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Lack of Gene- and Strand-Specific DNA Repair in RNA Polymerase III-Transcribed Human tRNA Genes

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UV light induces DNA lesions which are removed by nucleotide excision repair. Genes transcribed by RNA polymerase II are repaired faster than the flanking chromatin, and the transcribed strand is repaired faster than the coding strand. Transcription-coupled repair is not seen in RNA polymerase I-transcribed human rRNA genes. Since repair of genes transcribed by RNA polymerase III has not been analyzed before, we investigated DNA repair of tRNA genes after irradiation of human fibroblasts with UVC. We studied the repair of UV-induced cyclobutane pyrimidine dimers at nucleotide resolution by ligation-mediated PCR. A single-copy gene encoding selenocysteine tRNA, a tRNA valine gene, and their flanking sequences were analyzed. Protein-DNA footprinting showed that both genes were occupied by regulatory factors in vivo, and Northern blotting and nuclear run-on analysis of the tRNA indicated that these genes were actively transcribed. We found that both genes were repaired slower than RNA polymerase II-transcribed genes. No major difference between repair of the transcribed and the coding DNA strands was detected. Transcribed sequences of the tRNA genes were not repaired faster than flanking sequences. Indeed, several sequence positions in the 5′ flanking region of the $tRNA^{Val}$ gene were repaired more efficiently than the gene itself. These results indicate that unlike RNA polymerase II, RNA polymerase III has no stimulatory effect on DNA repair. Since tRNA genes are covered by the regulatory factor TFIIIC and RNA polymerase III, these proteins may actually inhibit the DNA's accessibility to repair enzymes.

Irradiation of DNA with UV light induces mutagenic DNA lesions, and exposure to sunlight has been linked to the development of human skin cancer. UVB (280 to 320 nm) and UVC (200 to 280 nm) irradiation of DNA produces two major photoproducts: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (for reviews, see references 6, 35, 44, and 61). CPDs are formed between two adjacent pyrimidines: 5′-TpT, 5′-TpC, 5′-CpT, or 5′-CpC. The rates of formation and repair of CPDs vary strongly due to structural features of chromatin and DNA sequence (53, 61). Since CPDs are repaired much more slowly than pyrimidine (6-4) pyrimidone photoproducts, CPDs are thought to be the most mutagenic UV lesions in mammalian cells (6, 44). Efficient DNA repair must occur before DNA replication to prevent the formation of stable transition mutations. C-to-T transitions are the major class of UV-induced mutations. UV-induced mutations were previously studied in RNA polymerase III-transcribed suppressor tRNA genes in *Saccharomyces cerevisiae* (1, 2) or tRNA marker genes propagated in human cells (7, 49). Intriguingly, there was a preference for UV-induced mutations to occur at sites where the dipyrimidine was on the RNA polymerase III-transcribed strand (1, 2). On the other hand, in genes transcribed by RNA polymerase II, mutations can be ascribed to dipyrimidine photoproducts on the nontranscribed strand (68). RNA polymerase II genes are repaired faster than the flanking chromatin, and the transcribed strand is repaired faster than the coding strand (5, 34, 50, 64, 65). In *Escherichia coli*, a transcription repair coupling factor encoded by the *mfd* gene is involved in strand-specific repair (50, 51). The Cockayne syndrome complementation group B gene product (63) and perhaps the general transcription factor TFIIH, which contains components of the nucleotide excision repair machinery (14, 47), seem to be essential links between RNA polymerase II transcription and DNA repair in mammalian cells (20, 61). Transcription-coupled repair could not be detected in RNA polymerase I-transcribed human rRNA genes (9, 15, 55, 67), although in yeast, repair of rRNA genes can be strand specific and probably transcription coupled as revealed in specific nucleotide excision repair mutants (66). The repair of genes transcribed by RNA polymerase III remains to be elucidated, since the genes are too small to be analyzed by Southern blot DNA repair assays.

tRNA genes, 5S rRNA genes, and some small nuclear RNA genes are transcribed by RNA polymerase III (for reviews, see references 18, 22, and 54). The general transcription factor TFIIIC binds to the intragenic box A and box B promoter elements of the tRNA genes and is an assembly factor for TFIIIB on TATA-less tRNA gene promoters (for a review, see reference 18). TFIIIB is the factor that contains the TATA-binding protein (TBP) and recruits RNA polymerase III for multiple rounds of transcription (13, 24). tRNAs are relatively stable, abundant RNA species found in most cell types. tRNA levels change in response to growth conditions and are generally lower in resting cells than in growing cells (25, 31). RNA polymerase III transcriptional activity is lower in the G_0 and G_1 phases of the cell cycle than in S and G_2 (10, 69), although the difference is often only twofold. Most tRNAs are encoded by dispersed multigene families of 10 to 20 members in eukaryotes, including gene variants and pseudogenes. More than 10 members of the human valine tRNA ($tRNA^{Val}$) gene family have been cloned and characterized (11, 52, 56). One member of the human $tRNA^{Val}$ gene family, designated clone pHtV8, is highly expressed in HeLa cells and human placenta (48). There is a human single-copy tRNA gene encoding the RNA polymerase III-transcribed selenocysteyl-$tRNA^{(Ser)Sec}$ ($tRNA^{Sec}$) gene, which was previously designated an opal suppressor (phospho)seryl-tRNA gene (28, 29, 33, 38). Its function is twofold: to serve as a carrier molecule upon which

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selenocysteine is biosynthesized and to serve as a direct donor of selenocysteine, which is the 21st naturally occurring amino acid, during protein synthesis (e.g., glutathione peroxidase) in response to specific UGA codons (27, 30). Genes coding for $tRNA^{Sec}$ were shown to be ubiquitous not only within the animal kingdom (27) but also in *E. coli* and in all other enterobacteria (21).

Earlier work from our laboratory demonstrated that ligation-mediated PCR (LMPCR) is a sensitive method for in vivo mapping of DNA adducts at nucleotide resolution (39–41). LMPCR is the preferred method to analyze CPD formation in small human genes or gene segments and to quantitate repair rates (16, 59, 64). In this work, we analyzed the formation and repair of CPDs in two tRNA genes and flanking regions in UV-irradiated human fibroblasts by LMPCR. Transcriptional activity of the $tRNA^{Sec}$ gene and a $tRNA^{Val}$ gene was determined by genomic footprinting, Northern blotting, and nuclear run-on analysis. In both tRNA genes, there was no preferential repair of the genes compared to the flanking regions, and a selective removal of DNA lesions from the transcribed DNA strand was not observed.

MATERIALS AND METHODS

Cell culture, UV irradiation, and genomic footprinting. The growth and UV irradiation of human foreskin fibroblasts was performed as previously described (62, 64). Briefly, contact-inhibited monolayers of fibroblasts were irradiated at a dose of 20 J/m^2 with a 254-nm-wavelength lamp (UVC). Nonirradiated cells and cells collected immediately after irradiation were used as negative controls (no UV) and positive controls (no repair), respectively. For DNA repair experiments, cells were incubated for various periods of time in the original medium.

For genomic footprinting experiments, human fibroblasts and naked DNA were treated with 0.2% dimethyl sulfate (DMS) as previously described (42, 45, 57).

