The Autoantigen Ku Is Indistinguishable from NF IV, a Protein Forming Multimeric Protein-DNA Complexes

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Summary

We have isolated a cDNA encoding the 84-kD subunit of NFIV. Tryptic peptide sequences were identified within the coding sequences, confirming its proper identity. The primary sequence of the protein is identical to that of the large subunit of the Ku autoantigen. A missing NFIV peptide sequence was identified within the sequence of the small subunit of Ku. In addition, the proteins are identical in immunological aspects. We suggest that the Ku and NFIV proteins are identical. This connection adds new biochemical data to our knowledge of the Ku autoantigen.

Nuclear factor IV (NFIV)[1] is a dimeric protein with subunits of 72 and 84 kD. It is an abundant protein in HeLa cell nuclei. As shown by combined biochemical and electronmicroscopical methods (1), NFIV recognizes the molecular ends of any double-stranded DNA molecule and can subsequently move freely, without energy input, on the DNA, until it encounters a blockade. When the DNA is fully covered, a stable regular DNA-multimeric protein complex with a spacing of 27–32 bp is formed. NFIV also binds to single-stranded DNA but no such regularity is detected in this complex. Based upon its properties, a role for NFIV in DNA replication, repair, or recombination has been proposed (1). The unique DNA binding properties of NFIV prompted us to investigate the protein in more detail.

The molecular weight as well as some other properties of NFIV are remarkably similar to those of the auto-antigenic protein Ku (2–4). This protein is also referred to as p70/p80 (5) and the 86–70-kD protein complex (6, 7). These antigens were identified on the basis of their reactivity with antibodies from patients with scleroderma-polymyositis overlap syndrome as well as with systemic lupus erythematosus and scleroderma. Like NFIV, the immunopurified Ku complex binds to the ends of double-stranded DNA for which the 70-kD subunit seems to be primarily responsible (2). However, a regular footprint pattern on double-stranded DNA, resulting from a translocation step, was not observed. Moreover, binding to single-stranded DNA was much less efficient than with NFIV.

We used a polyclonal antiserum against NFIV to isolate a cDNA clone of the 84-kD subunit of NFIV. The coding region of this sequence completely overlapped with that of the recently published 86-kD Ku subunit (8). In addition, peptides obtained from NFIV overlapped in sequence with the large Ku subunit as well as with the sequence of the Ku 70-kD DNA-binding subunit (9). In accordance with this, we show here that NFIV and Ku crossreact immunologically. These data indicate that NFIV and Ku are identical. This then provides new data on the mode of Ku antigen-DNA interaction as well as a detailed kinetic analysis of this interaction (1).

Materials and Methods

Protein Sequencing. NFIV was purified from HeLa cell nuclei using ion-exchange and DNA-cellulose affinity chromatography (1). The protein was found to be >95% pure as judged by SDS-gel electrophoresis and silverstaining. 50 μg of NFIV (corresponding to ~300 pmol) was digested with trypsin-TPCK (Worthington Biochemical Corp., Freehold, NJ) in a 100:1 mass ratio for 16 h at 37°C. The resulting peptides were separated on a HPLC μBondapak C18 column using a linear acetonitrile gradient in 0.1% trifluoroacetic acid. Four peptide-containing fractions were analyzed on a gas-phase sequenator (Applied Biosystems Inc., Foster City, CA). The results are shown in Fig. 1.

Isolation of the cDNA Clone and DNA Sequencing. The rabbit antiserum was preincubated with a nitrocellulose-immobilized *Escherichia coli* extract as well as with 20 ng/ml β-galactosidase to inhibit nonspecific binding. The antiserum (diluted 1:500) was then used to screen (10) a cDNA library in λgt11, made from polyadenylated RNA of NTera2D1 human teratocarcinoma cells (11) using horseradish peroxidase–conjugated second antibodies (SWARPO, DAKO immunoglobulins a/s, Denmark). From a screening of ~3.5 × 10^5 recombinant phages, five immunoreactive clones were isolated, plaque-purified, and amplified. *E. coli* extracts from cells infected with the λ-clones were prepared according to Landschulz et al. (12). Total infected cells were lysed in SDS-containing sample buffer and loaded on a 7%-polyacrylamide SDS-gel. The proteins were analyzed by immunoblotting.

