Radiation-activated DNA-binding Protein Constitutively Present in Ataxia Telangiectasia Nuclei*

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We have recently described the appearance of a specific DNA-binding protein in nuclei from human cells exposed to ionizing radiation which was not detected in nuclear extracts from unperturbed cells (Singh, S. P., and Lavin, M. F. (1990) Mol. Cell. Biol. 10, 5279–5285). We report here a similar activity which is constitutively present in nuclei of both unirradiated and irradiated cells from patients with the human genetic disorder ataxia telangiectasia (A-T). Activity was present in unirradiated nuclear extracts from 3 A-T cell lines of different complementation groups, but was not detected or was present only at a low level in 4 controls. Active protein was detected in the cytoplasm of both cell types. Exposure to ionizing radiation did not change the amount of DNA binding activity in A-T nuclei but led to an increase in nuclei from 4 control cell lines. Purification of the binding activities from A-T nuclei and control cytoplasm was carried out by affinity chromatography, as described previously for control extracts (Teale, B., Singh, S. P., Khanna, K. K., Findik, D., and Lavin, M. F. (1992) J. Biol. Chem. 267, 10295–10301). Southwestern analysis and UV cross-linking confirmed the presence of a major DNA-binding species at 70 kDa in both cases with a minor binding activity at 47 kDa also evident. It was not possible to distinguish between the binding activities from A-T and control cells under different conditions, and phosphorylation was required for binding activity in both cases. Footprint analysis revealed that the same sequence was being recognized by the control and A-T proteins. The constitutive presence of a specific radiation-responsive DNA-binding protein in A-T cells may be indicative of a continuous state of stress in these cells.

Ataxia telangiectasia (A-T) is characterized by immunodeficiency, developmental abnormalities, neurodegenerative changes, chromosomal instability, hypersensitivity to ionizing radiation, and predisposition to cancer (Boder, 1985; McFarlin et al., 1972; Peterson et al., 1964). While several reports using genetic linkage analysis of A-T families have allowed mapping of A-T genes to chromosomal region 11q 22–23 (Gatti et al., 1988; McConville et al., 1990; Foroud et al., 1991) and a candidate for the A-T gene has been cloned (Kapp et al., 1992), the nature of the biochemical defect remains unknown. A considerable body of evidence points to a defect at the level of chromatin or in DNA processing (Hanawalt and Sarasin, 1986). This evidence includes chromosomal instability and specific gene rearrangements (Russo et al., 1988), hypersensitivity to ionizing radiation (Chen et al., 1978; Taylor et al., 1975), radioreistant DNA synthesis (Lavin and Schroeder, 1988), defective DNA repair (Cornforth et al., 1985; Paterson et al., 1976), inappropriate gene expression (Baxter et al., 1988), altered DNA topoisomerase II activity (Davies et al., 1989; Singh et al., 1988), and a defect in one or more genes involved in the response of p53 to radiation (Kastan et al., 1992). The increased risk of developing a number of different tumors, also characteristic of heterozygotes (Pippard et al., 1988; Swift et al., 1987), makes this syndrome a useful model to investigate the molecular changes responsible for radiation sensitivity and cancer predisposition.

It is now evident that cells respond to radiation damage by the induction of specific genes and the synthesis of a range of proteins (Boothman et al., 1989; Domashenko et al., 1990; Fornace et al., 1989; Hallahan et al., 1991; Papathanasiou et al., 1991; Sherman et al., 1990). In addition to de novo protein synthesis, activation of pre-existing proteins has also been described in response to DNA damage (Glazer et al., 1989; Kingston et al., 1987; Frywyes et al., 1988). We have recently described the activation of a DNA-binding protein from a pre-existing protein in human cells exposed to γ-radiation (Singh and Lavin, 1990). This protein is present in active form in the cytoplasm of unirradiated cells and appears transiently in the nuclei of irradiated cells after exposure to γ-radiation. While this factor was first recognized by its ability to bind to a specific sequence in the SV40 enhancer, we have recently shown that it binds to several sequence elements in the human genome (Hobson et al., 1991). We have recently reported the purification of this binding activity from normal control cells (Teale et al., 1992). The role of this protein is not yet defined but since it responds to radiation damage it may be involved in the processing of DNA damage or regulation of gene expression. Since A-T cells show an abnormal response to ionizing radiation it was of interest to study the behavior of the DNA-binding protein in these cells. We show here that this protein is constitutively present in nuclei from unirradiated A-T cells of different complementation groups, contrary to that seen in control cells. The properties of the purified factor from A-T and control cells are compared.
**EXPERIMENTAL PROCEDURES**

