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Irreversible Repression of DNA Synthesis in Fanconi Anemia Cells Is Alleviated by the Product of a Novel Cyclin-Related Gene

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Primary fibroblasts from patients with the genetic disease Fanconi anemia, which are hypersensitive to cross-linking agents, were used to screen a cDNA library for sequences involved in their abnormal cellular response to a cross-linking challenge. By using library partition and microinjection of in vitro-transcribed RNA, a cDNA clone, pSPHAR (S-phase response), which is able to correct the permanent repression of semiconservative DNA synthesis rates characteristic of these cells, was isolated. Wild-type SPHAR mRNA is expressed in all fibroblasts so far analyzed, including those of Fanconi anemia patients. Correction of the abnormal response in these cells appears therefore to be due to overexpression after cDNA transfer rather than to genetic complementation. The cDNA contains an open reading frame coding for a polypeptide of 7.5 kDa. Rabbit antiserum directed against a SPHAR peptide detects a protein of 7.9 kDa in Western blots (immunoblots) of whole-cell extracts from proliferating, but not resting, fibroblasts. The deduced amino acid sequence of SPHAR contains a motif found in the cyclins, and it is proposed that SPHAR acts within the injected cell by interfering with the cyclin-controlled maintenance of S phase. In agreement with this proposal, normal cells transfected with an antisense SPHAR expression vector have a significantly reduced rate of DNA synthesis during S phase and a prolonged G_2 phase, reflecting the need for postreplicative DNA processing before entry into mitosis.

In the autosomal recessive genetic disease Fanconi anemia (FA), the increased frequency of spontaneous chromosomal aberrations (46), and particularly the aberrations induced by treatment with polyfunctional alkylating agents, has led to the suggestion that cells from these patients are deficient in the repair of DNA interstrand cross-links (9). Indeed, unrepaired cross-links are manifested as chromosome breaks even in mutagen-treated normal cells if they proceed to mitosis (51). However, the considerable clinical variability of FA is paralleled at the cellular level by a range of conflicting reports regarding the capacity of FA cells to repair DNA, which may partly be explained by genetic heterogeneity. We have previously used an immortalized, simian virus 40-transformed FA fibroblast as a partner for somatic cell fusion experiments to demonstrate that at least two different genes can lead to the clinical picture of FA (68). Such fusion experiments have been extended by Strathdee et al. (53), and they indicate that there are at least four complementation groups in FA (A, B, C, and D). Although the gene responsible for FA group C has been cloned (54), its function is as yet unknown.

The remarkable clinical variability of symptoms even within individual FA families (13) is generally attributed to modulating effects of the remaining genetic constitution. The implication is that other gene products influence the cellular processes in which the FA genes are involved. The identification of one or more of these phenotype-modulating genes might shed light on the nature of this process, which as yet is still largely a matter of speculation.

In the case of cells from several FA patients, persistence of cross-links over 24 h could be clearly demonstrated (8, 16). Cross-link persistence is often accompanied by an irreversible repression of DNA synthesis rates (35). A reduction in DNA synthesis arises by two interdependent mechanisms: firstly, the passive reduction caused by a physical block to the replication machinery, and secondly, the active inhibition of replicon initiation as a response to the presence of DNA lesions (17). The latter effect requires a signal transduction pathway that links the detection of lesions in DNA with the active regulation of replicon initiation. There is evidence that the response of FA cells, in terms of repression of DNA synthesis after cross-linking treatment, correlates with the genetic complementation group. Cells from group A, but not cells from groups B and D, show permanent repression of DNA synthesis (4, 36). Cells from group C have not yet been investigated.

Rather than the repair of DNA lesions, it may be the avoidance of such lesions that is deficient in FA cells (44). Under hypoxic conditions, cultured FA cells show a nearly normal phenotype in terms of in vitro life span and cloning efficiency. At elevated oxygen levels FA cells accumulate in the S and G_2/M phases. Either increased production of activated oxygen species or a failure of cellular detoxification processes has been suggested to be responsible for the chromosome damage in FA cells. Joenje and Gille have proposed a model in which either a mutant cross-link repair–DNA replication function in FA is hypersensitive to the oxygen species $^1O_2$ or an abnormally high $^1O_2$ flux overwhelms the cell (23). Clearly these alternatives can be accommodated by the genetic heterogeneity of FA, and

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they represent systems which could be influenced by a variety of modulating gene products.

A diffusable factor has been repeatedly suggested to be involved in FA, since cocultivation of normal fibroblasts with FA fibroblasts can lead to a reduction in the clastogen sensitivity of the latter (42). The nature of this factor is unknown; however, abnormalities in the production of tumor necrosis factor alpha and interleukin-6 in FA cells have been reported. The cellular hypersensitivity of FA cells to cross-linking treatments in vitro is corrected by addition of interleukin-6 to their growth medium (43). The involvement of hematopoietic growth factors in the basic defect of an inherited anemia, such as FA, is highly suggestive.

One of the most well established and consistent characteristics of FA cells is their disturbed cell cycle. In particular, their G_2 phase is unusually long, and many cells remain permanently arrested within this phase (44, 47, 60). G_2 arrest may reflect the accumulation of DNA damage and the need to repair this damage before mitosis. However, since cell cycle disturbance is observed even without experimental mutagenic insult, it seems likely that the cell cycle defect is independent of DNA repair processes.

In an attempt to identify the FA gene, or modulating genes, we screened cDNAs for their ability to correct the abnormal response of FA group A cells in terms of DNA synthesis rates after cross-linking. Correction can be achieved by microinjection of protein (14) or mRNA (3) from normal cells, paralleling the earlier somatic cell fusion experiments. Correction by microinjection of mRNA was restricted to an mRNA fraction of 650 nucleotides in length (3). In order to isolate the single correcting mRNA from this fraction, we constructed a cDNA library from which RNA could be transcribed in vitro, and this RNA was tested for its effect on FA cells after microinjection. By partitioning of the library into sublibraries we have been able to isolate a cDNA that codes for a small protein (SPHAR; S-phase response) able to restore the rate of DNA synthesis during S phase. The SPHAR sequence contains a motif found in the cyclin family, and examination of its expression and role in normal cells indicates that it is involved in the progression of the cell cycle.

MATERIALS AND METHODS

Cell culture and microinjection. The FA fibroblast line FA1BER (laboratory no. 1424) has been described previously (52). These cells belong to complementation group A (patient A, fibroblast line FA-A in reference 68), are deficient in the removal of interstrand cross-links (16), and show permanently reduced DNA synthesis rates after cross-linking (4). FA4BER (laboratory no. 3557) fibroblasts also belong to complementation group A, and FA0BER fibroblasts belong to complementation group non-A (patients B and C, respectively, in reference 68). FA0BER cells show normal recovery of DNA synthesis after cross-linking (4). FA8BER, FA9BER, and FA13BER are fibroblast cell cultures from FA patients diagnosed by our institute. The FA fibroblast lines GM1746 and CA178 were from the Institute for Medical Research, Camden, N.J., and E. Wunder (Heidelberg, Germany), respectively. Control fibroblasts, MATCON and LN9CON, were obtained from skin biopsy samples from two healthy men. The FA group D lymphoblastoid cell line HSC62 was kindly provided by Manuel Buchwald (Hospital for Sick Children, Toronto, Canada).

