the N-terminal regulatory domain of the protein. Furthermore, the NLS is immediately preceded by a 115 amino acid region required for the association of the enzyme with the replication factories. We propose that *in vivo* the activity of DNA ligase I could be modulated through the control of its sub-nuclear compartmentalization.

Keywords: DNA ligase I/DNA replication/factories/nuclear localization signal

Introduction

DNA ligases play essential roles in both DNA replication and DNA repair by joining single- and double-stranded breaks in an ATP-requiring reaction (Kornberg and Baker, 1991). Three distinct DNA ligases, whose function is not yet completely understood, have been biochemically identified in mammalian cells (Lindahl and Barnes, 1992). New perspectives to elucidate the role of DNA ligase I came from the isolation of both the human (Barnes *et al.*, 1990) and the murine (Savini *et al.*, 1994) cDNAs. DNA ligase I (~102 kDa) is divided into two clearly distinct regions: a highly conserved C-terminal region containing the active site (Tomkinson *et al.*, 1990), and a less conserved N-terminal portion dispensable for catalytic activity and probably corresponding to a regulatory domain (Kodama *et al.*, 1991; Savini *et al.*, 1994). The analysis of the human and murine protein sequences (Prigent *et al.*, 1992; Savini *et al.*, 1994) suggests the presence of a (nuclear localization signal) NLS and of putative phosphorylation sites in this domain.

Several observations indicate an involvement of DNA ligase I in DNA replication: (i) DNA ligase I level is higher in proliferating cells (Elder and Rossignol, 1990; Montecucco *et al.*, 1992); (ii) the human DNA ligase I cDNA complements the replicative defect of the *Saccharomyces cerevisiae cdc* 9 DNA ligase mutant (Barnes *et al.*, 1990); (iii) the enzyme co-purifies with a protein complex able to replicate SV40 DNA *in vitro* (Congjun *et al.*, 1994); (iv) it cannot be substituted for by DNA ligase III in an *in vitro* SV40 DNA replication assay (Waga *et al.*, 1994). The strongest evidence of a role in DNA replication derives from the human 46 BR cell line in which a mutation in the DNA ligase I gene (Barnes *et al.*, 1992) correlates with a delay in the joining of the Okazaki fragments (Prigent *et al.*, 1994). Interestingly, the same cell line is hypersensitive to a wide range of DNA-damaging agents (Lehmann *et al.*, 1988), providing genetic evidence for a role of DNA ligase I in DNA repair. Consistent with this, both the catalytic activity and the mRNA levels of DNA ligase I increase after UV treatment of human fibroblasts (Montecucco *et al.*, 1995). Taken together, these data suggest that DNA ligase I is involved in different aspects of DNA metabolism, probably depending upon associations with different enzymatic complexes.

The long half-lives of both DNA ligase I mRNA (Montecucco *et al.*, 1992) and its protein (Lasko *et al.*, 1990) indicate that transcriptional regulation takes place in the long-range period and suggests the existence of a short-term regulation at the post-translational level. Short-term regulation could control the recruitment of the enzyme in different complexes involved in chromosome replication and DNA repair as already suggested for PCNA (Bravo and Macdonald Bravo, 1987), DNA methyl transferase (Leonhardt *et al.*, 1992), cyclin-A and cdk2 (Cardoso *et al.*, 1993). All these proteins localize *in vivo* at specific dense structures, called replication factories, that undergo characteristic changes during S phase and where DNA synthesis occurs (Hozak *et al.*, 1993, 1994).

In this paper we analyse the nuclear and sub-nuclear localization of DNA ligase I during the cell cycle by means of indirect immunofluorescence experiments and report the identification of the functional NLS and of a replication factory targeting sequence.

Results

Specific localization of DNA ligase I at sites of cellular DNA replication throughout S phase

The subcellular distribution of DNA ligase I was assessed by indirect immunofluorescence experiments. Methanol-fixed HeLa cells were incubated with a mouse monospecific antiserum directed to human DNA ligase I. Binding to the antigen was visualized using a RHODOS-conjugated sheep anti-mouse Ig antibody. In agreement with results obtained by Lasko *et al.* (1990), the antigen was exclusively localized to the nucleoplasm (Figure 1)

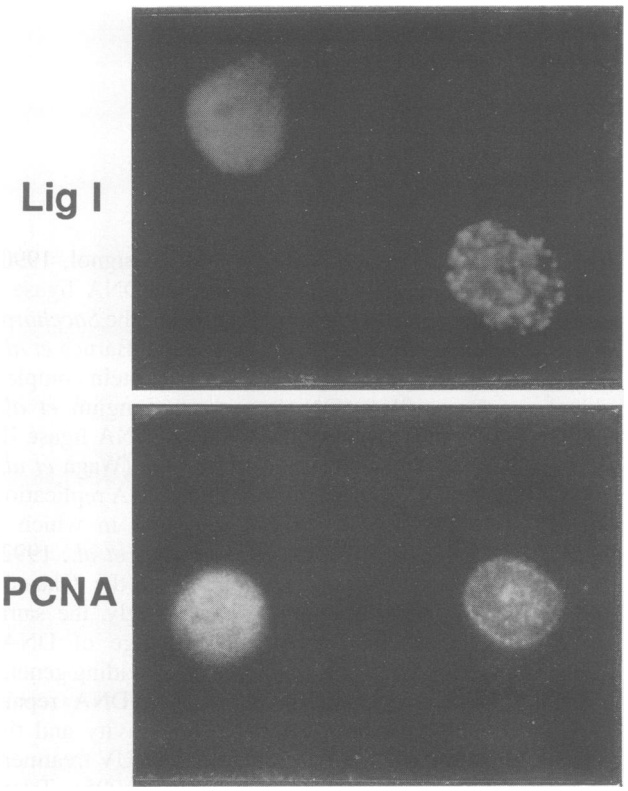

Fig. 1. Immunolocalization patterns of endogenous DNA ligase I (Lig I) and PCNA in exponentially growing HeLa cells stained with either mouse polyclonal antiserum against human DNA ligase I or PCNA(PC10) mAb. Lig I was revealed with a RHODOS-conjugated sheep anti-mouse Ig secondary antibody; PCNA with a FITC-conjugated sheep anti-mouse Ig antibody.

