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The *Drosophila melanogaster* Homologue of the Xeroderma Pigmentosum D Gene Product Is Located in Euchromatic Regions and Has a Dynamic Response to UV Light-induced Lesions in Polytene Chromosomes

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The *XPD/ERCC2/Rad3* gene is required for excision repair of UV-damaged DNA and is an important component of nucleotide excision repair. Mutations in the *XPD* gene generate the cancer-prone syndrome, xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy. XPD has a 5′- to 3′-helicase activity and is a component of the TFIIH transcription factor, which is essential for RNA polymerase II elongation. We present here the characterization of the *Drosophila melanogaster* XPD gene (*DmXPD*). *DmXPD* encodes a product that is highly related to its human homologue. The DmXPD protein is ubiquitous during development. In embryos at the syncytial blastoderm stage, DmXPD is cytoplasmic. At the onset of transcription in somatic cells and during gastrulation in germ cells, DmXPD moves to the nuclei. Distribution analysis in polytene chromosomes shows that DmXPD is highly concentrated in the interbands, especially in the highly transcribed regions known as puffs. UV-light irradiation of third-instar larvae induces an increase in the signal intensity and in the number of sites where the DmXPD protein is located in polytene chromosomes, indicating that the DmXPD protein is recruited intensively in the chromosomes as a response to DNA damage. This is the first time that the response to DNA damage by UV-light irradiation can be visualized directly on the chromosomes using one of the TFIIH components.

INTRODUCTION

Transmission of the genetic information contained in the DNA molecule requires the maintenance of its integrity from generation to generation. This fidelity can be affected by chemical and physical factors that cause diverse types of damage. The DNA molecule is particularly sensitive to these factors during replication, transcription, and recombination. To ensure the integrity of the genetic information, all organisms have evolved mechanisms for repairing or tolerating diverse types of DNA damage (for reviews, see Lehmann, 1995, 1998; Friedberg, 1996a; Sancar, 1996).

In eukaryotes, the nucleotide-excision-repair (NER) mechanism operates in many types of base damage caused by the covalent addition of methyl groups, the covalent linking of adjacent bases, and the cross-linking of bases in opposite DNA strands (Lehmann, 1987; Friedberg, 1996a; Wood, 1996). These kinds of lesions generate a distortion of the helical structure and are efficiently recognized and repaired by the NER system. During the function of the NER mechanism, damaged DNA in one strand is removed as an oligonucleotide, and then new DNA is synthesized using the undamaged DNA strand as a primer and template. This requires the products of ≤35 genes involved in the recognition of the damaged region, in the excision of the oligonucleotide, and in the repair of the mole-

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cule (for recent reviews see Friedberg, 1996a; Wood, 1996; Lindahl *et al.*, 1997). Some of the products of these genes are also components of the TFIIH complex.

NER has been extensively studied in yeast and in humans. In yeast, the characterization of mutants with higher UV sensitivity has allowed the identification of all the components that participate in NER. In humans, defects in NER are related to three genetic disorders. One of these disorders, xeroderma pigmentosum (XP), clearly correlates with defects in DNA-repair mechanisms. Patients affected with XP have an increased sensitivity to sunlight and they develop carcinoma. Patients with the other two disorders, tricothiodystrophy (TTD) and Cockayne's syndrome (CS), have sun sensitivity, but are not cancer prone as the XP patients. It has been suggested that the defects presented by individuals with these two latter syndromes are not completely related to DNA repair and are caused by defects in the transcription machinery (see below). On the other hand, TTD and CS are characterized by multiple defects, some shared by both disorders, especially defects in the development of the nervous system (Lehmann, 1987; Nance and Berry, 1992; Friedberg, 1996b; Taylor *et al.*, 1997; Lehman, 1998).

The biochemical function of some of the proteins has been identified. In particular, the products of the *XPD* and *XPB* genes have 5′- to 3′- and 3′- to 5′-ATP–dependent DNA helicase activities, respectively.

The NER complex has a dual role in DNA repair and in RNA polymerase II transcription (Buratowski, 1993; Feaver *et al.*, 1993; Schaeffer *et al.*, 1993). This link demonstrated that some alleles of *Rad25 (XPB)* and *Rad3 (XPD)* in yeast were deficient in RNA polymerase II transcription. These two proteins are subunits of the TFIIH RNA polymerase II transcription complex (Guzder *et al.*, 1994a,b; Schaeffer *et al.*, 1993). On the other hand, the TFIIH complex is essential for a proper DNA repair in vitro (Aboussekhra *et al.*, 1995). These findings suggest a transcription-coupled DNA repair mechanism in which the TFIIH factor is involved.

The TFIIH factor has several components. It is formed by the DNA helicases XPB and XPD, the p62, p52, p44, and p34 polypeptides of unknown function, and by the complex known as cdk-activating kinase or CAK, which is formed by three proteins, CDK7, CYCH, and MAT1. CDK7 and CYCH are involved in RNA polymerase II transcription (Feaver *et al.*, 1994; Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995) and the MAT1 protein functions as an assembling factor that could modulate the CDK7 kinase activity together with other TFIIH components (Rossignol *et al.*, 1997; Yankulov and Bentley, 1997). The CDK7 kinase phosphorylates the C-terminal domain (CTD) of the RNA polymerase II. This phosphorylation is necessary for elongation of the mRNA (Gerber *et al.*, 1995). The important role of the TFIIH factor in transcription and NER explains the highly pleiotropic phenotypes observed in XP, TTD, and CS disorders.

As mentioned before, these genes have been studied in yeast and in humans. In *Drosophila melanogaster* the *XPB* homologue has been identified as the *haywire* (*hay*) gene (Mounkes *et al.*, 1992). Alleles of *hay* mimic some of the defects of XP and CS. This finding suggests that the fly, a complex animal system, could be an interesting, easily manipulated model for the study of NER, in which double or triple mutants in genes encoding for components of the TFIIH factor could be analyzed. In *Drosophila*, some genes involved in the recognition of damaged DNA have been identified, including *XPA*, *XPC*, and the *XPF (mei-9)* gene, which encodes for a structure-specific nuclease that cleaves the damaged DNA region (Henning *et al.*, 1994; Sekelsky *et al.*, 1995; Shimamoto *et al.*, 1995; Sugasawa *et al.*, 1998). However, in addition to *haywire*, no other genes encoding TFIIH components have been identified in this organism.

In this paper we report the cloning of the *D. melanogaster XPD* homologue (*DmXPD*). *DmXPD* is 73% identical to its human homologue. The DmXPD product is ubiquitously distributed during embryonic development; at the onset of transcription in somatic and germ cells, it is localized in the nucleus. An analysis of the distribution of the *Drosophila* XPD product in polytene chromosomes indicates that it is mostly concentrated in the interband regions and that it is enriched in the puffs as discrete bands. UV irradiation of third-instar larvae induces an increase in the intensity and in the number of sites where the DmXPD product is present in the polytene chromosomes, indicating that the DmXPD protein is recruited intensively in the chromatin as a response to DNA damage.