The 3.0-kb EcoRI insert of clone λ-1 was subcloned in pUC18.

[1] *Abbreviation used in this paper*: NFIV, nuclear factor IV.

peptide number	amino acids
I	K_{40} A_{30} S_{45} F_{15} E_{20} E_{20} A_{20} S_{30} N_{15} Q_{13} L_{15} I_{10} N_9 H_6 I_5 E_7 Q_5 F_7 L_6 D_5 T_7 N_4 E_3 T_3
IIa	- K_{24} P_{34} S_{30} G_{25} T_{40} A_{25} A_{30} V_6 F_{10} E_{15} E_{25} G_{15} G_{12} D_{10} V_5 D_7 D_{12} L_3 L_8 D M I
IIb	- L_{31} M_{35} L_{33} P_{42} F_{32} D_{35} L_{20} L_{20} E_{15} D_{20} I_{20} E_5 S_{20} K_5
IIIa	K_{50} K_{30} D_{30} Q_{20} T_{20} A_{17} Q_{15} E_{10} I_8 F_7 Q_8 D_6 N_4 H_3 E_3 D_4 G_3
IIIb	T_{10} W_5 T_{15} V_5 D_{10}
IVa	- G_{30} L_{20} E_{20} I_{12} V_{12} K_{11}
IVb	- E_8 Y_5 S_6 E_3 E_9 E_6 L_4 K_6 - - I S K

Figure 1. Sequence of the tryptic peptides obtained from NFIV. The amount of amino acids (in picomoles) observed during the sequencing steps are indicated in the subscript. HPLC peaks 2, 3, and 4 contained overlapping peptides that were aligned according to their match with the nucleotide sequence (Fig. 2).

Its sequence was determined by shotgun cloning of TaqI and Sau3A restriction fragments in M13mp18 and M13mp19. Dideoxy chain-termination sequencing was carried out using Sequenase components (U.S. Biochemical Corp., Cleveland, OH) according to the specifications of the manufacturer.

Antibodies and Immune Precipitation. A rabbit antiserum against NFIV was prepared by primary injection of antigen (100 μg) in the popliteal lymph nodes, followed by two intramuscular booster injections. The serum was highly specific for NFIV, as shown by immunoblotting. Subunit-specific antibodies were prepared as described (13). This procedure yielded antisera that were highly specific for each of the two subunits. The patient serum containing antibodies to the Ku autoantigen was kindly donated by Drs. Griffith and Hardin (Yale University, New Haven, CT). A hybridoma supernatant containing the mAb RN3 directed against the 80-kD Ku subunit, was a gift of R. Verheijen and W. van Venrooij (University of Nijmegen) (14). Immunoprecipitations were performed as described (3). For precipitation with the RN3 mAb the protein A–sepharose beads were coated with rabbit anti–mouse IgG (RAM, Nordic Sciences, Tilburg, The Netherlands) as described (14).

Results

Structural Identity Between the Large Subunits of NFIV and Ku. A teratocarcinoma λgt11 library was screened with a rabbit antiserum raised against purified NFIV. This revealed five reactive clones which were plaque-purified and amplified. An *E. coli* extract from phage-infected cells was then analyzed by SDS-gel electrophoresis and immunoblotting. One clone encoded a polypeptide that reacted with the NFIV antiserum. Immunoblotting with the subunit-specific antisera (see Materials and Methods) indicated that the clone contained information for the 84-kD subunit of NFIV (data not shown). The insert DNA was 3.0 kb in size and consisted of a single EcoRI fragment, which was subcloned in pUC18 and sequenced. The clone contained 2,969 bp consisting of a single 1,885-bp long open reading frame and a 1,084-bp 3′-untranslated region. Analysis of the open reading frame showed it to be completely identical to the region encoding amino acids 105–732 of the 86-kD subunit of the Ku protein (8). The identity extends to the 3′-untranslated region with one difference, G-A at position 2683. Compared with the Ku cDNA we find a 263 bp extended 3′ sequence (Fig. 2). In spite of this, no poly(A) tail or poly(A) signal was found, indicating that priming of the cDNA must have occurred internally. The combined information of the Ku and NFIV-encoding clones spans 3,313 bp, which is close to the 3.1-kb Ku-86 mRNA detected in Northern blots (8).

