Nuclear Extracts Lacking DNA-dependent Protein Kinase Are Deficient in Multiple Round Transcription*

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The DNA-dependent protein kinase is the only protein kinase that is known to be directly regulated by double-stranded DNA (1). It is composed of a 470-kDa catalytic subunit and a 70-80-kDa heterodimeric regulatory protein, Ku (2–4). Mammalian cells deficient in either the catalytic subunit of DNA-PKcs (V30T) or Ku80 (xrs-6cvec) contain a 470-kDa catalytic subunit and/or Ku protein, or individually purified general transcription factors. We conclude that extracts from DNA-PK-deficient cells lack a positively acting regulatory factor or a complex of factors not readily reconstituted with individual proteins. We have also investigated the mechanistic defect in the deficient extracts and have found that the observed differences in transcription levels between Ku-positive and Ku-negative cell lines can be attributed solely to a greater ability of the Ku-positive nuclear extracts to carry out secondary initiation events subsequent to the first round of transcription.

The abbreviations used are: DNA-PK, DNA-dependent protein kinase; Ku80, the 80-kDa regulatory subunit of DNA-PK; AdMLP, adenovirus major late promoter; hsp70, heat shock protein 70; CHO, Chinese hamster ovary; RNAP II, RNA polymerase II; cs, catalytic subunit; PMSF, phenylmethylsulfonyl fluoride; XRCC, x-ray repair cross-complementation; TBP, TATA-binding protein; PIC, preinitiation complex; O-Me-G, 3'-O- methyl-GTP.

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1 The abbreviations used are: DNA-PK, DNA-dependent protein kinase; Ku80, the 80-kDa regulatory subunit of DNA-PK; AdMLP, adenovirus major late promoter; hsp70, heat shock protein 70; CHO, Chinese hamster ovary; RNAP II, RNA polymerase II; cs, catalytic subunit; HTLV-I, human T cell leukemia virus type I; DTT, dithiothreitol; Tris-Cl, pH 7.9, 1 mM DTT, 60 mM PMSF.

To investigate directly the involvement of DNA-PKcs and/or Ku protein in RNAP II-mediated transcription, we measured in vitro transcription levels in nuclear extracts prepared from DNA-PK-deficient CHO cell lines. We found that cells deficient in either DNA-PKcs (V30T) or Ku80 (xrs-6cvec) consistently show a 2–7-fold decrease in transcription with a number of promoters. Surprisingly, in vitro transcription levels cannot be restored to the deficient nuclear extracts by supplementing with purified catalytic subunit or Ku protein. Rather, restoration of activity requires a positively acting factor or complex derived from a DNA-PK-containing cell line. In addition, we found that the decreased levels of transcription in DNA-PK-deficient cells lie in a decreased ability to carry out secondary initiation events consequent to the initial round of transcriptional initiation.
and then lysed by 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 3000 × g for 5 min, and the supernatant was discarded. The pellet was washed in 2 packed cell volumes of lysis buffer. The washed pellet was resuspended in 4 packed cell volumes of extract buffer (50 mM Tris-Cl, pH 7.9, 0.1 M MgCl₂, 0.5 mM EDTA, 20% glycerol, 10% sucrose, 2 mM DTT, 0.5 mM PMSF), dialyzed twice against TM 0.1 M buffer, and centrifuged for 30 min at 15,000 × g. The supernatant was collected and protein concentration determined by Bradford assay (Bio-Rad), using bovine serum albumin as a standard. HeLa and LTLo3 cells were grown in suspension in S-minimum Eagle’s medium (Joklik-modified) containing 10% newborn calf serum, 1% nonessential amino acid supplement, and 100 mM sodium pyruvate in 5% CO₂, 95% air at 37°C. Cells were harvested and nuclear extract prepared as described.