MATERIALS AND METHODS

Fly Stocks

Wild-type *D. melanogaster* was the Oregon R strain. The mutant alleles, *l(2)57Cc-3/SM1*, *l(2)57Cd-2/SM1*, *l(2)57Ce-4/SM1*, *PuRz2/SM1*, and *Df(2R)PF1 c px sp/SM1*, were a gift from Jannis O'Donnell and have been previously described (O'Donnell *et al.*, 1989). *Df(2R) Pu-D17*, which uncovers 57B5–58B2, was used in immunostaining experiments (O'Donnell *et al.*, 1989). The flies were raised on a standard yeast–dextrose–propionic acid medium at 25°C.

Recombinant DNA

Two degenerate oligonucleotides (23 mers, located at the 5′ of the coding region) were designed based on the high identity between the human XPD and the yeast RAD3 proteins. The oligonucleotide sequences correspond to amino acids 40–50 (5′-GAA/G-ATG-CCC/G-TCC/G-ACC-TCC/G-GGC-AA-3′) and 234–242 (5′-TT-CTC-GAT-A/GTT-GTG-TAT-T/CTC-GTC-3′) of the human protein. These oligonucleotides were used in typical RT-PCR reactions using total RNA from adult *Drosophila* wild-type females. First-strand cDNA was synthesized with oligo-dT as a primer and then incubated with the degenerate oligonucleotides (20 pmol of each

primer) in PCR reactions using the following conditions: 94°C, 1 min; 58°C, 1 min; 72°C, 2 min in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, and 200 μM dNTPs (Boehringer Mannheim). Reaction products were electrophoresed on 2% agarose TBE gel and visualized by ethidium bromide staining. To confirm the amplification of the *D. melanogaster* XPD homologue, the PCR products were cloned in the M13 vector and sequenced with the sequenase version 2.0 DNA sequencing kit using T3, T7, reverse, KS, and SK −20 and −40 primers according to manufacturer protocols (United States Biochemical, Cleveland, OH; Amersham catalogue no. 70770, Amersham, Arlington Heights, IL). This DNA was then used as a probe to screen a λUni-Zap XR Stratagene library (catalogue no. 937602; Stratagene, La Jolla, CA) prepared with Canton-S 2–14 h embryo poly A^+ RNA. Hybridization conditions were 50°C in 5× SCC, 5× Denhardt's, 0.5% SDS, 0.5 mg/ml salmon sperm DNA with 6×10^5 cpm/ml of the [^{32}P]dCTP–labeled probe. After hybridization, the filters were washed in 0.1× SSC, 0.1% SDS at 50°C. Several clones were identified, and a clone with an insert of ~3 kb encoding for the complete XPD protein was sequenced. All routine cloning procedures, as well as Northern and Southern blot procedures, were carried out essentially as described by Sambrook *et al.* (1989).

Expression of Recombinant Protein, Production of Polyclonal Antibodies, and Whole-Mount Immunolocalization in Embryos

To make the DmXPD-glutathione-*S*-transferase fusion, which encodes a 309-bp fragment of the *D. melanogaster* XPD cDNA, the DmXPD carboxyl-terminal region from residues 680 to 783 in Figure 1 was cloned in the *Bam*HI site of the pGEX2 vector (Smith, 1993). The recombinant protein was purified as described by Smith (1993) and used to elicit polyclonal antibodies in Balb-C mice, as described by Harlow and Lane (1988). Total soluble protein extracts were prepared according to Reynaud *et al.* (1997). The concentration of these extracts was standardized according to Bradford (1976), and the samples were electrophoresed in 10% SDS-PAGE gels (Leammli, 1970), blotted into nitrocellulose, and immunostained according to Burnette (1981). The DmXPD protein was revealed using a 1:10,000 dilution of the anti-XPD polyclonal antibody, followed by peroxidase-conjugated goat anti-mouse immunoglobulin G as secondary antibody (Boehringer Mannheim, Indianapolis, IN). Embryos were immunostained essentially as described by Lee *et al.* (1993). The tissue was mounted in methyl salicylate, and images were photographed using Nomarski illumination.

Immunolocalization and Quantification of DmXPD on Polytene Chromosome Spreads

Fixation and spreading of the chromosomes essentially followed the protocol reported by Engels (1986). The chromosome immunostaining was modified to reduce background. The slides were washed several times in Tris-buffered saline (TBS) and then transferred to a blocking solution (10% BSA, 10% dry milk, 0.2% NP-40, and 0.3% Tween 20 in PBS) and incubated at least 5 h at room temperature. The slides were then washed several times in TBS, and the chromosomes were incubated with the primary antibody at a 1:10,000 dilution in blocking solution overnight. Slides were washed once in TBS and several times in 250 mM NaCl, 0.2% NP-40, and 0.2% Tween 20 in PBS. The slides were washed several times with PBS and blocked with the blocking solution. The chromosomes were incubated with the secondary antibody for 1 h at room temperature using FITC-conjugated anti-mouse antibody costained with propidium iodide (1:15,000 dilution of a 1 mg/ml solution). The chromosomes were visualized in a Bio-Rad (Richmond, CA) MRC-600 confocal microscope. Costaining of polytene chromosomes using an anti-human-p62 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; catalogue no. sc.292) and DmXPD was performed simultaneously; p62 was detected using a rabbit secondary antibody coupled to rhodamine.

For the DmXPD immunostaining in polytene chromosomes after UV irradiation, third instar larvae were collected and placed in a wet filter paper in one half of a Petri dish. The larvae were then irradiated with a Stratalinker (Stratagene) with 150, 300, and 600 J/m^2. After 30 min of recovery at room temperature, the salivary glands were dissected and polytene chromosome squashes prepared and stained with anti-XPD antibody and propidium iodide, as has been mentioned before. As control, similar chromosomes were stained with a commercial anti-histone antibody (Boehringer Mannheim), a commercial human anti–TATA-binding protein monoclonal antibody (Santa Cruz Biotechnology catalogue no. sl-421), and propidium iodide.

The pixel-intensity distribution in chromosmes was measured in transects 75 μm long, using the photon-counting and length-profile programs from the Bio-Rad confocal operating system (Bio-Rad MRC-600). These values were plotted in a histogram representing the number of pixels against arbitrary intensity units. In this case the maximum value is 255. What is considered in this kind of analysis is the difference in the number of pixels with the maximum value, which represent the accumulation of more target bound to the secondary antibody in the preparations.