**Cell Culture and Irradiation**—Five Epstein-Barr virus-transformed lymphoblastoid cell lines, C8ABR, C13ABR, C24ABR, C25ABR, and C27ABR, and 3 A-T cell lines AT1ABR, AT2ABR, and AT4ABR were used in this study. Cells were routinely cultured in RPMI 1640 medium with 10% fetal bovine serum at 37°C under an atmosphere of 5% carbon dioxide, 95% air. Cultures were harvested at a density of 2 x 10^6 cells/ml. Flow cytometry was performed regularly to ensure diploidy within the cultures and to discriminate between control and A-T cells (Bates and Lavin, 1986). Cells were exposed to γ-rays using a 137Cs source (Atomic Energy of Canada) at a dose rate of 4 gray/min. Incubation was carried out at 37°C for indicated times prior to preparation of extracts.

**Preparation of Cellular Extracts**—Nuclear extracts were prepared from lymphoblastoid cells essentially by the method of Dignam et al. (1983). Cells were centrifuged in Sorvall GSA tubes, and pellets were suspended in 1.5 ml of ice-cold buffer A (10 mM HEPES, pH 8.0, 50 mM NaCl, 0.5 mM sucrose, 1 mM EDTA, 0.25 mM EGTA, 0.6 mM spermidine, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 7 mM 2-mercaptoethanol) and subjected to Dounce homogenization (10-15 strokes until 80% lysed). The suspension was then transferred to bench centrifuge tubes, and nuclei were recovered by centrifugation at 1,000 x g for 10 min. Nuclear pellets were washed twice in buffer A, suspended in buffer A containing 400 mM NaCl and 25% glycerol (buffer B), and stirred for 30 min. Nuclear debris was discarded after centrifugation at 2,000 x g for 10 min. The supernatant was recovered after further centrifugation at 250,000 x g relative to 55% (v/v) with (NH4)2SO4, and centrifuged at 12,000 x g for 10 min. The pellet was suspended in a minimum volume of buffer B, and dialysis was carried out with the same buffer.

Cytoplasmic extracts were prepared by collecting the supernatant from nuclear pellets prepared from cells homogenized in buffer A containing 0.5% (v/v) Triton X-100. In this case also a 55% (NH4)2SO4 cut was carried out, followed by dialysis against buffer B. In some experiments cytosolic fractions were prepared from cells suspended in hypotonic solution and homogenized. Nuclear and cytoplasmic samples were stored at -20°C.

**Protein Purification**—DNA-binding protein was purified from A-T nuclear extracts (unirradiated) and from control cytoplasmic extracts (unirradiated) essentially as described in Teale et al. (1992).

**Sephacryl and Heparin-agarose Chromatography**—The dialyzed extract (90–80 mg) was applied to a column (35 x 260 mm) of preswollen Sephacryl S-200, equilibrated with buffer B containing 10% glycerol, and stirred for 16 h. The column was washed with 5 volumes of buffer B containing 100 mM NaCl for 30 min at 4°C prior to adding to a column (10 x 800 mm). The column was sequentially washed with 10 ml of buffer B containing 100 mM NaCl followed by dialysis against buffer B containing 100 mM NaCl.

**Sequence-specific DNA Affinity Chromatography**—The method was essentially the same as described by Kadonaga and Tjian (1986). The double-stranded DNA oligonucleotide (50 μg),

5'-GATCGTCAGTTAGGGTG-3'
3'-CAGCTATCCACCCTAG-5'

**SEQUENCE 1**

corresponding to the DNA-binding site with additional CTAG sequences attached at the 5' ends, was phosphorylated with T4 polynucleotide kinase (50 units) in 30 μl containing 20 mM ATP for 2 h at 37°C followed by self-ligation in 20 μl containing ligase buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl2, 100 mM DTT, and 5 mM ATP) and T4 DNA ligase (10 units) at 14°C for 16 h. This resulted in a 100–100 unit length product which was subsequently coupled to CNBr-activated Sepharose 4B (Johnson and McKnight, 1989). Dialyzed extract (1–5 mg of protein) in the presence of 2–5 μg of poly(dI-dC) was applied to a PolyPrep column (10 x 20 mm) equilibrated in buffer B containing 100 mM NaCl at a flow rate of 10 ml/h. The column was washed with 5 volumes of buffer B containing 100 mM NaCl followed by stepwise elution with 200 mM, 500 mM, and 1 M NaCl. Most of the binding activity was recovered in the 1 M NaCl fraction. The active fraction was dialyzed against buffer B containing 100 mM NaCl and the DNA-affinity step was repeated.

The purified protein was stored at 4°C and was stable for several months.