The human cell line EJ30 was obtained from R. Johnson (University of Cambridge, Cambridge, United Kingdom), and it is a pseudodiploid immortal line derived from line EJ, which was established from a bladder carcinoma (21).

Fibroblasts were cultured in Eagle's minimal essential medium with 10% fetal calf serum and 10 μg of penicillin and streptomycin per ml. Cultures were examined routinely for mycoplasmal contamination. Verification of cell identities was made by regular examination of DNA fingerprints by using synthetic oligonucleotides (38). Cells were released from confluence by trypsin digestion and seeded onto gridded coverslips 2 days before microinjection. The microinjection system has been described previously (3), and it was implemented by using procedures originally described by Grässmann et al. (15). For each pool of cDNA transcripts, approximately 200 cells were injected, and 30 to 40 of these were in S phase at the time of the assay for DNA synthesis rates.

Cross-linking treatment and DNA synthesis assays. Microinjected cells and control cells were treated in 0.5 μM 8-methoxypsoralen (8-MOP) with 3.9 kJ of UVA per m^2 as described previously (4). Control sham-irradiated cultures were treated only with 8-MOP. DNA synthesis rates were measured at 12 h after cross-linking by incubation for 30 min in [^{3}H]thymidine at 12 kBq/ml followed by fixation and autoradiography of the cells according to standard procedures. Autoradiographs were examined microscopically, and the silver grains above cell nuclei were scored. Distributions of grain counts per nucleus were compared as cumulative proportional frequency plots and by using the Kolmogorov-Smirnov nonparametric test for determination of significant differences (67).

For examination of DNA synthesis rates in larger cell populations, the assay described in reference 7 was used. Briefly, 2×10^5 cells were plated in quadruplicate into 3-cm-diameter petri dishes, one of which contained a coverslip. Newly synthesized DNA was labeled for 30 min by addition of [^{3}H]thymidine (48 kBq/ml) to the medium. At the end of the incubation period, the cells were washed and the pools of cytoplasmic [^{3}H]thymidine in three cultures were extracted in cold 5% trichloroacetic acid (TCA). [^{3}H]thymidine incorporated into cellular DNA was then extracted overnight in 1 N NaOH. The TCA-soluble and TCA-insoluble fractions were counted in a Beckman scintillation counter. DNA synthesis rates were expressed as TCA-insoluble cpm/TCA-soluble cpm, thus correcting for variations in cell numbers. The coverslip culture was processed for autoradiography as detailed above, and 200 cells were scored for [^{3}H]thymidine incorporation; this provided a measure of the proportion of cells in S phase at the time of labeling.

cDNA library construction and screening. The HeLa mRNA fraction, previously shown to correct the abnormal response of FA1BER fibroblasts (3), was used for cDNA synthesis essentially according to the method of Gubler and Hoffman (18) and our previously described strategy for cDNA cloning for microinjection (2). Briefly, first-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Karlsruhe, Germany) from 5 μg of mRNA fraction by using the oligonucleotide pCGGGCCCGCGGC $CGC(T)_{20}$ (primer 3) as a primer. The primer 3 oligonucleotide primes from the poly(A) tail of the mRNA and, in addition, contains recognition sequences for the *Not*I and *Apa*I restriction endonucleases. Second-strand cDNA was synthesized with DNA polymerase I after RNase H digestion of the RNA. The cDNA was blunted with T4 DNA polymerase and inserted into the *Sma*I site between the phage T3 and T7 RNA polymerase promoters of pT7T3-U19 (Pharmacia, Freiburg, Germany). *Escherichia coli* DH5-α cells were made competent (2×10^8 colonies per μg) by the method of Hanahan (19) and transformed with the cDNA library. Colonies were grown on GeneScreen Plus nylon filters (Du Pont, Bad Homburg, Germany), and replicas were made with the same material.

The screening methodology has been described previously (2). Briefly, bacteria were washed from fragments of frozen replica filters and grown in 20-ml cultures. Plasmid DNA was prepared and linearized by digestion with *Not*I—the site for which is provided at the 3′ ends of cDNA inserts by the oligonucleotide primer used for first-strand cDNA synthesis. Capped in vitro transcripts were synthesized by using phage T3 and T7 RNA polymerases as described previously (26). After transcription, plasmid DNA was removed by DNase I digestion, and the RNA was extracted and resuspended at approximately 200 ng/μl in 10 mM Tris-HCl, pH 7.2, for analytical gel analysis and microinjection. After identification of a complementing sublibrary, the appropriate frozen filter was removed from storage at −70°C and cut into pieces for the next round of injections. At the final stages of screening, individual colonies were picked and grown on agar in the wells of a microtiter plate; the final complementing clone was called pSPHAR.

Subcloning and sequencing. T7 DNA polymerase and the kit from Pharmacia were used for DNA sequencing. Subclones were constructed by using conveniently placed restriction enzyme sites: pSPHAR-M was constructed by *Mbo*II-*Not*I digestion of pSPHAR, insert preparation, blunting, and insertion into *Sma*I-cut pT7T3. Sequencing was performed with plasmid DNA or single-stranded DNA produced from the f1 origin of pT7T3 clones after infection with the phage M13 KO7.

RT-PCR. For amplification, RNA was extracted from fibroblast cultures by using the miniprep method of Wilkinson (61). A 100-ng portion of total cellular RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase after being primed with either random hexanucleotides or primer 3 (described above). The reverse transcriptase products were diluted directly into PCR buffer and amplified with either primer 1 (pCTCTGGGAAATGACTAG; positions 227 to 243 in Fig. 3) and primer 2 (pGCTGTTCAAAAATGAGC AGC; complementary to positions 432 to 413 in Fig. 3) or primer 1 and the *Not*I-*Apa*I portion of primer 3. Primers were used at 0.15 μM, and amplification was done for 35 cycles: 2 min at 55°C for annealing and extension and 1 min at 95°C. All reverse transcription PCR (RT-PCR) components were from Perkin-Elmer. mRNA integrity was checked by RT-PCR with primers which amplify a 500-bp region from glyceraldehyde phosphate dehydrogenase cDNA under the same conditions.

