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Identification of Nuclear Factor IV/Ku autoantigen in a human 2D-gel protein database

Modification of the large subunit depends on cellular proliferation

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Nuclear Factor IV (NFIV) is a heterodimeric DNA-binding protein from HeLa cells, recognizing molecular ends and is identical to the autoantigenic target Ku. We have identified the two NFIV/Ku subunits, by comigration, in the 2D-gel database of transformed human amnion cell (AMA) proteins. We observed that the large subunit of NFIV/Ku consists of at least 3 charge variants that correspond to SSP IEFs 5705 (81.2 kDa, p*I* 5.74), 6707 (81.2 kDa, p*I* 5.67) and 6706 (81.9 kDa, p*I* 5.60) in the AMA catalogue. The relative amounts of the 2 major variants (IEFs 5705 and 6707) was dependent on the state of cell proliferation. Inhibition of DNA-synthesis by hydroxyurea also changed the relative levels of the variants, whereas aphidicolin or a thymidine block had no effect. These results suggest a possible role for NFIV/Ku in DNA replication.

Nuclear Factor IV; Ku antigen; Primatin; Human protein database; 2D-gel electrophoresis; Hydroxyurea

1. INTRODUCTION

Nuclear Factor IV was isolated as a HeLa nuclear protein that recognized both ds- and ssDNA [1]. Unlike most DNA-binding proteins, NFIV needs molecular ends to recognize dsDNA. It recognizes either 3′-overhanging, 5′-overhanging or blunt ends, and after binding it migrates towards inward positions on the DNA. At sufficiently high concentrations, the protein is able to form a regular dsDNA-multimeric protein complex. NFIV from HeLa cells is a heterodimer consisting of a 72 kDa and a 84 kDa subunit [1]. Cloning of a cDNA encoding the 84 kDa subunit, as well as immunological evidence have demonstrated that the protein is identical to the autoantigen Ku [2]. The Ku protein is an autoantigenic target in patients bearing scleroderma, Graves' disease and systemic lupus erythematosus [3,4,5].

The cellular function of the protein is not known. Its ssDNA and dsDNA end-binding activity strongly suggests a role in DNA replication, repair or recombination. Ku [6] or a related protein [7] has also been implied in transcriptional control. Here we have identified both NFIV/Ku subunits in the 2D-gel database of transformed human amnion cell (AMA) proteins [8] by a comigration experiment. Furthermore, we present studies on the relative abundance of different charge variants of NFIV/Ku as a function of cell proliferation.

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Abbreviations: IEF, isoelectric focusing; SSP, sample spot number

2. MATERIALS AND METHODS

2.1. *Cells*

Transformed human amnion cells (AMA) were grown in Dulbecco's modified Eagle's medium containig 10% fetal serum and antibiotics (penicillin, 100 U/ml; streptomycin 50 μg/ml).

2.2. *2D-gel electrophoresis*

Standardized 2D (IEF-SDS) gel electrophoresis was performed as described [8,9].

2.3. *Inhibition of DNA synthesis*

Mitotic AMA cells were obtained by mechanical detachment essentially as described [10]. Inhibition of DNA synthesis was accomplished by addition of aphidicolin (5 μg/ml), hydroxyurea (10 mM) or thymidine (2 mM) 1 h after plating of the cells. Proteins were labelled for 1.5 h with [^{35}S]methionine starting 15.5 h after plating. This period corresponds to S-phase in non-treated cells [11]. Subsequently the cells were collected and resuspended in lysis buffer [9].

2.4. *NFIV/Ku preparations*

NFIV/Ku was isolated from HeLa cells as described [1] using ion-exchange and ssDNA- and dsDNA-affinity chromatography. The resulting preparation is more than 95% pure as judged by 2D-gel electrophoresis and silver staining.