and nucleoli were not stained. DNA ligase I distribution varied from homogeneous to granular-like encompassing the wide range of intermediate possibilities. A similar pattern could be observed when a monoclonal antibody (mAb) to PCNA was used (Figure 1). To test whether the different staining patterns were related to cell cycle progression, the enzyme localization was analysed in HeLa cells synchronized at the G_1/S border by a double block with thymidine and aphidicolin, and processed at different times after releasing the block. Cell nuclei were revealed by DAPI DNA staining. As exemplified in Figure 2, at each time-point a definite fluorescent pattern could be observed in most of the cells. In early S phase, 1 h after the release from the block, a fine, punctate pattern could be observed, evenly distributed throughout the nucleoplasm. In the mid-S, 5 h after release, dots appeared more concentrated around nucleoli and in the nuclear periphery. In the late S phase, 9 h after release, only a few, large dots could be observed, on a homogeneous staining of the nucleoplasm. The change in staining pattern was reminiscent of the time-course of DNA replication, suggesting an association of DNA ligase I with sites of DNA replication. To prove this, HeLa cells were pulse-labelled for 1 h with 50 μM BrdU and immediately costained with anti-DNA ligase I antibodies and FITC-conjugated anti-BrdU mAb. In non-replicating cells, evidenced by the lack of BrdU incorporation, DNA ligase I appeared homogeneously dispersed throughout the nucleoplasm. Vice versa, in replicating cells the staining patterns of BrdU and DNA ligase I were identical (Figure 3A). The colocalization was confirmed by optical dissection with confocal laser scanning microscopy for a better visualization of the punctate structure (Figure 3B). This analysis demonstrates that DNA ligase I indeed localizes at sites of ongoing DNA replication.

The DNA ligase I NLS resides in the N-terminal domain of the protein

In order to identify the DNA ligase I NLS, we produced fusions of chloramphenicol acetyltransferase (CAT) with various portions of the enzyme (Figure 4). CAT is a small protein that can passively diffuse through the nuclear membrane and evenly distributes in the cell volume. We have previously described that the specific subcellular accumulation of CAT depends on either canonical NLS or retention motifs. Moreover when the fusion protein is larger than 50–60 kDa nuclear accumulation can be observed only in the presence of a functional NLS (Weighardt *et al.*, 1995).

Specific human DNA ligase I cDNA fragments were cloned into the pA1-CAT vector (Weighardt *et al.*, 1995) and transfected into NIH 3T3 cells with the calcium phosphate coprecipitation technique. After 48 h, cells were immunostained with anti-CAT antibodies. Three chimeric genes were initially tested: one coding for the catalytic domain (C-ter) and the others for two regions spanning the entire N-terminal domain (N-ter and EF). As shown in Figure 5, the CAT/N-$ter_{(1-119)}$ fusion was evenly distributed in the whole cell volume whilst the CAT/C-$ter_{(202-919)}$ protein, because of its large size, remained confined in the cytoplasmic compartment. In contrast, nuclear staining was observed in cells transfected with the CAT/$EF_{(52-203)}$ construct. The same pattern was observed when the corresponding region of the murine DNA ligase I was tested (not shown). Further constructs (Figure 4) allowed us to identify a 13 amino acid peptide ($_{119}$PKRRT-ARKQLPKR$_{131}$), 100% identical between mouse and man, showing nuclear targeting activity [CAT/$DF_{(119-131)}$, Figure 5]. This sequence shows some classical features of the NLS so far described, such as lysine/arginine clusters immediately preceded by a proline (Dingwall and Laskey, 1991).

The function of this putative NLS was directly assessed by means of specific DNA ligase I mutants. To distinguish the transfected from the endogenous enzyme we developed a vector (pA1-HUC, Figure 6) that directs the expression in mammalian cells of proteins tagged at their C-terminus with a 15 amino acid muscular actin epitope recognized by the HUC1-1 mAb. The tagged enzyme was nuclear and during S phase colocalized at sites of BrdU incorporation as demonstrated by confocal laser scanning analysis (Figure 7). Therefore, the presence of the actin epitope did not affect the sub-nuclear localization of the protein, making this system suitable for the subsequent mutation analysis.

When the putative NLS was removed (ΔNLS, Δ119–135) nuclear localization was no longer observed in most of the transfected cells (Figure 8A), indicating that this region is indeed necessary for nuclear targeting. In an attempt to dissect this sequence, we produced two additional mutants carrying substitutions in the basic amino acids (K/R). In one mutant (NLS-M1) the cluster $_{(119)}$PKRR– –KR$_{(126)}$ was substituted with the sequence

