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DNA ligase I gene expression during differentiation and cell proliferation

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ABSTRACT

We have studied the regulation of mammalian DNA ligase I gene by using a cDNA probe in Northern blot experiments with RNA extracted from several cell types in different growth conditions. DNA ligase I mRNA is detected in all analysed cell systems, regardless of their proliferation state, including mature rat neurons. A significant increase in DNA ligase I mRNA level is observed when cells are induced to proliferate, in agreement with the raise of DNA joining activity found in the same cell systems. The increase parallels the start of DNA synthesis, but the messenger remains at high level beyond the end of the S fase and is detected also in the presence of aphidicolin. A decrease in DNA ligase I mRNA is observed in HL-60 and NIH-3T3 cells after differentiation. The high stability of DNA ligase I mRNA in both resting and proliferating human fibroblasts suggests a cell proliferation dependent rate of transcription. On the other hand the presence of a basal level of DNA ligase I in nondividing cells, strongly suggests an involvement of this enzyme in DNA repair. This conclusion is supported by a threefold increase in DNA ligase I observed 24 h after UV irradiation of human confluent primary fibroblasts.

INTRODUCTION

Different forms of DNA ligases have been isolated from microorganisms and higher eukaryotes (1–2). In recent years up to three forms of DNA ligases have been identified in mammalian cells (3–4), while in lower organisms a single activity is still supposed to accomplish all the polynucleotide joining reactions required during replication, recombination and repair.

All known DNA ligases catalyse the formation of phosphodiester bonds between adjacent DNA fragments with appropriate termini and structure. This reaction takes place upon the formation of a covalent enzyme-adenylate intermediate with the concomitant release of pyrophosphate or nicotinamide mononucleotide, depending on their cofactor specificity (1,5). Known DNA ligases differ one from the other for a number of biochemical properties which have been reviewed elsewhere (1–3). A role in DNA replication has been assigned to prokaryotic and eukaryotic type I DNA ligases (1–2). Additional findings suggested that the enzymes from prokaryotes and yeast are involved also in DNA repair (1,6), while a role of the human DNA ligase I in DNA repair is still an open question (7–8). Interestingly only prokaryotic and eukaryotic type I DNA ligases contain an intrinsic AMP-dependent DNA topoisomerase (topoligase) activity (9–11), suggesting a specific requirement for topoligation events in processes in which these enzymes are involved (12). It is therefore important to assess any involvement of mammalian DNA ligase I in the different processes in which DNA joining is required.

New perspectives derive from the isolation and cloning of the human DNA ligase I cDNA recently reported by Barnes et al. (13). The same authors suggest a role for this enzyme in DNA repair on the basis of DNA ligase I gene mutations found in a human cell line hypersensitive to DNA-damaging agents (8).

We thought that the description of the regulation of the DNA ligase I gene expression could help to understand the physiological role of this enzymic activity. To this aim we have investigated the level of DNA ligase I specific messenger in different cell types and growth conditions using a PCR amplified partial cDNA (14) as probe in Northern blot experiments.

MATERIALS AND METHODS

Probes

The 1257 bp partial cDNA of human DNA ligase I was prepared according to the procedure previously reported (14) except that the two oligonucleotides utilised as primers were: I, 5′-GAATTC-CTTAGTAGGTATCTTCAGGG-3′ (2773–2792); II, 5′-GAA-TTCATCCTGAAGCAGACGTTCTG-3′ (1535–1554).

The *c-myc* probe was derived from plasmid pMC41 (15); the *c-fos* probe was extracted from plasmid pc-fos-1 (16).

Cells and cell treatments

Peripheral blood lymphocytes were obtained from healthy donors and purified by Ficoll/metrizoate method as described by Freshney et al. (17). Lymphocytes were grown in RPMI 1640 (GIBCO, U.S.A.), 10% fetal calf serum, 50 μg/ml gentamicin, 2 mM L-glutamine and 50 μg/ml phytohemagglutinin.

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Human primary fibroblasts were cultured in DMEM medium (GIBCO, U.S.A.) containing 10% fetal calf serum, 50 μg/ml gentamicin and 2 mM L-glutamine. To obtain resting cells, confluent fibroblasts were grown for 5 days in DMEM medium supplemented with 0.25% fetal calf serum.

Human myeloid leukemia HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin and 2 mM L-glutamine. Their differentiation was induced with 1.3% DMSO for 2 or 5 days according to Siebenlist et al. (18).