3. RESULTS

3.1. *2D gel analysis of NFIV*

We analyzed purified NFIV on a 2D (IEF-SDS) gel. As shown in Fig. 1A, silver staining of the gel revealed

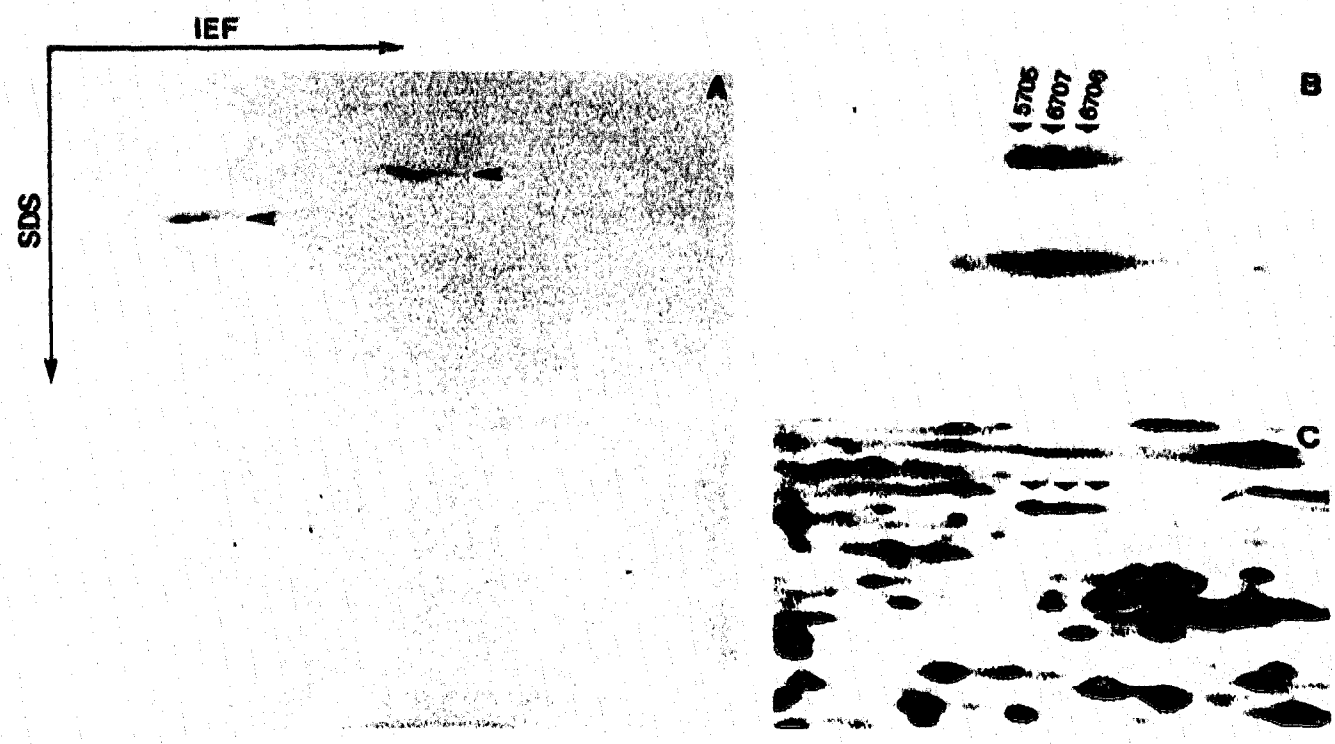

Fig. 1. Nuclear Factor IV large subunit comigrates with proteins IEF 5705 and IEF 6707. Purified NFIV was analyzed on a 2D (IEF-SDS) gel (A). Large and small subunit isoforms are indicated by arrows. In a separate experiment purified NFIV was mixed with a [^{35}S]methionine labelled AMA protein extract and analyzed on a 2D (IEF-SDS) gel. (B) Shows part of the gel after silver staining. The additional spots are derived from proteins in the AMA extract. (C) Shows the same part of the gel after autoradiography. The numbering of the proteins is according to [8].

that the protein consists of a series of polypeptides. The large subunit resolves into 3 polypeptides with nearly identical apparent molecular weights, but with different p*I*. The small subunit, on the other hand, is less well resolved, and may also consist of different modified forms (Fig. 1A).