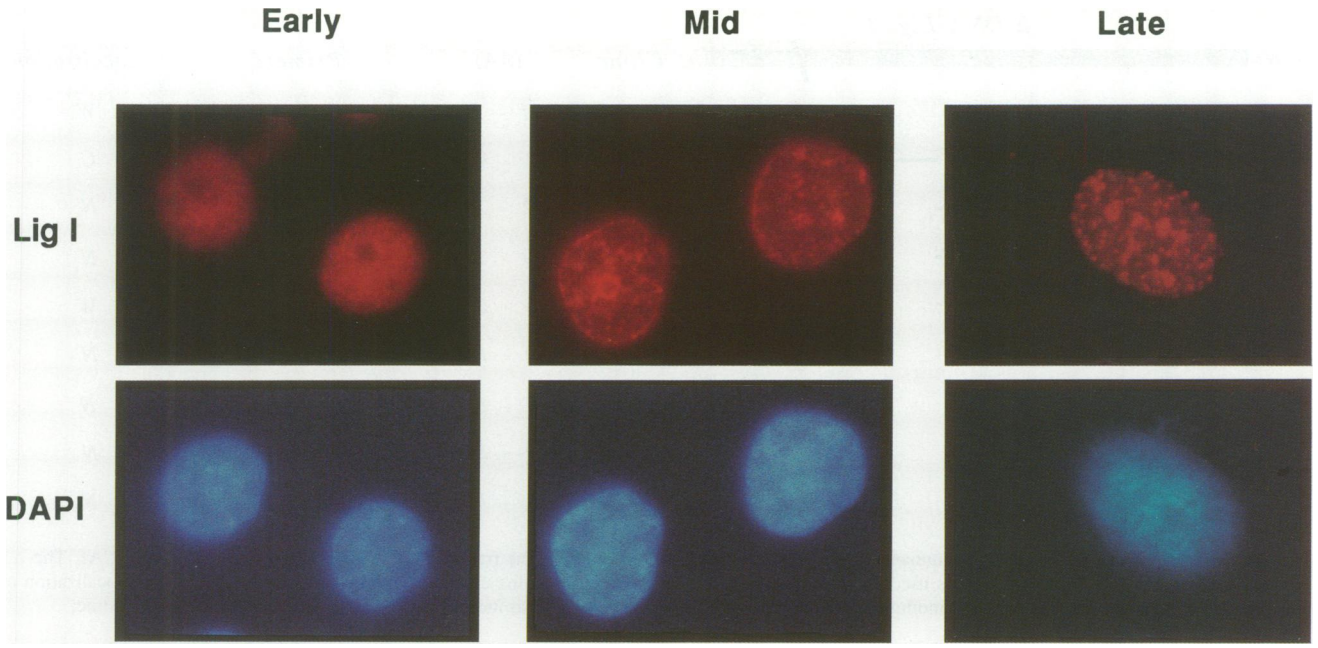

Fig. 2. Immunolocalization of endogenous DNA ligase I (Lig I) in different moments of S phase. HeLa cells were synchronized at the G_1/S border. At different times after release from the block (1 h: Early, 5 h: Mid; 9 h: Late) cells were immunostained with mouse polyclonal antiserum against human DNA ligase I and RHODOS-conjugated sheep anti-mouse Ig secondary antibody. Cell nuclei were stained with DAPI.

PEGG– –GE, without any appreciable effect (Figure 8A). In the second mutant additional substitutions, changing the $_{(129)}$PKRT$_{(132)}$ motif to PEGL (NLS-M2), produced an effect comparable with that observed with the ΔNLS protein (Figure 8A). Unexpectedly, both the ΔNLS and the NLS-M2 proteins gave a punctate nuclear staining pattern in ~10% of the transfected cells. BrdU incorporation experiments demonstrated that nuclear accumulation of both proteins occurred only in S phase nuclei (Figure 8B).

Identification of a DNA ligase I replication factories targeting sequence N-terminal to the NLS

The cell cycle dependent changes in distribution and the localization at replication foci suggested that DNA ligase I could be assembled in a functionally active complex, possibly through protein–protein interactions. In a preliminary attempt to identify sequences responsible for the recruitment of DNA ligase I at replication factories, we focused our attention on the N-terminus of the protein, already suggested to contain either phosphorylation sites or protein–protein interaction signals (Prigent *et al.*, 1992; Savini *et al.*, 1994). We therefore constructed a tagged DNA ligase I mutant in which residues 2–115 upstream of the NLS had been deleted, while the NLS itself was maintained. This construct was transfected into NIH 3T3 cells. Cells were pulse-labelled with BrdU immediately before costaining with HUC1-1 and BrdU mAb, and analysed by both conventional and confocal laser microscopy. Δ115 protein accumulated in the nucleus, demonstrating that in the presence of the NLS the first 115 N-terminal amino acids are dispensable for the nuclear accumulation of the enzyme. In contrast, the same mutant protein was unable to produce the punctate pattern in S phase cells, and always appeared evenly distributed in the nucleoplasm (Figure 9). The failure of the mutated protein to be recruited at the replication factories suggests a major role for the N-terminus in the cell cycle dependent association of DNA ligase I with the replication foci.

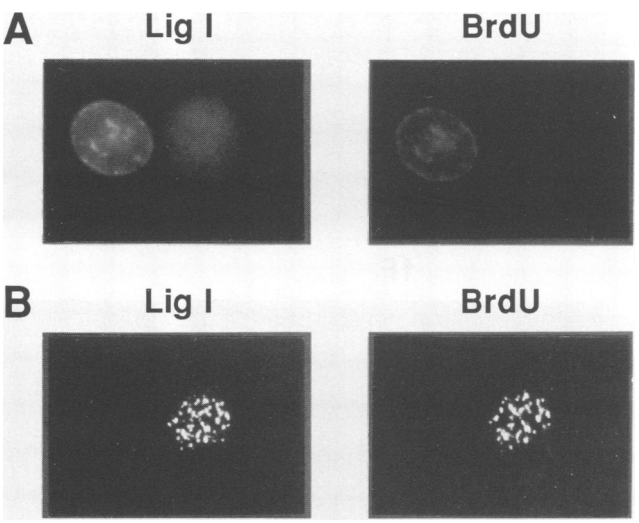

Fig. 3. Endogenous DNA ligase I colocalizes at sites of ongoing DNA replication. Exponentially growing HeLa cells were pulse-labelled with BrdU just prior to methanol fixation. Cells were co-stained with a mouse polyclonal antiserum against human DNA ligase I and with FITC-conjugated anti-BrdU mAb. Lig I localization was revealed with a RHODOS-conjugated sheep anti-mouse Ig secondary antibody. (**A**) Micrographs of the same field, showing the correspondence between DNA replication foci (BrdU) and Lig I structures. In non-replicating nuclei Lig I is dispersedly distributed. (**B**) Confocal laser scanning microscopy visualization of the punctate patterns.