**DNA-Protein Binding and Gel Electrophoresis**—DNA binding studies were performed using a double-stranded oligonucleotide corresponding to the DNA-binding site from the SV40 enhancer (or a heptamer of this oligonucleotide) as the binding template. The oligonucleotide was synthesized on an Applied Biosystems 380B model DNA synthesizer. End-labeling of the oligonucleotide was carried out with [32P]dGTP using Klenow polymerase.

Incubations were performed using 25 ng of 32P-labeled oligonucleotide, an appropriate amount of protein and a corresponding amount of poly(dI-dC)-poly(dI-dC) (competitor DNA) and 60,000 cpm of 32P-labeled DNA fragment in the presence of 5 mg/ml Tris-HCl, pH 8.3, 50 mM NaCl, 1 mM EDTA. The incubations were performed at room temperature for 15 min and a gel mobility shift assay was then performed on a 5% polyacrylamide gel in TBE (50 mM Tris-HCl, pH 8.3, 50 mM NaCl) at a dose rate of 4 gray/min. The gel was then dried and autoradiographed overnight.

**Southwestern Analysis and UV Cross-linking**—A heptamer sequence of the oligonucleotide corresponding to the binding site was used as a probe. Purified extract (10–50 ng) was separated by SDS-PAGE (4% (v/v) acrylamide stacking gels and 8% (v/v) acrylamide separation gel). The gel was then removed from the assembly and incubated in transfer buffer (250 mM HCl, pH 6.5, 190 mM NaCl, 20% (v/v) methanol, 0.1% (v/v) SDS) for 30 min prior to electrophoretic transfer to a nitrocellulose filter. The nitrocellulose filter was then soaked in a BLOTTO solution (5% (w/v) Carnation milk powder, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl; 1 mM EDTA, 1 mM DTT) for 1 h at room temperature. The filter was then washed in two changes of TNE-50 (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 500 mM KCl, 1 mM EDTA, 0.5% (w/v) Carnation milk powder) and then placed in a denaturing solution (5 M guanidine HCl, 50 mM Tris-HCl, pH 8.3, 2 mM EDTA, 50 mM DTT, 0.25% (w/v) Carnation milk powder) for 1 h at room temperature. The filter was subsequently washed with two changes of TNE-50 and then placed in a renaturing solution (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.1% (v/v) Nonidet P-40, 0.25% (w/v) Carnation milk powder) for 16 h at 4°C. The filter was washed as before and incubated in a solution containing 5 ml of TNE-50, 3.3 μg/ml of poly(dI-dC)-poly(dI-dC) (competitor DNA) and 60,000 cpn of 32P-labeled probe for 2 h. The filter was washed, air dried, wrapped in polyethylene film, and placed in an autoradiography cassette overnight at -70°C.

For UV cross-linking the synthetic oligonucleotide probe was end-labeled using bromoacetoxystyryl (50 μM). The binding reaction was performed as described above. DNA-binding proteins were cross-linked to 32P-labeled oligonucleotide by exposure to 300 nm of UV light (a time course was initially performed to determine the optimum length of exposure). The cross-linked proteins were mixed with 6 μl of 5 x SDS and gel loading buffer (0.01% (w/v) bromophenol blue, 6% (w/v) SDS, 15% (v/v) glycerol, 70 mM DTT, 0.3 M Tris-HCl, pH 6.8). The cross-linked proteins were heat denatured (100°C for 2 min), separated by SDS-PAGE (Laemmli, 1970), and autoradiographed.

**Footprint Analysis**—DNase footprints of the DNA-binding site was performed using a variation of the method in the Promega Core Footprinting System. This kit utilizes the KpnI/HindIII (300 base pairs) region of the SV40 promoter/enhancer as the binding template. An excess of purified cellular extracts, 80 ng (determined by mobility shift assay as the amount necessary to retard >100% of available DNA), were incubated with labeled SV40 fragment in the presence of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA) at room temperature for 15 min. The reaction was then supplemented with 2.5 mM CaCl2, 5 mM MgCl2 and digestion initiated with 5 units of RQ1 DNase. This was allowed to act for 80 s before addition of the stop solution (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 13.5 mM EDTA). The reaction was then subjected to phenol/chloroform before being desalted in a Pharmacia NAP-5 column, and then precipitated in isopropl alcohol (0.6 volumes). The resulting pellet was then resuspended in loading dye and applied to a 6% acrylamide, 6 M urea sequencing gel and autoradiographed.