Northern blot analysis. Total cellular RNAs from nonirradiated fibroblasts and from fibroblasts at various times after UV irradiation were isolated by the guanidinium isothiocyanate method (RNAgents; Promega, Madison, Wis.). Northern blot analysis was done similarly as previously described (37). Two micrograms of RNA was separated through 8% polyacrylamide–7 M urea gels and electroblotted onto a nylon membrane (GeneScreen) by using an electrotransfer device (Owl Scientific, Cambridge, Mass.). A radiolabeled 10-bp DNA ladder (GibcoBRL, Gaithersburg, Md.) was used as a length marker. Hybridization and prehybridization were performed at 50°C in a mixture containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM NaH_2PO_4, 2× Denhardt solution, 100 μg of salmon sperm DNA per ml, and 0.2% sodium dodecyl sulfate (SDS). The radiolabeled SCN-1 and V8N-3 oligonucleotides (see below) were used to probe for $tRNA^{Sec}$ and $tRNA^{Val}$, respectively. The oligonucleotides were complementary to tRNA sequences between box A and box B. All 5′ end labeling was performed by using T4 polynucleotide kinase and [γ-^{32}P]ATP (46). After overnight hybridization, the blots were washed four times for 10 min each at 50°C in 2× SSC–0.2% SDS and analyzed by autoradiography.

Nuclear run-on assays. Fibroblasts were grown as contact-inhibited monolayers and were UV irradiated as described above. Nuclear run-on transcription was carried out by the method of Wright and Bishop (71). Cell pellets (8×10^6 cells per assay) were lysed in 4 ml of reticulocyte standard buffer (10 mM Tris-Cl [pH 7.7], 10 mM NaCl, 5 mM $MgCl_2$) with 0.5% Nonidet P-40. Nuclei were resuspended in 250 μl of 25 mM dithiothreitol–90 mM KCl–5 mM $MgCl_2$–1 mM $MnCl_2$–10 mM Tris-Cl [pH 7.7]–1 mM ATP–1 mM CTP–1 mM GTP–25% (vol/vol) glycerol–250 μCi of [α-^{32}P]UTP and incubated at 28°C for 30 min. RNA was isolated by a guanidinium isothiocyanate method (RNAzol B; Tel-Test, Friendswood, Tex.) and further purified by passage over a Sephadex G-50 column.

Plasmid DNAs containing the various inserts were constructed by cloning PCR products into the pCRII vector (Invitrogen, San Diego, Calif.). To analyze the rate of transcription of the $tRNA^{Sec}$ gene, PCR products made with primers SCB-3 and SCB-6 (see below) were cloned, and for the transcripts of the $tRNA^{Val}$ gene, PCR products made with primers V8A-3 and V8A-6 were used. To analyze the transcription of the *JUN* gene, the 5′ end of the gene was amplified with primers E-3 and F-3 and cloned into the pCRII vector. Linearized, denatured DNA probes (10 μg) were applied to GeneScreen Plus nylon membrane filters with a dot blot apparatus (GibcoBRL). Hybridization and prehybridization were performed at 42°C in a mixture containing 5× SSC, 50% formamide, 5× Denhardt solution, 100 μg of salmon sperm DNA per ml, and 1% SDS. Labeled nuclear RNAs were hybridized to filters in a volume of 2 ml for 48 h at 42°C. Filters were washed in 0.1× SSC–1% SDS at 65°C and then treated with RNase A (10 μg/ml) in 2× SSC at 37°C for 15 min. Filters were washed in 0.1× SSC at 65°C and analyzed by autoradiography.

DNA isolation and cleavage at CPDs. Cells were lysed and DNA was purified as described previously (62). DNA was dissolved in Tris-EDTA buffer to a concentration of 0.2 μg/μl. The DNA was cleaved to completion with T4 endonuclease V for 1 h at 37°C and then treated with *E. coli* photolyase to generate ligatable ends (41, 62). The digested DNA was purified by phenol-chloroform extraction and ethanol precipitation and was dissolved in Tris-EDTA buffer at a concentration of 1 μg/μl. Damage and repair of CPDs in the total genome were determined by running digested DNA through 0.6% alkaline agarose gels.

LMPCR. LMPCR of the cleaved DNA was performed as previously described (42, 62). The amplified fragments were separated on 8% polyacrylamide–7 M urea gels. The gels were electroblotted onto nylon membranes, and the sequences were visualized by autoradiography after hybridization with a single-stranded gene-specific PCR probe (58, 62). Chemical DNA sequencing reactions (32), performed with genomic DNA from HeLa cells, were used as controls with all LMPCRs and to provide markers.

Oligonucleotide primers. All primers were designed with the Oligo 4.0 computer program (43). The following oligonucleotides were used to analyze the $tRNA^{Sec}$ gene: SCB-1 (5′ATGAGTGGGGAGAACTTATAGC), SCB-2 (5′GCCCTTGAAGTCAACCATCTCACACCTTTC), SCB-3 (5′CTTTCCAAAGGACGCGACCATAACTCTA), SCB-4 (5′CGGGCAACAAGCAAAATA), SCB-5 (5′GGGCAACAAGCAAAATAAGTCCGGTTCG), and SCB-6 (5′GTCCGGTTCGATAAGTAAGATTCAAGGC). For the $tRNA^{Val}$ gene, family member pHtV8, the following primers were used: V8A-1 (5′TCTGGGGAAGAATGGATCTA), V8A-2 (5′TGGATCTAAGGGCCATGACTCCATG), V8A-3 (5′AAGCCGACACCTGGATGTTGACTAT), V8A-4 (5′TTAATTGGCAATGGATTGG), V8A-5 (5′AATCCGGCCGGTGTCTAAGTGTAGC), and V8A-6 (5′TGTAGCAAAACTTGTGAACGTTTATACACA). To analyze the 5′ flanking region of the $tRNA^{Val}$ gene, these primers were used: V8C-1 (5′TAATTGCTGGAAGTGACTGG), V8C-2 (5′CCCTTAGAGGTCACGCTTTACAGTTTGA), V8C-3 (5′TACAGTTTGACAAACGGCATTACGC), V8C-4 (5′TGAACGTTTATACACATAGACTCC), V8C-5 (5′CCACACTAAGAAACCTTTTTGAACTGGAAG), and V8C-6 (5′TGAACTGGAAGGAAAACACAGTCTTGTT). Oligonucleotide primers numbered 1, 2, and 3 and primers numbered 4, 5, and 6 were used to analyze the lower and the upper strands of the genes, respectively. For the Northern blot analysis primers SCN-1 (5′TGTCGCTAGACAGCTACAGGT) and V8N-3 (5′GTGTTAGGCGAACGTGATAAC) were used to detect $tRNA^{Sec}$ and $tRNA^{Val}$, respectively. To clone the 5′ end of the *JUN* gene, primers E-3 (5′ACTGGTAGCAGATAAGTGTTGAGCTCGG) and F-3 (5′CTCTGGACACTCCCGAACCACCAG) were used.

Quantitation of repair rates. Nylon membranes were exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and radioactivity in CPD signals was determined with the ImageQuaNT computer program as previously described (64). Background values were subtracted. A repair curve was established for each position that gave a sufficient signal above background. The time at which 50% of the initial damage was removed was determined from this curve. This value was incorporated into Fig. 7.

RESULTS

Expression of $tRNA^{Sec}$ and $tRNA^{Val}$ genes. To investigate transcription of the two tRNA genes in human cells, we UV irradiated fibroblasts with 20 J/m^2 and determined the levels of $tRNA^{Sec}$ and $tRNA^{Val}$ by Northern blot analysis (Fig. 1a). Total RNA was isolated from fibroblasts, separated on a denaturing 8% polyacrylamide–7 M urea gel, electroblotted, and hybridized with specific radiolabeled primers. Figure 1a shows $tRNA^{Sec}$ and $tRNA^{Val}$ levels in fibroblasts. There was no significant change in the transcript levels of the single-copy $tRNA^{Sec}$ gene or the $tRNA^{Val}$ genes before and after UV irradiation.