Discussion

In this report we describe the nuclear and sub-nuclear localization of human DNA ligase I during the cell cycle.

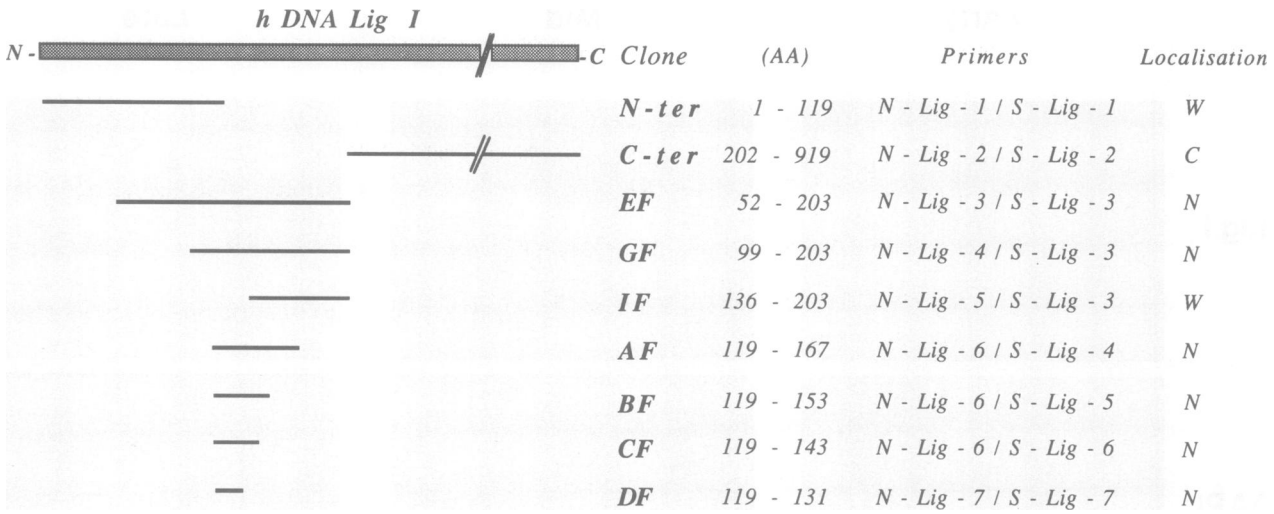

Fig. 4. Strategy for the identification of human DNA ligase I NLS. Black bars indicate the fragments of human DNA ligase I fused to CAT. The name of the clones and the set of primers used for PCR amplifications of the corresponding cDNA fragments are shown. The cellular localization of each fusion protein, as revealed by immunofluorescent staining of transfected cells, is also indicated. W, whole cell staining; C, cytoplasmic; N, nuclear.

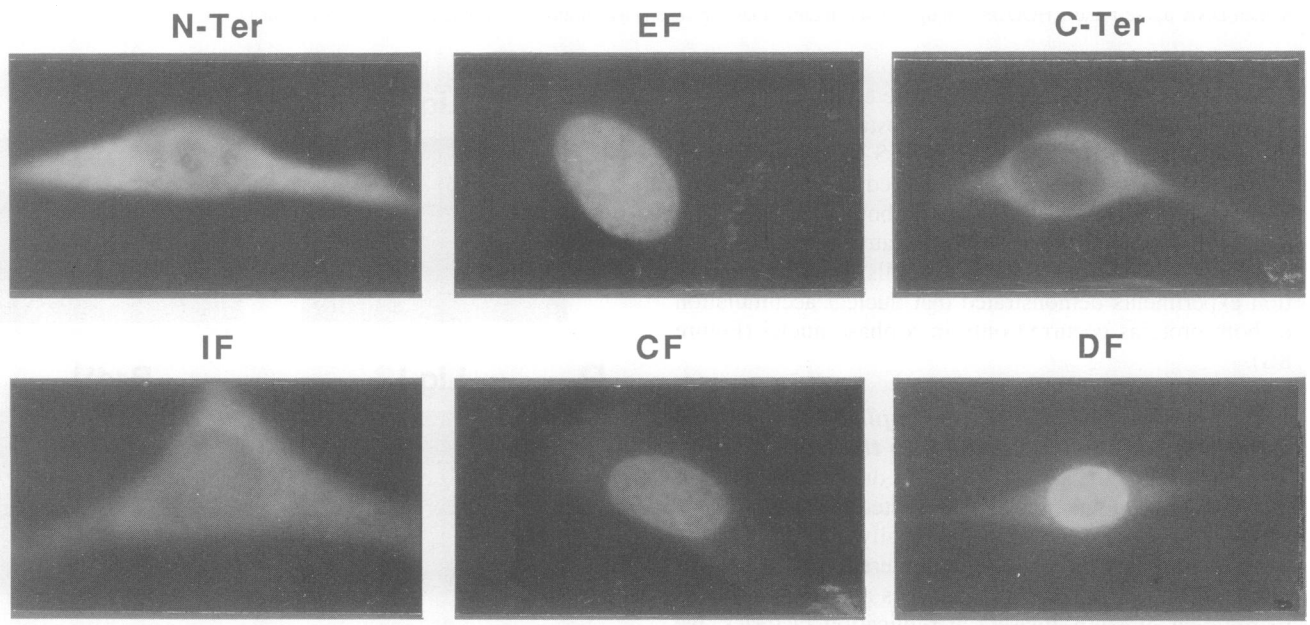

Fig. 5. Sub-cellular localization of different CAT–DNA ligase I fusion proteins in transfected cells, as revealed by immunofluorescence with anti-CAT antibodies.

