Review Article

Characterisation of the promoter region of the human DNA-repair gene *Rad51*

L. Hasselbach[1], S. Haase[1], D. Fischer[2], H.-C. Kolberg[2], H.-W. Stürzbecher[1]

[1]Institute of Pathology, University Clinic Schleswig-Holstein, Campus Luebeck
[2]Clinic for Gynaecology and Obstetrics, University Clinic Schleswig-Holstein, Campus Luebeck, Luebeck (Germany)

Summary

Purpose of investigation: Regulatory elements of the 5'-flanking region of the DNA-repair gene Rad51 were analysed to characterise pathological alterations of Rad51 mRNA expression during tumour development.

Methods: Various fragments of the Rad51 promoter were cloned into the pGL3 reporter vector and the respective promoter activity was determined by luciferase assays in transfected U2-OS cells. Transcription factor binding was identified using Protein/DNA arrays.

Results: The region encompassing base pairs –204 to –58 was identified as crucial for Rad51 gene transcription. Down regulator sequences are present upstream (–305 to –204) and downstream (–48 and +204) of this core promoter element. Promoter activity is significantly enhanced by substituting G at the polymorphic positions +135 and +172 for C and T, respectively. Transcription factors Ets1/PEA3, E2F1, p53, EGR1, and Stat5 were identified as relevant for regulating expression of Rad51.

Conclusion: We identified three separate cis-sequence elements within the Rad51 transcriptional promoter, one ensuring basal levels of expression and two elements limiting expression to relatively low levels. The characterisation of transcription factor binding might help to explain high-level expression of Rad51 in a variety of solid tumours. The polymorphic sites appear important for the increased risk of breast and/or ovarian cancer for BRCA2 mutation carriers.

Key words: Recombination factor Rad51; Promoter analysis; Gynaecological tumours; Single nucleotide polymorphism.

Introduction

Homologous recombination (HR) is important for the generation of genetic diversity and for chromosome segregation during meiosis, as well as the repair of complex forms of DNA damage, such as double-strand breaks (DSBs), interstrand crosslinks, or DNA adducts in close proximity on opposite strands. DSBs are considered the principal lethal DNA damage resulting from treatment with radiomimetic and crosslinking drugs. In addition, DSBs can arise from endogenous processes, like replication fork stalling during attempted replication over a single-strand break. HR-repair mechanisms are known to resolve such replication fork-associated DSBs [1]. Cell death or impaired cell function and genetic instability can occur if these lesions are left unrepaired or are repaired inaccurately. Thus, maintenance of genetic stability relies heavily on the high fidelity of HR-mediated repair. Cells with mutations in HR genes (e.g. BRCA1, BRCA2, Rad51, Rad54, XRCC2, or XRCC3) exhibit high levels of genetic instability and sensitivity to crosslinking agents and ionising radiation (IR) [2]. Conversely, hyperactivity of the HR pathway also could contribute to genetic instability by causing inappropriate recombination (e.g. between repeat sequences or mutant alleles), resulting in translocations, deletions, duplications, or loss of heterozygosity [3, 4]. Over-expression of Rad51, the key factor of homologous recombination, has been observed in a number of tumour cells [5-8], thus causing elevated rates [9, 10], which may account for some of these observed instabilities. Elevated expression of wild-type Rad51 protein is correlated with histological grading of invasive ductal breast carcinoma [7]. Additionally Rad51 protein interacts with a variety of tumour suppressor proteins like the products of the breast cancer susceptibility genes BRCA1 and BRCA2 [11, 12] and the 'guardian of the genome', p53 [13], indicating a possible association of Rad51 to the process of tumourigenesis.

Despite extensive studies there are only few reports about tumour-derived mutations in the *Rad51* gene [14]. Obviously, mutations in the coding sequence of *Rad51* play only a minor role in tumourigenesis. Several reports indicate loss of heterozygosity (LOH) at the chromosomal location of *Rad51* [15, 16]. Levy-Lahad and co-workers showed that a single-nucleotide polymorphism (SNP) at nucleotide 135 of the non-coding exon-1 of the *Rad51*-gene increases breast cancer risk for *BRCA2*-mutation carriers [17]. Thus, Rad51-mediated repair activity presumably must be tightly regulated to maintain genetic stability.

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In order to understand the regulatory mechanisms governing pathological expression of the *Rad51* gene in gynaecological and other tumours we need information on the organisation of the *Rad51* gene promoter. In the present study we analysed molecular elements involved in the transcriptional regulation of the human *Rad51* gene (HsRad51). We demonstrate that the DNA elements specifically recognised by transcription factors E2F and Ets1/GABP are essential for human *Rad51* gene promoter activity and the transcription factor EGR could be a very important down regulator. Two different SNPs in the first exon of the *Rad51* gene affect *Rad51* gene promoter directed expression *in vitro*. This work should bring important insights not only into homologous recombination mechanisms but also into cell proliferation regulation as well as cancer therapy.

Materials and Methods

Screening of PAC genomic library and sequencing of the 5'- region of the HsRad51

A human genomic PAC library (RPCI1, 3-5 Human PAC Library No.:704, Pieter de Jong, Rosewell Park Cancer Institute) was kindly provided by Resource Center/Primary Database of the German Human Genome Project, Berlin, Germany. This library was screened with a random labelled PCR fragment amplified from human genomic DNA by using the Rad51 specific primers 5'-ATGGCAATGCAGATGCAGCTTGAAGC-3' and 5'-TGGCTTCACTAATTCCCTTA-3'. PAC clone, RPCIP704I24767 was identified as containing the Rad51 gene. The isolated clone was fragmented with restriction enzymes Pst I, Hind III and Eco RI/Eco RV and the fragments were subcloned into the pBluescript SK vector (Stratagene). Clones positive for the target gene fragments were plated on a Hybond-C Nitrocellulose membrane (Amersham) and mapped by means of hybridisation with oligonucleotides spanning the human Rad51 cDNA sequence (DDBI accession no. D14134, Yoshimura *et al.*, 1993). The 5'-flanking region of the human Rad51 gene was sequenced. DNA from target subclones was extracted using the Qiagen DNA purification kit (Qiagen) and sequenced on a MWG automated sequencer (MWG LI-COR LI-4200 system) under the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 50°C for 15 sec and 70°C for 1 min.