3.2. *Assignment of NFIV subunits to proteins in the AMA 2D-gel protein database*

In order to identify NFIV in the AMA protein database [8], we mixed [^{35}S]methionine labelled protein extract from AMA cells with purified NFIV. Silver staining of the gel (Fig. 1B) followed by autoradiography revealed that the 3 spots which constitute the large subunit comigrate with a series of 3 radiolabelled proteins (Fig. 1C), which corresponded to SSP IEFs 5705 (81.2 kDa, p*I* 5.74), 6707 (81.2 kDa, p*I* 5.67) and 6706 (81.9 kDa, p*I* 5.60) in the AMA 2D-gel protein database [8]. SSP IEFs 5705 and 6707 are also known as primatin [8] and correspond to IEFs 8z30 and 8z31 in the HeLa protein catalogue [12].

The small subunit is not visible in this part of the gel, but careful analysis of the silver-stained gel and the autoradiograph revealed that it is identical to AMA protein SSP IEF 2505. It has an apparent M_r of 64.2 kDa and a p*I* of 6.77.

3.3. *Proliferation-specific distribution of charge variants of the large NFIV subunit*

Two charge variants of the large subunit (proteins IEF 5705 and 6707) have been studied previously, and were referred to as 8z30 and 8z31, respectively [13,14]. Their identical mobility in a SDS-gel, as well as their reactivity with the same monoclonal antibody, already suggested that they correspond to charge-variants of a single polypeptide. Interestingly, the relative levels of the 2 protein variants differ considerably between quiescent and transformed cells. In quiescent human fibroblasts (MRC-5) IEF 6707 is present at 2.3-fold higher levels than the more basic variant IEF 5705. In proliferating normal MRC-5 cells this ratio is about 1.2, while in their SV40-transformed counterparts the ratio is 0.6. The results indicate that - dependent on growth - the relative amounts of the charge variants change. In this analysis [13], the other protein variant (SSP IEF 6706) was not examined, because it is synthesized at much lower levels, and therefore undetectable at the exposure times used.

The apparent dependence on the state of cellular proliferation suggested that the protein may be modified during progression through the cell cycle. Analysis of the [^{35}S]methionine labelled proteins synthesized throughout the cell cycle of AMA cells failed to show a

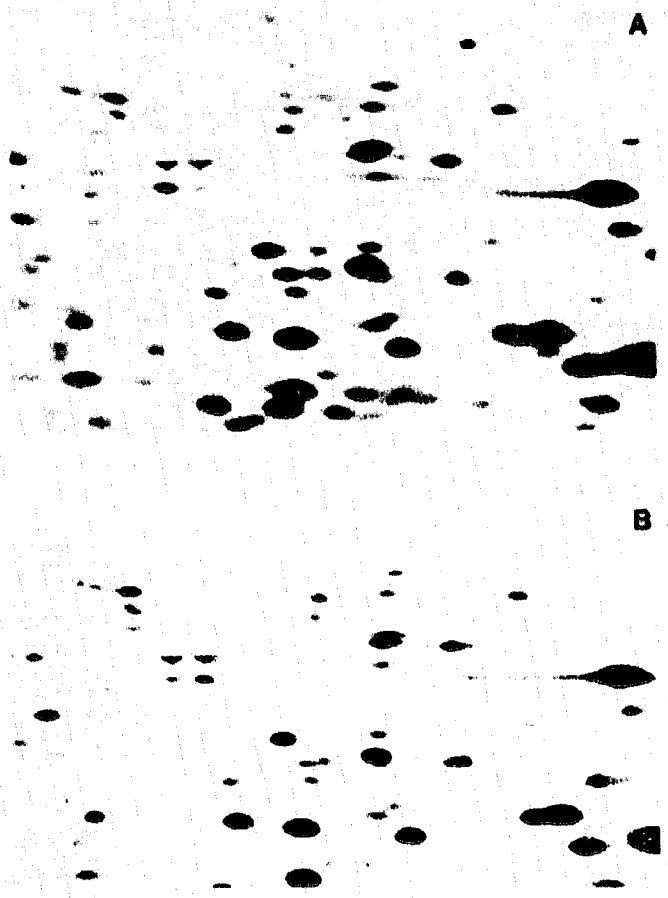

Fig. 2. Synthesis of charge variants IEF 5705 and IEF 6707 (indicated by triangles) in normal and hydroxyurea-treated transformed human amnion (AMA) cells (A) Shows the pattern of synthesis of [^{35}S]methionine labelled proteins in untreated AMA cells. (B) Shows the pattern of synthesis in hydroxyurea-treated cells.