Although at the UV dose used, an inhibition of transcription elongation is unlikely (the probability of a CPD in the tRNA gene is approximately 1%), an effect of UV on transcription initiation could not be ruled out. In addition, the stability of the tRNA species in contact-arrested fibroblasts is not known. For these reasons, we carried out nuclear run-on assays to demonstrate transcription of the tRNA genes. Figure 1b shows that both genes are actively transcribed in contact-arrested fibroblasts before and after UV irradiation. While the rate of transcription of the $tRNA^{Sec}$ gene does not change noticeably after UV irradiation, the signal for $tRNA^{Val}$ increases at the later time points, although this is not reflected in increased mRNA levels. There is a slight increase in transcription of the *JUN* gene immediately after UV irradiation (64), which served as a

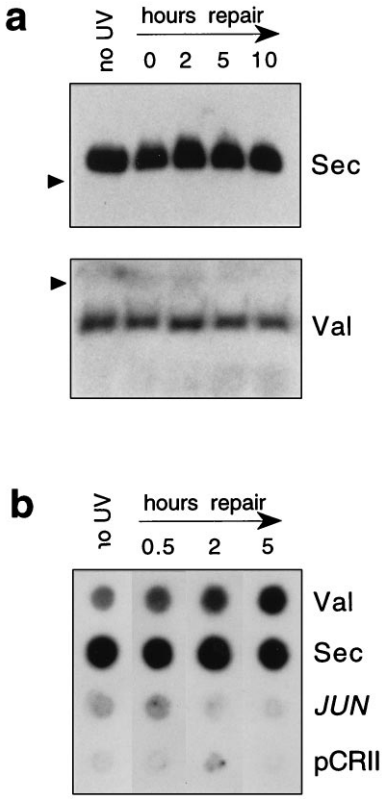

FIG. 1. RNA analysis of tRNAs in UV-irradiated human fibroblasts. Cells were harvested at the indicated time points after 254-nm irradiation at 20 J/m^2. (a) Northern blot analysis of tRNAs. Total RNA was isolated and separated on 8% polyacrylamide–7 M urea gels, electroblotted to nylon membranes, and hybridized with radiolabeled oligonucleotides specific for the indicated tRNAs. The arrowheads indicate the position of a comigrating 80-bp single-stranded DNA marker. The oligonucleotide SCN-1 was used as a hybridization probe for tRNA^{Sec}, and oligonucleotide V8N-3 was used for tRNA^{Val}. (b) Nuclear run-on assay to assess the transcription of the tRNA^{Sec} and tRNA^{Val} genes. Nuclei (8×10^6) were isolated and incubated under run-on conditions. The labeled nuclear RNAs were hybridized to 10 μg of dot-blotted DNA probes.

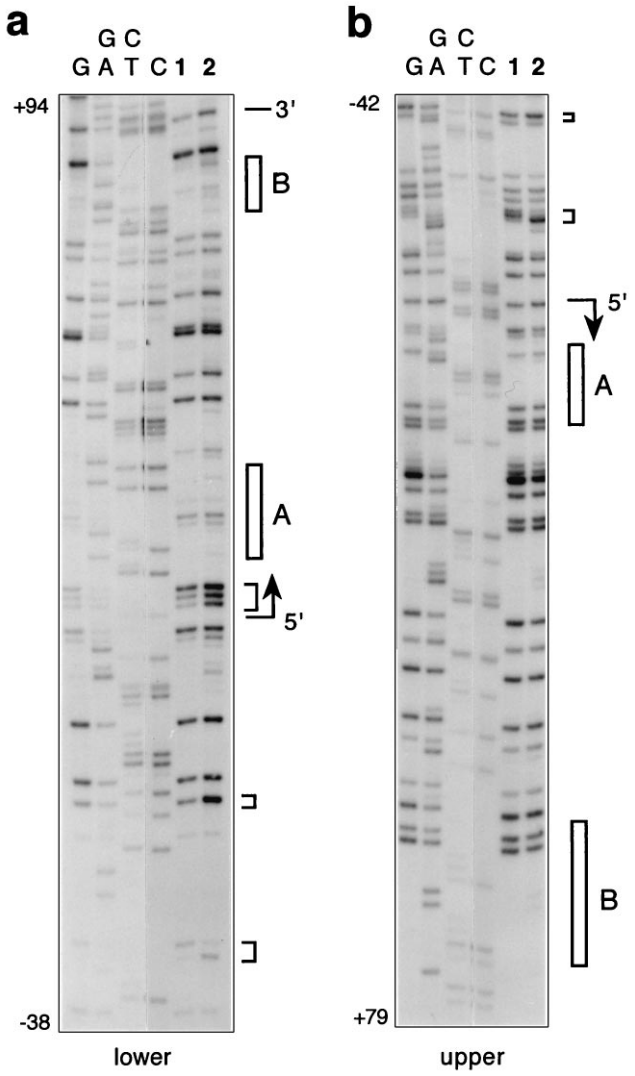

FIG. 2. Genomic footprinting of the tRNA^{Sec} gene in human fibroblasts. Lanes 1, fibroblast DNA treated with DMS in vitro; lanes 2, DNA isolated from fibroblasts treated with DMS in vivo. Maxam-Gilbert control sequences are in lanes G, GA, CT, and C. The 5′ and the 3′ ends of the tRNA^{Sec} gene are indicated. The open boxes A and B indicate the DNA binding sites of transcription factor TFIIIC. DMS footprints are indicated with brackets. (a) The autoradiography shows the lower (transcribed) strand of the region spanning nt −38 to +94. Primers SCB-1 to SCB-3 were used for LMPCR. (b) The sequences cover the upper (nontranscribed) strand of the region spanning nt −42 to +79. Primers SCB-4 to SCB-6 were used for LMPCR.

control. The rates of transcription of the tRNA genes appear to be significantly higher than that of the *JUN* gene.

Genomic footprinting of the tRNA^{Sec} and tRNA^{Val} genes. In tRNA genes of eukaryotes, two transcription factors are essential for RNA polymerase III transcription (17–19). TFIIIC binds to the internal control sequences (box A and box B). This complex is necessary for the binding of TFIIIB to a proximal upstream site. TFIIIB serves for the correct positioning of RNA polymerase III over the transcription start site. We used DMS footprinting in vivo to detect specific transcription factors bound at regulatory sequences of the tRNA^{Sec} and tRNA^{Val} genes. Fibroblasts were treated with DMS, which reacts predominantly with guanines (Gs). DNA was isolated and cleaved at modified bases with piperidine. The resulting DNA fragments were amplified by LMPCR and compared to DMS-treated purified DNA. Protein-DNA interactions are indicated by different G lane patterns (protection or hyperreactivity) for the amplified DNA fragments treated in vivo versus in vitro.

Figure 2 shows genomic footprinting analyses of the lower strand (Fig. 2a) and the upper strand (Fig. 2b) of the tRNA^{Sec} gene. The brackets in Fig. 2 indicate significant differences between the DMS-treated DNA in vitro (lanes 1) and in vivo (lanes 2). In Fig. 2a, lane 2, the nucleotides (nt) at positions −32, −20, and +2 to +4 are hyperreactive in vivo compared to the in vitro DMS modification pattern (lane 1), whereas the G at position −31 is protected. In Fig. 2b, the sequence of the upper (nontranscribed) strand spanning nt −42 to +79 is shown. There are hyperreactive Gs at positions −37 and −15, comparing the in vivo-treated DNA (lane 2) with the in vitro sample (lane 1). Positions −17 and −16 are protected in vivo. The results indicate protein-DNA interactions near the TATA box in both strands of the tRNA^{Sec} gene. However, no genomic footprints could be detected near boxes A and B.