Characterisation of the transcription initiation site

Total RNA from the human osteosarcoma U2-OS cell line was isolated with PAXgene/RNAeasy (Qiagen) and mRNA was purified with an Oligotex mRNA kit (Qiagen). To obtain full-length 5' ends of the Rad51 cDNA, full-length RNA-Primer extension was performed with the GeneRacer kit (Invitrogen). A specific primer of Rad51 cDNA, locating downstream of the translation start site, Race-long: 5'-CTCTAACCGTGAAATGGGTTGTGGGCCAAAGC-3' was hybridised to mRNA of Rad51. The procedure was performed as described by the manufacturer. DNA fragments resulting from the RLM–RACE PCR were analysed by electrophoresis on a 3% (w/v) agarose gel, cloned with a TOPO TA Cloning kit (Invitrogen) and sequenced (MWG LI-COR LI-4200 system).

Construction of the reporter gene vectors for promoter analysis

Nested deletions were first constructed using the Erase-a-Base System (Promega) in the fragments F II (-965 to +1430 bp; with regard to the transcription initiation site) to minimise the predicted core promoter region. Polymerase chain reaction was used to generate specific sequence fragments of human Rad51 promoter. Different reactions with special set of primers were run. Each pair of primers contained Kpn I and Hind III restriction sites as linkers to facilitate subcloning (Table 1). The PCR products were cloned in the promoter less pGL3-basic vector (Promega) digested with Kpn I and Hind III. The nucleotide sequences of all constructs were confirmed by sequence analysis (MWG LI-COR LI-4200 system).

Cell maintenance and transfection and detection of luciferase activity

The human osteosarcoma cell line U2-OS was cultured as an adherent monolayer in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal calf serum, Glutamax-1, glucose, non-essential amino acids and penicillin/streptomycin (Life Technologies) at 37°C in an incubator containing air/CO_2 (19:1).

Transfection was carried out by calcium phosphate co-precipitation, as described by Ausubel *et al.* [18]; 1 x 10^6 U2-OS cells were seeded in 6 cm tissue culture plates in DMEM complex medium for 10-15 hours prior to transfection. The cells were treated with 1 M CaCl /HEBS and then transfected with 10 μg pGL3 promoter-luciferase reporter construct (recombinant plasmid). The cells were incubated for an additional 56 hours. Then the cells were washed with PBS and harvested in Reporter Lysis Buffer (Promega). After incubation for 30 min, cell extracts were centrifuged at 12,000 x g for 30 min to pellet the cell debris and protein quantification was performed by BCA Protein Assay (PIERCE, Rockford, USA). Luciferase activity was measured with the Luciferase Assay System (Promega) on a luminometer and expressed as a percentage relative to the luciferase activity obtained with the promoterless vector pGL3-basic. The experiments were performed in duplicate and similar results were obtained from at least three independent experiments.

Plasmid construction and function test of the SNP in the promoter region

Mutagenesis was performed using a direct PCR approach. The following oligonucleotides were used as primers: 5'-GTAAATAAGCTTCGCTCCGACTTCACCCCGCGGG$_g$GTGGCA-3' for the polymorphism in exon 1 at the position 172, and 5'-GTAAATAAGCTTCGCTCCGACTTCACCCCGCGGGCGTGGCACGCGCCCGACCCGCACGGCCCCCAACGCCC$_g$TGGCTTACGCTCCAC-3' for the polymorphism in exon 1 at the position 135. Mutated nucleotides are indicated by small lower-case letters. Reporter vectors containing the respective polymorphism were chosen for large-scale DNA preparation and used in transfection experiments.

Screening of transcription factor binding in the promoter region

Screening of the transcription factors in the promoter region was performed using the TranSignal Protein/DNA Arrays (Panomics, Inc. Redwood City, CA, U.S.A.) according to the instructions of the manufacture. The array allows identification of potential tran-

Table 1. — *Primers used for PCR amplification of Rad51 gene DNA fragments cloned into the pGL3 reporter vector. The primers were designed with the restriction enzyme cut sites Kpn I and Hind III, respectively, at the 5' end.*