Isolation of SPHAR genomic clones. Restriction digestion and Southern blots indicated that the SPHAR sequence is located on a 3.7-kbp *Taq*I fragment (data not shown). In order to isolate a 5′ genomic clone, we used PCR after adding a polyadenine tail to the ends of the genomic *Taq*I fragments to provide a complementary sequence for a nonspecific oligo(dT) primer. HeLa genomic DNA was digested to completion with *Taq*I, and the products were extended at the 3′ end in a reaction volume of 50 μl (100 mM K cacodylate [pH 7.2]–10 mM

$CoCl_2$–1 mM dithiothreitol) containing 3 pmol of the 3′ ends, 1 nmol of dATP, and 15 U of terminal deoxynucleotide transferase (Gibco-BRL). Incubation was performed at 37°C for 30 min. A 100-ng portion of A-tailed DNA was amplified in a 100-μl solution (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin) with 200 μM each deoxynucleoside triphosphate and 0.15 μM primer 4 (pCCTAGTCATTTCCCAGAG; complementary to positions 244 to 227 in Fig. 3) and primer 3 (described above), for 30 cycles of 55°C for 1 min, 72°C for 2 min, and 95°C for 1 min. Samples (10 μl) of the products were separated on a 1.5% agarose gel, denatured, transferred to Hybond-N nylon membranes (Amersham, Braunschweig, Germany), and hybridized with a 5′ SPHAR probe (positions 1 to 243 in Fig. 3). The 5′-flank probe was produced by PCR amplification of sequences between positions 1 and 243 of the clone pSPHAR with primer 4 and T7 primer (pCTAATACGACTCACTAT), agarose gel preparation of the amplified product, removal of vector sequences with *Bam*HI, and labeling with digoxigenin-dUTP by using the kit from Boehringer (Mannheim, Germany). Hybridized probe was detected by using alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer) after reaction with 5-bromo-4-chloro-3-indolyl-phosphate and Nitro Blue Tetrazolium. The remaining genomic PCR products were blunted by using T4 DNA polymerase and phosphorylated in a 10-μl solution (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 50 mM dithiothreitol) with 1 mM ATP and 0.5 U of T4 polynucleotide kinase for 60 min at 37°C. The products were then ligated into the *Sma*I site of pT7T3 (Pharmacia) and transformed into *E. coli* DH5-α. Minipreps of colonies were made, and the presence of the SPHAR insert was detected by hybridization with the 5′ probe described above. A positive clone, pSPHAR-G (1.2-kb insert), and its two *Eco*RI subclones, pSPHAR-G7 (0.7 kb) and pSPHAR-G11 (0.5 kb), were sequenced as described above.

Peptide synthesis, antiserum production and Western blots (immunoblots). The SPHAR peptide, ILFQKVSEANSQTELL, was synthesized as an octameric antigenic peptide on a branching lysine core as described by Posnett et al. (40). The peptide was dissolved in phosphate-buffered saline and used to immunize a New Zealand White rabbit with weekly boosters. After 3 weeks, serum was collected every 2 weeks and tested in comparison to preimmunization serum for specific recognition of the peptide, which was spotted onto nitrocellulose filters. Sera were purified by affinity chromatography through protein A-agarose (Pharmacia).

Cell extracts were prepared from fibroblast cultures by three freeze-thaw-vortex cycles in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9)–60 mM KCl–1 mM dithiothreitol–1 mM EDTA–4% Ficoll–1 mM phenylmethylsulfonyl fluoride–1 mM aprotinin–1 mM leupeptin (10^7 cells per 100 μl) and loaded onto sodium dodecyl sulfate (SDS)–15% polyacrylamide gels (28). Proteins were electroblotted onto nitrocellulose membranes, and the membranes were blocked by incubation for 1 h at room temperature in TNTW buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) with 5% dried milk powder. Membranes were then incubated for 1 h at room temperature with serum or preimmunization serum diluted 1:4 to 1:20 with TNTW buffer. Filters were washed twice with TNTW buffer for 15 min at room temperature and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; Amersham) for 1 h at room temperature. After the filters were washed as described above, bound goat IgGs were detected by enhanced chemiluminescense (Amersham). Some filters were stripped of antibody by incubation for 30 min at 50°C in 62.5 mM Tris-HCl (pH 8.0)–100 mM 2-mercaptoethanol–2% SDS and reprobed with an actin-specific mouse monoclonal antibody (Boehringer Mannheim).

SPHAR antisense expression vectors and transfection. A PCR product containing the SPHAR open reading frame, 10 bp of the 5′ untranslated region, and 8 bp of the 3′ untranslated region was inserted behind the simian virus 40 early promoter, and the T7 RNA polymerase promoter, in the eukaryotic expression vector pSVExT7 (Fig. 7). The sense or antisense orientations of the inserts in individual clones were confirmed by *Xmn*I restriction analysis. The SPHAR plasmids were cotransfected with pSV2-neo into exponentially growing EJ30 cells after complex formation with a cationic lipopolyamine mixture (1) (Transfectam; IBF-Biotechnics). Transfectants were selected in 400 μg of G418 per ml (Geneticin; Gibco), and individual clones were analyzed for sense or antisense SPHAR expression by RT-PCR using primers 1 and 2 (described above) and a primer complementary to the T7 RNA polymerase promoter.

Analysis of cell cycle kinetics. EJ30 sense and antisense SPHAR transfectants were allowed to reach confluence, and then they were grown for 3 days in minimal essential medium with 0.1% fetal calf serum. As a control, EJ30 cells transfected with pSV2-neo only were similarly serum starved. The cells were then released by trypsinization and plated onto petri dishes in minimal essential medium with 10% fetal calf serum, 400 μg of G418 per ml, 0.6×10^{-4} M 5′-bromo-2′-deoxyuridine, and 0.6×10^{-4} M 2′-deoxycytidine. Aliquots of cells were harvested at various time points (3×10^5 cells at time zero) and frozen in minimal essential medium with 10% fetal calf serum and 10% dimethyl sulfoxide prior to analysis by flow cytometry. The analysis of cell cycle parameters by flow cytometry has been described previously (39). Cells were stained first with Hoechst 33258 and then with ethidium bromide. The cells were then passed through an ICP-22 arc lamp instrument interfaced via the Ahrens Cytometry Analysis System (Hamburg, Germany) to an IBM AT computer. The intensity of the ethidium bromide fluorescence reflected the amount of DNA and, therefore, the passage of individual cells through the cell cycle. Simultaneous measurement of of Hoechst fluorescence, which is quenched by incorporated bromodeoxyuridine, allowed quantitation of the numbers of cells in each phase of three consecutive cell cycles. Data were analyzed by using specialized software from Phoenix Flow Systems (San Diego, Calif.).

Nucleotide sequence accession number. The sequence data reported here appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number X82554.

RESULTS

Molecular cloning of SPHAR by functional expression. FA1BER cells and cells from other FA complementation group A patients have a permanently reduced DNA synthesis rate after DNA-cross-linking treatment (4, 36). A cDNA library was constructed from an mRNA fraction previously shown to correct FA1BER fibroblasts after microinjection (3). Within this cDNA library of 55,000 recombinants, a sublibrary of 6,000, in vitro-transcribed RNA from which increased the DNA synthesis rate of microinjected FA1BER cells after treatment with 8-MOP–UVA, was identified. In vitro RNA transcripts from other sublibraries showed no effect in the assay. Clones from the correcting sublibrary were further divided into 5 subgroups, and their effects in the cross-link repair assay were measured after they were injected into FA1BER cells. Figure 1A shows the results of one experiment in which FA1BER cells were injected with RNA transcribed from these sublibraries. The results are expressed as the DNA synthesis rate of injected FA1BER fibroblasts in comparison to that of sham-irradiated FA1BER cells (100% DNA synthesis) at 12 h after an 8-MOP–UVA cross-linking challenge. Only one of the subgroups (SL4.2) was able to correct the FA1BER response. Figure 1B summarizes the remaining library partition steps. However, the single cDNA clone, pSPHAR, still only partially corrected the abnormal response. DNA synthesis was measured by determining the level of [^{3}H]thymidine incorporation by quantitative autoradiography. The distributions of grain counts for the populations of untreated, uninjected, and SPHAR-injected FA1BER cells are shown in Fig. 2 as cumulative proportional frequencies. The correction of the FA1BER response by the cDNA clone pSPHAR was statistically significant ($P < 0.05$) by the two-sided Kolmogorov-Smirnov test (67).