**RESULTS**

**Cellular Distribution of DNA-binding Protein in A-T and Control Cells**—A specific DNA-binding protein appears in the nuclei of control lymphoblastoid cells exposed to ionizing radiation (Singh and Lavin, 1990). Since cells from patients with ataxia telangiectasia (A-T) show abnormal responses to ionizing radiation (Singh and Lavin, 1990).
DNA-binding Protein in Ataxia Telangiectasia

In order to investigate this phenomenon in more detail, DNA binding activities were purified from A-T nuclei (unirradiated) and from the cytoplasm of unirradiated control cells. Purification was carried out by affinity chromatography as described recently for control extracts (Teale et al., 1992). As observed in that study, 4 major protein bands of approximately 70, 55, 47, and 31 kDa were detected by SDS-PAGE for both A-T and control (Fig. 3A). Increasing protein concentration over the range 1-20 ng revealed the same degree of DNA binding activity in A-T and control (data not shown). The specific activity of the AT1ABR purified extract was 4.16 × 10^6 units/mg of protein, approximately the same as previously published for C3ABR (Teale et al., 1992). Southwestern analysis showed that two binding species were present in both A-T and control preparations, one at 70 kDa and a second with lesser activity at 47 kDa (Fig. 3B). Photoactivated cross-linking of the purified protein to ^32P-labeled oligonucleotide probe containing 5-bromodeoxyuridine residues revealed the presence of only the 70-kDa species (Fig. 3C).

Properties and Comparison of Binding Sites—The purified proteins from the two cell types were heat labile to the same extent with binding activity decreasing markedly above 40 °C (Fig. 4A). Loss of activity occurred at the same rate for both proteins as the salt concentration was increased to 1 M in the binding reactions (results not shown). Dephosphorylation of the proteins caused a similar pattern of loss of activity in A-T and control (Fig. 4B). DNase-I footprint analysis was carried out to verify that the binding activities from the two cell types were recognizing the same sequence. A protected region of 5 nucleotides with the sequence CACTT, located between nucleotides 256 and 252 in the 5'-flanking region of the distal repeat of the SV40 enhancer, was evident with both control and A-T proteins (Fig. 5).

DISCUSSION

In this report we have demonstrated that a specific DNA-binding protein is abnormally distributed between the nucleus and cytoplasm in cells from patients with the human genetic disorder A-T. In 4 control cell lines this DNA-binding protein is either absent or present at low levels in nuclear extracts from unirradiated cells. Exposure of control cells to ionizing radiation causes the binding factor to appear in nuclei (Singh and Lavin, 1990). These results suggest that the binding protein is normally present in control cells sequestered in the cytoplasm and is translocated to the nucleus by some unknown mechanism.

In order to check the generality of this observation, extracts were prepared from a number of control and A-T cell lines representing three different complementation groups (Chen et al., 1993). As observed previously with C3ABR, nuclear extracts from C24ABR and C25ABR (unirradiated) showed negligible binding activity (Fig. 1A, lanes 1-4) whereas activity was present in nuclear extracts from a second A-T cell line, AT4ABR (Fig. 1A, lanes 7 and 8). Exposure of C24ABR and C25ABR to 10 gray of γ-rays caused the activity to appear in nuclear extracts, and binding increased with increasing protein concentration (Fig. 1B, lanes 1-4). Again in the case of this A-T cell line, AT4ABR, comparable binding activity was present in both unirradiated and irradiated extracts (Fig. 1A, B, lanes 7 and 8). Data from 2 other control cell lines demonstrate binding activity is either absent or present at low levels in nuclear extracts from unirradiated cells (C13ABR and C27ABR, data not shown). In all cases exposure to ionizing radiation increased the amount of nuclear DNA binding activity. Unirradiated nuclear extracts from AT1ABR (representing a third complementation group) also possessed the specific DNA binding activity and irradiation did not appreciably alter the amount of binding activity (data not shown). It seems likely that binding activity was translocated from the cytoplasm to the nucleus in response to radiation (Singh and Lavin, 1990). Again in this study activity was detected in the cytoplasm of unirradiated controls (Fig. 2, lanes 3 and 4) but was present in both compartments for unirradiated A-T cells (Fig. 2, lanes 5-8). Overall there was a high level of DNA binding activity in nuclear extracts from all 3 unirradiated A-T cell lines, whereas 4 controls showed undetectable or low levels of activity.

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known form of anchorage-release mechanism in response to cellular radiation damage. While its role remains unknown it has been shown to bind to many sequences in human genomic DNA characterized by a consensus motif (Hobson et al., 1991; Teale et al., 1992). The transcriptional regulators NF-κB and glucocorticoid receptor are also localized to the cytoplasm as part of macromolecular complexes in the absence of stimuli (Leonardo et al., 1989; Picard and Yamamoto, 1987). The appropriate stimulus, the presence of hormone in the case of the steroid receptor, causes dissociation from heat shock protein 90 (Nigg, 1990), whereas for NF-κB, dissociation is achieved by phosphorylation of an inhibitor, IκB (Baeuerle and Baltimore, 1988).