F1	5'-CTG ACT GGT ACC AAA GAC GAG GTT TCA CCA CG-3'
	5'-CTA AGC TTC AAG AGG TAA TGC GTC-3'
F2	5'-CAG GTA CCC TCC TGC AAA TCT CCA-3'
	5'-CTA AGC TTC AAG AGG TAA TGC GTC-3'
F3	5'-CAG GTA CCT TCT CGA GCT TCC TCA-3'
	5'-CTA AGC TTC AAG AGG TAA TGC GTC-3'
F4	5'-CAG GTA CCC TCC TGC AAA TCT CCA-3'
	5'-CTA AGC TTC GGC AGT ATC TGA ATG-3'
F5	5'-CTG ACT GGT ACC AAA GAC GAG GTT TCA CCA CG-3'
	5'-CTA AGC TTG TTC ACG CCA GTA ATC-3'
F6	5'-CTG ACT GGT ACC AAA GAC GAG GTT TCA CCA CG-3'
	5'-CTA AGC TTA GGA GTT CAG AGG AAG-3'
F7	5'-CAG GTA CCA CTG CTG GGA TTA CTG-3'
	5'-CTA AGC TTA GGA GTT CAG AGG AAG-3'
F8	5'-CAG GTA CCT GCC GAA ACA AAC CAC-3'
	5'-CTA AGC TTC AAG AGG TAA TGC GTC-3'
F9	5'-CAG GTA CCC TCC TGC AAA TCT CCA-3'
	5'-CTA AGC TTC TCG AGA AGA TGG ATA G-3'
F10	5'-CAG GTA CCT TCT CGA GCT TCC TCA-3'
	5'-CTA AGC TTC GGC AGT ATC TGA ATG-3'
F11	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA CAG AAG CTT CTC ACG CGT GTA GTC C-3'
F12	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA CAG AAG CTT CCA GCG GCT TTC AGA A-3'
F13	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC C-3'
F14	5'-GTA TTA GGT ACC GAC GAT ACT CTC GCC T-3'
	5'-GTA CAG AAG CTT CCA GCG GCT TTC AGA A-3'
F15	5'-GTA TTA GGT ACC GAC GAT ACT CTC GCC T-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC C-3'
F16	5'-GTA TTA GGT ACC TGG GCG AGA GGG TTT G-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC C-3'
F17	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'- GTA CAG AAG CTT TAC TCC TAC CCC AGT C-3'
F18	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA CAG AAG CTT TTC TGC GCA CAA CCC A-3'
F19	5'- GTA TTA GGT ACC TCC CGT CTT GGG TTA G -3'
	5'-GTA CAG AAG CTT TTC TGC GCA CAA CCC A-3'
F20	5'-GTA TTA GGT ACC GAC GAT ACT CTC GCC T-3'
	5'-GTA CAG AAG CTT TTC TGC GCA CAA CCC A-3'
F21	5'-GTA TTA GGT ACC GTT GCC GTG GTT AGC-3'
	5'-TA CAG AAG CTT CCA GCG GCT TTC AGA A-3'
F22	5'-GTA TTA GGT ACC GTT GCC GTG GTT AGC-3'
	5'-GTA CAG AAG CTT CCT GCG CGA GTT TAC-3'
F23	5'-GTA TTA GGT ACC GAA GAG GGC AGT CTG-3'
	5'-GTA CAG AAG CTT CCA GCG GCT TTC AGA A-3'
F24	5'-GCA TCG GGT ACC AAG TTT GAA TTA GTC C-3'
	5'-GTA CAG AAG CTT CGA CGT AAC GTA TCC-3'
F25	5'-GCA TCG GGT ACC AAG TTT GAA TTA GTC C-3'
	5'-GTA AAT AAG CTT AGG GCT CGG TCT CTG-3'
F26	5'-GTA TTA GGT ACC GTT GCC GTG GTT AGC-3'
	5'-GTA CAG AAG CTT CGA CGT AAC GTA TC-3'
F27	5'-GTA TTA GGT ACC GAA GAG GGC AGT CTG-3'
	5'-GTA CAG AAG CTT CGA CGT AAC GTA TCC-3'
F28	5'-GCA TCG GGT ACC AAG TTT GAA TTA GTC C-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GCG TGG CA-3'
F32	5'-GTA TTA GGT ACC CGA CGC ATT ACC TCT TGG-3'
	5'-GTA CAG AAG CTT CTC ACG CGT GTA GTC C-3'
F33	5'-GTA TTA GGT ACC CGA CGC ATT ACC TCT TGG-3'
	5'-GTA CAG AAG CTT CCT GCG CGA GTT TAC-3'
F34	5'-GTA TTA GGT ACC CGA CGC ATT ACC TCT TGG-3'
	5'-GTA CAG AAG CTT CGA CGT AAC GTA TCC-3'
F35	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GCG TGG CAC GCG CCC GAC CCG CAC GGC CCC CAA CGC CCG TGG CTT ACG CTC CAC-3'
F36	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GAG TGG CA-3'
F37	5'-GTA CAA GGT ACC TGC CGA AAC AAA CCA C-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GCG TGG CA-3'
F38	5'-GTA TTA GGT ACC GCG GAC CGC GCG CA-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GCG TGG CAC GCG CCC GAC CCG CAC GGC CCC CAA CGC CCG TGG CTT ACG CTC CAC-3'
F39	5'-GTA TTA GGT ACC GCG GAC CGC GCG CA-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GAG TGG CA-3'
F40	5'-GTA TTA GGT ACC GCG GAC CGC GCG CA-3'
	5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC CCG CGG GCG TGG CA-3'

scription factor binding to the promoter under investigation by competition experiments. The array membrane is covered with 54 different oligonucleotides representing known transcription factor consensus binding sites. The corresponding 54 biotinylated DNA binding oligonucleotides were pre-incubated with U2-OS nuclear extracts to allow formation of DNA/protein complexes. The DNA/protein complexes were separated from the free probes, and the bound probes in the complexes were than extracted and hybridised to the TranSignal membrane. Eight PCR products (F41~F48) from different regions of Rad51 promoter and containing potential transcription factor binding sites were used as inhibitors (Table 2). In competition experiments, these PCR fragments were incubated first with U2-OS cell nuclear extract, and the biotinylated DNA binding oligonucleotides from the array were added subsequently to the system. After hybridisation, the membranes were washed and the signals were detected via chemo-luminescence detection system (Panomics, Inc. Redwood City, CA, USA), following the protocol of the manufacturer.

Table 2. — *PCR fragments used as a competitor for TranSignal Protein/DNA Array. The PCR-fragments encompass Rad51 gene sequences predicted to contain binding sites for the transcription factor mentioned.*