NIH-3T3 mouse fibroblasts were grown in DMEM medium, 10% newborn calf serum, 50 μg/ml gentamicin and 2 mM L-glutamine.

Rat neurons were obtained as previously described (19).

UV irradiation of human confluent primary fibroblasts was done on cells grown in 14.5 cm plastic Petri dishes in 10% FCS containing medium as described by Nocentini and Mezzina (7). After removal of culture medium cells were irradiated with a single dose (20 J/m^2) of 254 nm light, the medium was readded to the cells and total RNA was extracted at different time intervals.

Analysis of mRNA levels steady-state

Total RNA was prepared from cells as previously described (20). RNA gels: 10 μg of total RNA were lyophilized, resuspended in 15 μl of sample buffer (50% formamide, m6% formaldehyde, 20 mM NaH_2PO_4/Na_2HPO_4 pH 7.0, 5% glycerol, 0.02% bromophenol blue, 0.02% xylene cyanol and 0.2 mg/ml ethidium bromide) and incubated 10 min at 68°C. RNA was electrophoresed onto a 1.5% agarose, 6% formaldehyde gel. At the end of the run, the gel was photographed on a UV transilluminator and then transferred to a Zetabind membrane (Cuno Lab. Prod. U.S.A.). Processing of membranes was as reccomended by the supplier.

Northern blot hybridisations were performed as described by Sambrook et al. (21). Prehybridisation and hybridisation solution: 4× SSC, 4× Denhardt's, 50% formamide, 0.5% SDS, 7.5 mM $Na_4P_2O_7$, 12.5 mM NaH_2PO_4, 0.3 mg/ml yeast tRNA. Probes were labeled at a specific activity of 10^9 cpm/μg with the Multiprime DNA Labeling System (Amersham, UK) and added to the hybridisation solution at a concentration of $2-4\times10^6$ cpm/ml. After an overnight hybridisation, membranes were washed twice for 30 min each in 2× SSC, 1% SDS at room temperature, once in 2× SSC, 1% SDS at 68°C and once in 0.2× SSC, 0.1% SDS at 68°C. In order to rehybridise membranes, probes were stripped by boiling 10 min in 10 mM Tris−HCl pH 7.5, 1 mM EDTA, 1% SDS.

In experiments in which we compared relative transcript levels, samples were normalized to equal amounts of total RNA.

In vivo DNA synthesis

To monitor DNA synthesis *in vivo* during serum stimulation, duplicate microdilution wells were plated at equal cell density for each experimental time point. Cells were pulse-labeled with [^{3}H] thymidine (20 Ci/mmol; Amersham UK) at 0.025 mCi/ml for 1 h before harvesting. Cell layers were carefully drained, rinsed twice with phosphate-buffered saline, resuspended in 0.1 ml phosphate-buffered saline, spotted onto a Whatman GF/C filter and batch washed with ice-cold 5% trichloroacetic acid according to Bollum (22).

Preparation of neuronal crude extract

In order to test DNA ligase I activity, neurons were first resuspended in 3 volumes of 10 mM Tris−HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 4 mM sodium metabisulfite, 1 μM pepstatin and 1 mM PMSF, then sonicated on ice 4 times for 4 s at intervals of 20 s in a Branson sonifier at 70 watt. The extract was centrifuged at 8000× g for 10 min in an Eppendorf centrifuge. Ligase-[^{32}P]AMP adducts were prepared as previously described (23). The cell extract was incubated at room temperature for 20 min in 50 mM Tris−HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA and 20 μCi α[^{32}P]ATP (3000 Ci/mmol; Amersham) in a total volume of 70 μl. DNA ligase-adenylate complex was separated from unreacted α[^{32}P]ATP by gel filtration on a spun-column (21), lyophilised, resuspended in 25 μl of loading buffer, boiled and loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis the gel was stained with Coomassie Brillant Blue, dried and exposed to X-ray film at −70°C.