We then analyzed the tRNA^{Val} clone pHtV8 of the human tRNA^{Val} gene family (56) by in vivo footprinting. Because the pre-tRNA of this tRNA^{Val} gene is highly expressed in HeLa cells and human placenta (48) and this copy is also highly

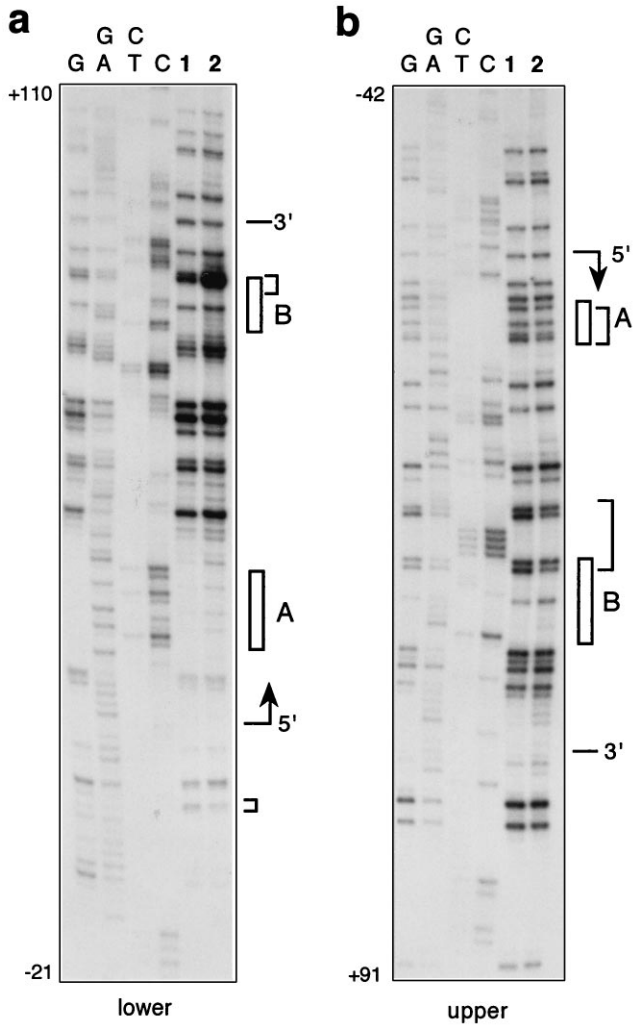

FIG. 3. Genomic footprinting of a tRNA^{Val} gene in human fibroblasts. Lanes and symbols are as in Fig. 2. (a) The sequences cover the lower (transcribed) strand of the region spanning nt −21 to +110. Primers V8A-1 to V8A-3 were used for LMPCR. (b) The data are for the upper (nontranscribed) strand spanning from nt −42 to +91. Primers V8A-4 to V8A-6 were used for LMPCR.

transcribed by RNA polymerase III in vitro (56), we decided to analyze this particular family member. The LMPCR primers used for genomic footprinting and DNA repair assays reveal only the sequences of this specific gene copy. Figure 3a shows a genomic footprint analysis from nt −21 to +110 of the transcribed (lower) strand. Two Gs in box B at positions +61 and +62 are strongly hyperreactive, and another G at position −7 shows some protection against DMS modification in vivo. In the upper strand, we could detect protection near boxes A and B (Fig. 3b and confirmed by densitometric scanning [data not shown]). The Gs at positions +12, +15, +17, and +18 in box A (Fig. 3b, lane 2) are less reactive to DMS than the same Gs in vitro (lane 1). The same was found for Gs at positions +45, +46, +52, and +53 next to box B.

The results indicate protein-DNA interactions near boxes A and B in the tRNA^{Val} gene and suggest that this gene copy is occupied by the regulatory factor TFIIIC.

Repair of CPDs in the tRNA^{Sec} gene. We first analyzed the rates of repair of UV-induced CPDs at single-nucleotide resolution along the human tRNA^{Sec} gene by LMPCR. Human foreskin fibroblasts were grown as contact-inhibited monolayers and were UV irradiated at a UV dose of 20 J/m^2. After incubation to allow DNA repair, DNA was isolated and cleaved with T4 endonuclease V to generate single-strand breaks at CPDs and then digested with *E. coli* photolyase to generate ligatable ends (41). Repair of CPDs in the total genome was analyzed by separation of T4 endonuclease V-cleaved DNA fragments on alkaline agarose gels. Repair was approximately 50 to 60% complete after 8 to 10 h and approximately 80 to 90% complete after 24 h (12), which is consistent with data on genomic repair of CPDs as determined by others (3, 4, 26).

Figure 4 shows repair of CPDs along the tRNA^{Sec} gene. The 5′ and 3′ ends of the tRNA^{Sec} (38) and boxes A and B are indicated. Figure 4a shows the sequences of the transcribed (lower) strand from nt −26 to +135, whereas Fig. 4b shows the upper strand spanning the region from nt −91 to +77. Lanes 0 show the initial CPD formation at dipyrimidines after UV irradiation. UV-specific signals are seen almost exclusively at dipyrimidine sites. Repair of CPDs at a particular site is indicated by a decrease in the intensity of the corresponding gel band compared with its intensity at 0 h. When we compare the signals in Fig. 4a of the transcribed sequences and the flanking regions, the kinetics are almost identical. Although there are significant differences from site to site, the average repair of CPDs within this gene is relatively slow, and only about 50% of the lesions are removed after 6 to 10 h (see Fig. 7 for quantitation). Very similar repair kinetics were detected when we analyzed the upper (nontranscribed) strand (Fig. 4b). The CPDs in the 5′ flanking region and in the coding sequence are repaired at the same rate, with a half-life of about 6 to 10 h. In both strands of the tRNA^{Sec} gene, most CPDs are removed after 24 h.

Repair of CPDs in the tRNA^{Val} gene. We analyzed rates of repair of CPDs in the tRNA^{Val} clone pHtV8 of the human tRNA^{Val} gene family (56) by LMPCR. Figure 5a shows analysis with a primer set that reads the lower strand from nt +1 to +138. The 5′ and 3′ ends of the gene and boxes A and B are indicated. When we compare the transcribed strand and the 3′ flanking sequence within this gel, there is no significant difference in repair rates. The average half-life of CPDs was calculated at approximately 9 h for both regions. The upper strand from nt −31 spanning the whole coding sequence and the 3′ region up to nt +102 was analyzed in Fig. 5b. Most CPDs are repaired, with a half-life of approximately 11 h for the coding region and 12 h for the 3′ region. There are two CPD hot spot positions. One thymidine (T) located inside a stretch of 10 pyrimidines 11 nt downstream of the 3′ end shows strong photoproduct formation (arrowhead in Fig. 5b). Further, there is a strong signal at a sequence between the 5′ end and box A. Several CPDs upstream of the 5′ end (hatched box in Fig. 5b) were repaired about three times faster than the sites between the 5′ end and box A.

To reveal the differences near the 5′ end of the tRNA^{Val} gene at a better resolution, we designed primer sets to analyze the repair rates in the 5′ flanking sequences. Figure 6a shows the lower strand of the tRNA^{Val} gene from nt −77 to +33. We calculated an average half-life of 8 h. In Fig. 6b, we analyzed the upper (coding) strand from nt −74 to +29. In general, 50% of the CPDs were removed after about 7 h, but there is a CPD hot spot between the 5′ end and box A for which a half-life of 12 h was estimated. The second prominent site for CPD formation, only 10 nt upstream, was 50% repaired in only 4 h (Fig. 6b, striped box). In fact, many sites on the upper strand were repaired faster in the 5′ flanking sequences than in the coding sequence (Fig. 5b and 6b).