NFIV Peptides Map Both in the Large and Small Ku Subunit. To confirm the identity of our clone, we purified NFIV to apparent homogeneity and determined the amino acid sequence of tryptic peptides separated by reversed-phase chromatography. Due to the large size of NFIV, three of the four peaks contained a mixture of two peptides (Fig. 1). In two cases, IIIa/b and IVa/b, the amount of detected amino acids could be used to identify the two different peptides in the mixed sequences. Peptides I, IIa, IIIa, IIIb, and IVa matched the predicted amino acid sequence of Ku/NFIV, while peptide IIb was present in the first 104 amino acids of the Ku sequence, not present in the NFIV clone (see Fig. 2). All peptides are preceded by an arginine or lysine residue, confirming their proper tryptic origin. This indicates that we have cloned the cDNA encoding the 84-kD subunit of NFIV, confirming that the large subunits of the Ku and NFIV proteins are identical. The only unassigned NFIV-derived peptide, IVb, was found to match the 70-kD Ku subunit, at positions 557–570 (9). This suggests that the small subunits of Ku and NFIV are identical too.

Ku and NFIV Are Immunologically Indistinguishable. We examined the relationship of the Ku and NFIV proteins by immunoprecipitation. Using a rabbit antiserum against purified NFIV we immunoprecipitated both the 72- and 84-kD subunits from a HeLa extract. The migration of the radiolabeled proteins on the SDS-gel was indistinguishable from that of purified NFIV, as visualized by silverstaining (see Fig. 3, lanes *1* and *3*). Immunoprecipitation with a human anti-Ku serum revealed polypeptides of identical size (lane *5*). The mAb RN3 (14), which is specific for the 86-kD subunit of Ku, also precipitated proteins indistinguishable from NFIV (lane 7). These experiments indicate that NFIV and the Ku antigen are indistinguishable in electrophoretic behavior, but do not exclude the possibility that NFIV represents a immunologically distinct subpopulation of the Ku antigen or