In Vitro Transcription—500 ng of DNA template was incubated for 30 min at 30°C with 30-μg of the appropriate nuclear extract in 25 mM Tris-Cl, pH 7.9, 0.05 M KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, and 20 units of RNasin in a final volume of 46.5 μl. The nuclear extract was then added to a final concentration of 1 μM ATP, 250 μM CTP, 12.5 μM UTP, 12.5 μM 3′-O-methyl-GTP and 10 μCi of [α-32P]CTP and the mixture incubated for another 45 min at 30°C. In some reactions, 10 μg/ml heparin was added 2-5 min after the initiation of transcription to prevent further increase in transcription. In some cases, endogenous GTP permitted extension of transcripts beyond the G-less cassette. To digest these to a uniform size, 40 units of ribonuclease T1 was added, and the reactions were incubated for 10 min at 37°C. All reactions were stopped by the addition of 150 μl of 0.1 M NaCl, 200 μg/ml tRNA, 0.02 mM EDTA, and 1% SDS and 100 μl of 10 mM Tris-Cl, pH 7.9, 1 mM EDTA, and 0.1 M NaCl. The reactions were extracted with 300 μl of a 1:1 (v:v) mixture of phenol and 96% chloroform, 4% isoamyl alcohol, and the RNA was precipitated by the addition of 750 μl of cold ethanol-containing 0.5 M NH₄OAc. The resulting pellets were washed with 500 μl of 70% cold ethanol, dried, and resuspended in 20 μl of 7 M urea dilaureate. Samples were loaded onto a prerun 5% urea polyacrylamide gel and electrophoresed at 700 V for 45 min. Incorporated radiolabel was visualized and quantitated by PhosphorImage analysis (Molecular Dynamics).

Protein Purification—Recombinant Ku protein was purified as described previously (26). Native DNA-PKcs was purified from HeLa cell nuclear extracts as described previously, except that the phenyl-Superose and Mono S steps were omitted (24). The αδ and γ subunits of TFIIA were purified individually using Ni²⁺-nitrilotriacetic acid-agarose chromatography (25), TFIIA was further purified by gel filtration on a Superdex 200 column to isolate assembled complexes from free subunits. Recombinant TFIIH (26) and recombinant TFIIH 30- and 74-kDa subunits were purified individually using phosphorylase b affinity chromatography. Recombinant TFIIIE 34- and 56-kDa subunits were purified as described (28). RNAP II (29) was purified from a HeLa cell nuclear pellet by DE52, heparin-agarose, and DEAE hydrocell 1000 (Raimin) chromatography. TFIIH was purified from HeLa cell nuclear extracts by phosphocellulose and DE52 chromatography, followed by GST-VP16 affinity chromatography as described previously (30). TFIIH was also purified from HeLa nuclear extracts using phosphocellulose, DE52, Superdex 200 (in 0.1 mM KCl buffer), DEAE hydrocell, phenyl-Superose, and Superdex 200 (in 0.3 mM KCl buffer) chromatography. For both purification schemes, TFIIH fractions were identified by Western blots using anti-TFIIH p62 antibody (Santa Cruz) and anti-TFIIH p40 antibody (Zymed Laboratories Inc.). Native TFIIH was purified as described previously (31). The peak from the Mono S column was further purified on a PureCel strong cation exchange resin. Highly purified preparations of epitope-tagged affinity purified TFIIH (eIIH) were obtained from a HeLa cell line expressing an influenza hemagglutinin peptide-TBP fusion protein. The eIIH was purified as described previously (32) except that the 1.0 M KCl phenocellulose fraction containing the eIIH complex was passed over a column (1 ml of bed volume) of starting volume of culturing positive control of a eIIH peptide antibody coupled to protein A. After washing, eIIH was eluted with 3 ml of 1 mg/ml influenza epitope peptide. Fractions containing eIIH were identified by Western blots probed with anti-TBP antibody (Santa Cruz Biotechnology). Individual factors were assayed for activity by comparing levels of in vitro transcription in their absence to levels seen in reactions containing the complete complement of general transcription factors.

RESULTS

In Vitro Transcription—To investigate the possible involvement of DNA-PKcs, Ku protein, or both in RNA polymerase II-mediated transcription, we measured in vitro transcription levels in nuclear extracts prepared from matched pairs of DNA-PK-containing and DNA-PK-deficient CHO cell lines. The V30T cell line is radiation-sensitive and DNA-PKcs-deficient, and AA8 is a matched wild-type, parental CHO cell line (5, 33). The xrs-6ecvec cell line is radiation-sensitive and -deficient in the 80-kDa subunit of Ku protein, and xrs-6ecKu80 is a matched cell line rescued with the cDNA encoding the human Ku80 protein (34).