RESULTS

Molecular Characterization of the D. melanogaster XPD Homologue

The *XPD* human gene and its yeast homologue *Rad3* have been previously characterized; these studies showed that they are highly conserved (Weber *et al.*, 1990). To clone the *D. melanogaster* homologue, synthetic–degenerate oligonucleotides were designed based on some of the regions with the highest identity between the human and yeast genes to amplify a region of ~600 bp (see MATERIALS AND METHODS). Typical RT-PCR reactions were performed, and a specific band of the expected size was isolated. This PCR fragment was sequenced, and it was confirmed to contain the predicted *XPD* region. This DNA was used to screen a *D. melanogaster* embryonic cDNA library. A full-length clone was isolated, sequenced, and analyzed. A conceptual open reading frame (ORF) of 783 amino acids with high identity to XPD proteins from other organisms was found (Figure 1). This ORF starts just after a stop codon in the same frame, indicating that it is the beginning of the protein. The global identity between the human and the *Drosophila* homologue is 73%; the identity to *Saccharomyces cerevisiae Rad3* and *Schizosaccharomyces pombe Rad 15* is 52% and 51%, respectively. This identity is particularly high (almost 100%) in the nucleotide-binding box and the proposed DNA–DNA and DNA–RNA helicase domains (Figure 1).

The *DmXPD* gene maps to polytene chromosome interval 57C5–9 in the right arm of the second chromosome (Figure 2A). Several loci have been identified in this region. *Punch (Pu)*, which encodes for a guanosine triphosphate cyclohydrolase, maps at 57C4–5, *tudor (tud)*, a maternal effect grandchildless gene, maps at 57C9, and between these two genes there are at least three other complementation groups

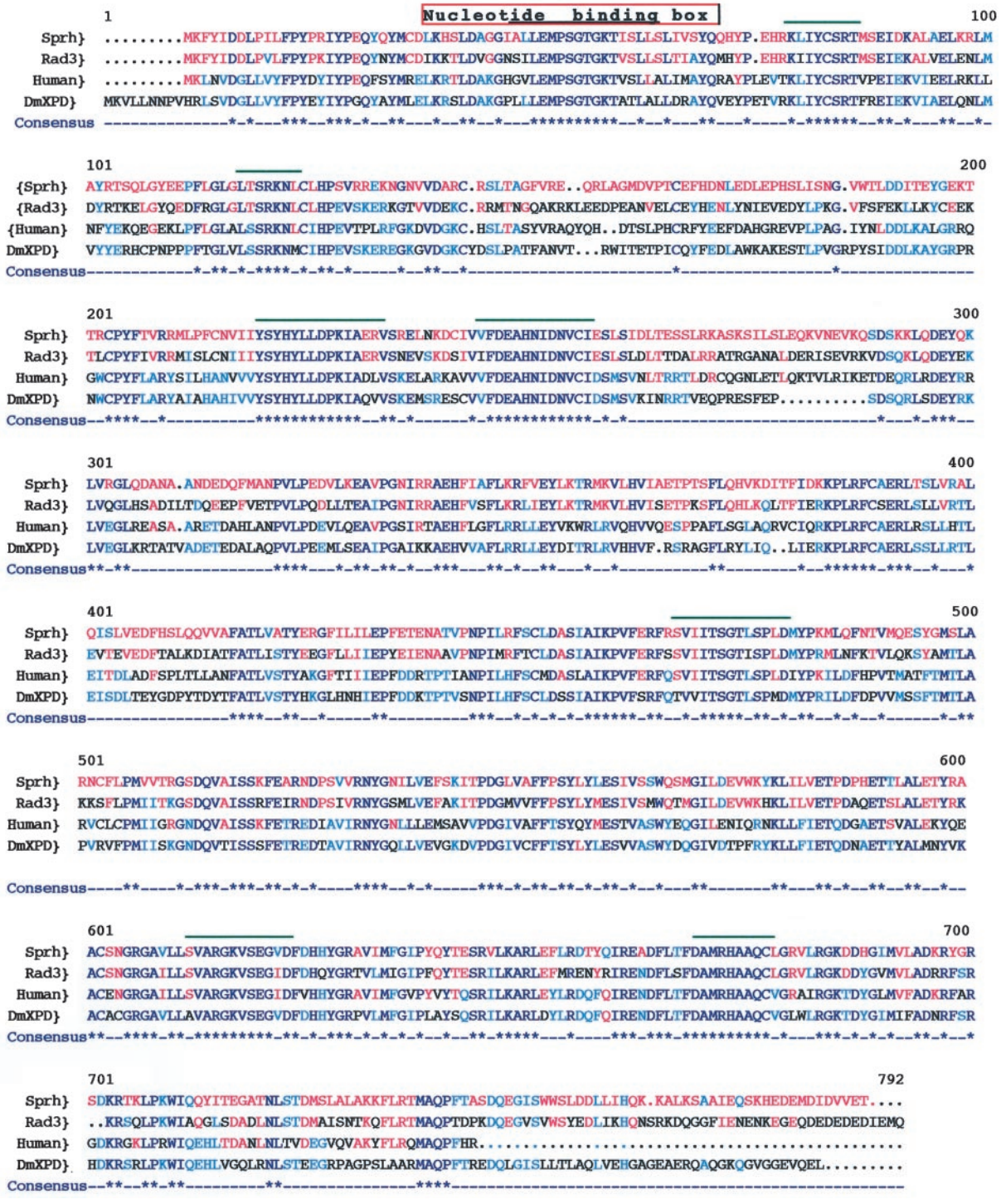

Figure 1. Sequence alignment of the conceptual DmXPD protein with representative XPD proteins from other organisms. Sprad is XPD from *S. pombe* (accession nos. G5022 and G5044); *Rad3* is from *S. cereviseae* (accession no. G603412) and the human XPD (accession no. G296645). *DmXPD* is the homologue from the fly. The amino acids in dark blue are present in all the XPD proteins; residues in red are in at least two proteins but not in DmXPD; residues in cyan are the ones that are in DmXPD and in at least one other homologue. Identical residues in all the proteins are labeled with an asterisk. The helicase domains are indicated with green lines, and the nucleotide-binding box is in a red box.

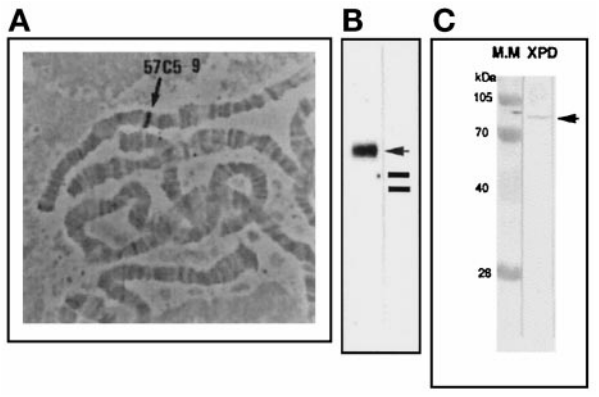

Figure 2. Panel A shows the *DmXPD* localization in polytene chromosomes after in situ hybridization. The arrow indicates the position at 57C5–9 in the right arm of the second chromosome. Panel B shows a Northern blot using total RNA from 0- to 24-h embryos using the DmXPD full-length cDNA as a probe. The arrow indicates a band of 3 kb, and the bars indicate the rRNA position in the gel. Panel C shows a Western blot using total protein extracts from embryos of 0–24 h and developed with an anti-DmXPD polyclonal antibody (see MATERIALS AND METHODS). The lane M.M. shows the molecular weight markers; in the DmXPD lane is shown the antibody-detected band from the total embryo extract with the expected DmXPD molecular weight.

