Nuclear factor IV (NFIV) 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 electron microscopical 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 antisem 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 sequence in overlap 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) 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 an 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 cDNA library in Agt11, 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 × 10⁶ recombinant phages, five immune-reactive 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.
Its sequence was determined by shotgun cloning of TqI 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 Immunoprecipitation.** 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-agarose 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 Xgt11 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 by which 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 Subunits.** 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
vice versa. To examine this possibility, we depleted radiola\-beled 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. See legend on following page.
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.
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 MgCl₂. 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 x 10⁵ 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.

We thank P. Burbach, A. van der Kleij, and R. Amons for help with the amino acid sequence analysis; J. Skowronski and W. van Venrooij for the Agt11 library; R. Verheijen for the RN3 antibody; and J. A. Hardin for the anti-Ku serum. Discussions with E. de Vries and W. van Driel are gratefully acknowledged.
This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial support from the Netherlands Organization for Scientific Research (NWO).

Address correspondence to Dr. Peter C. van der Vliet, Laboratory for Physiological Chemistry, Vondellaan 24A, 3521 GG Utrecht, the Netherlands.

Received for publication 20 April 1990 and in revised form 29 June 1990.

References

1. De Vries, E., W. van Driel, W.G. Bergsma, A.C. Arnberg, and P.C. van der Vliet. 1989. HeLa nuclear protein recognizing DNA termini and translocating on DNA forming a regular DNA-multimeric protein complex. J. Mol. Biol. 208:65.

2. Mimori, T., and J.A. Hardin. 1986. Mechanism of interaction between Ku protein and DNA. J. Biol. Chem. 261:10375.

3. Mimori, T., J.A. Hardin, and J.A. Steitz. 1986. Characterization of the DNA-binding protein antigen Ku recognized by autoantibodies from patients with rheumatic disorders. J. Biol. Chem. 261:2274.

4. Mimori, T., M. Akizuki, H. Yamagata, S. Inada, S. Yoshida, and M. Homma. 1981. Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap. J. Clin. Invest. 68:611.

5. Reeves, W.H. 1985. Use of monoclonal antibodies for the characterization of novel DNA-binding proteins recognized by human autoimmune sera. J. Exp. Med. 161:18.

6. Yaneva, M., R. Ochs, D.K. McRorie, S. Zweig, and H. Busch. 1986. Purification of an 86-70 kDa nuclear DNA-associated protein complex. Biochim. Biophys. Acta. 841:22.

7. Yaneva, M., and H. Busch. 1986. A 10S particle released from deoxyribonuclease-sensitive regions of HeLa cell nuclei contains the 86-kilodalton-70-kilodalton protein complex. Biochemistry. 25:5057.

8. Yaneva, M., J. Wen, A. Ayala, and R. Cook. 1989. cDNA-derived amino acid sequence of the 86-kD subunit of the Ku antigen. J. Biol. Chem. 264:13407.

9. Reeves, W.H., and Z.M. Sthoeeger. 1989. Molecular cloning of cDNA encoding the p70 (Ku) lupus autoantigen. J. Biol. Chem. 264:5047.

10. Mierendorf, R.C., C. Percy, and R.A. Young. 1987. Gene isolation by screening λgt11 libraries with antibodies. Methods Enzymol. 152:458.

11. Sen Gupta, D.N., B.Z. Zmudzka, P. Kumar, F. Cobianchi, J. Skowronski, and S.H. Wilson. 1986. Sequence of human DNA polymerase β mRNA obtained through cDNA cloning. Biochem. Biophys. Res. Commun. 136:341.

12. Landschulz, W.H., P.F. Johnson, E.Y. Adashi, B.J. Graves, and S.L. McKnight. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. 2:786.

13. Smith, D.E., and P.A. Fischer. 1984. Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in Drosophila embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J. Cell Biol. 99:20.

14. Verheijen, R., H. Kuijpers, W. van Venrooij, and F. Ramaekers. 1988. The 80 kilodalton component of the Ku autoantigen complex is associated with the nuclear matrix. Ph.D. Thesis. University of Nijmegen, The Netherlands. 129-149.

15. Williams, J.G., and C.M. Radding. 1981. Partial purification of an exonuclease inhibitor induced by bacteriophage Mu-1. J. Virol. 39:548.

16. Mimori, T., Y. Ohosone, N. Hama, A. Suwa, M. Akizuki, M. Homma, A.J. Griffith, and J.A. Hardin. 1990. Isolation and characterization of cDNA encoding the 80-kDa subunit protein of the human autoantigen Ku (p70/p80) recognized by autoantibodies from patients with scleroderma-polyarthritis overlap syndrome. Proc. Natl. Acad. Sci. USA. 87:1777.