Another interpretation of the data obtained here is that the protein is perinuclear rather than cytoplasmic, being lost to the cytoplasm during nuclear extraction for control cells but retained in A-T because of a modification to the protein or the nuclear envelope. Angulo et al. (1991) have recently cloned the gene for a 44.6-kDa DNA-binding protein, KIN17, which is localized to the perinuclear region. The apparent compartmental change after radiation exposure might also be explained by a conformational change in a protein or configurational change at the level of the nuclear envelope. The
DNA-binding Protein in Ataxia Telangiectasia

maldistribution of the binding protein in A-T cells might be due to a mutation in the protein itself, or in a protein it associates with or as a result of changes at the level of the nucleus or envelope. It seems unlikely that the latter is the case unless such a change is of a very specific nature and does not affect the distribution of the majority of cellular proteins between the nucleus and the cytoplasm.

Purification of the binding activity from A-T and control cells did not reveal any obvious differences in binding to DNA or in the pattern of proteins observed. However, the recognition motif 5′-CAGTT-3′, determined by DNase footprinting, was considerably smaller than that observed with crude nuclear extracts (5′-GTCAGTTAGGTG-3′). The reduction in the size of the protected region may be due to the loss of other proteins (during purification) that associated with the binding protein and influenced the nature or affinity of binding. There exists a precedent for this in that the footprint for the human upstream binding factor is extended when a second species-specific protein (SLI) is present in the DNA-binding reaction (Jantzen et al., 1990). It seems likely that the binding activity described here is complex and is composed of more than one DNA-binding factor and possibly other proteins which may modulate or regulate specific binding in the cell. The existence of such complexes is well described for transcription factors and specificity of binding or in altering the binding affinity to DNA. The two major proteins without specific DNA-binding activity from cytoplasmic (control) and nuclear (A-T) extracts suggests that this may be due to specific association. The two major proteins without specific DNA-binding activity, 31 and 55 kDa in size, may play a role in ensuring specificity of binding or in altering the binding affinity to DNA. The interaction of these proteins with one another or with other factors may determine their cellular localization. The cellular distribution pattern observed in several A-T cells could be due to mutations in one or more of these proteins. Alternatively, localization of the binding activity in both the nucleus and cytoplasm of unirradiated A-T cells may be a consequence of specific secondary changes such as post-translational modification. This could be due to phosphorylation changes which are known to alter the localization of cellular proteins (Baueuerle and Baltimore, 1988; Dale et al., 1989; Hunt, 1989; Prywes et al., 1988). We have demonstrated that phosphorylation alters the binding activity in both control and A-T cells but provided no evidence that this was important in cellular localization.

The mechanism by which radiation modulates cell behavior remains unclear. This is complicated by observations that radiation can cause marked changes not only at the level of chromatin structure but also to the plasma membrane (Leyko and Bartosz, 1986; Painter, 1986). Consequently, signalling pathways could be initiated at two levels, the plasma membrane or the nucleus. Several genes are induced in response to radiation exposure (Kramer et al., 1990), in some cases preferential induction by radiation is observed (Fornace et al., 1989), whereas in others a single gene is induced by different genotoxic agents (Stein et al., 1989). It seems likely that damage to DNA and subsequent alteration in chromatin structure is the initiating event for gene activation. De novo protein synthesis is not always required for gene induction since it has been demonstrated that new synthesis of the transcription factors fos and jun is not required for activation of the collagenase enhancer by UV radiation (Kramer et al., 1990).

In the example provided here new protein synthesis is not required since pre-existing DNA-binding protein is translocated to the nucleus in response to radiation damage (Singh and Lavin, 1990).

It is not clear how the abnormal distribution of a radiation-responsive specific DNA-binding protein relates to the defect in A-T. However, it is worthy of note that this protein, which is translocated to the nucleus in control cells after radiation exposure, is constitutively present in all 3 radiation-hypersensitive A-T cell lines of different complementation groups assayed. In control cells it is possible that the protein plays a role in DNA processing after radiation damage (Hanawalt and Sarasin, 1986). Its continuous presence in A-T nuclei may be counterproductive and may contribute to the variety of abnormalities in this syndrome. Finally it will be of interest to determine whether the maldistribution of the binding activity is related to the abnormality in p53 response seen in A-T cells after exposure to radiation (Rastan et al., 1992).

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