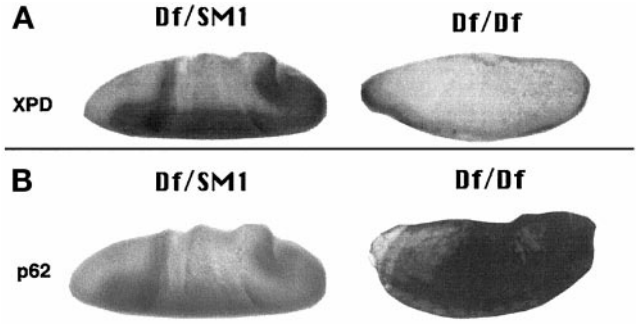

Figure 3. Specificity of the DmXPD antibody. Heterozygous (*Df/SM1*) and homozygous (*Df/Df*) embryos for the *Df(2R) Pu-D17* (break points 57B5–58B2), which does not carry the DmXPD-genomic region, were immunostained with an antibody raised against the DmXPD-GST fusion protein (panel A). In panel B, embryos of the same genotypes as in panel A were immunostained with an anti-p62 antibody.

identified by O'Donnell *et al.* (1989). Transgenic flies containing the DmXPD cDNA under the control of the *hsp70* promoter failed to rescue lethal mutations from the three complementation groups between *Pu* and *tud* (our unpublished data). This negative result suggests that none of the *loci* identified so far inside 57C5–9 is the *DmXPD* gene.

The DmXPD Protein Is Cytoplasmic in the Early Embryonic Stages, and Its Nuclear Localization Starts at the Onset of Transcription in Somatic and Germ Cells

The *DmXPD* gene encodes a unique transcript of ~3 kb (Figure 2B), which is present at all developmental stages (our unpublished data). Even though the DmXPD transcript is always present during development, there is no information of the in vivo distribution of any of the TFIIH components during embryogenesis in any organism. To analyze the distribution of the DmXPD protein during embryogenesis, we raised antibodies against a GST fusion protein containing the DmXPD carboxy-terminal region (see MATERIALS AND METHODS). As shown in Figure 1, this region is not present in the *XPD* homologues from other species and does not contain helicases or deoxynucleoside triphosphate-binding domains that could cause nonspecific antibody recognition. Figure 2C shows that the antiserum specifically recognizes a unique protein of the expected DmXPD molecular weight. Moreover, to test the specificity of the DmXPD antibody, we used flies carrying the deficiency *Df(2R) Pu-D17* (O'Donnell *et al.*, 1989), which uncovers the *DmXPD* genomic region (57C5–9). Homozygous embryos with the deficiency die during late embryogenesis and are easily recognizable because they have a defect in the ventral region (our unpublished observations). These embryos show very low DmXPD staining (Figure 3A), probably caused by maternal contribution. In contrast, the Df/$^{+}$ embryos show higher DmXPD staining (Figure 3A) with a distribution pattern that is essentially similar to the one seen in embryos stained with monoclonal antibodies raised against another TFIIH component, the human p62 protein (Figure 3B and MATERIALS AND METHODS), which recognizes preferentially a p62 *Drosophila* protein (Figure 6A). These results indicate that the antibodies raised against the DmXPD–GST fusion protein specifically recognize the DmXPD product.

Next, we analyzed the DmXPD protein distribution in wild-type embryos. At the syncytial blastoderm stage, the protein is distributed throughout the embryo, and its source is probably maternal. However, no nuclear localization is detected at this stage (Figure 4A). The protein starts to be concentrated in the embryo periphery just before cellularization (Figure 4, B and B'), and at the cellular blastoderm it is mostly nuclear in somatic cells; meanwhile, there is no signal in the pole cells (Figure 4, C and C'). After this stage the protein is preferentially nuclear and is found in the nuclei of both somatic and pole cells (Figure 4D and our unpublished results).

Immunofluorescence experiments with third-instar larval tissues show that cells with polytene chromosomes have a very strong DmXPD nuclear signal (Figure 4, E and F). However, some cytoplasmic DmXPD was detected in all developmental stages we have analyzed (our unpublished results and Figure 4F and