PCR fragment	Primer for PCR	Sequence position of AF203691	Sequence position of Rad51 gene promoter region	Fragment contains predicted binding sites for transcription
F41	Rad-Kpn-429up: 5'-CAG GTA CCT GCC GAA ACA AAC CAC-3´			
	Rad-Hind-553down: 5'-CTA AGC TTC AAG AGG TAA TGC GTC-3´	1003~1127	-543/-419	p53
F42	F11-p53: 5'-GTA TTA GGT ACC CGA CGC ATT ACC TCT TGG-3´			
	Rad-Hind-662down: 5'-GTA CAG AAG CTT TAC TCC TAC CCC AGT C-3´	1111~1236	-435/-310	Myc-Max (Mammalian C-Type LTR poly A downstream element)
F43	F11-p53: 5'-GTA TTA GGT ACC CGA CGC ATT ACC TCT TGG-3´			
	Rad-Hind-750down: 5'-GTA CAG AAG CTT CTC ACG CGT GTA GTC C-3´	1111~1324	-435/-222	EGR1
F44	Rad-Kpn-967up: 5'-GTA TTA GGT ACC GCG GAC CGC GCG CA-3´			
	Rad-Hind-1165down: 5'-GTA AAT AAG CTT CGC TCC GAC TTC ACC C-3´	1541~1739	-5/+204	EGR1; CP2
F46	PCP-Kpn-1342up: 5'-GCA TCG GGT ACC AAG TTT GAA TTA GTC C-3´			
	Last2-Hind-1401down: 5'-GTA CAG AAG CTT CCT GCG CGA GTT TAC-3´	1342~1401	-204/-145	E2F; GKLF
F47	Rad-Kpn-841up: 5'- GTA TTA GGT ACC TCC CGT CTT GGG TTA G-3´			
	Last4-Hind-1488down: 5'-GTA CAG AAG CTT CGA CGT AAC GTA TCC-3´	1415~1488	-131/-58	c-Ets-1, SP1
F48	CEBP-F: 5'-TTA CGT CGA CGC GGG CGT GA-3´			
	Rad-Hind-967down: 5'-GTA CAG AAG CTT CCA GCG GCT TTC AGA A-3´	1480~1541	-66/-5	NF1, Stat5
F49	Last1-Kpn-1241up: 5'-GTA TTA GGT ACC GTT GCC GTG GTT AGC-3´			
	Rad-Hind-750down: 5'-GTA CAG AAG CTT CTC ACG CGT GTA GTC C-3´	1241~1324	-305/-222	AP4

Results

Cloning and characterisation of the 5'-flanking region of the human Rad51 gene

Screening of a human genomic library with a 5'-fragment of the human Rad51 cDNA identified one bacterial phage clone (RPCIP704I24767) encompassing the complete genomic sequence of the human Rad51 gene. Using the method of primer walking we obtained an 8.1 kb genomic fragment upstream of the second intron of human Rad51 gene. (Data submitted to DDBJ/EMBL/GenBank databases, accession no. AF203691). Computer analysis of this genomic fragment revealed that the 5'-UTR involves the first exon. A 3.3 kb nucleotide sequence encompasses the first intron. We did not identify a TATA box, indicating that the human Rad51 gene belongs to a TATA-less GC rich housekeeping gene family. The translation start appears located immediately at the beginning of the second exon.

To confirm the location of the transcription start site and to investigate if other variations of the transcriptional initiation of the Rad51 gene exist, we performed an RNA-based 5'-end-primer extension analysis. For this purpose, full-length RLM–RACE was applied on mRNA isolated from the human osteosarcoma cell line U2-OS with a specific primer of the coding sequence of rad51 (cDNA Primer 5'-CTCTAACCGTGAAATGGGTTGTGGGC-CAAAGCTTTCTTCT-3' (Race-long)). Contrary to reports about several alternative transcriptional initiation sites of the rad51 gene (Strausberg R. GDB BC001459; NCBI Annotation Project, GDB XM_031515), in U2-OS cells no significant extension products of a size different from the expected 319bp product were obtained (data not shown). In agreement with the data reported by Yoshimura (GDB D14134) we identified nucleotides CCGCGC as the start sequence.

To analyse the promoter activity of the cloned 5' region, three DNA fragments of different lengths upstream of ATG were subcloned into the pGL3-Basic vector in front of a luciferase reporter gene and the constructs were named FII (pGL3-rad-(-965/+1430)), FIII (pGL3-rad-(+402/+3564)) and FIV (pGL3-rad-(+402/+1619)), respectively. These constructs were transfected into U2-OS cells. Promoter activity was analysed with the Luciferase Reporter System described in *Materials and Methods*. As shown in Figure 1A, cells transfected with FII (pGL3-rad51-(-964/+1430)) displayed a more than 25-fold luciferase activity compared to the pGL3-Basic vector and the other two constructs containing fragments FIII and FIV. Activity of FII was comparable to the basic activity of the *waf-1* gene promoter in U2-OS cells, used as a positive control.