We performed transcription assays using extracts from these cell lines and a supercoiled DNA template containing either the AdMLP, hsp70, or HTLV-I promoter incorporated upstream of a G-less cassette. Nuclear extracts were prepared and stored under identical conditions as described under “Materials and Methods.” Equal amounts of total nuclear extract protein were added to each transcription reaction. Representative transcription assays are shown in Fig. 1. In each case, the extracts from DNA-PK-deficient cells (xrs-6ecvec and V30T) showed less transcription than the matched, wild-type counterparts (xrs-6ecKu80 and AA8). Results from this and four similar, independent experiments are summarized in Table I. These data show a clear defect in the transcriptional activity of DNA-PK-deficient nuclear extracts, ranging from 2.4- to 7.7-fold, when compared with their matched controls. The defect appears to be general, in that it is observed with all promoters tested.

Overall levels of transcription were generally higher with the hsp70 promoter than with the others, and this promoter also showed the greatest fold difference between the paired nuclear extracts. Similar results were obtained with a template containing only the hsp70 core promoter and lacking the heat shock elements (data not shown).

Nuclear Extract Mixing Experiments—To determine if the in vitro transcriptional activity of the DNA-PK-deficient nuclear extracts could be rescued, a series of mixing experiments was performed. In these experiments, 7.5, 15, 22.5, or 30 μg of a test nuclear extract was mixed with 30 μg of the deficient nuclear extract, and in vitro transcription assays were carried out using the AdMLP template as described under “Materials and Methods.” Transcription levels of the mixed nuclear extracts were compared with the appropriate DNA-PK-positive control. Results of these experiments are shown in Fig. 2. As can be seen in A and D, addition of xrs-6ecKu80 nuclear extract resulted in the complete rescue of in vitro transcription levels of both xrs-6ecvec and V30T nuclear extracts. The decrease in transcription levels observed in reactions containing greater than 45 μg of total nuclear extract protein may be due to an excess of protein. Similarly, addition of AAS nuclear extract resulted in the complete rescue of transcription levels in the DNA-PKcs-deficient V30T nuclear extracts (Fig. 2B). However, AAS gave less than maximal restoration in the Ku80-deficient xrs-6ecvec extracts (Fig. 2C). The xrs-6ecKu80 cells express a human Ku80 gene under control of the strong cytomegalovirus promoter. It may be that expression of this gene at higher than endogenous levels makes the extracts even more active than would otherwise be the case.
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We have also seen that V30T extracts failed to rescue xrs-6cvec nuclear extracts (Fig. 2E), despite the fact that these cells represent different genetic complementation groups (XRCC5 versus XRCC7) (35). There was greater, but still incomplete, rescue when the experiment was performed in the opposite direction, with xrs-6cvec extracts added to V30T. These results suggest that both mutant cell lines lack a common factor, not DNA-PK itself, the absence of which results in decreased levels of in vitro transcription. This was confirmed by the experiments described in the next section.

In Vitro Transcription Assays Supplied with Purified DNA-PK—Initially, we attempted to restore transcription levels to the DNA-PK-deficient nuclear extracts by the addition of purified recombinant Ku and DNA-PKcs purified from HeLa cells. However, we observed no increase in transcription levels in the presence of these purified proteins (Fig. 3). Similar results were obtained when proteins were added individually over a wide range of concentrations (data not shown). It is possible that the human Ku70 subunit or DNA-PKcs subunit is not capable of functioning in the hamster cell lines, explaining the lack of complementation between species. However, the amino acid sequences of DNA-PK components are relatively conserved between species (reviewed in Ref. 36). Also, previous studies have shown that cross-species complementation occurs between human and yeast Ku70 (37) and between human and mouse DNA-PKcs (38). Thus it is likely that human DNA-PK components can function in hamster cells.

Assuming that human DNA-PK can, in principle, function in hamster cells, then the failure to rescue transcription by the addition of purified DNA-PK suggests that the defect may not be directly attributable to the absence of Ku or DNA-PKcs from the extract but rather to the absence of some other common factor. This could be an individual protein that lies downstream of DNA-PK in a signaling pathway or a complex of proteins that fails to assemble in the absence of Ku and DNA-PKcs. When this positively acting factor or complex is supplied to the DNA-PK-deficient cell lines, there is a complete restoration of in vitro transcription levels.