Sequence analysis of pSPHAR. The sequence of the 641-bp cDNA insert in pSPHAR is shown in Fig. 3. Examination of the sequence indicates the presence of an open reading frame of 192 bp coding for a protein of 63 amino acids. The ATG start codon is preceded at position −3 by a purine; this is the only agreement with the consensus sequence around start codons (27). There are four other ATGs upstream from the putative start codon; this is unusual, but not unique, for a mammalian mRNA. The human DNA repair gene *ERCC2* has two upstream ATGs (59), and the recently identified oncogene *ect2* has no fewer than eight (33). The short open reading frame in pSPHAR ends with a TGA stop codon followed by 175 bp of noncoding sequence. A classical polyadenylation signal is located 15 bp upstream from the polyadenine tail.

The amino acid sequence of the translation product is indicated below the nucleotide sequence in Fig. 3. The calculated molecular weight of this polypeptide is 7,515. Secondary structure analysis performed by the method of Garnier et al. (11) suggested that 60% of the polypeptide is in a helical structure divided into three blocks by extended regions. A computer search of the EMBL library indicated that the sequence is nearly identical (96% identity) to the expressed sequence, IB776, identified by single-pass sequencing of human brain cDNAs (24). A closer examination of the SPHAR protein sequence has revealed a motif found in region III of the so-

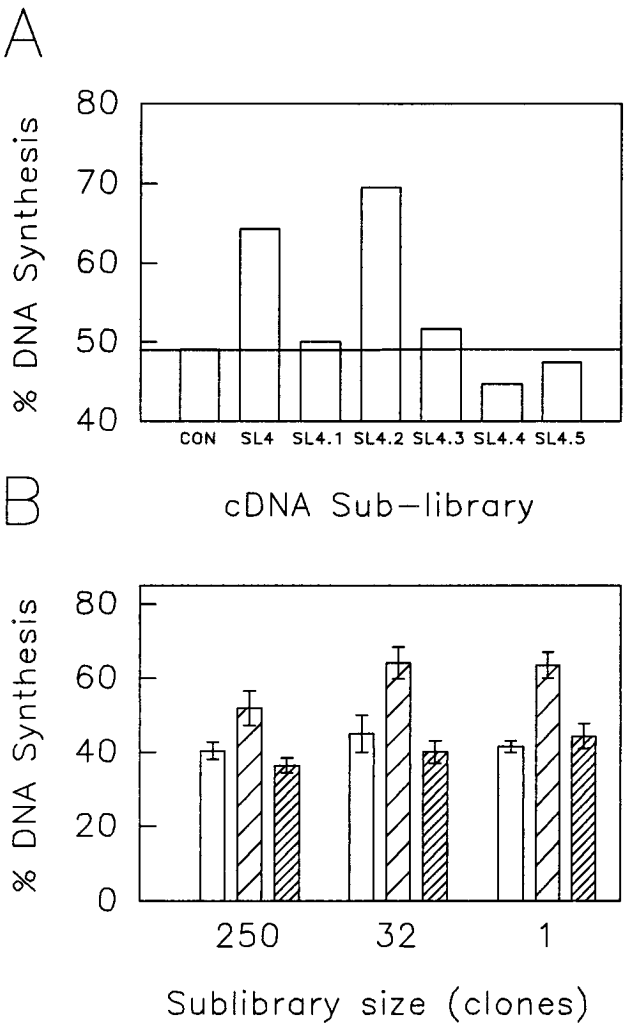
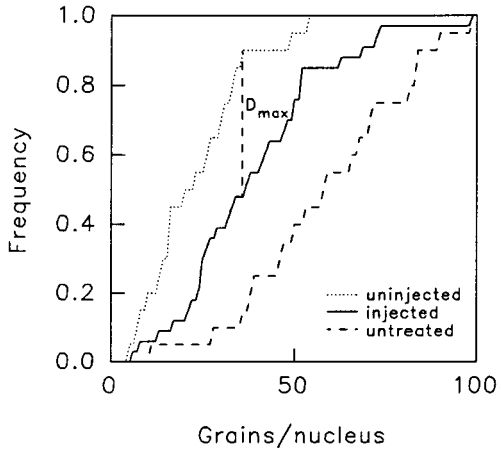


FIG. 1. cDNA library partition. (A) The DNA synthesis rate of psoralen-UVA-treated FA1BER fibroblasts after microinjection with in vitro-transcribed RNA from cDNA clones relative to that of untreated FA1BER cells (100%) is shown. Sublibrary number 4 (SL4) contained 6,000 recombinants, and its five sublibraries (SL4.1 through SL4.5) each contained 1,200 recombinants. CON, control, uninjected psoralen-UVA-treated FA1BER cells. (B) The rates of DNA synthesis in 8-MOP–UVA-treated FA1BER cells injected with in vitro-transcribed RNA from sublibraries is shown (shaded columns) in comparison to those of synthesis in uninjected cells (open columns) for each partition step. Coarsely shaded columns represent the correcting sublibrary at each partition step, and finely shaded columns represent the noncorrecting sublibraries at each step. The numbers under the columns give the numbers of recombinants in the correcting sublibrary. Each partition step was verified at least once by independent injection of coded transcripts.



D_{max} = 0.42

Values of D_{max} required for significance at :

p<0.10	p<0.05	p<0.01
>0.34	>0.38	>0.45

FIG. 2. Complementation of FA1BER cells by pSPHAR in vitro transcripts. Cumulative proportional frequencies of grains per nucleus for FA1BER fibroblasts after microinjection of in vitro transcripts from the HeLa cDNA clone pSPHAR. Dotted line, uninjected, 8-MOP–UVA-treated cells; dashed line, uninjected and untreated cells; solid line, 8-MOP–UVA-treated cells injected with SPHAR RNA transcripts. The value D_{max} is used to measure the significance of the shift in distributions as shown.

called "cyclin box" of the large cyclin gene family. The alignment in Fig. 4 shows the positions of identical or similar amino acids within this motif in SPHAR and 10 representative cyclins. Altogether, 41 cyclins have been aligned (data not shown). The closest similarity of SPHAR to a single cyclin group was found with the D-type cyclins (59 to 64%). Remarkably, cyclin D1 showed 50% identity to SPHAR from position 1 to 14 of the alignment. IME1, the most distant member of the cyclin family, showed the highest level of similarity (also 50%) to a single cyclin within this same region (45).