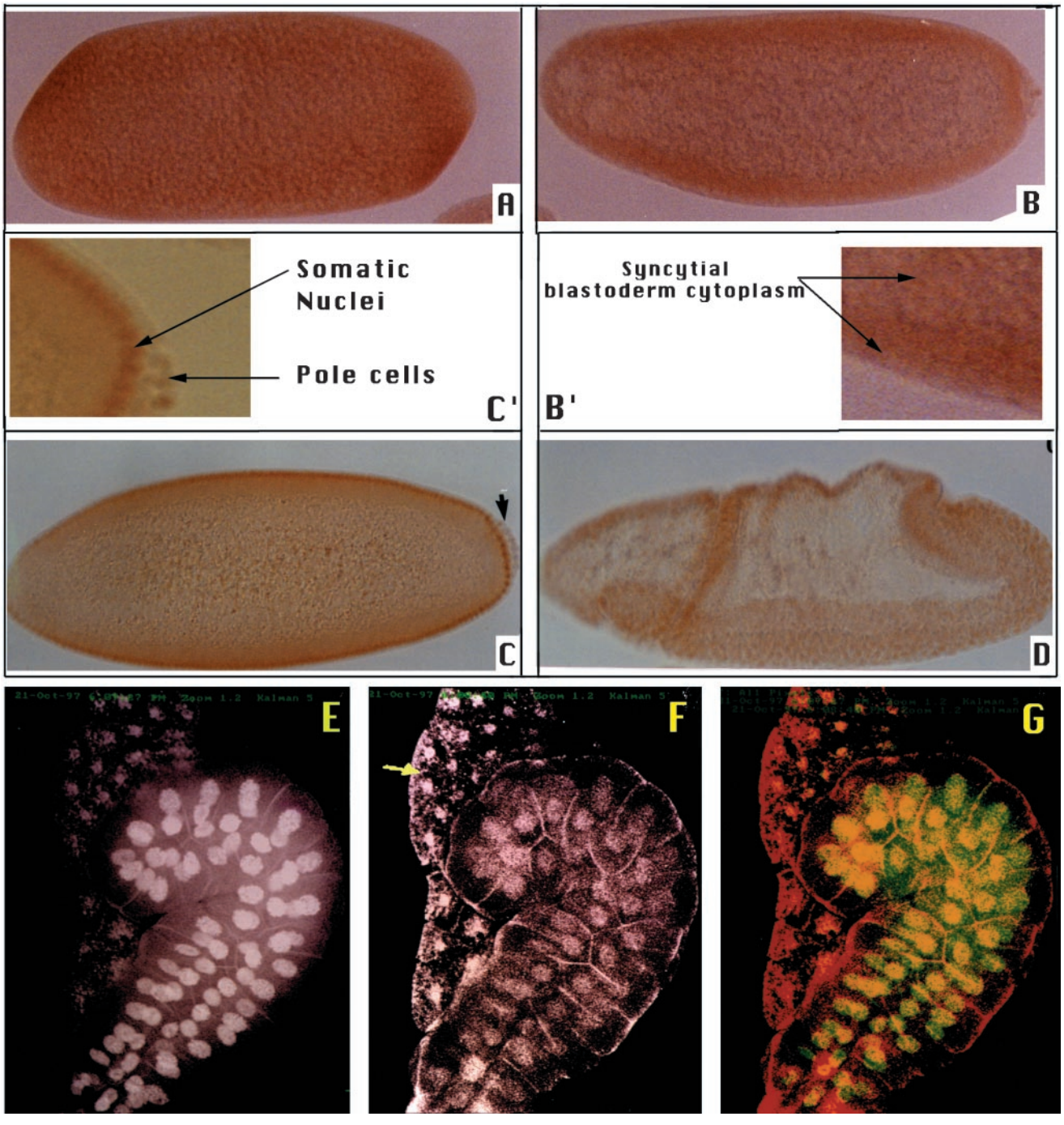

Figure 4. DmXPD distribution in wild-type embryos and larval tissues. Immunostaining with the anti-DMXPD antibody of embryos in different developmental stages: (A) syncytitial blastoderm; DMXPD is abundant and homogeneously distributed, probably from maternal contribution; (B) cellularization; DMXPD is concentrated in the embryo periphery; (B′), an amplification of panel B; (C) cellular blastoderm; the germ cells are indicated with an arrow, and DmXPD is mostly located in the nuclei of the somatic cells; (C′) an amplificaton of panel C; (D) gastrulation; DmXPD is nuclear in all cells; (E) larval-salivary gland and fat body nuclei stained with cytogreen (which stains DNA); (F) the same preparation as in panel E immunostained with the anti-DmXPD antibody. The arrow indicates cytoplasmic DmXPD in the fat body cells; (G) overlay of panels E and F. Cytogreen-stained DNA appears in green, anti-DmXPD signal is in red, and the overlapping of both signals is in yellow.

red signal in Figure 4G). In larval tissues, this cytoplasmic signal was more evident in the fat body (arrow in Figure 4F); no signal was detected in similar preparations with the preimmune antiserum in the cytoplasm (our unpublished results). Our findings, showing that DmXPD is nuclear, correlate with the nuclear location of other TFIIH components (CDK7, CYCH, MAT1, and p62), which are preferentially found in coiled bodies in human cultured cell nuclei (Grande *et al.*, 1997; Jordan *et al.*, 1997). Nevertheless, no cytoplasmic location is mentioned for XPD or other TFIIH components in these reports. Our results indicate that a DmXPD pool exists in the cytoplasm.

XPD Is Preferentially Located in the Interbands of Salivary Gland Polytene Chromosomes, and the Number of Stained Bands Increases after UV Irradiation

The TFIIH complex has a dual role in RNA polymerase II transcription and in DNA repair. The protein members of the complex should be found where RNA polymerase II transcription or DNA repair are occurring in the chromosomes. We performed a series of experiments to determine the DmXPD location in relation to these two cellular processes. First, we tested the DmXPD location in standard polytene chromosome preparations. These chromosomes were double labeled with propidium iodide, which stains heterochromatin regions more intensively (bands) (Figure 5, A and B) and with the DmXPD antibody detected with a secondary antibody coupled to fluorescein (Figure 5, C and D). The results show that the DmXPD product can be identified in discrete regions, mostly in the interbands and concentrated in the puffs (Figure 5, E and F). Little staining is also observed in the nuclear matrix and in regions that could be heterochromatin, judged by their propidium iodide staining (Figure 5, A–F). It is important to note that DmXPD is not found nonspecifically all through the puff but is localized in specific zones that can be seen as sharp bands (Figure 5D), suggesting that even though the whole region in the puff is being highly transcribed, DmXPD is found only in particular positions. Costaining of polytene chromosomes with the anti-DmXPD antibody and with the anti-p62 antibody shows that both proteins colocalize (Figure 6B), suggesting that DmXPD is present mostly as a component of the TFIIH complex in the chromosomes.

XPD has an important role in the NER mechanism, and defects in this gene in humans and yeast cause a dramatic increase in UV sensitivity. The fact that we can detect the DmXPD protein directly in polytene chromosomes allows us to determine the amount and/or the distribution of the protein in the chromosomes after UV damage. With this goal, third-instar larvae were irradiated with UV doses of 150 (half-LD, Mounkes *et al.*, 1992), 300, or 600 J/m^2, and after 30–40 min of recovery, the salivary gland polytene chromosomes were dissected and stained with the DmXPD antibody. The individual's lethality is observed at the pupae stage 36–48 h after irradiation. The chromosomes were visualized and quantified by photon counting. The DmXPD signal generates a distribution of the total number of pixels with different intensities. In the control experiment with no irradiation (Figure 7A and its respective graph in Figure 7E), the DmXPD signal had a normal distribution with a minor peak of 3% of pixels at the maximum arbitrary units (255) of intensity (see figure legend). In contrast, as UV doses were increased, more DmXPD was associated with diverse sites of the chromosomes, and the quantified signal was displaced to higher arbitrary units of intensity (13% in the chromosomes that received 600 J/m^2, the maximum dose tested; Figure 7, B–D, and their respective graphs in Figure 7E). The behavior of the response follows a typical dose–response curve as shown in Figure 7F.

These results indicate that the number of sites in the chromosomes with a DmXPD signal is increased in UV-irradiated larvae, suggesting that DmXPD is recruited to damaged chromosomal regions in a higher concentration. Alternatively, UV-damaged chromosomes may have lost some properties, thereby permitting nonspecific antibody binding, which produces a higher signal. However, we discarded this possibility because no significant changes in the signal intensity in irradiated polytene chromosomes (150–600 J/m^2) were detected using anti-histone and anti–TATA-binding protein antibodies after UV irradiation in our samples (our unpublished results and see MATERIALS AND METHODS).

The increase in chromatin-associated DmXPD protein after UV irradiation could be due to synthesis of new DmXPD protein or to relocation of preexisting DmXPD found in the nuclear lumen or in the cytoplasm to chromosomes. Northern and Western blot experiments indicated that there is not a significant increase in the DmXPD RNA or in DmXPD protein after UV irradiation (our unpublished results). Thus, it is more likely that the increase in the amount of DmXPD in the chromatin after UV irradiation is due to a relocation of the protein from the nucleoplasm or the cytoplasm to the chromosomes (see DISCUSSION). If chromosome preparations are made 24 h after UV irradiation, a decrease in signal intensity is observed (our unpublished results), indicating a decay of the presence of DmXPD in the chromosomes with time.