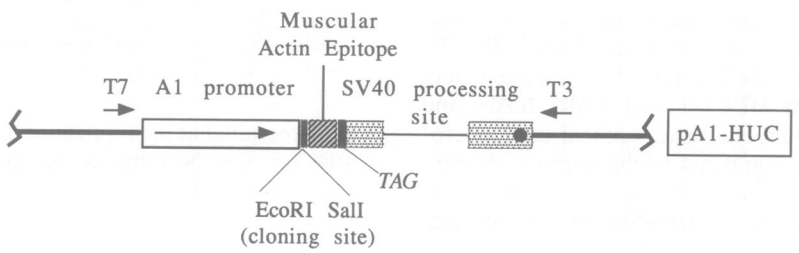

Fig. 6. Schematic rapresentation of pA1-HUC vector. The T7 and T3 promoters in the pBluescript SK(+) plasmid flank a cassette formed by: the hnRNP A1 gene promoter (A1 promoter), the *Eco*RI–*Sal*I cloning sites, the muscular actin epitope encoding region, followed by the TAG stop codon and by the SV40-derived RNA processing site.

We show that the enzyme has a diffuse nucleoplasmic distribution in non-S phase nuclei, while being specifically localized at sub-nuclear sites of ongoing DNA replication throughout S phase. Colocalization of DNA ligase I at sites of BrdU incorporation suggests that the enzyme is assembled in the DNA replisome at replication foci *in vivo*. A similar pattern has been reported for other replication factors such as DNA polymerase α, PCNA (Hozak *et al.*, 1993) and DNA methyl transferase (Leonhardt *et al.*, 1992). All these proteins appear to be relatively stable and their intracellular level remains essentially unchanged during the cell cycle (Wahl *et al.*, 1988; Morris and Mathews, 1989; Leonhardt *et al.*, 1992). Thus the sub-nuclear redistribution of DNA replication proteins may reflect the strategy by which cells control assembly and disassembly of their replication machinery without affecting the overall concentration of the proteins involved. At present there is no evidence concerning the nature of the signals that regulate this phenomenon; however, the modulation of either unique interactions among replication factors or associations with an organising scaffold, or

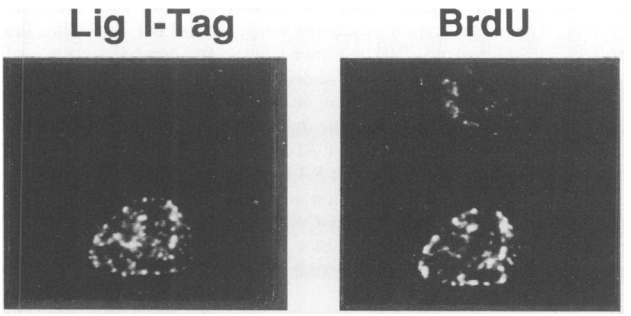

Fig. 7. Tagged DNA ligase I colocalizes at sites of ongoing DNA replication. Exponentially growing NIH 3T3 cells were transfected with pLig I-Tag plasmid and 48 h later pulse-labelled with BrdU just prior to methanol fixation. Cells were co-stained with HUC1-1 mAb and with FITC-conjugated anti-BrdU mAb. Lig I-Tag localization was revealed with a RHODOS-conjugated sheep anti-mouse Ig secondary antibody. Confocal laser scanning microscopy of the same field shows the correspondence of DNA replication foci (BrdU) and Lig I-Tag structures. S phase nuclei of cells non-expressing the tagged protein are also detectable.

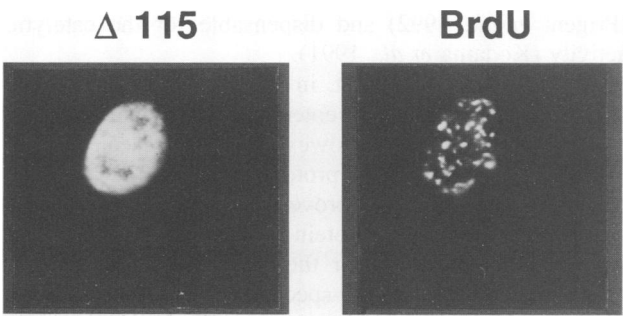

Fig. 9. Δ115 mutant fails to colocalize with sites of ongoing DNA replication. Exponentially growing NIH 3T3 cells were transfected with pΔ115 plasmid and 48 h later pulse-labelled with BrdU just prior to methanol fixation. Cells were co-stained with HUC1-1 mAb and with FITC-conjugated anti-BrdU mAb. Δ115 localization was revealed with a RHODOS-conjugated sheep anti-mouse Ig secondary antibody. Confocal laser scanning microscopy of the same field shows that the Δ115 mutant is homogeneously dispersed in the nucleoplasm of a replicating cell visualized by BrdU labelling.