Transcription in the Presence of the DNA-PK Inhibitor Wortmannin—Since DNA-PK has intrinsic protein kinase activity, it was of interest to know whether ongoing phosphorylation activity of endogenous DNA-PK contributed to the higher level of transcription seen with extracts from DNA-PK-containing cells. The catalytic subunit of DNA-PK falls into the phosphatidylinositol 3-kinase family, and its activity has been shown to be effectively inhibited by approximately 250 nM of the phosphatidylinositol 3-kinase inhibitor wortmannin (39). If the enzymatic activity of DNA-PK is responsible for the higher levels of in vitro transcription observed in the DNA-PK-containing nuclear extracts, one would expect that the difference in transcription levels between xrs-6cKu80 and xrs-6cvec nuclear extracts would be eliminated in the presence of wortmannin. Transcription reactions were performed in the presence of various concentrations of wortmannin, with results shown in Fig. 4. The approximately 2-fold difference in transcription levels between the paired nuclear extracts was maintained throughout a range of 0 to 1000 nM wortmannin. In control experiments, we show that the enzymatic activity of purified DNA-PK is almost completely abolished at a concentration of 500 nM wortmannin (Fig. 4C).

We conclude from these experiments that ongoing DNA-PK enzymatic activity is not required for transcription. Consistent with this, we have compared linear and circular templates under the conditions of our assays, and we find that the difference between extracts from DNA-PK-positive and -negative cells is maintained in both cases (data not shown). It is established that linear DNAs are much more effective than circular DNAs in stimulating DNA-PK phosphorylation activity (40, 41). The finding that both types of templates give equivalent results is one more line of evidence that ongoing enzymatic

### Table 1

| Extract            | Template | AdMLP | hsp70 | HTLV I |
|--------------------|----------|-------|-------|--------|
| xrs-6cKu80 vs. xrs-6cvec | 5.5 ± 2.9 | 7.7 ± 2.2 | 4.3 ± 1.6 |
| AAS vs. V30T       | 2.4 ± 2.3 | 4.3 ± 1.1 | 2.9 ± 2.2 |

Values are means ± S.D. n = 5 in all cases.
activity of DNA-PK does not contribute to the difference between extracts.

In Vitro Transcription Assays Supplemented with Purified Transcription Factors—Because the transcriptional defect of the DNA-PK-deficient cell lines occurred at all promoters tested, one possibility was that the missing component was a general transcription factor. We attempted to rescue transcription by the addition of purified transcription factors to our in vitro transcription assays. Recombinant TFIIA, recombinant TFIIB, recombinant TBP, epitope-tagged TFIID, recombinant TFIIE, recombinant TFIIF, RNAP II, and TFIIH were purified and assayed for activity as described under “Materials and Methods.” No single factor, when added in the amounts indicated (Fig. 5A), was capable of restoring transcription levels in the xrs-6cKu80 nuclear extract to the levels seen in the xrs-6cKu80 control. Transcription was also not restored by addition of a combination of purified transcription factors (Fig. 5B). Thus, the observed decrease in transcription levels of DNA-PK-deficient nuclear extracts cannot be attributed to limiting amounts of a general transcription factor.

Most of the transcription factors used in this experiment were from recombinant sources and may lack post-transla-
Heparin, in low concentrations, has been shown to prevent the formation of new initiation complexes without disrupting transcriptionally active complexes already present on the template (23).

In order to determine whether the xrs-6cvec nuclear extracts are defective in primary or secondary transcriptional initiation, the transcription assay illustrated in Fig. 6 was performed. Both the rate of PIC formation and the number of productive PICs formed were measured by varying the length of time that the nuclear extract was incubated with template prior to the addition of ribonucleoside triphosphates. The ability of the extracts to undergo secondary initiation events was monitored by comparing the level of RNA synthesis in the presence and absence of heparin.

These experiments produced a clear but unanticipated result. When transcription was limited to primary initiation events by the addition of heparin, little or no difference was observed in the ability of either nuclear extract to initiate transcription (Fig. 6; compare intensity of bands at 30 min preincubation in presence of heparin). The rate of preinitiation complex formation and the number of productive complexes formed were similar in both extracts. In fact, preinitiation complex formation was actually higher in the xrs-6cvec extracts when compared with xrs-6cKu80 extracts. In contrast, the two extracts differed considerably in levels of transcription in the absence of heparin. This difference could be solely attributed to the approximately 4-fold greater ability of the xrs-6cKu80 extracts to carry out secondary initiation.

**Trapping of RNA Polymerase II by G-less Cassette Template**—In order to better understand the mechanistic differences between primary and secondary initiation events in the xrs-6cvec versus the xrs-6cKu80 nuclear extracts, we have taken advantage of a technique first described by Szentirmay and Sawadogo (42), and later refined by Lei et al. (43) that involves “trapping” of the RNA polymerase II at the end of a G-less cassette by the addition of high concentrations of the chain terminator 3'-O-methyl GTP (O-Me-G). As can be seen in lanes 3 and 8 of Fig. 7A, addition of O-Me-G to a final concentration of 500 μM is sufficient to compete with endogenous GTP present in the nuclear extracts, preventing elongation beyond the G-less cassette, and resulting in the formation of stalled RNAP II complexes. The majority of these complexes can be chased to yield long RNAs by the addition of GTP (Fig. 7B).