DISCUSSION

The NER mechanism, given its association to severe genetic syndromes, is of special relevance for hu-

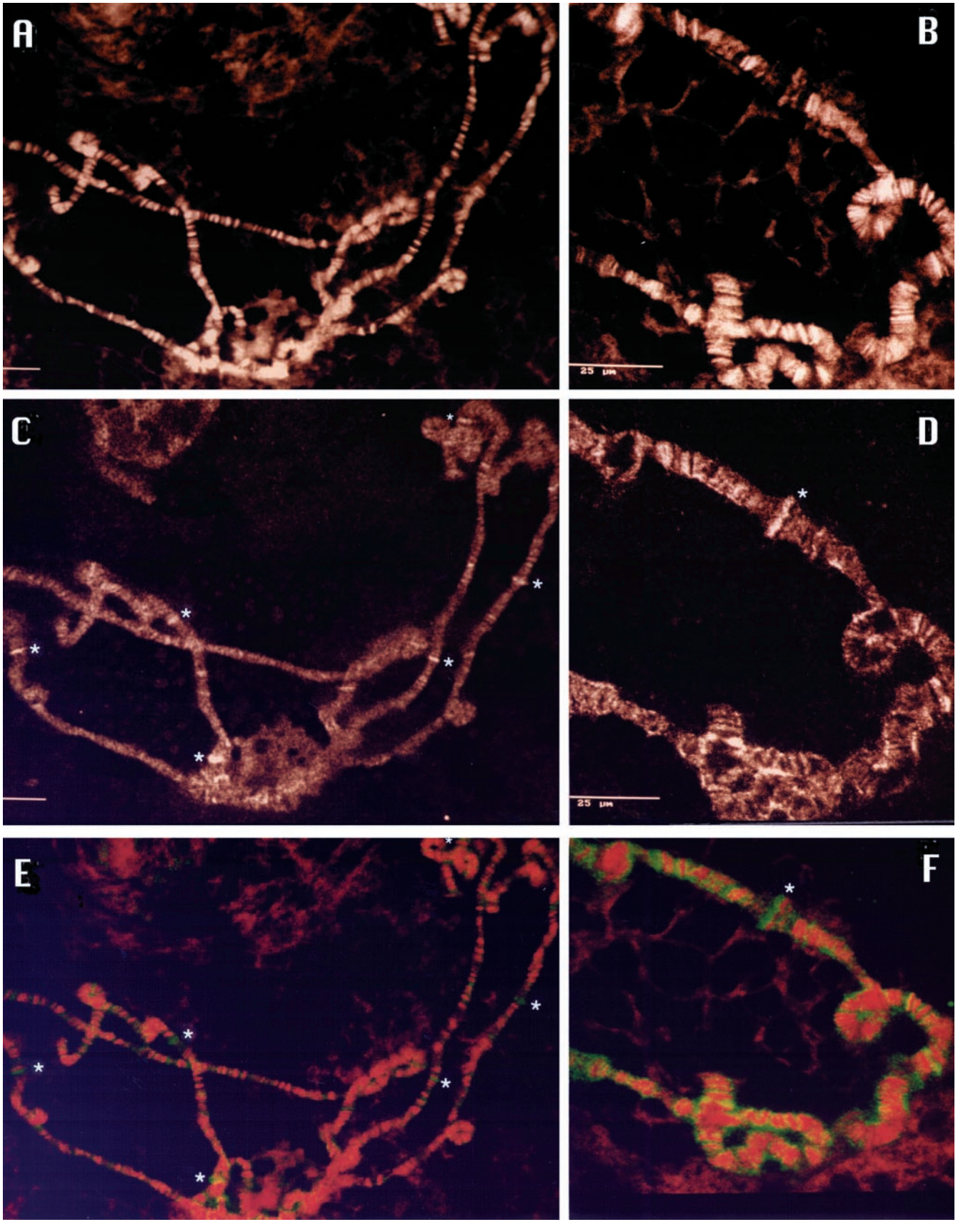

Figure 5.

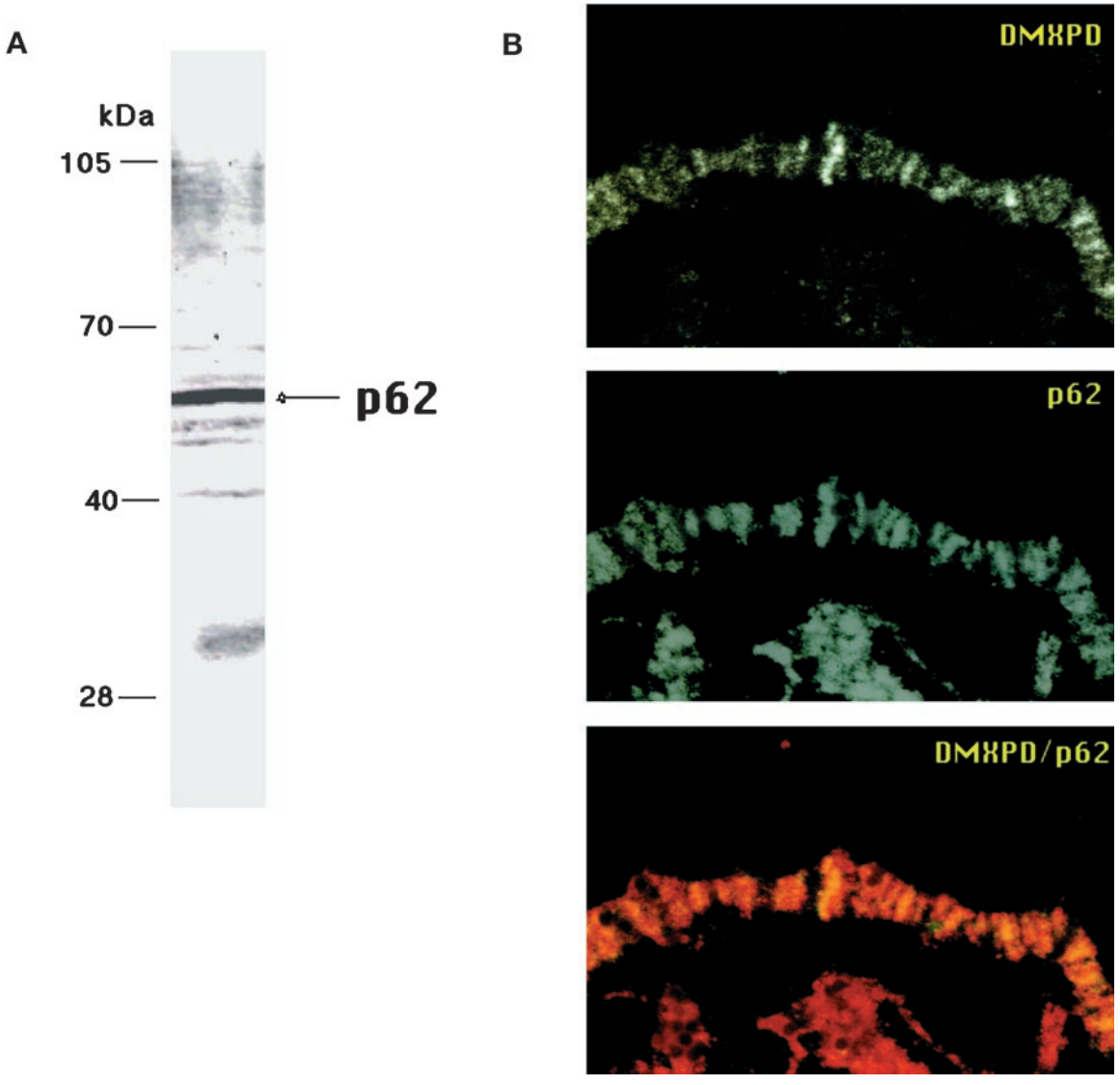

Figure 6. Colocalization of DmXPD and p62 in polytene chromosomes. (A) Western blot with a human anti-p62 monoclonal antibody against a total soluble *Drosophila* embryo extract. The arrow indicates the *D. melanogaster* p62 protein. (B) Immunolocalizations in polytene chromosomes of DmXPD (top panel), p62 (middle panel), and colocalization of both signals (bottom panel). Notice that most of the signal in both stainings colocalize (yellow in the bottom panel).