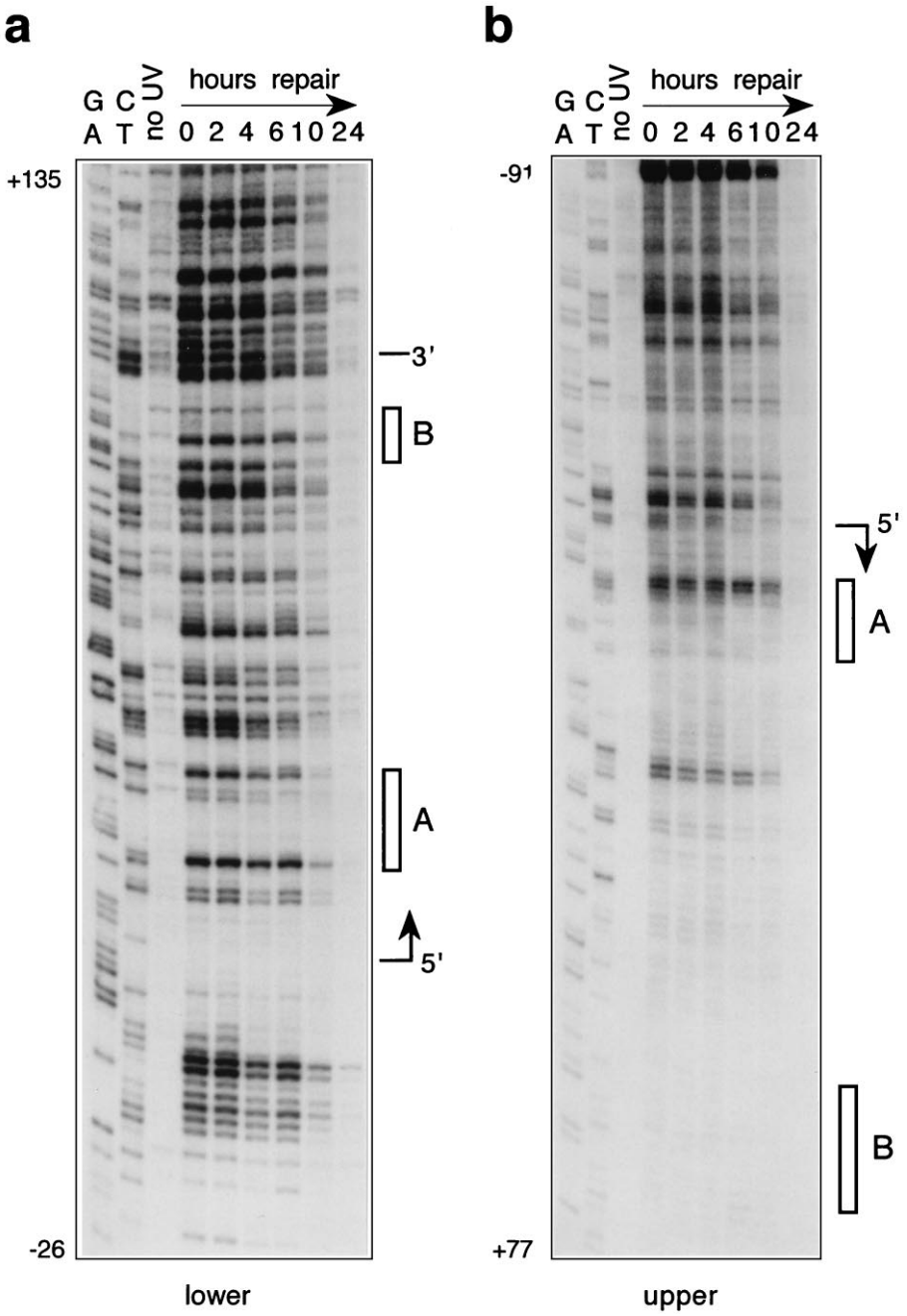

FIG. 4. Repair of UV-induced CPDs along the tRNA^{Sec} gene. Confluent fibroblasts were UV irradiated at 20 J/m^2 and allowed to repair for the indicated periods of time. Lanes GA and CT are Maxam-Gilbert sequencing reactions. Symbols are as in Fig. 2. (a) The autoradiography shows the lower (transcribed) strand from nt −26 to +135. Primers SCB-1 to SCB-3 were used for LMPCR. (b) The sequences cover the upper (nontranscribed) strand of the region spanning nt −91 to +77. Primers SCB-4 to SCB-6 were used for LMPCR.

Summary of CPD repair in tRNA genes. Figure 7 represents a summary and quantitation of all obtained DNA repair data and the genomic footprinting analysis. Genomic footprints are indicated with brackets. Since many CPDs are located in a stretch of pyrimidines and the repair kinetics within these clusters were often similar, they are grouped together as one single rectangle. The calculated average repair rate of each domain is shown as a dashed line. Note that Fig. 7 does not show differences in rates of CPD formation or in repair kinetics. In Fig. 7a, we show the analysis of the tRNA^{Sec} gene. There are only small differences between the repair of the coding and transcribed strands. The half-life of CPDs in the coding strand was on average 7.5 h, whereas the transcribed strand was repaired to 50% in about 5.5 h. However, some sites in the transcribed strand are repaired more slowly than sites in the nontranscribed strand. For the upper 5′ flanking sequence of the tRNA^{Sec} gene, the average half time was 8 h. For the lower strand, we observed a site of fast repair upstream of the 5′ end, with a half time of 4 h. Figure 7b shows the analyzed sequences of the tRNA^{Val} gene. The coding strand was repaired to 50%