RESULTS

DNA ligase I mRNA levels vary with cell proliferation

DNA ligase I is the major polynucleotide joining activity present in proliferating cells (2). Its activity raises up to 100 times in human lymphocytes upon PHA stimulation, with a slight delay respect to the onset of DNA synthesis (24), thereafter remaining expressed at high levels (25). The recent reported isolation and cloning of the cDNA encoding for the human DNA ligase I (13) offers the opportunity to verify at transcriptional level the relationship between cell proliferation and expression of the gene. In this perspective we have PCR amplified a 1257 bp partial cDNA (as described under 'Materials and Methods') to study by Northern blot analysis the levels of DNA ligase I mRNA in total RNA extracted from human and mouse cell cultures and from rat tissues. From the results shown in Figure 1, three main conclusions can be drawn: 1) the DNA ligase I messenger is present, though at different levels in all analysed cell types; 2) the size of the detected mRNA appears identical in man and mouse, suggesting that no major rearrangements have occurred during mammalian evolution of the gene; 3) there is a strong

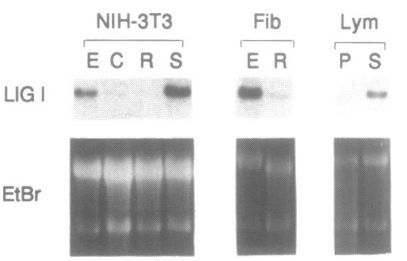

Figure 1. DNA ligase I mRNA levels vary with the rate of cell proliferation. Total RNA was prepared from mouse and human cells collected under different growth conditions. NIH-3T3 cells: esponential growth (E), confluence (C), 2 days starvation in low serum (0.25%) after confluence (R), or the same after 24 h serum (10%) stimulation (S); human primary fibroblatsts (Fib): exponential growth (E) or starved for 5 days in low serum (0.25%) after confluence (R); human lymphocytes: peripheral (P) or induced to proliferate with PHA (S). The RNAs (10 μg) were analyzed by Northern blot hybridization with the DNA ligase I-specific probe (LIG I). Total amount of rRNA was quantitated by ethidium bromide staining (EtBr).

correlation between DNA ligase I gene expression and the rate of cell proliferation. In fact in all test systems (human lymphocytes, human primary fibroblasts and mouse NIH-3T3 cells) the amount of DNA ligase I mRNA is considerably higher in proliferating cells than in resting cells. In particular the results obtained with human lymphocytes before and after PHA stimulation, are in agreement with the data of enzymatic activity (24) and of Northern blot analysis reported by Petrini et al. (26) .

On the other hand in NIH-3T3 mouse cells the evident decrease in the level of DNA ligase I mRNA produced by confluence (Figure 1, lane C) and serum starvation (Figure 1, lane R) can be reverted by a successive 24 h period of serum stimulation (Figure 1, S).

On the basis of these results we have investigated the kinetics of the mRNA increase in resting human primary fibroblasts induced to proliferate by serum (Figure 2). Human primary fibroblasts, starved for 5 days in 0,25% FCS containing medium, were induced to replicate by serum feeding. Total RNA was prepared from cells removed after 6, 12, 18, 24, 30 h and analysed in Northern blot with DNA ligase I and *c-myc* specific probes. The results of the hybridization were quantitated by densitometric scanning as reported in Figure 2. [^{3}H]TdR incorporation was followed (Figure 2) to determine both the entry in the first S phase and the level of cell synchronization. DNA ligase I mRNA basal level starts to increase 12 h after serum stimulation, reaching a plateau level 6 h later. As expected, the *c-myc* mRNA level shows a only transient increase with a peak at the 6 h after serum stimulation. The lack of significative reduction in the DNA ligase I specific signal even after S-phase completion, is similar to what previously reported for other replicative enzymes mRNAs (27). Although DNA ligase I mRNA raises during S-phase, DNA synthesis does not seem to play a role in this process. In fact, the presence of the DNA synthesis inhibitor aphidicolin during serum stimulation (Figure 3, lane A), does not affect the large increase in the steady state observed in a similar experiment performed with NIH-3T3 cells. This result is in agreement with the observation reported by Petrini et al. (26) with peripheral blood T cells in the presence of hydroxyurea.