Cyclin motifs are found in several other proteins; protein tyrosine phosphatases, for example, share a short region of similarity to B-type cyclins in the N-terminal portion of the cyclin box (10). The same region is missing in cdc25, the cdc2 tyrosine phosphatase, and the interaction between cyclin B and cdc25 is postulated to provide in *trans* an activating domain which in other protein tyrosine phosphatases functions in *cis.*

Genomic organization of the SPHAR sequence. Use of PCR to amplify the open reading frame of SPHAR from human genomic DNA produced a product of the same size as that from the cDNA clone pSPHAR, indicating that the sequence

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-119 ttatggcttctgcctgaatacttaaaatgccttactacagttatccagttgacatgttt
 -59 ttaattcatataaggtatattgggtatattgaagtatatatattgtattacaaagactt
  -1 gTTCTTGTATTTTAAAATGTCAGTGCAAAAAATATATGGTGGAACCTTTCTTTAAAGTT
  59 GAAATGCAGTATTATTTAAATCTGAAAGGTTAAAAAGCTTTCTTCACCTTATATATGTT
 118 CTACCACTGTGACTTTTTAGTTGAAGACTAGTAAATTAACTTTTAGTTAGAAGATGCCT
 177 ACTACTTTTGTTGTTTATTTTAATCAGCAGAGCACAGAGACACATAAAAACTCTGGGAA

 236 ATG ACT AGG ATA AAA ATA TCA GTA TGT ATC TGT TTT AGA TAT TTT
   1 MET Thr Arg Ile Lys Ile Ser Val Cys Ile Cys Phe Arg Tyr Phe

 281 GAG TTT TGC TTT TTT TAT GCC TTG AAT ATT TTA TTT CAA AAA GTA
  16 Glu Phe Cys Phe Phe Tyr Ala Leu Asn Ile Leu Phe Gln Lys Val

 326 TCT GAA GCA AAT TCT CAG ACT GAA CTA CTT CTT AGA CCT CAC TGT
  31 Ser Glu Ala Asn Ser Gln Thr Glu Leu Leu Leu Arg Pro His Cys

 371 AAG AAT ATT TTA TTC AAT GTC TCA TTT ATG ATA GAT TTG CAA GCT
  46 Lys Asn Ile Leu Phe Asn Val Ser Phe Met Ile Asp Leu Gln Ala

 416 GCT CAT TTT TGA ACAGCTTTTTGCATGGGATAGGAGCATGTCTATTCTAACACAT
  61 Ala His Phe ---

 471 CAGCTTATTAAAAAGCAAGAATTTTAAAAATAAGATAAATGTAAAGTTGTTTTATAAAC
 530 GATCCTGTTAATTAAACCACAGACACCATATATCCTTCTGCATCCTTTGGCCAATAAAA
 589 GTTGCTGGAGAACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIG. 3. Nucleotide and deduced amino acid sequences for the cDNA clone SPHAR. The first nucleotide of the insert in pSPHAR is assigned to position 1. Uppercase letters are the cDNA sequence, with the predicted polypeptide sequence given underneath. Lowercase letters are from the 5′ genomic SPHAR clone (the sequence shown represents only a small portion of the sequenced DNA). The polyadenylation signal at position 582 is underlined. The asterisk at position 180 indicates the first nucleotide of the deletion clone pSPHAR-M used for sequencing. The underlined nucleotides in the genomic sequence represent possible promoter structures. The underlined amino acids make up the motif found in cyclin sequences.

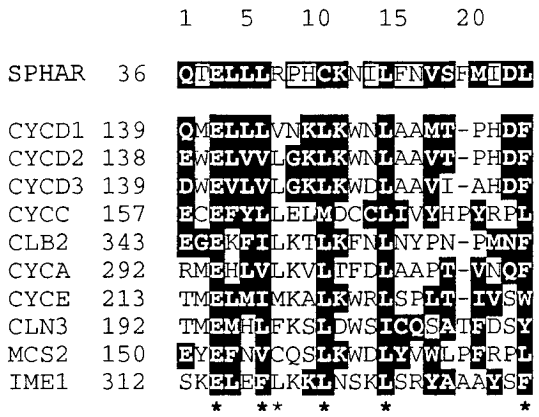

FIG. 4. A cyclin-related motif in SPHAR: sequence alignment of SPHAR with a conserved region of various cyclins. The aligned region is part of cyclin homology box III (30), and it includes the only invariant amino acid found in all known cyclins (glutamic acid at position 3 of the alignment). Identical and similar matches of these cyclins to SPHAR are highlighted by inverse text. Boxed residues in SPHAR indicate similarity or identity in up to 50% of the cyclins; SPHAR residues in inverse type indicate similary or identity in 50% or more of the cyclins. Highly conserved positions (occupied by residues of one similarity group only) are marked under the alignment by asterisks; note that SPHAR shares five of these (bold asterisks). The similarity groups used were RHK, ASTGP, IVLMFYWC, and EDQN. The sources of the sequences were as follows: CYCD1, human D1-type cyclin (63); CYCD2, human D2-type cyclin P3 (65); CYCD3, human D3-type cyclin H34 (65); CYCC, human C-type cyclin (31); CLB2, *Saccharomyces cerevisiae* B-type cyclin (55); CYCA, human A-type cyclin (58); CYCE, human E-type cyclin (25); CLN3, *Saccharomyces cerevisiae* G_1 cyclin (37); MCS2, *Schizosaccharomyces pombe* mitotic catastrophe suppressor 2 (34); and IME1, *Saccharomyces cerevisiae* inducer of meiosis 1 (45, 50).

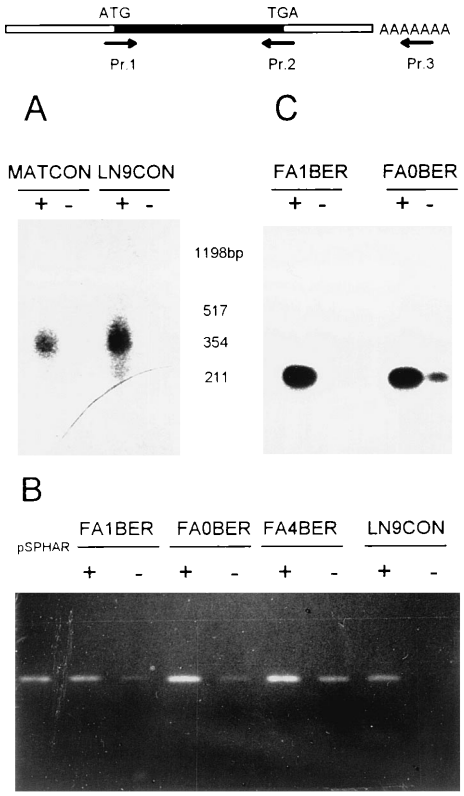

FIG. 5. Analysis of SPHAR mRNA expression in fibroblasts. The diagram shows the primers used for PCR amplification. (A) Two independent mRNA preparations, from control fibroblast lines MATCON and LN9CON, were reverse transcribed with primer 3 and amplified with primers 1 and 3 in order to exclude amplification of contaminating genomic DNA. PCR products were separated on a 1.5% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled probe for the SPHAR open reading frame. +, amplification after reverse transcription; −, amplification without prior reverse transcription. The minimum size of the expected product was 390 bp. (B) RNA from FA1BER, FA0BER, and FA4BER fibroblasts and the control fibroblast line LN9CON was transcribed with random hexanucleotides and SPHAR cDNA was amplified with primers 1 and 2. Products were loaded onto a 2% agarose gel and visualized by ethidium bromide staining. pSPHAR, product of PCR with primers 1 and 2 from the plasmid clone pSPHAR; +, amplification after reverse transcription; −, amplification without prior reverse transcription. The expected size of the product was 209 bp. The weak signals from some samples without reverse transcription were due to low-level contamination of these RNAs with genomic DNA. (C) RT-PCR products with primers 1 and 2 from FA1BER and FA0BER fibroblasts were separated on a 1.5% agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled probe for the SPHAR open reading frame. Other details were as described for panel B.

is not interrupted by an intron at the genomic level. The same product was obtained with DNA from eight FA fibroblast or lymphoblastoid cultures (data not shown). PCR with genomic DNA was used to amplify and clone a longer 5′ genomic fragment containing the SPHAR sequence from human DNA. Sequence analysis (sequence shown partially in Fig. 3) confirmed the colinearity of this sequence with the SPHAR cDNA sequence. Three TATATT sequences were located 21, 35, and 44 bp upstream from the first base of the cDNA sequence. Zoo blots have shown that SPHAR is conserved within the class Mammalia (unpublished data).