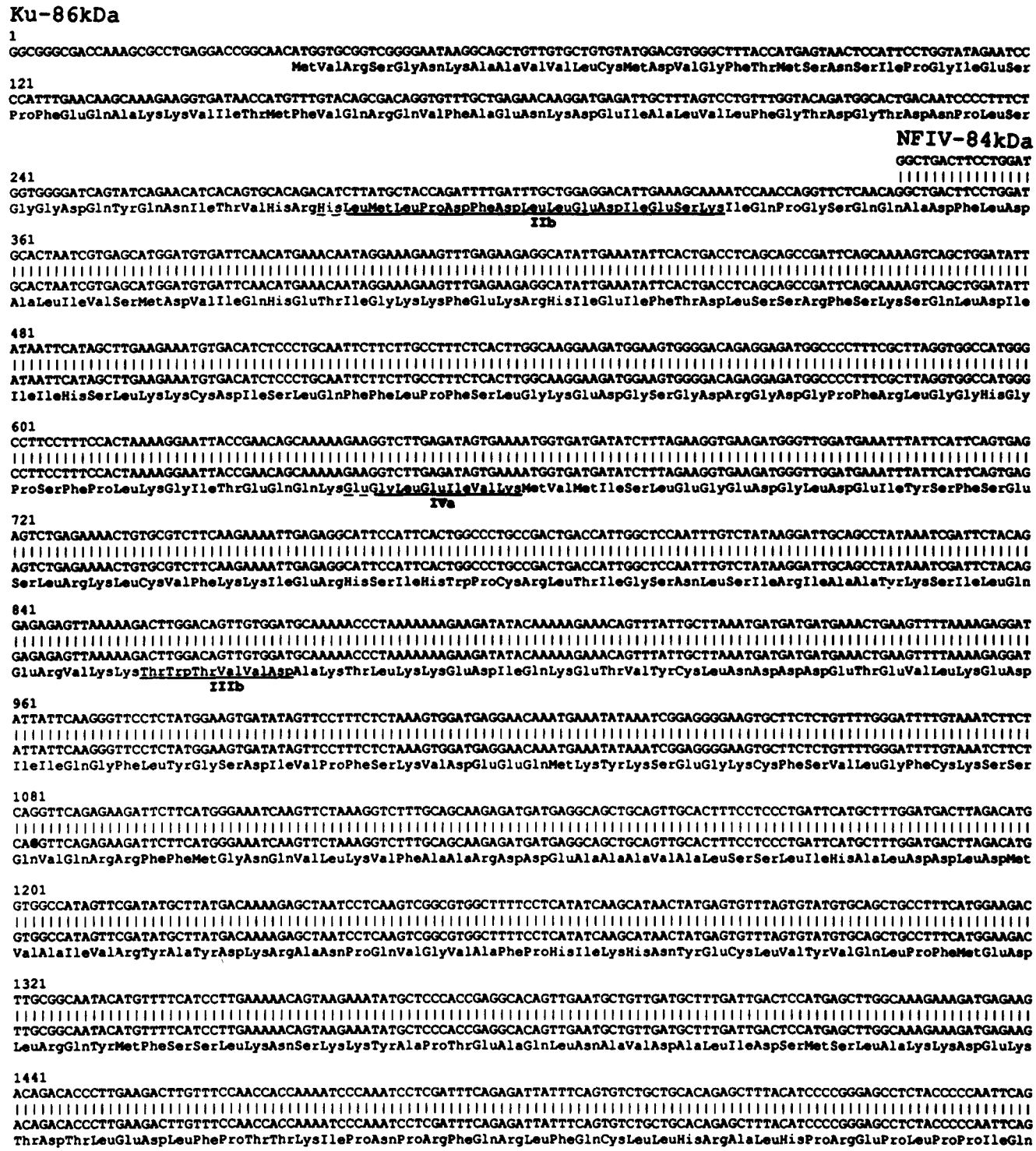

Figure 2. See legend on following page.

vice versa. To examine this possibility, we depleted radiolabeled extracts of NFIV by immunoprecipitation with anti-NFIV serum and incubated the supernatant with an anti-Ku serum or mAb RN3 to detect any Ku-specific proteins. As shown in Fig. 3 *B*, lanes *2* and *4*, Ku polypeptides could not be detected in these supernatants. The reverse experiment, depletion of Ku polypeptides (lane *5*) followed by immunoprecipitation with anti-NFIV serum, also failed to reveal any residual polypeptide (Fig. 3 *B*, lane *6*). Thus we conclude that NFIV and Ku antigen are immunologically crossreac-