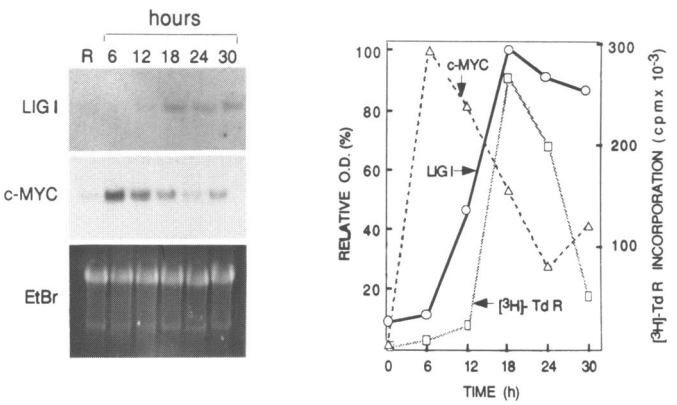

Figure 2. Effect of serum stimulation on DNA ligase I mRNA level. Exponentially growing human primary fibroblasts were starved for 5 days in 0.25% serum (R) and then stimulated with 15% serum for the indicated times. Left: Total RNA (10 μg) was probed in Northern blot hybridization with DNA ligase I-specific probe (LIG I) and with a probe specific for the *c-myc* proto-oncogene (c-MYC). EtBr: rRNA quantitation. Right: The autoradiograms were analyzed with the help of the Bioprofil software (Vilber Lourmat) to quantitate the intensity of the hybridization signals. The relative intensity of each resultant autoradiogram is presented as% of the maximal O.D. value. In the same plot the [^{3}H]-TdR incorporation curve is shown.

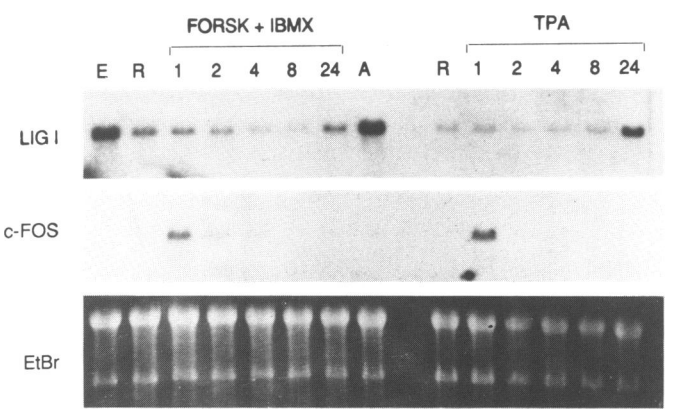

Figure 3. Effect of increased c-AMP levels and of TPA or aphidicolin treatments on DNA ligase I gene expression. Exponentially growing NIH-3T3 cells (E) were starved for 48 h in 0.25% serum (R) and then treated with Forskolin and IBMX (10 μM and 0.5 mM, respectively), or with TPA (100 ng/ml) in 0.25% FCS containing medium for the indicated times (h). NIH-3T3 starved cells were also serum stimulated with 15% FCS in the presence of 5 μg/ml of aphidicolin (A). Total RNA (10 μg) was probed in Northern type hybridizations with the DNA ligase I-specific probe (LIG I) and with a probe specific for the *c-fos* proto-oncogene (c-FOS). EtBr: rRNA quantitation.

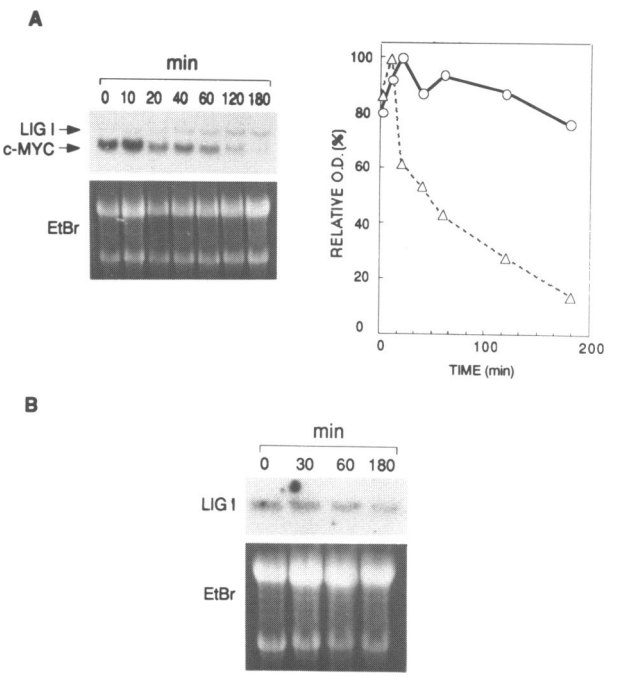

Figure 4. DNA ligase I mRNA stability. Exponentially growing (Panel A) or resting (Panel B) human primary fibroblasts were treated for the indicated times with Actinomycin D (5 μg/ml). Panel A . Left: total RNA (10 μg) was probed in Northern type hybridization with the DNA ligase I-specific probe (LIG I) and with a probe specific for the *c-myc* proto-oncogene (c-MYC). EtBr: rRNA quantitation. Right: The autoradiograms were analyzed with the help of the Bioprofil software (Vilber Lourmat) to quantitate the intensity of the hybridization signals. The relative intensity of each resultant autoradiogram is presented as% of the maximal O.D. value. (Δ): c-MYC. (O): LIG I.