This approach allows one to identify whether multiple rounds of transcription are limited by the number of active templates, by the concentration of active RNAP II, or by some other kinetic phenomenon. If initiation of transcription is
occurring via the reuse of templates, the presence of stalled RNAP II forces subsequent RNAP II molecules to pile up at the end of the G-less cassette resulting in a ladder of shortened transcripts at intervals of approximately 30 nucleotides. We do not observe such a ladder in the presence of O-Me-G in either lane 3 or lane 8 nuclear extracts. Therefore, few, if any, templates are being reused in our assays. Consequently the differences in observed transcription levels cannot be directly attributed to differences in the ability of the two nuclear extracts to recycle template.

Likewise, if the recycling of RNAP II and/or tightly associated transcription factors is required for later rounds of transcription, trapping of RNAP II at the end of the G-less cassette should inhibit new initiation. Although there is some inhibition of transcription with O-Me-GTP (compare lanes 2 and 3 and lanes 7 and 8), it does not ablate the difference between xrs-

FIG. 5. Purified general transcription factors do not restore the in vitro transcription levels of DNA-PK-deficient nuclear extracts. Transcription reactions were performed using nuclear extract from DNA-PK-deficient (xrs-6cvec, V30T) or DNA-PK-containing (xrs-6cKu80, AA8) cell lines. Amount of protein from each extract is indicated in micrograms. Additional proteins, purified as described under “Materials and Methods,” were added as indicated. Template was supercoiled plasmid containing the AdMLP promoter. A, addition of either 10–15 ng of recombinant TFIIB, 7 ng of recombinant TFIIB, 3 ng of recombinant TBP, 1–2 ng of epitope-tagged TFIIH, 50 ng of the 56-kDa subunit plus 30 ng of the 36-kDa subunit of TFIIIE, 30 ng of the 58-kDa subunit plus 15 ng of the 26-kDa subunit of TFIIIF, 50–100 ng of RNA polymerase II, or 0.5–1 μl of TFIIH. B, addition of same purified transcription factors as in A in combination. Txn, transcription; arb, arbitrary.

FIG. 6. Differences in transcription levels between xrs-6cvec and xrs-6cKu80 extracts occur at the level of multiple round transcription. Transcription reactions were performed using 30 μg of nuclear extract prepared from xrs-6cvec or xrs-6cKu80 cell lines. Extract was preincubated (Preinc.) with 500 ng of the hsp70 supercoiled template at 30 °C for the indicated times. Ribonucleoside triphosphates were added, followed after 5 min by 10 μg/ml heparin. This allowed the initiation rate for a single round of transcription to be measured. Transcription was also carried out in the absence of heparin to allow comparison of single versus multiple round transcription.

FIG. 7. Direct comparison of xrs-6cvec and xrs-6cKu80 transcription when RNA Pol II is “trapped” by a G-less cassette template. A, transcription reactions were performed using 50 μg of either xrs-6cvec or xrs-6cKu80 nuclear extract and the hsp70 G-less cassette template in the absence or presence of 500 μM O-Me-G. Readthrough RNAs were trimmed to the size of the G-less cassette by the addition of RNase T1. Single round transcription was measured by the addition of 10 μg/ml heparin to some reactions. B, GTP chase-transcription reactions using 50 μg of xrs-6cKu80 extract were carried out as described above in the presence of 500 μM O-Me-G followed by a 30-min chase with 2 mM GTP.

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6cKu80 and xrs-6vec nuclear extracts (lanes 3 and 8) nor does it reduce transcription to the level seen in the presence of heparin (lanes 5 and 10). This suggests that whereas RNAP II and/or some tightly associated factor is partially limiting in the reaction, this limitation is not responsible for the difference of in vitro transcription levels between these two nuclear extracts. This result is consistent with our earlier findings that addition of purified RNAP II does not rescue transcription in the Ku-negative nuclear extract. Thus the difference between extracts falls into the category that Lei et al. (43) described as “kinetic limitation.” The Ku80 nuclear extracts must contain an activity that promotes multiple rounds of transcription but this activity is not RNAP II itself.