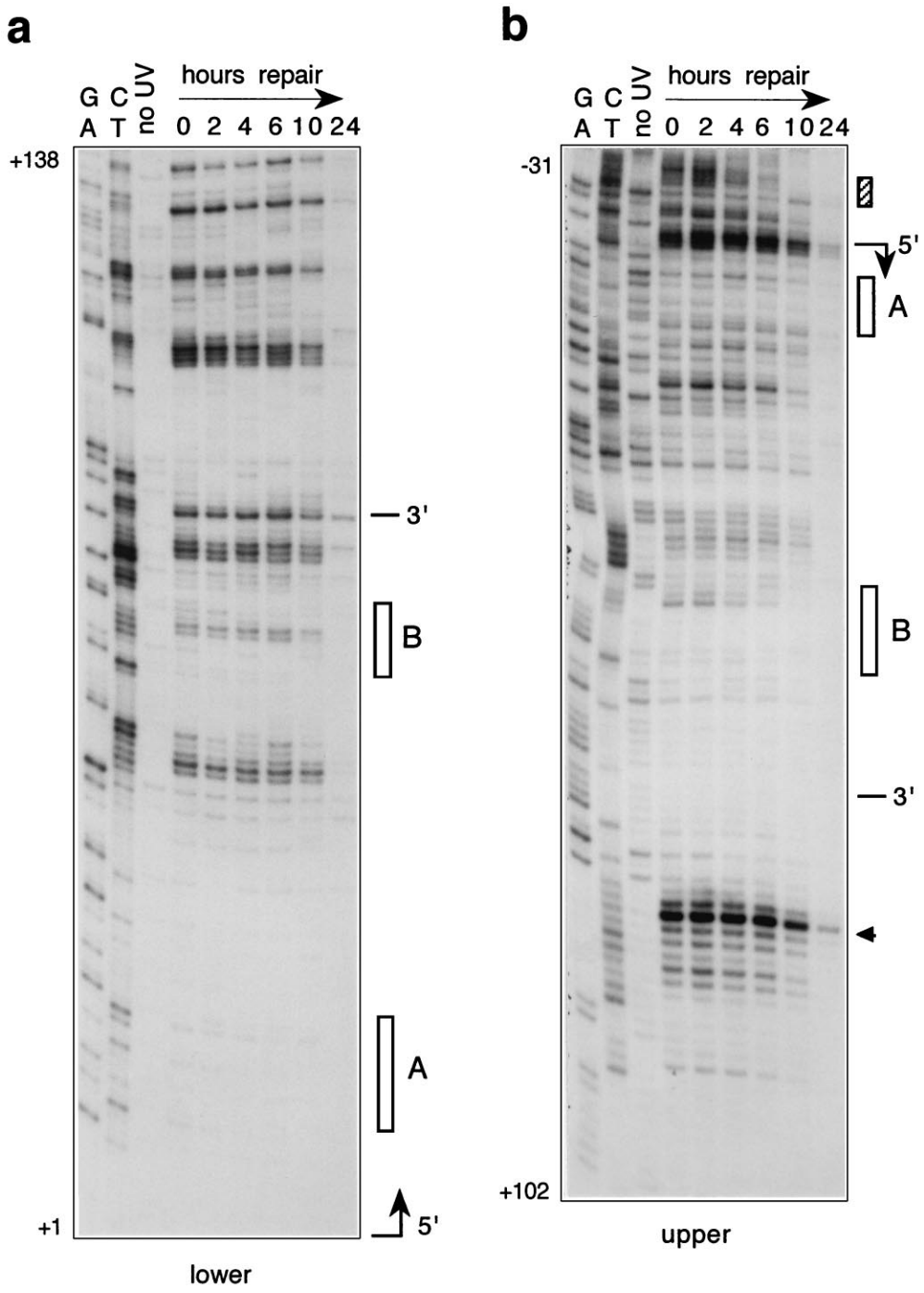

FIG. 5. Repair of UV-induced CPDs along a $tRNA^{Val}$ gene. Lanes and symbols are as in Fig. 4. (a) The gel shows the lower (transcribed) strand from nt +1 to +138. Primers V8A-1 to V8A-3 were used for LMPCR. (b) The sequences cover the upper (nontranscribed) strand of the region spanning nt −31 to +102. Primers V8A-4 to V8A-6 were used for LMPCR. The stippled box shows a site of fast repair upstream of the transcription initiation site. The arrowhead indicates strong CPD formation in the RNA polymerase III terminator region.

in 11 h, whereas the half-life of CPDs was 9 h in the transcribed strand. Again, some sites in the transcribed strand are repaired more slowly than sites in the nontranscribed strand. Fifty percent of the CPDs of the 5′ and 3′ flanking lower-strand sequence were repaired in 8 h. Further, we calculated an average half-life of 7 h for the CPDs of the upper strand in the 5′ flanking region.

DISCUSSION

Transcriptional status of the analyzed tRNA genes. Each of the 60 to 90 different isoaccepting species of tRNA appears to be encoded by human tRNA gene families of about 10 to 20 members (11, 48). The expression of these TATA-less tRNA genes depends on the presence of two highly conserved intra-

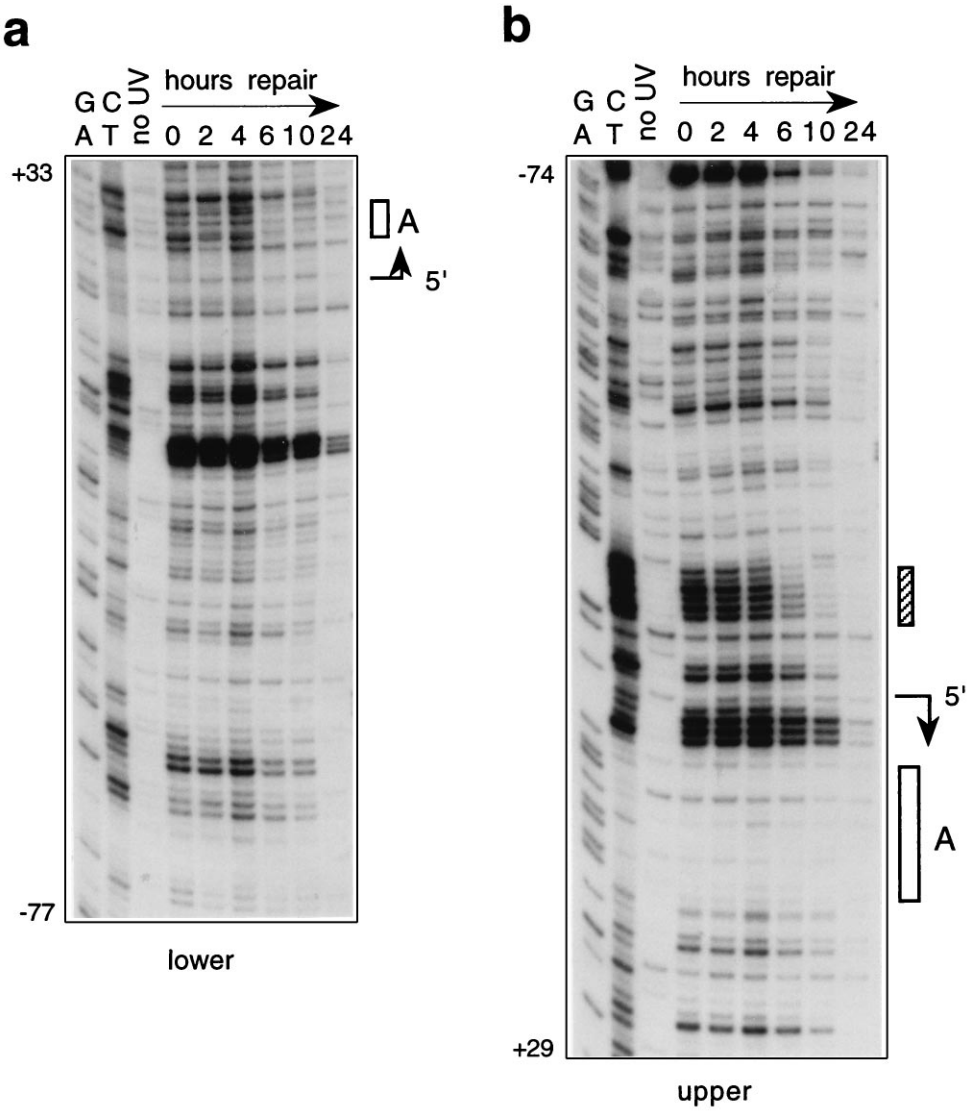

FIG. 6. Repair of UV-induced CPDs along the 5′ upstream region of the tRNA^{Val} gene. Lanes and symbols are as in Fig. 4. (a) The gel shows the lower (transcribed) strand from nt −77 to +33. Primers V8C-1 to V8C-3 were used for LMPCR. (b) The sequences cover the upper (nontranscribed) strand of the region spanning nt −74 to +29. Primers V8C-4 to V8C-6 were used for LMPCR. The stippled box indicates a site of fast repair upstream of the transcription initiation site.

genic promoter sequences (box A and box B) which are bound by TFIIIC (for reviews, see references 17 and 70). TFIIIC directs TFIIIB, a TBP-containing multisubunit complex, to its position upstream of the transcriptional start site (23). Then, RNA polymerase III is positioned over the transcriptional start within 10 nt upstream from the coding gene and terminates within an oligo(T) stretch near the 3′ end of the tRNA gene (19). Such pre-tRNAs are cleaved in the nucleus to mature tRNAs by 5′ and 3′ processing nucleases before they enter the cytoplasm. The fact that all isoacceptor species of tRNA^{Val} appear to have almost the same nucleotide sequence (56) makes analysis of a single tRNA copy difficult. Therefore, the Northern blot and nuclear run-on data (Fig. 1) for tRNA^{Val} cannot distinguish family members. However, the family member used for DNA repair studies (clone pHtV8) is highly expressed in HeLa cells and human placenta and is also strongly transcribed in vitro (48, 56). For the genomic footprinting analysis we used primers which correspond to the sequence of the tRNA^{Val} gene of clone pHtV8 isolated from a human genomic library (56). Because LMPCR primers are specific for this family member, DMS footprinting will reveal the DNA-protein interaction of this tRNA^{Val} gene only (Fig. 3). The upper strand of the tRNA^{Val} gene in box A and next to box B showed protection against DMS modification. On the lower strand, there was a hypersensitive site within box B and protection immediately upstream of the 5′ end. This in vivo modification pattern indicates the binding of TFIIIC to boxes A and B. The footprint upstream of the 5′ end suggests binding of TFIIIB and RNA polymerase III to the tRNA^{Val} gene. Therefore, this tRNA^{Val} gene is most likely transcriptionally active in human fibroblasts.