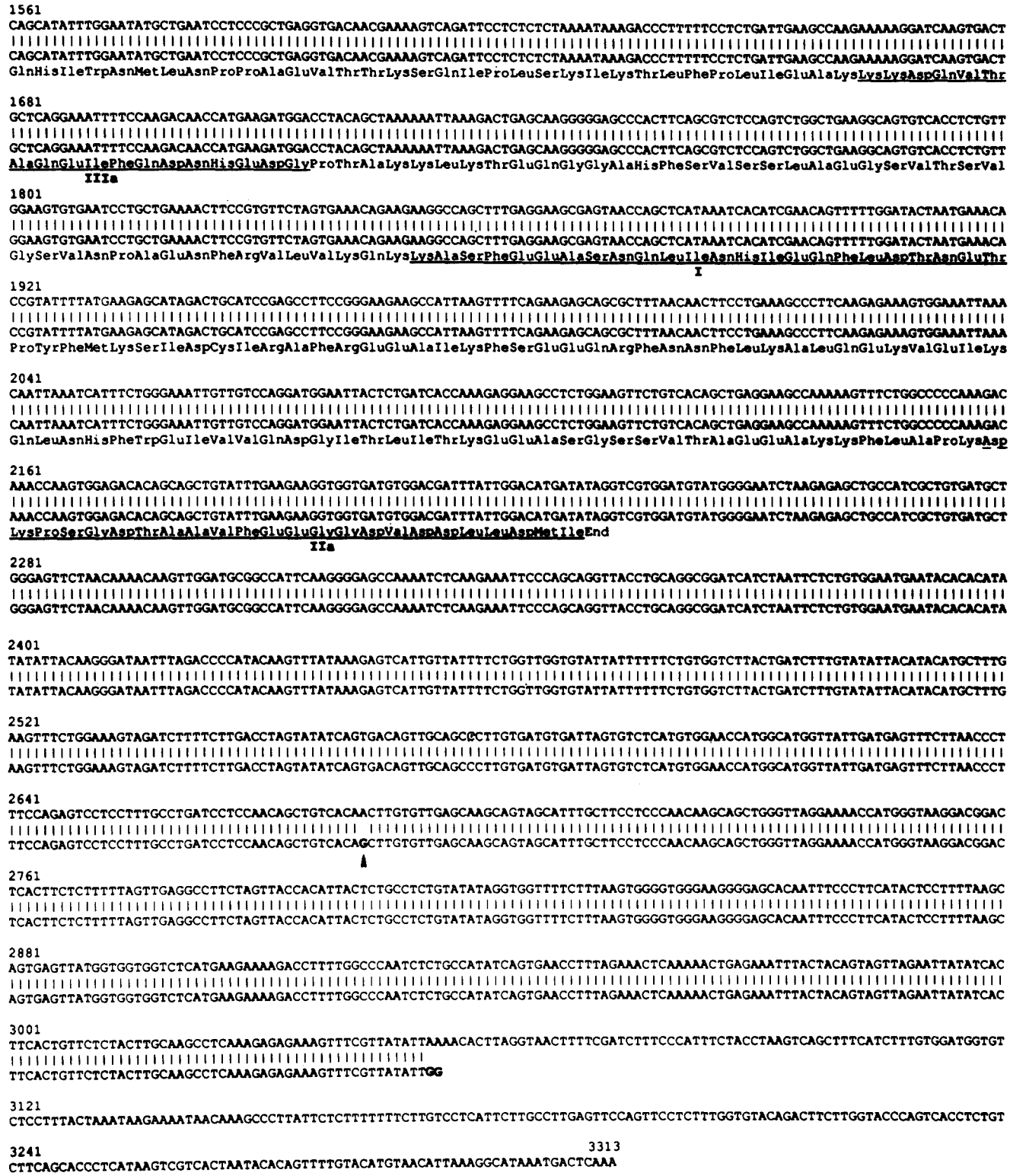

Figure 2. Sequence similarity between the cDNAs encoding the large subunits of Ku and NFIV. NFIV 84 kD and Ku 86 kD cDNA sequences are shown in top and bottom strand, respectively. Note the single G-A difference at position 2683. The NFIV-derived peptides are indicated in the deduced amino acid sequence.