SPHAR is expressed as a polyadenylated transcript in FA and normal cells. Since the level of SPHAR mRNA expression is too low for detection on Northern (RNA) blots, reverse transcription of mRNA, followed by PCR amplification with SPHAR-specific primers, was used to examine the expression of SPHAR mRNA in human fibroblasts. Since amplification of intronless genes from mRNA is highly sensitive to DNA contamination, reverse transcription was primed from the oligo(dT) portion of primer 3, and the same primer was used, together with a primer specific for SPHAR, for amplification. Products were separated on agarose gels, blotted, and hybridized to a labeled SPHAR probe for identification (Fig. 5A). The coding sequence of SPHAR was clearly present in RNA from both normal fibroblast lines tested.

cDNA synthesis was also primed by using hexanucleotides before PCR with primers 1 and 2, which flank the SPHAR open reading frame, as shown in Fig. 5B. Products were readily detected from control fibroblast RNA, FA1BER and FA4BER (complementation group A) RNA, and FA0BER (complementation group non-A) RNA in ethidium bromide-stained gels. Products were again transferred to nylon membranes and hybridized with a SPHAR probe to confirm that the amplified product was indeed SPHAR (Fig. 5C). We have tested fibroblasts from one further group A patient and fibroblasts from patients belonging to group B and group D and have found SPHAR transcripts in these cells too (data not shown). Sequence analysis of the SPHAR open reading frame amplified from the DNA of eight individuals (six FA patients and two controls) has revealed the presence of only silent or conservative base changes in comparison to the pSPHAR sequence: A_{250} to G (Lys-5 remains Lys) in one control DNA, A_{403} to G (Ile-56 to Met) in FA0BER DNA, and T_{379} to A (Ile-48 remains Ile) and A_{394} to G (Ser-53 remains Ser) in DNA from a group D patient.

A 7.9-kDa protein is detected in human cell extracts by an antiserum to SPHAR. Analysis of the SPHAR amino acid sequence for antigenic determinants, according to the method of Hopp and Woods (22), indicated a peak of hydrophilicity around the sequence Lys-29–Glu-32. A polyclonal rabbit anti-

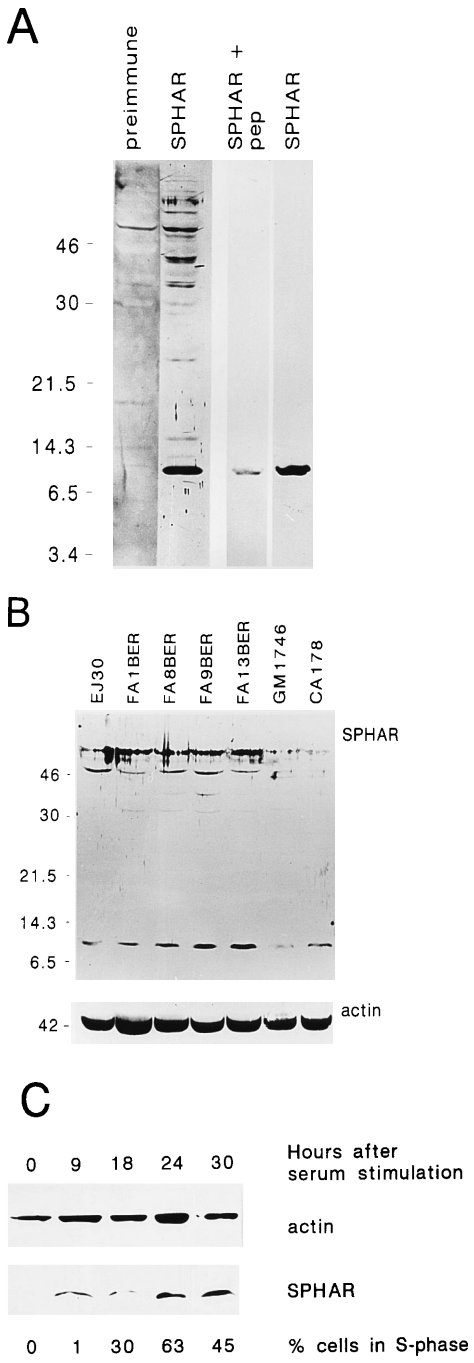

FIG. 6. Immunodetection of SPHAR sequences in whole-cell extracts. (A) Whole-cell extracts were prepared from normal human fibroblasts and separated on an SDS–15% polyacrylamide gel. Proteins were electroblotted to a nitrocellulose filter and incubated with either preimmune serum (diluted 1:10) or antiserum (diluted 1:10 in lane 2 and 1:60 in lanes 3 and 4) directed against the SPHAR peptide, with or without addition of synthetic peptide, as indicated. Bound rabbit IgGs were detected by goat anti-rabbit IgG conjugated to horseradish peroxidase and by chemiluminescence. The sizes of marker proteins are shown at the left. The anti-SPHAR-peptide serum detected a protein of 7.9 kDa. (B) Whole-cell extracts were prepared from EJ30 cells and fibroblasts from FA patients and separated on an SDS–15% polyacrylamide gel. Proteins were electroblotted to a nitrocellulose filter and incubated with rabbit antiserum directed against the SPHAR peptide. The filter was subsequently stripped of antibody and reincubated with an antiactin monoclonal antibody to allow semiquantification of the SPHAR protein level. (C) Confluent EJ30 cells were arrested by serum starvation and then were stimulated to proliferate by being plated in medium with 10% fetal calf serum. At the various time points after stimulation indicated, aliquots of cells were processed for flow cytometry or immunodetection with the antiserum to SPHAR peptide and, after stripping, a monoclonal antibody directed against actin. Only the appropriate areas of the electrotransferred 15% acrylamide gel are shown. The numbers below the blots show the proportions of cells in S phase as determined by measurement of ethidium bromide fluorescence by flow cytometry.

body, raised against a synthetic 16-amino-acid peptide spanning this putative antigenic determinant, detected a protein of 7.9 kDa on Western blots of extracts from normal human fibroblasts (Fig. 6A). Antibody binding was attenuated by excess synthetic peptide, indicating congruence between the amino acid sequence deduced from the cDNA and the immunodetected protein. A 7.9-kDa protein was also detected in extracts from FA cells, with no significant differences in levels of expression (Fig. 6B). The SPHAR mRNA detected in normal and FA fibroblasts (Fig. 5) is indeed translated in vivo. SPHAR was not detected in serum-starved cells as shown by the Western blot in Fig. 6C. Serum stimulation of the cells is followed by expression of SPHAR and entry into S phase, as determined by flow cytometry. Proliferation-dependent expression of SPHAR was similarly found in primary diploid fibroblasts (data not shown).