To ensure that we investigated DNA repair rates only in transcriptionally active tRNA genes, we also analyzed a tRNA^{Sec} gene, which is present as a single-copy gene in the human genome (27, 33). This gene serves as a donor of selenocysteine, which is the 21st naturally occurring amino acid, to selenoproteins and suppresses the nonsense codon UGA (27, 30). This tRNA gene has some unique features. Transcrip-

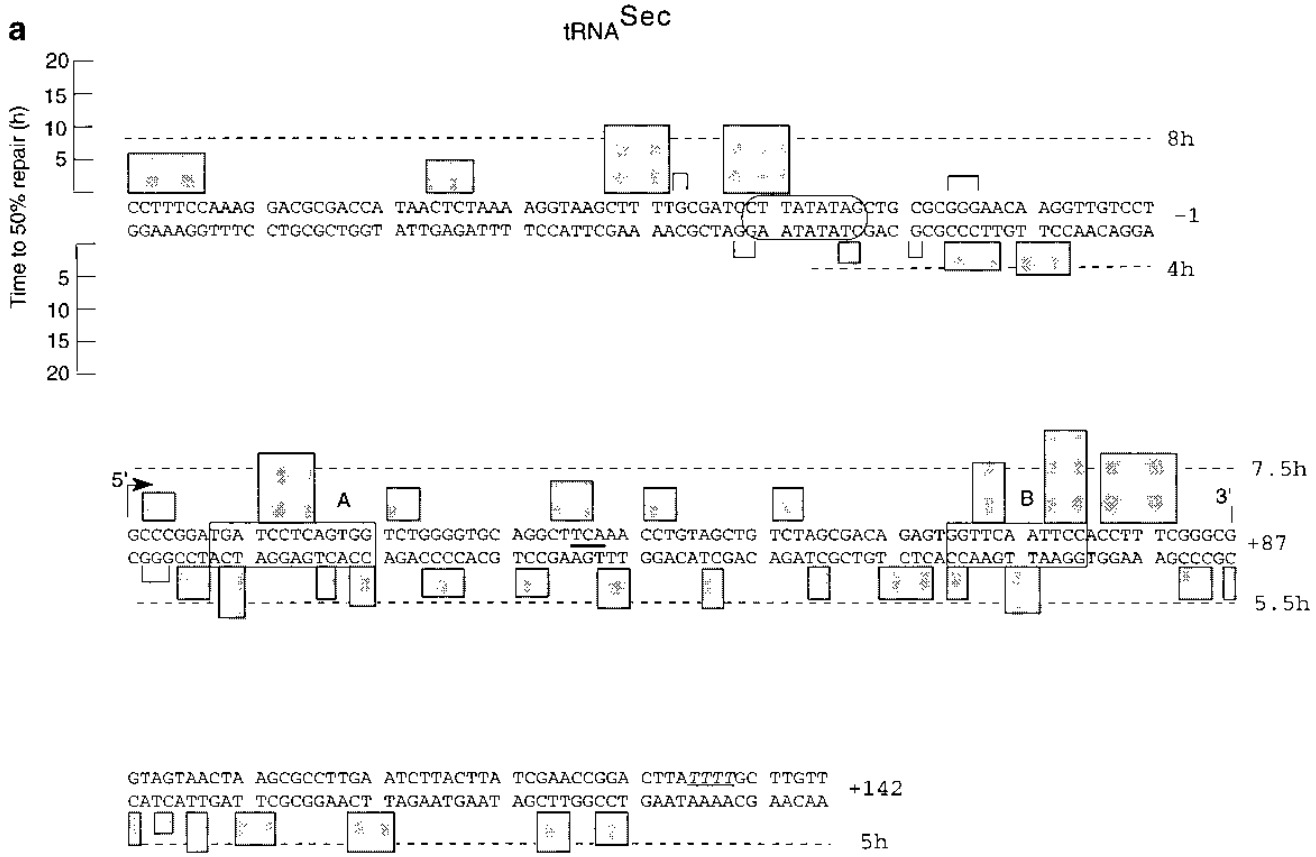

FIG. 7. Summary of DNA repair along the human tRNA genes. The 5′ and the 3′ ends of the tRNAs are indicated. The open boxes A and B indicate the DNA binding sites of transcription factor TFIIIC. Brackets show the presence of genomic DMS footprints. A thick line represents the position of the anticodon. The underlined italic Ts indicate the transcription termination sites. Repair rates, determined as the time at which 50% of the CPD signal was removed, were estimated for each CPD site with a visible signal above background and are represented by rectangles. The calculated average repair of each domain is shown as a dashed line. (a) Sequences of the $tRNA^{Sec}$ gene. The position of the TATA box is marked by an ellipsoid box. (b) Data for the $tRNA^{Val}$ gene.

tion starts at the first nucleotide within the coding sequence of the gene (27). Further, in contrast to the classical TATA-less tRNA genes, the eukaryotic $tRNA^{Sec}$ gene possesses a strong TATA box (8, 36). This observation supports a model in which TBP interacts directly with the TATA element (23, 36). In fact, we could detect prominent genomic footprints in vivo near the sequence 5′CTTATATAG at nt −24 (TATA box) upstream of the transcription start site and between the transcription start site and box A (Fig. 2). However, we could not detect DMS modification near box B. This result suggests binding of TFIIIB and RNA polymerase III directly to the transcription initiation site. Binding of TFIIIC to boxes A and B may be abolished, since in this gene the functional TATA box can promote direct binding of TBP-TFIIIB near the transcription initiation site. This is consistent with results obtained by others (27, 36). These authors showed that the information required for transcription of the $tRNA^{Sec}$ gene is only in the 5′ flanking region. To confirm the transcriptional activity of the $tRNA^{Sec}$ gene, we also performed Northern blot and nuclear run-on analyses. We found high transcription rates and high levels of $tRNA^{Sec}$ before and after UV irradiation in fibroblasts. In fact, the rates of transcription of the tRNA genes appear to be much higher than that of the *JUN* gene (Fig. 1b), which is transcribed by RNA polymerase II and for which we had previously found very high repair rates (64). Taking all data together, we conclude that both tRNA genes are transcriptionally active in fibroblasts and that transcription is not inhibited after UV irradiation (Fig. 1b). Thus, the lack of preferential repair that we have observed cannot be explained by a lack of transcription.