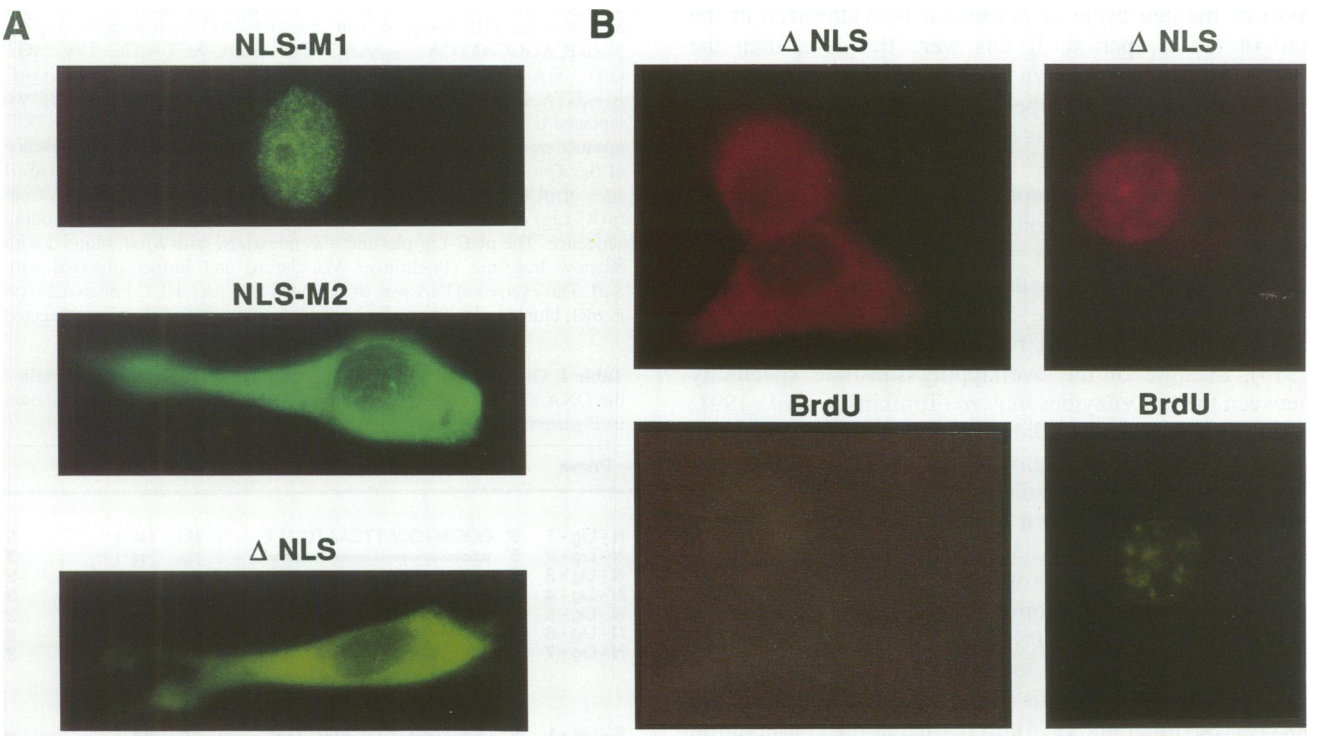

Fig. 8. (**A**) Subcellular localization of different tagged DNA ligase I mutants as revealed by immunostaining of transfected NIH 3T3 cells with HUC1-1 mAb and FITC-conjugated sheep anti-mouse Ig secondary antibody. (**B**) Different subcellular localization of the ΔNLS mutant during the cell cycle. Exponentially growing NIH 3T3 cells were transfected with the pΔNLS plasmid and 48 h later pulse-labelled with BrdU just prior to methanol fixation. Cells were co-stained with HUC1-1 mAb and with FITC-conjugated anti-BrdU mAb. ΔNLS localization was revealed with a RHODOS-conjugated sheep anti-mouse Ig secondary antibody. Micrographs of the same fields show the correspondence between DNA replication foci (BrdU) and ΔNLS structures and the absence of DNA replication in the cells with cytoplasmic distributed ΔNLS.

both, could have a major role. In this regard, it is important to identify the protein determinants involved. Thus far, only DNA methyl transferase has been analysed in detail and its association with replication foci was found to depend on a sequence of ~200 residues downstream of the NLS (Leonhardt *et al.*, 1992) in the N-terminus of the protein that corresponds to a large regulatory region dispensable for the enzymatic activity (Bestor, 1990).

The results reported here demonstrate that the DNA ligase I determinants for nuclear and sub-nuclear accumulation are also located in the N-terminal regulatory domain, which is both a target of post-translational modifications (Prigent *et al.*, 1992) and dispensable for the catalytic activity (Kodama *et al.*, 1991).

A DNA ligase I mutant, in which the NLS has been deleted (ΔNLS), failed to enter the cell nucleus in most of the expressing cells. However, an unforeseen nuclear localization of the ΔNLS protein was detected in ~10% of transfected cells and proved to be S phase-specific. Interestingly, the ΔNLS protein localized at the replication foci, ruling out a role for the NLS in the sub-nuclear recruitment. This S phase-specific nuclear accumulation could be due to the presence of a cell cycle dependent NLS that we failed to detect, although its physiological significance would be difficult to understand. We favour an alternative explanation which takes into account the ability of DNA ligase I, and of its ΔNLS mutant, to associate with the nuclear factories. This behaviour suggests the occurrence of specific protein interaction. We propose that a protein able to bind DNA ligase I only enters the cell nucleus immediately before the S phase, either because it is sequestered in the cytoplasm during most of the cell cycle or because it is synthesized at the end of the G_1 period. If this were the case, then the cytoplasmic ΔNLS protein would be 'piggybacked' into the cell nucleus as described for other proteins with their NLS deleted (Zacksenhaus *et al.*, 1993). According to this hypothesis, ΔNLS DNA ligase I would interact with an as yet unidentified replication factor whose nuclear localization is strictly controlled during the cell cycle. This interaction could be relevant for the recruitment of the wild-type protein at the replication foci and could explain why DNA ligase III is unable to replace DNA ligase I in a SV40 DNA replication assay (Waga *et al.*, 1994), in spite of the overlapping substrate specificity between the two enzymes *in vitro* (Tomkinson *et al.*, 1991; Elder *et al.*, 1992). A validation of this hypothesis could derive from the identification of the region of DNA ligase I involved, a possible candidate being the 115 N-terminal residues that seem to be a replication factories targeting sequence. This sequence has no homology with the functionally analogous region of the DNA methyl transferase. The 115 amino acid region is rich in charged residues, in prolines and in (SP) and (PS) motifs, all features conserved in evolution from mouse to man, in spite of the fact that the N-terminal domain is quite different in these two species (Savini *et al.*, 1994). In particular the conservation of (SP) and (PS) residues could have a functional significance since these motifs are present in the recognition site of many protein kinases (Kemp and Pearson, 1990). Phosphorylation of the DNA ligase I N-terminal domain has been shown to modulate the catalytic activity (Prigent *et al.*, 1992). Moreover it could have a role in modulating the interaction at replication foci.