Stability of DNA ligase I mRNA

As it is shown above, the steady state for the DNA ligase I messenger can fluctuate between two distinct values. The higher one is typical of rapidly dividing cells, while the other is characteristic of resting cells. The passage from the latter to the former occurs late in the first G1 phase. The cell can achieve these two levels of DNA ligase I mRNA modifying either the rate of gene transcription or the stability of the messenger. To discriminate between these two possibilities we measured by Northern blot analysis the effect of Actinomycin D on the amount of DNA ligase I mRNA in exponentially growing and resting fibroblasts. The Figure 4 shows that, regardless of the cell proliferation state, the mRNA for DNA ligase I appears to be very stable, its level being reduced to only 81,5% and 93% respectively in resting and proliferating fibroblasts grown for 3 h in the presence of the drug. On the contrary the amount of *c-myc* mRNA in rapidly dividing cells is reduced to 50% and 16% of the initial value after 1 and 3 h of treatment with Actinomycin D. This result strongly suggests that the increase of mRNA for DNA ligase I observed after serum stimulation of quiescent cells is mainly due to a higher rate of gene transcription.

The steady state level for DNA ligase I mRNA varies with cell differentiation

The demonstration that the level of DNA ligase I mRNA decreases when growing cells are induced to quiescence by serum starvation, lead us to investigate the effect of terminal differentiation. For this reason we have analysed two different systems. In the first one, human promyelocytic HL-60 cells were induced to differentiate to granulocytes by treatment with 1,3% DMSO (18). Northern blot analysis on RNA extracted after 2 or 5 days of treatment indicates that the DNA ligase I expression already reaches a lower basal level at the second day of treatment and remains thereafter unchanged (Figure 5). This behavior parallels that of *c-myc* gene usually utilised as a marker of HL-60 cells differentiation. This result prompted us to investigate the more physiological *in vivo* differentiation system of developmental neurons. We investigated the level of DNA ligase I mRNA in rat neurons isolated from animals sacrified between −3 and +9 days from birth. This system is characterised by the disappearance of G2+M phase cells at birth (−3 d, 6% G2+M cells; 0 d, 0% G2+M cells) and by the drastic decrease in the activity of typical replicative enzymes such as DNA polymerase α and δ/ϵ (28,29). The results of Northern blot hybridization are shown in Figure 6A. The level of DNA ligase I mRNA remains essentially constant from −3 days before birth up to birth, weakly decreasing at day 9 after birth to reach the level observed in adult brain (data not shown). The permanence of DNA ligase I mRNA after birth is reflected also at protein level. In fact, as shown in Figure 6B, DNA ligase I self-adenylating activity remains essentially unchanged between −2 days before and +9 days after birth. In this respect, DNA ligase I resembles DNA polymerase β (28), which is involved in DNA repair synthesis and is constitutively expressed in resting cells, including adult neurons (28,30).

We asked whether DNA ligase I could be coordinately expressed with DNA polymerase β upon DNA damaging treatments. DNA polymerase β promoter contains an ATF/CRE

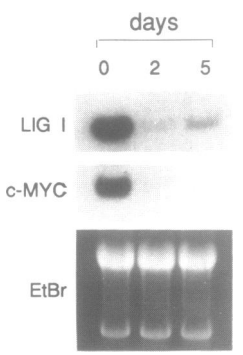

Figure 5. Effect of HL-60 cells differentiation on DNA ligase I gene expression. Total RNA (10 μg) extracted from HL-60 cells before (0) or after differentiation (2 and 5 days in the presence of 1.3% DMSO) was probed in Northern blot hybridization with the DNA ligase I-specific probe (LIG I) and with a probe specific for the *c-myc* proto-oncogene (c-MYC). EtBr: rRNA quantitation.