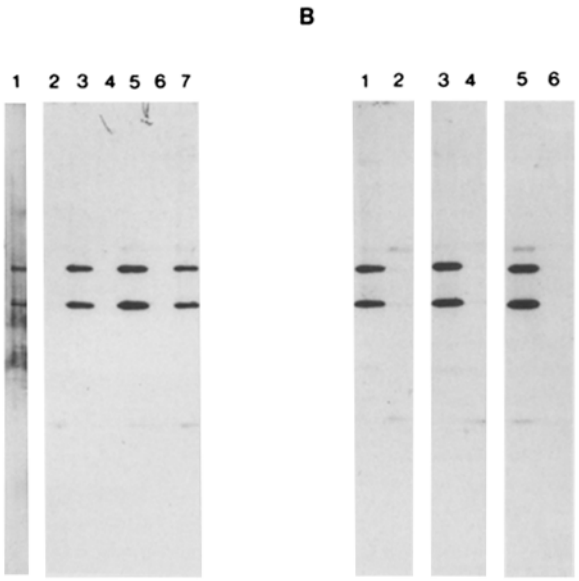

Figure 3. (*A*) Immunoprecipitation of Ku and NFIV polypeptides. Lane *1* shows a silver-stained part of the gel containing purified NFIV. Lanes *2* to *7* show the autoradiograph of immunoprecipitated [^{35}S]methionine-labeled proteins. (Lane *2*) Pre-immune rabbit serum; (lane *3*) anti-NFIV serum; (lane *4*) normal human serum; (lane *5*) patient anti-Ku serum; (lane *6*) rabbit anti-mouse IgG serum; (lane *7*) mAb RN3. (*B*) Radiolabeled HeLa extracts were depleted of NFIV by immunoprecipitation with anti-NFIV serum. The pellets were analyzed (lanes *1*, *3*). The supernatants were analyzed by immunoprecipitation using anti-Ku serum or RN3 (lanes *2*, *4*). Lanes *5* and *6* show the depletion of Ku polypeptides by the anti-Ku serum followed by immunoprecipitation with anti-NFIV serum.

tive and do not represent immunologically distinct subpopulations of each other. Taken together these results strongly indicate that Ku and NFIV are identical proteins.

Discussion

Based upon several criteria, NFIV and Ku appear to be indistinguishable. First, an 84-kD NFIV cDNA clone was identical to the Ku 86-kD cDNA sequence (8), except for a single point mutation. Second, all NFIV peptides that were analyzed fit the Ku protein sequence. In accordance with this, antibodies against NFIV quantitatively remove Ku polypeptides and vice versa. Northern blot experiments using the Ku 70-kD encoding cDNA as a probe reveal only one mRNA (9). This indicates again that also the small subunits of Ku and NFIV may be indistinguishable. However, we cannot exclude that NFIV and Ku have the same primary structure but are modified differently, since both Ku peptides can be phosphorylated at serine residues (7). Such a modification may go undetected in immunological or electrophoretic analysis.

NFIV and Ku show differences in DNA-binding properties. Most interestingly, NFIV is able to translocate over dsDNA (1), whereas Ku is not (2). This difference may be related to a DNA-binding specificity of NFIV. We observed that A + T-rich DNA-ends are covered by NFIV with a higher affinity than G + C-rich DNA-ends. The use of a different DNA molecule may provide an explanation for the detection of only a terminal footprint with the Ku protein. These differences may of course also be due to a difference in modification of the proteins or to different isolation procedures.

Ku is isolated by immuno-affinity chromatography, necessitating elution with 3.5 M $MgCl_2$. Considering the limited stability of Ku (4), this procedure may functionally change the Ku molecule. During purification of NFIV, only buffers with neutral pH and moderate salt concentrations (up to 0.5 M NaCl) are used. Thus, we consider it possible that the differences between Ku and NFIV are due to different isolation procedures.

By gel filtration analysis, the Ku complex in a crude extract was shown to behave as a molecule of ~300 kD, suggesting a tetramer. Purified NFIV was shown by electron microscopy to behave as a 150-kD heterodimer in DNA-bound and unbound form (1). Possibly the Ku-containing, 300-kD particle represents a complex with another protein or contains Ku proteins that are linked together by DNA remaining from the isolation procedure. We noticed that most of the protein in the cell is present in a DNA bound form that can be extracted by 0.3 M NaCl. However, as we have not analyzed NFIV by gel filtration, we can not exclude that the different values are due to different methods used.

The function of the Ku/NFIV protein is presently unknown. Several other proteins, involved in DNA replication, repair, or recombination are able to recognize molecular ends. A remarkable similarity in DNA binding properties involving an ends-specific DNA interaction as well as a presumed translocation step was noted within the bacteriophage Mu pgam protein, that is involved in recombination control (15). However, several other functions are possible, like a role in DNA replication (1) or in chromatin structure (7). This would be in agreement with the high level of active protein found in HeLa cells (5×10^5 molecules per cell). The rapid inactivation of Ku/NFIV in the absence of DNA (4; and van Driel, W., personal communication), coupled to the high stability in the presence of DNA (1) also suggests that most of the molecules in the cell are present in a DNA-bound form.

While this article was under review, Mimori et al. (16) reported the cloning of cDNAs encoding the larger subunit of the Ku protein. Their sequence overlaps the sequences described in this paper.

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