Materials and methods

Construction of CAT–DNA ligase I fusions

Recombinant plasmids were produced by cloning either oligonucleotides or PCR products into the *NsiI–SalI* sites of the pA1-CAT vector (Weighardt *et al.*, 1995). Different portions of human DNA ligase I were obtained by PCR amplification of the cDNA [ATCC No. 65857 (Barnes *et al.*, 1990)] with suitable primers (Figure 4 and Table I). N (*NsiI*) designates sense sequence while S (*SalI*) designates antisense. N primers were designed to obtain C-terminal fusions in-frame with the CAT protein. The amplification reactions were carried out with the *Taq* polymerase (GeneAmp; Perkin Elmer Cetus); the correct sequence of the products was verified by the dideoxy method (Sequenase™ DNA Sequencing Kit, USB) after cloning into the TA vector (Invitrogen Co.). Inserts were then excised by double digestion with *NsiI–SalI* and cloned into pA1-CAT. To produce the CAT–DF fusion two complementary oligonucleotides, N-Lig-7 (+) and S-Lig-7 (–) (Table I), flanked by *NsiI* and *SalI* sites were annealed (5 min at 95°C and slow cooling at room temperature) and cloned into pA1-CAT. The correct size of the fusion proteins was verified by Western blot analysis of transfected cells total extract using anti-CAT antibodies (not shown).

Construction of pA1-HUC expression vector and of tagged DNA ligase I mutants

pA1-HUC expression vector was produced by cloning into *Eco*RI–*Pst*I sites of pA1-SV40 plasmid (Weighardt *et al.*, 1995), the fragment obtained by the annealing of the following oligonucleotides: (+) 5′-AATTC ATGCAT TATATATATA GTCGAC GGG *ATG TGG ATA TCC AAA CAA GAA TAT GAT GAA GCA GGG CCA AGT ATT* TAG CTGCA-3′; (–) 5′-G CTA AAT ACT TGG CCC TGC TTC ATC ATA TTC TTG TTT GGA TAT CCA CAT CCC GTCGAC TATATATATA ATGCAT G-3′. This fragment contains, from the 5′ end: *Eco*RI and *NsiI* sites (underlined) followed, after a 10 nucleotide (nt) spacer, by a *SalI* site (underlined); a Gly codon preceding the muscular actin epitope (italics) recognised by HUC1-1 mAb (ICN); a stop codon and a *PstI* site (underlined). To obtain the tagged DNA ligase I construct we first PCR amplified the cDNA region from nt 2503–2877 with primers: Lig-5′: 5′-GGCAGGCATTCA $_{(2503)}$AAG CTT GGA ACT GGC TTC AGT GAT GA$_{(2528)}$-3′ and Lig-3′: 5′-GGCAGGCATTCA GTCGAC $_{(2877)}$GTA GGT ATC TTC AGG GTC AG$_{(2858)}$-3′. In this way we replaced the original stop codon with a *SalI* (underlined) site. The PCR product was digested with *Hin*dIII and *SalI* and ligated in the presence of the *Kpn*I–*Hin*dIII hLig I cDNA fragment (nt 1–2503) into *Kpn*I–*Sal*I sites of pUC19 vector (Boehringer Mannheim). The resulting construct (pUC-Lig) contains 120 nt of the 5′-UTR followed by the entire coding sequence. The pUC-Lig plasmid was linearized with *Kpn*I, blunted with Klenow fragment (Boehringer Mannheim) and further digested with *SalI*. The excised cDNA was then cloned into pA1-HUC linearized with *Eco*RI, blunted and digested with *SalI*. A clone (pLig I-Tag) was selected

Table I. Oligonucleotide primers used in PCR amplification to obtain the DNA ligase I cDNA fragments encoding the fusion proteins shown in Figure 4