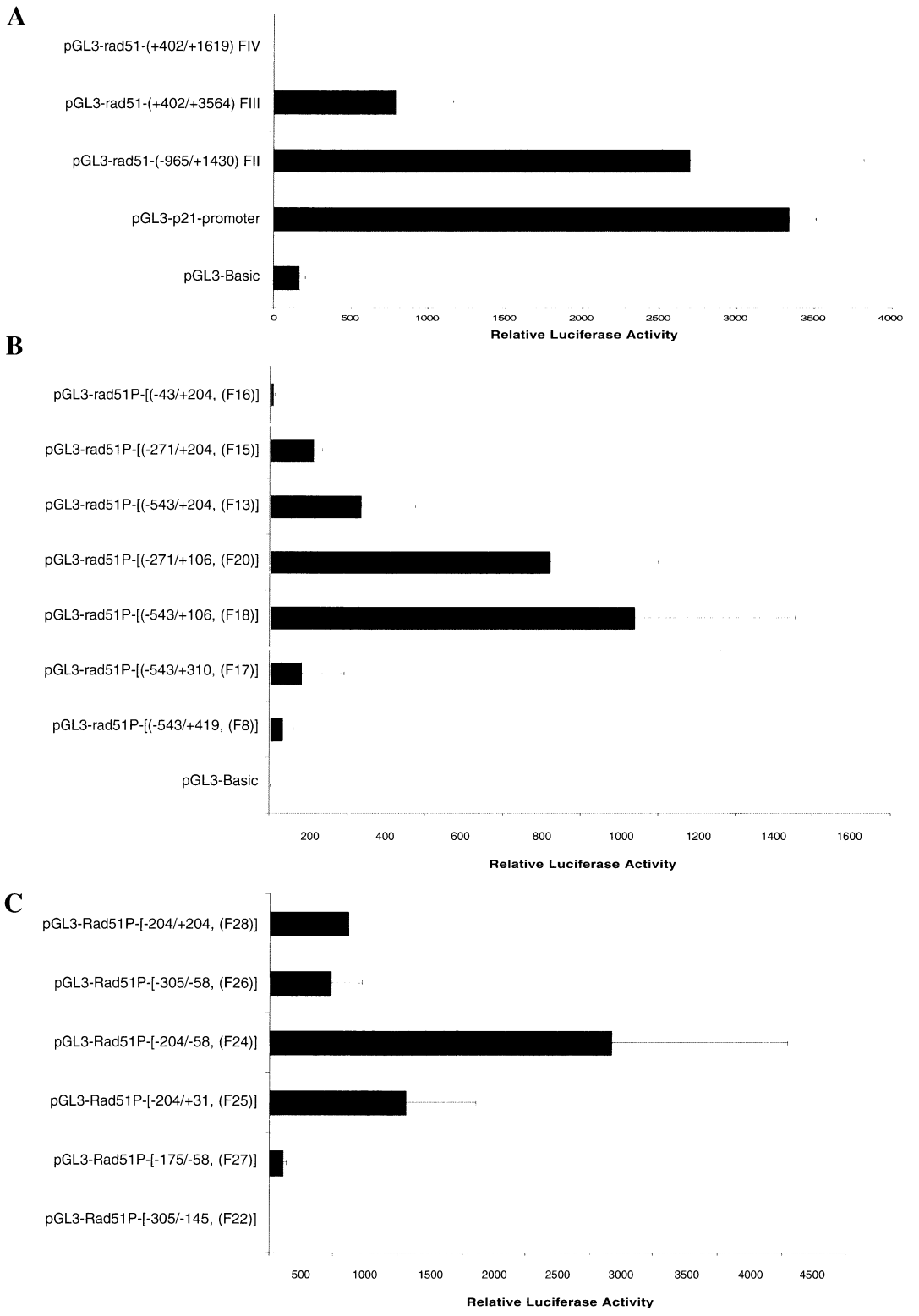

Figure 1. — Promoter activity of the 5'-region of the human Rad51 gene
The respective reporter constructs were transfected into U2-OS cells; 56 hours after transfection luciferase activity was quantified using the Luciferase-Assay-System as described in *Materials and Methods*. All results are means (± S.D.) of at least three independent experiments.
(A) A reporter construct containing the p21 promoter and the promoter-less-vector (pGL3-Basic) were used as positive and negative controls, respectively. Three overlapping fragments spreading 4.5 kb nucleotides upstream of the Rad51 translation start site were subcloned into the pGL3 reporter vector and transfected into U2-OS cells.
(B) Different pGL3-Rad51 constructs based on fragment FII were transiently transfected into U2-OS cells and tested for promoter activity using the Luciferase-Assay-System. The exact position of the Rad51 promoter fragments is given in the figure.
(C) The different pGL3-rad51 constructs were transiently transfected into U2-OS cells and tested for promoter activity using the Luciferase-Assay-System. The exact position of the Rad51 promoter fragments is given in the figure.

Regulatory sequences within the human Rad51 gene promoter

To define the exact DNA sequence involved in the regulation of Rad51 gene expression, we focused our analysis on fragment FII encompassing nucleotides -964 to +1430 of the 5'-flanking region of the Rad51 gene. To narrow down the core promoter region nested deletions were constructed using the Erase-a-Base System (Promega) or using specified polymerase chain reactions within fragment FII (Table 1) and cloning them into the pGL3 reporter vector. The resulting series of pGL3-Rad51P-DNA constructs was tested for promoter activity in transfection experiments. These analyses led us to the 747bp Rad51-reporter-gene construct F13 (pGL3-rad51P-(-543/+204)) (Figure 1B), which contains the 543 base pairs upstream and 204bp downstream of the transcription start site. Constructs F15 (pGL3-rad51P-(-271/+204)) and F16 (pGL3-rad51P-(-43/+204)), containing Rad51 fragments encompassing deletions from the 5'-end of F13 of 272 and 500bp, respectively, displayed reduced promoter activity compared to the full length F13 fragment. However, deletion of 98 and 426bp, respectively, from the 3'-end of F13 (constructs F18 (pGL3-rad51P-(-543/+106)) and F11 (pGL3-rad51P-(-543/-222)) resulted in a dramatic increase of promoter activity (Figure 1B). No significant promoter activity was detected in constructs containing bigger deletions of either the 3'-end of F13 (constructs F17 (pGL3-rad51P-(-543/-310)) and F8 (pGL3-rad51P-(-543/-419))) or of the 5'-end of F13 (construct F16 (pGL3-rad51P-(-43/+204)) (Figure 1B). From these results we conclude that the 649bp rad51 sequence of construct F18 (-543 to +106) contains significant elements of the rad51 gene promoter.

Detailed analysis of this region revealed that the sequence 5' of the transcription initiation site encompassing base pairs –204 to –58 (F24, Figure 1C) displays the highest promoter activity and is crucial for rad51 gene transcription (core promoter element). Shortening this sequence further results in complete loss of promoter activity (F27 and F22; Figure 1C). A significant decrease in luciferase activity was observed with constructs F26 (pGL3-rad51P-(-306/-58)) and F28 (pGL3-rad51P-(-204/+204)) (Figure 1C). These data imply the presence of down regulator sequences upstream (-305 to -204) and downstream (-48 and +204) of the core promoter element.