man disease. Since NER is highly conserved among eukaryotes, yeast has constituted a useful genetic model for isolation and study of the components of this system. Only a few homologues of these components have been characterized in *Drosophila*. Here we report the identification of the XPD homologue from the fly. It would be relevant to test whether mutations in the NER components in *Drosophila* would cause defects similar to the ones found in human syndromes caused by an affected NER mechanism. An initial demonstration that this may be the case is the phenotype presented by flies carrying defective alleles of the *haywire* (*XPB*) gene, which mimic to some extent the XP and CS defects in humans (Mounkes *et al.*, 1992).

Figure 5 (facing page). Distribution of the DmXPD protein in polytene chromosomes. (A and D) Polytene chromosomes stained with propidium iodide visualized in a confocal microscope at 40 and 100×, respectively. The propidium iodide preferentially binds regions where the DNA is more compact (bands). (B and C) The DmXPD immunostaining of the same chromosomes as in panels D and A, respectively. DmXPD is mostly located in the interbands and enriched in the puffs. (E and F) The overlay compositions of panels A and C and of panels B and D, respectively. The propidium iodide signal is shown in red, and the DmXPD signal is shown in green; yellow is shown where both signals overlay. The asterisks mark DMXPD signal at specific bands in diverse chromosome locations.

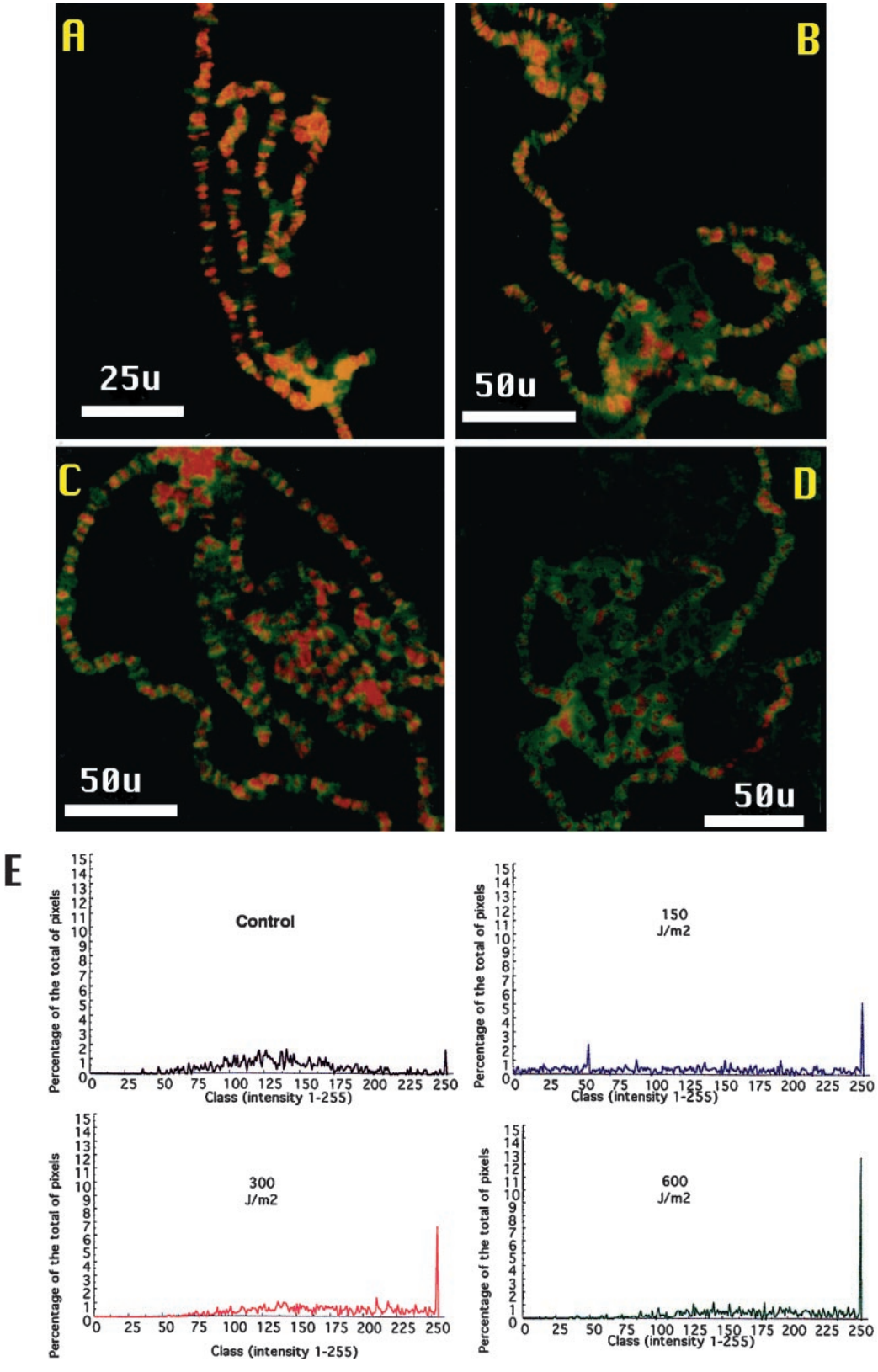
A
25u
B
50u
C
50u
D
50u
E
Control
150 J/m2
300 J/m2
600 J/m2
Percentage of the total of pixels
Class (intensity 1-255)

Figure 7.