Pyrimidine dimer formation. The formation of CPDs along the tRNA genes appears to be dictated mostly by the DNA sequence and possibly by protein-DNA interactions. There are strong hot spots for dimer formation in the flanking region of the $tRNA^{Val}$ gene at a 5′-TTTT sequence at position −29 in the lower strand, on the upper strand near position −74, and between the transcription start site and box A (Fig. 6). CPD hot spots at or near regulatory sequences may be caused by binding of sequence-specific proteins (41, 60). Generally, there was not much difference in the total CPD yield between the transcribed and nontranscribed DNA strands. A very prominent site of CPD formation was observed between two thymidines in the termination site of the $tRNA^{Val}$ gene sequence at position +81 (5′-TTTT) (Fig. 5b). This UV-hypersensitive site could be a consequence of secondary structure of the DNA formed by the RNA polymerase III machinery. No specific factors are involved in termination of RNA polymerase III. Only a run of four or more T residues is necessary as a termination signal, and this may reflect the border between the transcription machinery and the 3′ flanking inactive chromatin. The structure of this DNA site may be altered by its special function and therefore may be more sensitive for UV damage formation. Unfortunately, not enough sequence information about the $tRNA^{Sec}$ gene is available to reveal the CPD forma-

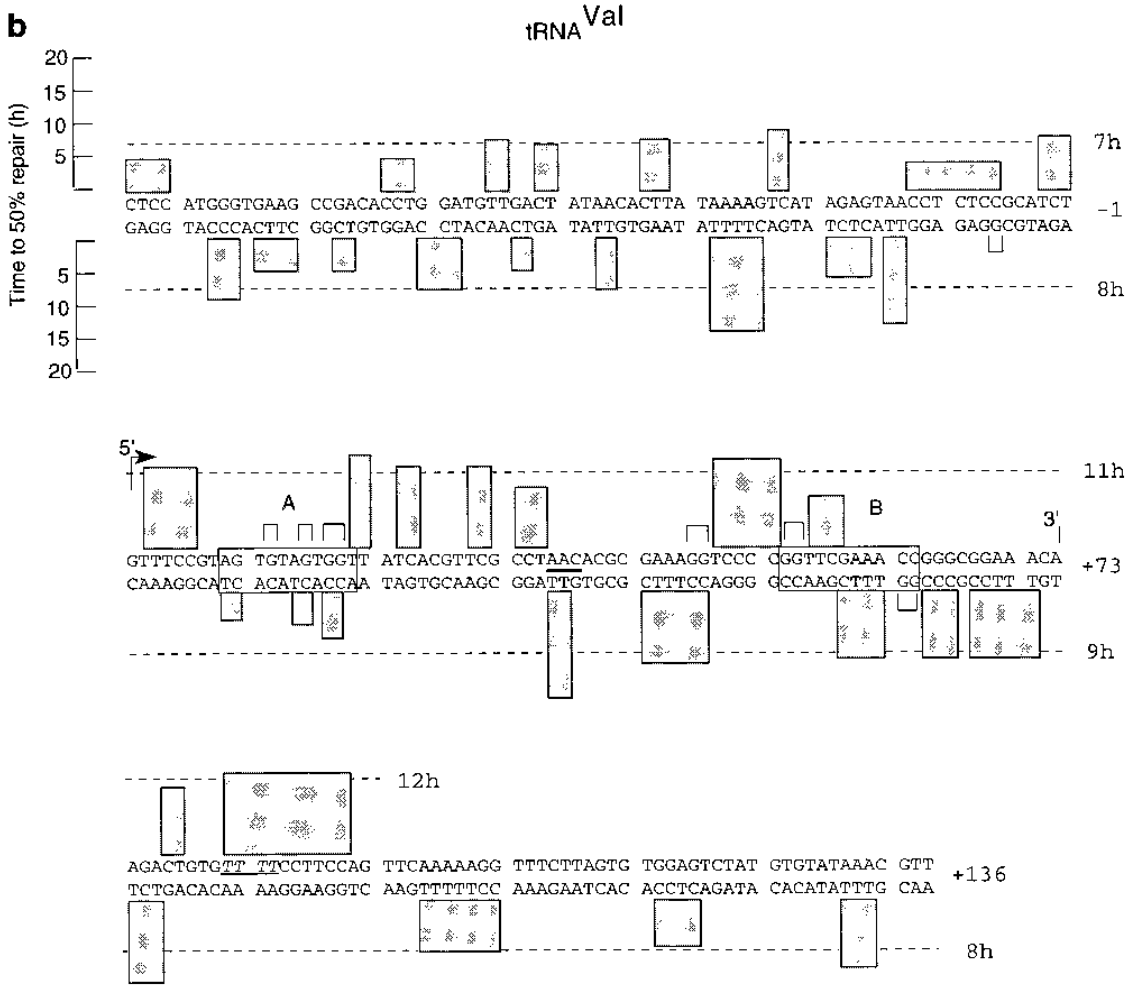

FIG. 7—*Continued.*

tion at this termination site by LMPCR. It would be interesting to determine if this UV-hypersensitive site is a general feature of the terminators in RNA polymerase III-transcribed genes.

Lack of gene- and strand-specific DNA repair. Rates of repair of CPDs were analyzed with the aid of LMPCR. This technique provides a sensitivity sufficient to study and to quantitate DNA repair processes at nucleotide resolution (16, 59, 64). The repair of CPDs can vary with DNA sequence position. Slow repair was observed at positions corresponding to binding sites of RNA polymerase II transcription factors (16, 64), and transcription-coupled repair was reported for many different RNA polymerase II transcribed genes (5, 16, 20, 34, 64). In this study, we investigated the rates of repair of two RNA polymerase III-transcribed tRNA genes and their flanking sequences (Fig. 7). In the $tRNA^{Sec}$ gene, CPDs in the upper nontranscribed strand have a half-life of 7.5 h. The upper-strand 5′ flanking sequences were repaired with the same efficiency as the coding sequences. The half-life of CPDs in the lower transcribed strand of the $tRNA^{Sec}$ gene was 5.5 h. However, many CPD positions between box A and box B of the upper nontranscribed strand are repaired at the same rates as positions within the transcribed strand. In the $tRNA^{Val}$ gene, the CPDs of the 5′ flanking upper strand are repaired faster (50% in 7 h) than those of the nontranscribed strand (11 h) and the lower transcribed strand (9 h). Fifty percent of the dimers of 5′ and 3′ flanking sequences of the lower strand are removed in 8 h. This is similar to the repair of the transcribed sequences. LMPCR analysis of the human *JUN* gene, which is transcribed by RNA polymerase II, showed that regions of transcription-coupled repair (between nt −40 and +100) are more than 90% repaired within 4 h on both strands in human fibroblasts (64). In both analyzed tRNA genes, we could not detect such high repair rates (despite apparently higher transcription rates [Fig. 1b]), and no significant differences between the rates of repair of the transcribed sequences and the flanking nontranscribed regions were apparent. The genes are repaired with the same kinetics as the surrounding noncoding regions and only slightly faster than bulk genomic DNA. Further, we could not detect significant strand-specific repair. The lack of transcription-coupled repair may be explained by absence of a transcription repair coupling factor that operates in conjunction with the RNA polymerase III machinery. The results are also consistent with a model in which tRNA genes are covered by the regulatory factor TFIIIC and RNA polymerase III, which may actually inhibit the DNA's accessibility to repair enzymes.

Since most RNA polymerase III-transcribed genes are present in multiple copies with a coding region of less than 100 bp, DNA damage within these genes may not be as detrimental as in RNA polymerase II-transcribed genes, which are in most

cases many kilobases long and are single copy. It appears that there was no evolutionary need to enhance DNA repair of multicopy genes like tRNA genes relative to the overall DNA repair efficiency. The limiting factor for cells to survive UV irradiation was to selectively repair single-copy genes by the development of a DNA repair machinery coupled only to RNA polymerase II transcription.

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