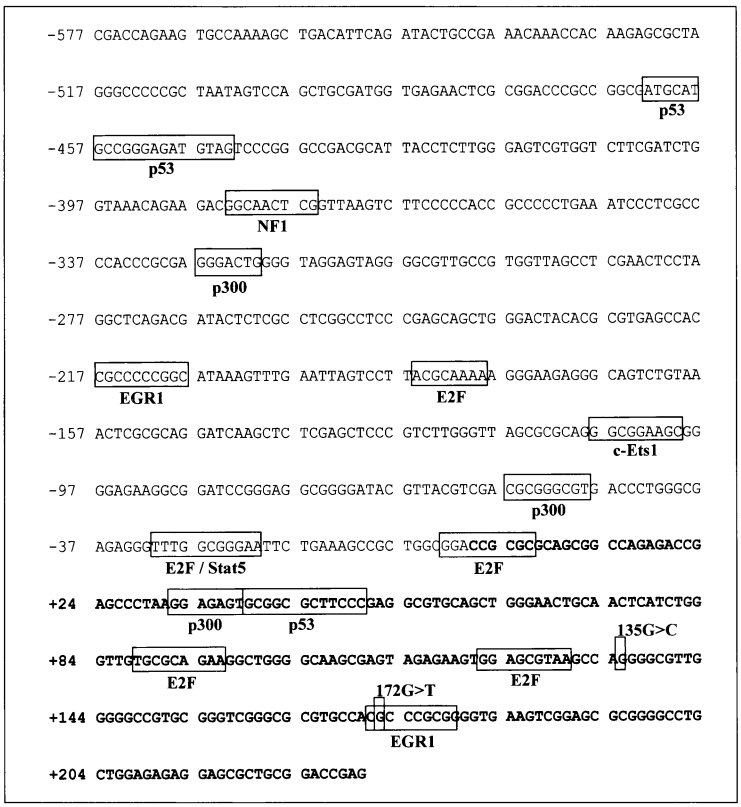

Figure 2. — Nucleotide sequence of the human *Rad51* gene promoter region.

The nucleotide sequence, derived from PAC clone (RPCIP704I24767), starting at base pair 577 bp upstream to nucleotide 204 downstream of the transcription start site is shown. Bold letters indicate the UTR sequence. Nucleotides upstream of the transcription start site have negative numbers. Computer-identified transcription factor binding sites are boxed. The single nucleotide polymorphisms are indicated with larger cursive bold letters. The sequence has been deposited in GenBank under Accession no. AF203691.

Identification of transcription factors that potentially regulate Rad51 gene expression

Computer analysis of fragment F13 (-543/+204) revealed that the sequence is rich in guanine and cytosine residues and contains a number of putative consensus transcription factor binding sites, such as sites recognised by factors GABP, E2F, p53, NF1, SP1 and EGR1 (Figure 2).

To determine experimentally transcription factors that interact with the rad51 gene promoter region the TranSignal Protein/DNA Array was used. This array system measures the interaction of a library of 54 specific transcription factors with a given promoter via competition of a constant number of transcription factor molecules for DNA binding sites displayed on the membrane provided and the target promoter sequence. The different Rad51 promoter sequences, which contained predicted transcription factor binding sites, were amplified by PCR (Table 2) and the mixture of resulting PCR fragments was pre-incubated with U2-OS cell nuclear extract as source of transcription factors. Subsequently, the biotinylated transcription factor oligonucleotide mixture provided by the manufacturer was added. Finally, the mixture was hybridised to the array membrane. Figure 3 shows that the binding signals for several transcription factors were significantly reduced compared to the control membrane indicating binding of these factors to the Rad51 promoter regions used as competitors. These results clearly identify the following transcrip-

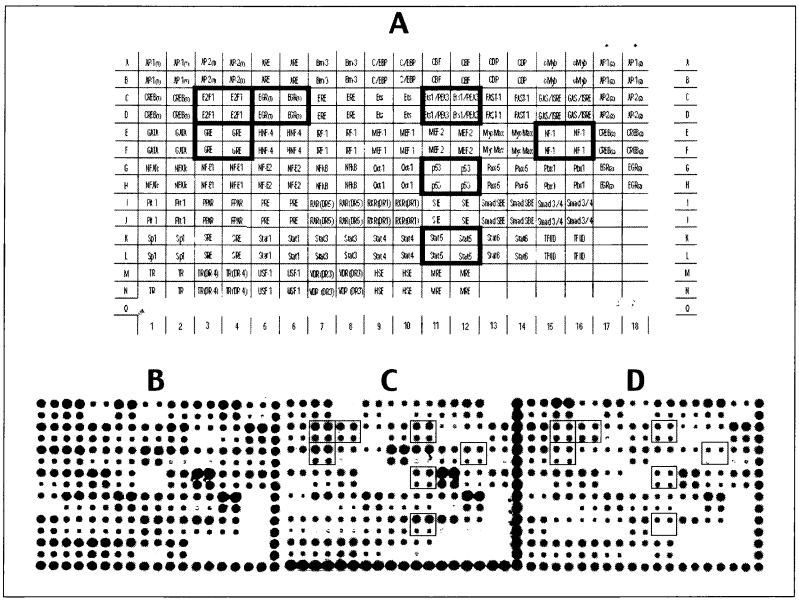

Figure 3. — Screening for transcription factor binding in the human Rad51 gene promoter region
(A) Schematic diagram of the TranSignal Protein/DNA Array. The transcription factors are listed in the order of spotting (in duplicate) on the array. The amount of DNA in the second row of each transcription factor binding site corresponds to a 1:10 dilution. The dark grey columns along the right and bottom sides of the array indicate where biotinylated DNA has been spotted. These spots are intended for alignment.
(B) Control experiment performed with a nuclear extract from HeLa cells, provided by the supplier.
(C) Binding experiment without competition. The nuclear extract from U2-OS cell line was incubated with a mixture of biotinylated oligonucleotides corresponding to the respective transcription factor binding sites (TF oligonucleotide mixture).
(D) The competition test. The cell nuclear extract of U2-OS cell line was first incubated with the mixture of PCR fragment S (F41 to F49) and subsequently the biotinylated TF oligonucleotide mixture was added. The DNA fragments F41 to F49 were amplified from different regions of the *Rad51* promoter. Signal reduction was used as a measure for binding of the respective transcription factor to the Rad51 promoter. Spots showing significant binding of the respective transcription factor to the Rad51 promoter are framed.

tion factors as potentially relevant for regulating the expression of the Rad51 promoter: Ets1/PEA3, E2F1, p53, NF1, EGR1, Stat5, and GRE (Figure 3).