Primer			Position (nt)	
N-Lig-1	5′	GGCAGGCATTCAATGCAT	- (121 - 148)	3′
N-Lig-2	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (724 - 748)	3′
N-Lig-3	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (274 - 300)	3′
N-Lig-4	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (415 - 439)	3′
N-Lig-5	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (526 - 550)	3′
N-Lig-6	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (475 - 499)	3′
N-Lig-7	5′	T	- (475 - 513) - G	3′
S-Lig-1	5′	GGCAGGCATTCAGTCGAC	- (477 - 453)	3′
S-Lig-2	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (2877 - 2854)	3′
S-Lig-3	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (729 - 706)	3′
S-Lig-4	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (622 - 598)	3′
S-Lig-5	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (579 - 558)	3′
S-Lig-6	5′	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬	- (550 - 526)	3′
S-Lig-7	5′	TCGAC	- (513 - 475) - ATGCA	3′

after restriction map analysis and partially sequenced. Mutated tagged enzymes were obtained by substituting an *Eco*RI fragment of pLig I-Tag (hLig I cDNA sequence nt 105–1543) with a mutated fragment obtained by PCR. To produce the pNLS-M1 clone, the *Eco*RI fragment was amplified with two different pairs of oligonucleotides according to a procedure previously described (Diviacco *et al.*, 1992). In the first amplification, sense primer MS (5′-CAGGAAGGGAGAATTCTG-ACGC-3′) (nt 95–116) and antisense primer Ma (5′-G CTG CTC CCC AGC TGT GCC ACC CTC CGG AAT CCC TGA TGG GGA AC-3′) (nt 502–458), were used to produce *Sa* fragment. Mutated nucleotides are underlined. In the second amplification, sense Mb primer (5′-CA GCT GGG GAG CAG CTC CCG AAA CGG ACC ATT CAG GAA GTC CTG G-3′) (nt 488–532) and antisense MA primer (5′-GC TGG TGG GAA TTC TTG GCC C-3′) (nt 1556–1536) were used to produce *bA* fragment. *Sa* and *bA*, which share only the 15 nt sequence (488–502) of the Ma and Mb oligonucleotides, were purified from agarose gel, mixed and further amplified with the external MS and MA primers. After digestion with *Eco*RI the PCR product was cloned into *Eco*RI site of pLig I-Tag. To produce the pNLS-M2, oligonucleotides Mb′ (5′-CA GCT GGG GAG CAG CTC CCG GAA GGA CTC ATT CAG GAA GTC CTG G-3′) (nt 488–532) and MA were used to amplify the *b′A* fragment. The *Sa* and *b′A* fragments, which share the 15 nt sequence 488–502, were processed as described above and amplified with the external MS and MA primers. The amino acid substitutions introduced in NLS-M1 and NLS-M2 proteins are described in the text. To obtain the pΔNLS, encoding a mutant lacking amino acids 119–135, oligonucleotides MS and MdA (5′-C CAG GAC-AAT CCC TGA TGG GGA ACT GTC C-3′) containing sequence 532–453(Δ526–474) were used in the first PCR to produce *SdA* fragment. Oligonucleotides MdS (5′-C CCA TCA GGG ATT-GTC CTG GAA GAG CAG AGT GAG G-3′) containing sequence 462–547(Δ474–526) and MA were used in the second one to produce *dSA*. Clone pΔ115, lacking amino acids 2–115, was obtained by a single PCR using the SD primer (5′-G GAATTCTGACGCCAAC ATG-TCA GGG ATT CCG AAG CG-3′) containing the sequence 105–482(Δ123–465) and the MA primer. Clones were analysed by restriction map and the presence of the mutated regions was checked by sequencing. The correct size of the fusion proteins was verified by Western blot analysis of transfected cells total extract using HUC1-1 mAb (not shown).

Cell culture and transfection

Human HeLa S3 and mouse NIH 3T3 cells were grown as described in Montecucco *et al.* (1992). Synchronization of HeLa cells was performed as described by Stein and Stein (1989). In short, cells were seeded in slide flasks (18×50 mm, NUNC) and the day after were grown for 14 h in 2 mM thymidine (Sigma) containing medium. After a 9 h release from the metabolic block, aphidicolin (Sigma) was added at a final concentration of 5 μg/ml (Pedrali-Noy *et al.*, 1980). After 12–16 h most cells were synchronized at G_1/S border. Transfections of mouse NIH 3T3 cells were performed as previously described (Weighardt *et al.*, 1995).

Immunofluorescence

Immunofluorescence of methanol fixed cells was performed as described in Weighardt *et al.* (1995). Fusion proteins were detected either with rabbit polyclonal antibodies to CAT (5′ prime-3′ prime Inc.) or anti-muscular actin mAb (HUC 1-1, ICN). Secondary antibodies included FITC-conjugated mouse anti-rabbit IgG (5′ prime-3′ prime Inc.), RHODOS-conjugated sheep anti-rabbit IgG F(ab′)$_2$ fragment (Boehringer Mannheim) and FITC- or RHODOS-conjugated sheep anti-mouse Ig (Boehringer Mannheim). Mouse polyclonal antibodies raised to recombinant human DNA ligase I (Gallina *et al.*, 1995) produce a immunostaining pattern indistinguishable from the one observed with 1A9 mAb to DNA ligase I (kindly provided by Prof. Tomas Lindahl). Simultaneous visualization of DNA ligase I and sites of DNA synthesis involved growing cells in 50 μM BrdU (Sigma) for 1 h immediately before methanol fixing. DNA ligase I was detected with polyclonal anti-DNA ligase I antibodies (1:600) and RHODOS-conjugated anti-mouse secondary antibody. Antibodies were then fixed in place with cold methanol for 10 min, coverslips were treated with 2 M HCl at 37°C for 1 h to denature DNA and neutralized in 0.1 M borate buffer pH 8.5. After washing, cells were incubated for 60 min at room temperature with the FITC-conjugated anti-BrdU mAb (Boehringer Mannheim). PCNA was revealed by PCNA(PC10) mAb (Santa Cruz Biotechnology). DNA was stained with 0.2 μg/ml DAPI (Sigma) before the last washing. Specimens were examined and photographed on Leitz Orthoplan microscope. Optical sections were obtained using the Bio-Rad MRC-1000 confocal microscope (BioRad). Micrographs were taken using a focus imagerecorder plus (Focus Graphics Inc., USA) on Kodak Tmax 100 film.

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