DISCUSSION

We present the first definitive evidence that nuclear extracts prepared from cells that are deficient in DNA-PK, a protein primarily known for its essential role in repair of DNA double-strand breaks, are also impaired in their ability to carry out basal transcription in vitro. The effect was seen with extracts of both Ku80-deficient and DNA-PKcs-deficient cells. The relative decrease in transcription levels was always greater for the Ku80-negative nuclear extracts than for the DNA-PKcs-negative extracts. This suggests that Ku protein may have a greater or perhaps more direct role than DNA-PKcs in transcriptional regulation.

Mixing experiments show that the impaired transcription is due to the lack of a positively acting factor or complex of factors in the deficient extracts. We have yet to identify this positively acting factor. Our inability to restore transcription by the addition of purified DNA-PK suggests that the role of DNA-PK in RNAP II-mediated transcription may be either less direct or more complex than we had initially anticipated.

Interestingly, it has been reported that nuclear extracts from DNA-PK-deficient cells also show a modest (2-fold) decrease in nucleotide excision repair activity. As with the transcription defect, the excision repair defect could not be restored by purified DNA-PK protein components (44). It remains to be established whether the transcription and nucleotide excision repair defects have any common mechanistic basis.

We have shown that the Ku-negative nuclear extracts are not defective in their ability to carry out single round transcription. Rather, the observed difference in in vitro transcription levels between Ku80-positive and Ku80-negative nuclear extracts can be attributed solely to the ability of the Ku80-containing nuclear extracts to undergo multiple rounds of transcription.

The role of secondary initiation, or reinitiation, in the overall levels of RNA synthesis has been overshadowed by the tremendous effort required to obtain our basic understanding of how primary initiation takes place. However, there is every reason to expect that the process of reinitiation is also a highly regulated step. Recently Oelgeschlager et al. (45) have shown that in HeLa cell nuclear extracts, TAF(I)Is (general transcription co-factors that associate with the TATA-binding protein of TFIIID and play a central role in RNAP II transcriptional regulation) (reviewed in Ref. 46) actually impair functional PIC assembly but elevate absolute levels of transcription by facilitating secondary initiation events. Activators of transcription such as heat shock factor and the estrogen receptor have also been shown to selectively enhance reinitiation (45, 46).

Reinitiation may be enhanced by recycling promoter and/or RNAP II and other transcription related proteins more efficiently (47). For example, a promoter might be “marked” as competent for reinitiation during the primary initiation event. Our RNAP II trapping experiments indicate, however, that under our conditions templates are in functional excess and are not being recycled. Similarly, whereas we did find that the concentration of RNAP II may be partially limiting in both extracts, recycling of RNAP II was not required in order to observe the difference in transcription levels between Ku80-negative and Ku80-positive nuclear extracts.

Our results thus fall into the category of “kinetic limitation” described by Lei et al. (43). In this respect, it is interesting that the multiple round transcription defect in DNA-PK-deficient extracts resembles that observed by these workers with carboxy-terminally truncated forms of the 74-kDa subunit of TFIIIF (43). One possibility is that the effect of DNA-PK on multiple round transcription is mediated through phosphorylation of TFIIIF in the carboxy-terminal region. The activity of the 74-kDa subunit of TFIIIF is known to be regulated by phosphorylation (48). TFIIIF is phosphorylated by the TAFII 250-associated protein kinase (49), but it is not known if other kinases also contribute to its phosphorylation. It will be of interest to determine whether the pattern of TFIIIF phosphorylation changes in the presence and absence of DNA-PK in vivo.

Alternatively, DNA-PK could affect transcription by phosphorylation of a different general transcription or through interactions with an unknown or novel protein. This protein could facilitate active reloading of transcription factors at new promoters or releases nonproductively loaded factors from DNA. The extraordinarily large DNA-PK holoenzyme could serve as a nucleus for assembly of a multiprotein complex that carries out these functions.

One of the important questions that remains to be answered, in addition to the identity of the missing factor, is whether DNA-PK-deficient cells are impaired in their ability to carry out transcription in vivo. In this respect, it is interesting to note that certain transcriptional activators are capable of binding to and stimulating the activity of DNA-PK (19). This interaction could provide a mechanism for increasing the level of reinitiation at specific promoters regulated by these factors. Moreover, mice lacking Ku 80 (50) or Ku 70 (51) genes are proportional dwarfs. A defect in the general transcription machinery is one possible explanation for this phenotype.

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