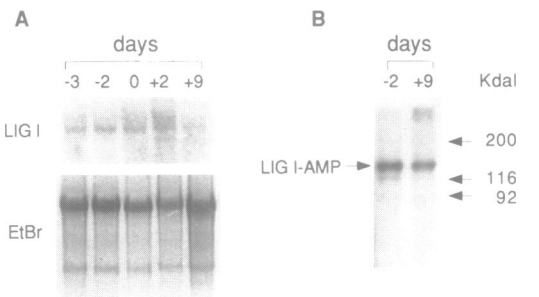

Figure 6. DNA ligase I expression in rat neurons. Rat neurons were prepared from animals sacrificed before birth (−3, −2 days), at birth (0), and after birth (2 and 9 days). Panel A: 3 μg of total RNA were analyzed by Northern blot hybridization with the DNA ligase I-specific probe (LIG I). EtBr: rRNA quantitation. Panel B: SDS-PAGE analysis of cellular extracts prepared from neurons extracted 2 days before or 9 days after birth. The adenylated form of DNA ligase I is reveled by autoradiography. The sizes and the positions of the molecular mass markers are indicated.

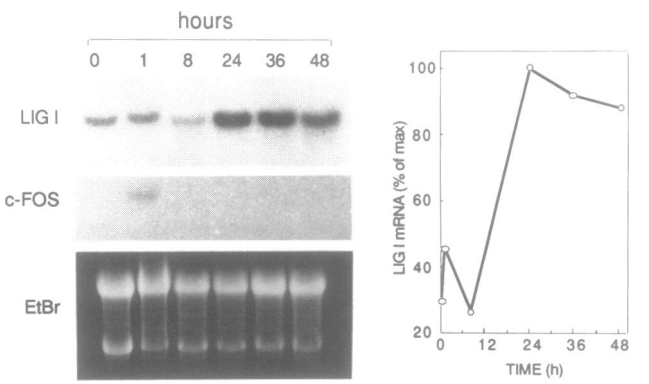

Figure 7. Effect of UV irradiation on DNA ligase I mRNA level. Confluent human primary fibroblasts were kept in 10% FCS containing medium for 5 days and then UV_{254nm} irradiated as described in 'Materials and Methods'. Left: Total RNA (10 μg) was probed in Northern type hybridization with the DNA ligase I-specific probe (LIG I) and with a probe specific for the *c-fos* proto-oncogene (c-FOS). EtBr: rRNA quantitation. Right: The autoradiograms were analyzed with the help of the Bioprofil software (Vilber Lourmat) to quantitate the intensity of the hybridization signals. The relative intensity of each resultant autoradiogram is presented as % of the maximal O.D. value.

binding site that makes it sensitive to both increases in the intracellular levels of c-AMP and treatment with mutagens (31,32). Recently a putative ATF/CRE binding site has been located by sequencing analysis in the DNA ligase I gene promoter (33). Therefore we have controlled whether agents that increase c-AMP levels (forskolin and IBMX) can also influence the level of expression of DNA ligase I gene. For this purpose, mouse NIH-3T3 cells were serum starved for 48 h, then treated with forskolin and IBMX. The Northern blot analysis revealed that while the level of the control gene *c-fos* increased transiently after 1h of treatment, DNA ligase I mRNA level did not significantly raise (Figure 3).

The sequence TGACGTCA (ATF/CRE) can mediate both c-AMP and TPA- induced transcriptional activation (34,35). We have therefore investigated the effect of TPA treament on the DNA ligase I gene expression. As shown in Figure 3, in NIH-3T3 resting cells an increase in the DNA ligase I mRNA level can be observed but only 24 h after TPA addition. This late responce indicates that the GACGTCT putative ATF binding site present in the DNA ligase I gene promoter (33) is unable to mediate both c-AMP and TPA responsiveness.

DNA ligase I mRNA is induced late after UV irradiation

The fact that the mRNA for DNA ligase I is detecteble in resting cells (neurons, peripheral lymphocytes, differentiated HL-60 cells, resting fibroblasts) suggests a role for this enzyme during DNA repair as well. In favor of this hypotesis is the observation by Barnes et al. (8) that mutations in the DNA ligase I gene produce hypersensitivity to DNA damaging agents. Moreover Nocentini and Mezzina (7) reported a threefold increase of DNA ligase I activity 24−48 h after UV irradiation of human fibroblasts. For this reason we decided to investigate whether UV irradiation could modulate DNA ligase I gene expression. We have measured the level of DNA ligase I transcript in human fibroblasts after UV_{254nm} irradiation. The *c-fos* specific induction at very early times in response to UV irradiation was taken as a measure of the efficiency of the treatment (36). The results, presented in Figure 7, are in agreement with previous observations on the increase of DNA ligase I activity (7). In fact the level of DNA ligase I mRNA raises approximately three fold reaching a maximum 24 h after damaging treatment.