Cells expressing a SPHAR antisense RNA have reduced DNA synthesis rates and a disturbed cell cycle. The physiological role of SPHAR was investigated by constructing stable transfectants expressing an antisense SPHAR RNA from a strong viral promoter. Figure 7A shows the plasmid vector utilized in these experiments. The production of plasmid-derived SPHAR RNA was checked by RT-PCR, as shown in Fig. 7B for the antisense transfectant clone EJ30.SPHAR2.4. When SPHAR primer 1 and a primer specific for the T7 RNA polymerase promoter were used, the PCR produced a product of 266 bp from antisense, but not sense, RNA. Examination of SPHAR polypeptide levels on Western blots indicated that there was a reduction in expression levels to about 70% in the antisense clones (data not shown).

Three independent antisense clones were examined for their rates of semiconservative DNA synthesis at 24 h after release from serum starvation. DNA synthesis was measured by determining the level of incorporation of [^{3}H]thymidine into DNA and normalizing this to the overall quantity of [^{3}H]thymidine taken up by the cells; this normalization corrects for any variations in cell numbers (7). All three clones showed significantly ($P < 0.05$) reduced DNA synthesis rates in comparison to those of the controls, which were cells transfected with the vector lacking SPHAR sequences (Fig. 8A). The reduction in DNA synthesis rates was not due to a failure to enter S phase, since comparable proportions of cells in all four cell lines were in S phase at the time of DNA labeling (Fig. 8B). The rates of DNA synthesis within the S-phase cells were clearly affected by an artificial reduction in SPHAR protein levels. This could be expected to have consequences for cell cycle progression, and this possibility was examined for one antisense transfectant by analysis of cell cycle kinetics.

When the passage of the antisense transfectant EJ30.SPHAR2.1 through three successive cell cycles was monitored by flow cytometry, a considerable accumulation of cells in G_2 phase was observed. Figure 9 presents the data from cell cycle analyses performed 72 h after serum stimulation of the synchronized cells. As a further control, a transfectant expressing sense SPHAR RNA, EJ30.SPHAR1.2, was also included in this analysis. The total fractions of cells in the G_1 and G_2 phases (first, second, and third cycles) are shown as the ΣG1/GF and ΣG2/GF ratios, where GF is the growth fraction (all cells not in G_0 phase). The ratios ΣG1/GF and ΣG2/GF are

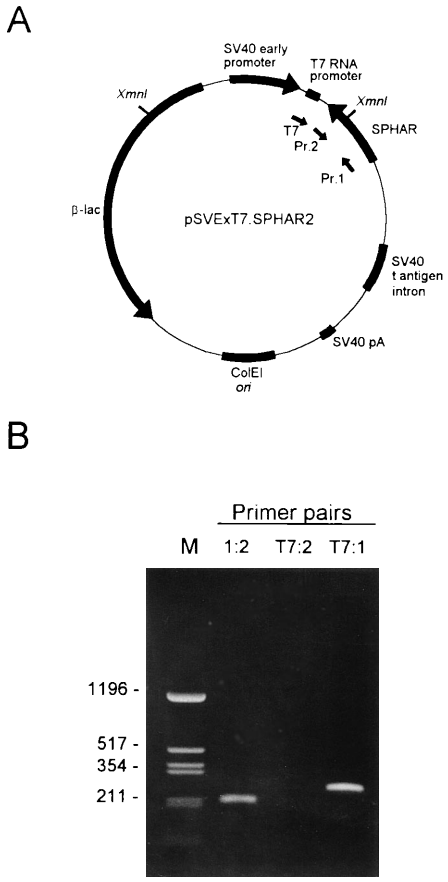

FIG. 7. Transfectants expressing antisense SPHAR RNA. (A) pSV-EX7 eukaryotic expression vector driving expression of an antisense SPHAR RNA from the simian virus 40 early promoter. A blunted and phosphorylated PCR product encoding the SPHAR open reading frame and 10 bp of the 5′ UTR was inserted into the *Eco*RV site of the vector. The orientation of clones was established by *Xmn*I restriction analysis. The sense (pSV-EX7.SPHAR1) and antisense (pSV-EX7.SPHAR2) constructs were used for transfection of EJ30 cells. (B) RNA was isolated from a G418-resistant EJ30 clone transfected with an antisense pSV-EX7.SPHAR2 construct. The RNA was reverse transcribed, and the cDNA was amplified as indicated with primers P1, P2, and T7 shown in panel A. Endogenous and plasmid-coded SPHAR RNA yielded the expected 209-bp product with primers 1 and 2; plasmid-coded antisense RNA yielded a 266-bp product with primers T7 and 1. PCR with no previous reverse transcription yielded no products with any primer pair (data not shown).

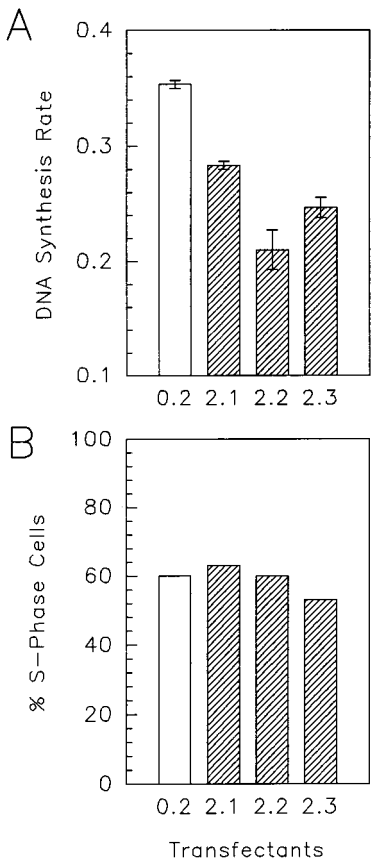

FIG. 8. DNA synthesis rates in antisense SPHAR transfectants. (A) The bar graph shows the incorporation of [^{3}H]thymidine into DNA (TCA-insoluble fraction) normalized to the cytoplasmic [^{3}H]thymidine pool (TCA-soluble fraction) for three independent antisense SPHAR transfectants (EJ30.SPHAR2.1, -2.2, and -2.3; shaded columns) and EJ30 transfectants carrying the vector only without SPHAR sequences (EJ30.SV0.2; open column)). Error bars show the standard deviations. (B) The proportions of cells in S phase in the experiment whose results are shown in panel A, as determined by [^{3}H]thymidine labeling, autoradiography, and scoring of 200 cells for incorporation of label, are shown.

thus proliferation-independent measurements which allow comparison of anti-SPHAR cells and control cells. While the proportions of cells in G_1 were similar for all three cell lines, significantly more of the cells expressing antisense SPHAR were in G_2 ($P < 0.05$).