Effect of single nucleotide changes at the position 135 and 172 of Exon 1 on Rad51 promoter activity

To determine whether the polymorphisms at the positions +135 and +172 of exon 1 of the human Rad51 gene influence Rad51 gene expression, six reporter constructs were analysed containing either the normal nucleotide (wild-type) or the respective base change (single nucleotide polymorphism (SNP)) at the respective positions. The constructs F13 (pGL3-rad51,-543/+204) and F16 (pGL3-rad51,-5/+204) were altered either at position +135 to exchange wild-type nucleotide G to C, found associated with elevated breast cancer risk for BRCA2 mutation carriers [17] or at position +172 to exchange wild-type nucleotide G to T, found in about 20% of the total population (Haase and Stuerzbecher, unpublished).

These constructs were transiently transfected into U2-OS cells and the corresponding luciferase activities were quantified. The results presented in Figure 4 demonstrate that the single nucleotide change in both positions significantly enhanced Rad51 promoter activity compared to the constructs with the corresponding wild-type nucleotide at the respective positions.

Discussion

In this study, we have cloned and characterised a promoter region of the human Rad51 gene. The basic promoter region is 747 nucleotides in length (-543 to +204 bp, relative to the transcriptional start site (F13)) and contains three separate cis-sequence elements within the Rad51 transcriptional promoter, not yet by comparative analysis, but by expression of various reporter constructs in vivo. The first element, positioned between -204 and -58 bp (F24) ensures basal levels of expression since truncation of this Rad51 promoter element to either -175 to -58 bp (F27) or to -305 to -145 bp (F22) completely eliminates significant expression of the luciferase reporter (Figure 1). A second sequence element, located within the 262 bp between -58 to +204, specifically limits Rad51 expression to relatively low levels. Progressively, larger truncations of this sequence from the 3' terminus of reporter constructs result in significantly higher basal luciferase activity. A third promoter element located between -305 to -204 bp is also required for down-regulating Rad51 expression in reporter assays (F26). Fragments encompassing base pairs -543 to -310 do not display significant promoter activity in reporter assays (F17 and F8).

The Rad51 promoter region contains sequence elements that are sites for transcription factor binding. Figure 1 and especially the transcription factor binding array shown in Figure 3 reveal that the Factors E2F and c-Ets1 play an important role in regulating Rad51 gene expression. Elimination of sequences encompassing the E2F site located between -204 and -175 or the c-Ets1 site -145 to -58 completely abolishes Rad51 promoter activity (Figure 1C).

Ets/PEA3 binding sites are common enhancer elements in eukaryotic genes and are also found near the transcriptional start sites of many TATA-less promoters. It functions as a minimal transcriptional initiator element as well as a transcriptional activator of genes like rat prolactin and BRCA1 [19-21].

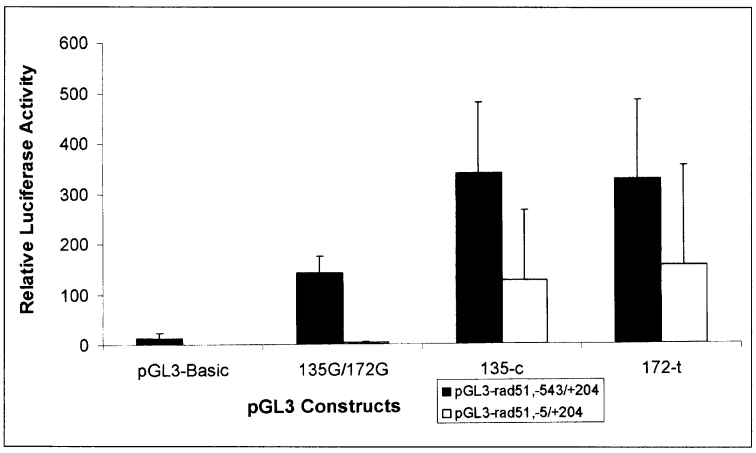

Figure 4. — Functional analyses of the single nucleotide polymorphisms in non-coding exon 1 of the Rad51 gene.
The constructs 135G/172G contain the wild-type sequence at the respective nucleotides. Using site-directed-mutation a G to C change at the position 135 (135-c) and a G to T change at position 172 (172-t) of exon 1 were introduced. The respective reporter constructs were transfected into U2-OS cells. 56 hours after transfection luciferase activity was quantified using the Luciferase-Assay-System as described in *Materials and Methods*. All results are means (± S.D.) of at least three independent experiments.

The E2F family of transcription factors plays a crucial role in the control of cell cycle progression by regulating the expression of genes required for G1/S transition including genes encoding DNA replication proteins, enzymes involved in nucleotide synthesis and components of the origin recognition complex. It is well known that E2F transcription factors determine whether or not a cell will divide by controlling the expression of key cell-cycle regulators, for example it regulates the cdk inhibitor p21 gene or induces the DHFR gene expression during G_1 to S phase transition [22–25]. In agreement with the data presented here Iwanaga and coworkers reported that Rad51 gene expression like the activity of the mismatch repair genes MSH2 and MLH1 is clearly growth regulated and that the Rad51 gene is mainly mediated by E2F [26]. Our data suggest that the E2F site located between -204 to -175 appears especially important for basic Rad51 expression and that other E2F sites located downstream of -58 might be involved in limiting expression under normal growth conditions. Alterations in transcription of genes regulated by members of the E2F family of transcription factors can be viewed as reflecting a constant battle between repressor and activator complexes [27]. Importantly, various chromatin regulatory complexes have been linked to E2F proteins and changes in histone modifications correlate with states of E2F-dependent transcription. CBP/p300 has been proposed to be involved in these processes as a co-activator of E2F [28]. Interestingly, there are several binding sites for p300 in the regions of the Rad51 involved in down-regulation of expression (Figure 1 and Figure 2).