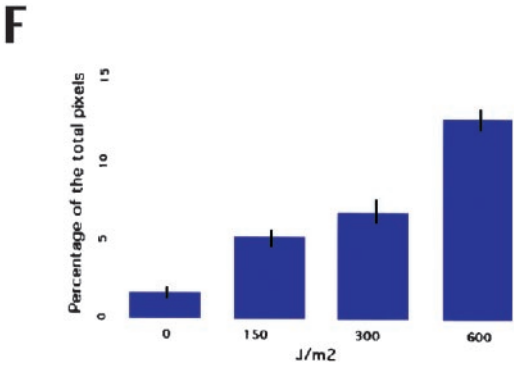

Figure 7 (cont). Distribution and quantification of DmXPD in polytene chromosomes after UV damage double stained with the anti-DmXPD antibody (green) and with propidium iodide (red). (A) Nonirradiated chromosomes from third-instar larvae as a control. (B–D) Polytene chromosomes UV irradiated with 150, 300, and 600 J/m^2 doses, respectively; the green signal corresponds to the places where DmXPD is located. (E) Quantification of the DmXPD signal observed in each one of the cases shown in panels A–D. Each graph represents averaged data from arbitrary scannings on 75-μm longitudinal sections of the three chromosomes found in 16 different nuclei per experimental condition. The percentage of total pixels at the maximum intensity was calculated using the photo-scan program from the confocal microscope. The maximum intensity value (255) is given in arbitrary units determined by the program. (F) Column representation of the percentage of pixels at maximum intensity of the DmXPD staining in relation to the UV irradiation dosage.

XPD is an important component of the TFIIH transcriptional complex. Mutations in the *XPD* yeast homolgue *Rad3* affect the transcription by RNA polymerase II (Guzder *et al.* 1994). In *D. melanogaster* embryos, there is a very low level of transcription at the preblastoderm stage, and it is dramatically increased after cellularization (Lamb and Laird, 1976; McKnight and Miller, 1976; Foe *et al.*, 1993). The transcription of some specific genes such as histones and some gap and pair-rule genes is initially detected during mitotic cycle 10, during the syncytial blastoderm. This is the first moment during embryo development at which transcription can be detected, probably as result of an elongation of interphase at mitotic cycle 10 (Edgar and Shubiger, 1986; Foe *et al.*, 1993). It is also known that the RNA polymerase II exists in nuclei that are still not fully transcriptionally active, and that the CTD domain is hypophosphorylated. The phosphorylation of the CTD is one of the important functions of the TFIIH complex, as it allows the polymerase to elongate after transcription initiation (Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995). This phosphorylation coincides with the onset of transcription in both somatic and germ cells (Seydoux and Dunn, 1997). When we analyzed the distribution of the DmXPD protein during early development, we found that DmXPD is located in the cytoplasm before the RNA polymerase II transcription is fully active. DmXPD is at the periphery of the embryo when the nuclei migrate to the periphery, and by the time the cellularization has been completed, DmXPD is nuclear (approximately mitotic cycle 14). Therefore, there is a correlation between the nuclear localization of DmXPD in the cellular blastoderm nuclei and the moment when the TFIIH complex phosphorylates the RNA polymerase II CTD. From this data we propose that TFIIH is not active in the nontranscribing nuclei during early development because all its components are still not present in the nucleus. In fact, it has been recently reported that, during mitosis, most of the human TFIIH components are phosphorylated and excluded from the mitotic nuclei (Long *et al.*, 1998).

An important question is raised from our work and from the previous work of Seydoux and Dunn (1997). They found that the earliest moment at which a significant phosphorylation of the CTD can be detected is at the cellular blastoderm stage. Here we show that one of the TFIIH components involved in the CTD phosphorylation is also detected in the nuclei at this specific developmental stage. Thus, how are the few genes that are expressed at mitotic cycle 10 in the syncytial blastoderm transcribed when there is no active RNA polymerase II? A possible explanation could be that the levels of the phosphorylated CTD, as well as the DmXPD levels in these nuclei, are very low and therefore very difficult to identify by immunological techniques. An alternative explanation could be that, at this stage, the polymerase does not require the TFIIH complex, and that it is relevant only when a full transcriptional activity is present in the cell, after cellularization. In agreement with this possibility, the transcription of some genes in yeast is independent of the TFIIH–kinase activity mediated by the RNA polymerase II (Dong-ki and Lis, 1998). In future experiments it will be important to determine the distribution of other TFIIH proteins during early embryogenesis as well as to establish whether they already exist as a complex in the cytoplasm or if they are recruited in the nuclei to be assembled in the TFIIH complex.

We found that some DmXPD is in the cytoplasm of embryos and in larval tissues. Interestingly, both XPB and XPD may have a function in the cytoplasm. The *XPB* yeast homologue *Ssl2* was initially identified as a dominant supressor of a mutation that prevents translation initiation because it generates a stable stem-and-loop structure in the 5′-untranslated region of the *ZHIS4* gene (Guylas and Donahue, 1992). *Ssl2 (XPB)* and *Rad3 (XPD)* are involved in antagonizing *Ty1* transposition in *S. cerevisiae*, probably by inhibiting reverse transcription or by destabilizing a *Ty1* cDNA (Lee *et al.*, 1998). These results and our DmXPD localization data suggest a role for this protein in the cytoplasm. Future studies in the fly, on the role of these proteins in the cytoplasm, will be important to

determine possible new functions of some of the TFIIH components.

Studies on the distribution of some of the TFIIH components in chromatin have been limited to their localization in cultured cell nuclei. Some of the TFIIH components are dispersed in the nuclear lumen, and others are found in particular regions in the nuclei in foci called "coiled bodies" (Grande *et al.*, 1997; Jordan *et al.*, 1997). DmXPD is located in the interbands, which are euchromatic regions with relaxed chromatin. DmXPD is mostly excluded from the bands (heterochromatic regions), although colocalization of DmXPD with propidium iodide in some band regions was also found. It is important to note that even though DmXPD is enriched in the puffs, it is not distributed throughout but is localized in sharp discrete bands inside these regions. This is consistent with evidence that shows that in vitro TFIIH is found only in the transcription initiation sites and does not travel with the RNA polymerase during the RNA elongation (Dvir *et al.*, 1997).

Studies on the analysis of the DNA response to UV damage, directly visualizing the chromosomes, have not been reported. The fact that we can localize DmXPD in polytene chromosomes opened the possibility for analyzing changes in the DmXPD distribution in response to DNA damage. We determined that there is an increment in the number and intensity of sites where DmXPD is located in the UV-irradiated chromosomes of third-instar larvae, but we did not observe an increase in the DmXPD transcript or protein after the treatment. The conclusions derived from these observations are that XPD, probably as part of the TFIIH complex, is highly dynamic in the chromosome architecture and that it is recruited to regions where it was not present before UV irradiation.

In vitro experiments have led to the proposal that transcription by RNA polymerase II is inhibited when there is UV DNA damage, because TFIIH is used for NER instead of being used for transcription (Zhaoyang *et al.*, 1998). We found that highly transcribed regions are rich in DmXPD. After UV damage, new regions are stained with the anti-DmXPD antibody, even heterochromatin, but the puffs still have DmXPD. However, it is possible that regions that are highly transcribed are more sensitive to suffering damage from different physical and chemical agents (Bohr *et al.*, 1985); therefore, the NER complex should be recruited to repair the puffs as well. Similar studies to the ones presented here with other NER components, not only from the TFIIH complex, including XPA, XPC, XPF as well as the use of thymine–dymers antibodies, will be important to determine whether they have a similar behavior in terms of response and localization dynamics. The eventual isolation of *DmXPD*-defective alleles, as well as other nonidentified fly genes that might encode components of the TFIIH complex, will be a useful approach by which to study the role of this complex during development.

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