DISCUSSION

SPHAR is not the basic defect in FA, but it is involved in its cellular phenotype. SPHAR is a single-copy sequence conserved throughout the class Mammalia and located on human chromosome 8 (data to be published elsewhere). We attribute the partial correction of the abnormal response of FA1BER fibroblasts to a cross-linking challenge by SPHAR to overexpression, since the sequence is present and not mutated in these cells. The SPHAR polypeptide increases the rate of semiconservative DNA synthesis, which, in FA cells, is characteristically irreversibly reduced after psoralen cross-linking. The effect is specific, as shown by its isolation through library partition: at each step, the correcting cDNA pool was identified among other pools taken from the same cDNA library.

We calculate that, at the concentration of RNA used (200 ng/μl), in the final stages of SPHAR isolation each cell received 2×10^4 copies of the SPHAR transcript, leading to an intracellular concentration several orders of magnitude above the endogenous level. We have additional evidence that removal of the long 5′ untranslated flank from SPHAR transcripts leads to a dramatic improvement in the efficiency of translation in vitro; possibly, secondary structure assumed by the RNA inhibits initiation. Posttranscriptional regulation via its long 5′ untranslated flank, which also contains several ATGs, has also been reported for the cyclin IME1 (48).

Expression of an antisense SPHAR RNA in normal cells leads to an approximately 40% increase in the time required to traverse G_2 phase, suggesting that SPHAR is necessary for normal cell cycle progression. Indeed, SPHAR protein is detected only in proliferating fibroblasts. G_2-phase arrest is characteristic for FA cells, and it probably reflects the requirement to complete DNA repair and replication before mitosis. The prolongation of G_2 found for the antisense transfectants is suggestive of a role for SPHAR protein in the preceding S phase.

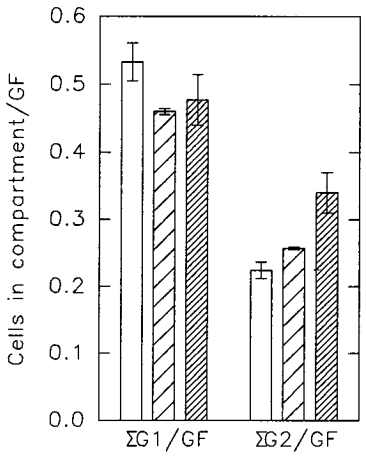

FIG. 9. Cell cycle analysis of SPHAR transfectants. The bar graph shows the proportions of cells in G_1 phase and G_2 phase relative to the total proliferating cell fraction (GF) for the control transfectant EJ30.SV0.2 (open columns), sense transfectant EJ30.SPHAR1.2 (coarsely shaded columns), and antisense transfectant EJ30.SPHAR2.1 (finely shaded columns) at 72 h after release from serum starvation. The data are averaged from three independent experiments, and the error bars show the standard deviations.

SPHAR affects initiation and/or elongation of replicons. The DNA synthesis assay used after microinjection of cDNA transcripts measures the rates of DNA replication in individual mitomycin-treated FA cells and then compares them as a population with those of other, untreated, cells. Since the proportion of FA cells in S phase remains constant at the level of damage applied, entry into S phase is not affected. The measured reduction in DNA synthesis rates was due to an effect in those cells already committed to S phase, and it is this effect which SPHAR is able to modulate.

Formally, two explanations for SPHAR's effect remain: either it leads to improved DNA repair and thus to increasing replicon elongation and initiation rates, or the rate of replicon initiation is increased despite the presence of unrepaired DNA lesions. The level of correction observed in FA cells injected with SPHAR is most compatible with the second explanation. The fact that SPHAR expression is specific for proliferating cells and that DNA synthesis and cell cycle progression are disturbed in normal cells expressing antisense SPHAR, even without mutagenic treatment, suggests that SPHAR functions independently of DNA damage and repair. This function may be in the regulation of replicon initiation within the replicating cell.

Does SPHAR compete with the D-type cyclins? Although SPHAR is much smaller than the true cyclins, the presence of a cyclin-related motif suggests that its function may be allied to that of the cyclins themselves. The cyclin sequence alignment shown in Fig. 4 can be extended to the N terminus of SPHAR, albeit with weak similarity. Interestingly, the position of the first residue of SPHAR then corresponds exactly to the beginning of the third exon of the yeast cyclin gene *mcs2* (34). Since exons often define the evolutionary borders of structural and/or functional domains, SPHAR probably represents an autonomous folding unit with a cyclin-related domain and, presumably, function. The minimal cyclin A sequence required for binding to, and activation of, cdc2 kinase resides in two blocks within the cyclin box and spans residues 189 to 241 and 275 to 320 (29). The cyclin motif in SPHAR corresponds to residues 293 to 314 of cyclin A and is thus within one of these functional domains.

The inhibition of replicon initiation induced by DNA damage might be mediated by cyclins. In the case of mutagen-treated mammalian cells, there is evidence that it is the direct interaction of the replication fork with DNA lesions which triggers the altered regulation of cyclin B-p34^{cdc2} via the cdc25 kinase (32, 56). Since activated cyclin B-p34^{cdc2} is an absolute prerequisite for mitosis, cell division is thus coupled to the completion of DNA repair and replication via a signal transduction pathway involving cyclin B.

A candidate cyclin for the control of DNA synthesis during S phase is type D cyclin, to which the cyclin-like motif in SPHAR shows the strongest similarity. The involvement of D-type cyclins in S phase was first suggested by their broad expression peak during G_1 phase and S phase. Treatment of quiescent diploid fibroblasts with platelet-derived growth factor or epidermal growth factor leads to DNA synthesis and induction of D-type cyclin mRNA. However, the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces D-type cyclin but not DNA synthesis (62). While cyclin D is clearly associated with DNA replication, its induction is not sufficient for entry into S phase; this is in contrast to the case with cyclin A, which is required for S-phase entry but not for S-phase maintenance (5, 12, 69). The presence of a D-type cyclin-related motif in SPHAR suggests that this protein might compete for the same effectors and induce effects similar, or contrary, to those of cyclin D itself.

Immunoprecipitation experiments have demonstrated that D-type cyclins are complexed with other proteins in the cell (66). In the case of nontransformed cells, these proteins include the protein kinase subunits CDK2, CDK4, and CDK5; a 21-kDa universal cyclin-dependent kinase (CDK) inhibitor, Cip1 (20); and, remarkably, proliferating-cell nuclear antigen (PCNA). The last protein is an auxiliary protein of DNA polymerase δ, and it is essential for both semiconservative DNA synthesis and DNA synthesis during excision repair (41, 49). In cells transformed to immortality by oncogenic viruses, only binary complexes of cyclins and CDKs are observed. Since Cip1 mRNA is upregulated after γ irradiation via the tumor suppressor p53 (6, 64), the quaternary complex of cyclin, p21, PCNA, and CDK is clearly an intermediary in coupling detection of DNA damage to cell cycle control. Furthermore, p21 is able to directly interact with PCNA and arrest DNA replication independently of cyclin and CDK (57). Several pathways for modulating DNA synthesis as a response both to cell cycle triggers and to environmental stress probably exist; it seems likely that these mechanisms use both common and particular components. We suggest that SPHAR, with its cyclin-like motif, is one such component.

Correction of one aspect of the FA group A phenotype by a protein with a cyclin-like motif indicates that the process probably affected by homozygous mutation of the *FA-A* gene is cell cycle control in response to environmental stress.

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