Another important factor involved in this aspect of rad51 regulation might be the tumour suppressor p53. We identified two potential p53 binding sites in the vicinity of the rad51 basal promoter and characterised p53 as interacting with these sites in our array analysis (Figure 3). As for E2F, local changes in histone acetylation have also been observed at the promoters of a number of p53 target genes [29]. Examples of p53-dependent transcriptional regulation by local chromatin remodelling include i) transcriptional repression by p53 through recruitment of histone deacetylase activity and targeted core histone deacetylation at the Map 4 promoter ii) p53 transcriptional activation at the $p21^{Waf-1}$ promoter mediated by displacement of HDAC1, recruitment of the histone acetyltransferases p300, CBP and TRRAP, targeted histone acetylation and nucleosomal remodelling by ATP-dependent remodelling components hSNF5 and hBRG1 [29]. These considerations suggest potential roles for p53 and E2F directed chromatin remodelling in the regulation of the Rad51 promoter which have to be tested experimentally.

We observed that reporter constructs containing EGR1 binding sites either upstream or downstream of the Rad51 minimal promoter region, displayed decreased promoter activity (Figure 1). These data together with Figure 3 imply that the transcription factor EGR1 could act as a repressor in the regulation of Rad51 gene expression. The product of the early growth response (EGR1) gene is a transcription factor known as a potential regulator of more than 30 genes playing a role in growth, development and differentiation. In addition, EGR1 exhibits significant transformation suppressing activity. Liu *et al.* reported that EGR1 suppresses growth of human HT-1080 fibrosarcoma cells by induction of transforming growth factor β1 (TGF-β1). The authors proposed that the induction of TGF-β1 could be one of the mechanisms of EGR1 tumour suppressor activity [30]. Kanamoto and colleagues presented that TGF-β can promote DNA instability through down-regulation of Rad51 gene expression and inhibition of DNA repair [31]. In view of their data and the results presented here we propose a TGF-β1-EGR1-Rad51 pathway which leads to down-regulation of Rad51 expression in response to TGF-β1. However, this TGF-β1-EGR1-Rad51 pathway might be more complex. Nair and coworkers reported that not only TGF-β1 but also p53 is involved in EGR1 dependent apoptosis, since EGR1 transactivates p53 gene expression and inhibition of p53 function, on the other hand, abrogates EGR1-dependent apoptosis [32].

The down-regulating element downstream of base pair -58 also contains a binding site for Stat transcription factors. In our screening for transcription factors using the Protein/DNA array, the signal reduction at the Stat5 spot clearly implies the involvement of this transcription factor in regulating expression of the Rad51 gene. This finding is in agreement with a report by Huang *et al.* who reported that Rad51 over-expression is mediated by Stat5-dependent transcription [33]. The transcription factor Stat5 is constitutively activated by tyrosine phosphorylation after transforma-

tion of hematopoietic cells by p210Bcr/Abl. BCR/ABL significantly enhances the expression of Rad51 and several Rad51-Paralogs. In cells expressing the BCR/ABL oncogenic tyrosine kinase, over-expression of Rad51 is important for cellular resistance to cisplatin and mitomycin C treatment [34]. High-level expression of Rad51 is characteristic of a variety of solid tumours [6, 7] as is drug resistance of Rad51 over-expressing cell lines [35]. Our data on positioning and characterising the Stat5 binding site in the rad51 promoter region might thus help to explain the physical and regulatory basis for these observations.

BRCA1 and BRCA2, the two major hereditary breast cancer susceptibility genes, are associated with early-onset breast and/or ovarian cancer, encode products that each interact with the rad51 gene product and participate in a common DNA damage response pathway associated with the activation of homologous recombination and DSB repair [11, 12]. In 2001 Levi-Lahad and co-workers presented evidence that a single nucleotide polymorphism (SNP) in the 5'-untranslated region of rad51 modifies cancer risk in BRCA2 carriers. This SNP, designated 135g/c, is a substitution of C to G at position 135 in the Rad51 cDNA. Survival analysis in BRCA2 carriers showed that 135C increased the risk of breast and/or ovarian cancer with a hazard ratio of 4.0 [17]. In our sequence analysis of the 5'-region of the Rad51 gene, we found another single nucleotide change at the position 172 of exon 1. The single nucleotide polymorphism either at position 135 or at position 172 of exon 1 from wild-type G to either C or T, respectively, appears to alleviate the repressing function of the region downstream of base pair -58 (Figure 4). Furthermore, eliminating just nucleotides +107 to +204 from Rad51 reporter constructs leads to a significant increase in promoter activity (F20, F18; Figure 1). Our analysis does not discriminate between effects mediated by transcription factors binding to this region of the 5'-untranslated region of the Rad51 gene and direct effects of the SNPs described. However, these results underline the importance of the sequence encompassing the polymorphic sites for Rad51 gene expression and might help to explain the molecular basis of the increased risk of breast and/or ovarian cancer for BRCA2 mutation carriers.

Conclusion

We identified three separate cis-sequence elements within the Rad51 transcriptional promoter. The first element, positioned between -204 and -58 bp ensures basal levels of expression, two sequence elements, located within the 262 bp between -58 to +204 and between -305 to -204 bp, specifically limit Rad51 expression to relatively low levels. Transcription factors E2F and c-Ets1 play an important role in regulating Rad51 gene expression. In addition, p53 and E2F directed chromatin remodelling might play a role in the regulation of the Rad51 promoter. Furthermore, we propose a TGFβ1-EGR1-Rad51 pathway which leads to down-regulation of Rad51 expression in response to TGFβ1. Our data on positioning and characterising the Stat5 binding site in the Rad51 promoter region might help to explain the physical and regulatory basis for high-level expression of Rad51 protein in a variety of solid tumours. The polymorphic sites at base pairs +135 and +172 significantly effect Rad51 expression and, thus, appear important for the increased risk of breast and/or ovarian cancer for BRCA2 mutation carriers. In summary, the data presented provide primary information about the organisation of the Rad51 promoter and its regulatory elements and will deepen our understanding of pathological alterations of Rad51 expression during cancer development.

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Address reprint requests to:
H.W. STUERZBECHER, Ph.D.
Institute of Pathology,
University Clinic Schleswig-Holstein
Campus Luebeck
Ratzeburger Allee 160
Luebeck D-23538 (Germany)