DISCUSSION

We have studied the expression of the DNA ligase I gene in several human, mouse and rat systems by Northern blot analysis using a human partial cDNA as a probe. DNA ligase I mRNA was detected in all test systems, including resting cells, but its relative cellular content is higher in proliferating cells. This analysis indicated that, in mammalian cells, maximal expression of DNA ligase I mRNA coincides with the first S phase after serum stimulation, thereafter remaining high despite the decline of DNA synthesis. In this regard the pattern of DNA ligase I gene expression resembles that of DNA polymerase α (37), supporting the general idea of an involvement of DNA ligase I in DNA replication.

Moreover the increase of DNA ligase I mRNA occurs also when resting fibroblasts are serum stimulated in the presence of aphidicolin. Aphidicolin specifically prevents replicative DNA synthesis by inhibiting DNA polymerases α, δ and ϵ, and, in our conditions, reversibly arrests the cells at the G1/S border without S phase citotoxicity (38). These observations are in agreement with those obtained in PHA stimulated peripheral blood T cells exposed to hydroxyurea (26).

On the other hand when actively replicating cells are induced to differentiate into nondividing cells, as in the case of HL-60 treated with DMSO, the DNA ligase I mRNA levels sharply decrease, although remaining detectable.

The high stability of DNA ligase I mRNA determined in the systems here described suggests that its steady state level is controlled mainly by the rate of gene transcription. Moreover the stability of both mRNA and protein (39) indicates that the variation of the intracellular content of DNA ligase I takes place in the long range period, supporting the hypothesis that the short term regulation of the enzymic activity occurs at post-translational level (40−41).

We have also shown that DNA ligase I is expressed at low but well detectable levels in cells unable to restart a replication cycle, such as rat neurons after birth. A DNA ligase activity is present in rat brain extracts at levels comparable to those found in liver, kidney and lung (42) and events of DNA ligation have been demostrated in neuronal and glial nuclei isolated from adult guinea pig (43−44). Our data definitely prove that the DNA ligase I gene is constitutively expressed in neuronal cells as revealed by detection of both mRNA and 130 kDa self-adenylating polypeptide (4). In addition, by measuring the perinatal levels of DNA ligase I mRNA, we have demonstrated that its expression is regulated differently from that of the replicative DNA polymerase α and δ/ϵ being more similar to that of DNA polymerase β. This finding strongly suggests that DNA ligase I could be involved also in processes different from nuclear DNA replication, such as repair DNA synthesis catalysed by DNA polymerase β. An intriguing observation is that in neurons the level of the uracil DNA-glycosylase drops during development (19). This observation suggested an attractive hypothesis on DNA aging (45). It could be interesting to investigate whether in neurons DNA ligase I and DNA polymerase β activities can be involved in a possible alternative pathway for the removal of uracil that results from spontaneous cytosine deamination. A role of DNA ligase I in both DNA replication and repair would not be unexpected since the human gene can complement also the repair defect of the *cdc9 S.cerevisiae* mutant (13). More recently Barnes et al. (8) suggested a correlation between the DNA ligase I mutations in the human 46BR cells and some defects in DNA excision repair. An additional indication for a role of DNA ligase I in excision repair comes from our observation that DNA ligase I transcription increases threefold in confluent fibroblasts 24 h after UV irradiation. This finding well matches the behaviour of the DNA joining activity in the same system (7). It is known that UV-induced DNA repair in normal fibroblasts occurs over long post-UV period with an overall rate that can be described as the sum of two first-order reactions characterised by different rate constants (46). When a dose of 20 J/m^2 is used, the 50−60% of dimers is removed in 24 h, the remaining damages being removed later with a slower kinetics. Some authors (47) suggested that the photoproducts removed with the slower kinetics may concern pyrimidine dimers that are less accessibile to excision repair enzymes. The observed increase in DNA ligase I activity could be important for this DNA repair pathway.

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