difference in the ratio of the 2 major variants (SSP IEFs 5705, 6707) of the large subunit (results not shown). In view of the DNA-binding properties of NFIV/Ku we investigated whether changes in the 2 major variants of the large subunit occur in cells arrested at the G1/S transition border of the cell cycle. For this purpose, synchronized AMA cells were treated with either aphidicolin, high concentrations of thymidine or hydroxyurea (Fig. 2). The drugs were administered at G1-phase, 1 h after plating mitotic cells. Proteins were then labelled with [^{35}S]methionine 15.5–17 h after plating. This timespan corresponds to S-phase in non-treated cells [11]. Control cells, which did not receive inhibitors, synthesize high levels of IEF 5705 and low levels of IEF 6707, characteristic of the transformed cells. Thymidine and aphidicolin-treated cells exhibit an almost identical pattern of protein synthesis (data not shown). However, the cells that received hydroxyurea have a low IEF 5707 level and a high IEF 6707 level (Fig. 2), just like the quiescent cells.

4. DISCUSSION

We have identified Nuclear Factor IV (NFIV), which is identical to the Ku autoantigen [2], in the AMA 2D-gel protein database [8]. The purified protein corresponds to SSP IEF 5705, 6707 and 6706 as charge variants constituting the large subunit, as well as SSP IEF 2505 corresponding to the small subunit.

The nature of the modification which distinguishes the charge variants is not known. Labelling with [^{32}P]orthophosphate 'in vivo' could not be detected for SSPs 5705 and 6707 [8,13]. However, phosphoamino-acid analysis of the Ku protein has indicated that both the small and large subunit of Ku may be phosphorylated at serine residues [6]. In addition, a DNA-dependent protein kinase was recently found that could phosphorylate the Ku autoantigen 'in vitro' [15]. The reason for this discrepancy is at present unknown. According to our data [8,13] it is possible that the difference between the IEF 5705 and 6707 polypeptides may be due to post-translational modifications other than phosphorylation. The detection of another large subunit variant (SSP IEF 6706) suggests that multiple modifications are possible.

Proteins 5705 and 6707 previously attracted attention because the relative level of the charge variants appeared to be influenced by the proliferative state of the cell [13]. We show here that in hydroxyurea treated cells the pattern of synthesis of the charge variants is altered. In contrast, aphidicolin treatment and high concentrations of thymidine do not influence the relative amount of synthesis of the charge variants. The drugs all cause accumulation of cells in and around the G1/S boundary, suggesting that the modification of the large subunit may take place close to the onset of DNA replication.

The target of hydroxyurea is ribonucleotide reductase [16], the key enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides. High concentrations of thymidine disturb, by allosteric interactions, the coordinated synthesis of deoxyribonucleotides [17]. Aphidicolin is a competitive inhibitor of DNA polymerases α and δ [18]. Presently, we do not understand why the various inhibitors show such contrasting effects on the synthesis of the charge variants.

Immunofluorescence studies using a monoclonal antibody (mAb 1C4C10) that recognizes both charge variants have revealed that the protein is present in the nuclei, especially in the nucleoli [13]. In addition, microinjection of mAb 1C4C10 in the cytoplasm of cells expressing NFIV leads to nuclear and nucleolar accumulation [14]. Cells that are about to enter M-phase show some antigen in the cytoplasm (data not shown), while M-phase staining is most apparent in the area surrounding the chromosomes, which agrees well with the immunofluorescence data on the Ku antigen. It should be stressed however that immunofluorescence data on

the Ku autoantigen have yielded conflicting results concerning the nucleolar staining [3,4,19,20,21]. One way to reconcile these data is to assume that NFIV/Ku has a cell stage-specific interaction with other cellular components, thereby shielding epitopes recognized by some of the available antibodies.

The analysis of NFIV/Ku in the human AMA 2D-gel protein database has provided us with data concerning proliferation- and cell cycle-specific modifications of the polypeptide. In combination with biochemical data on the nucleic acid binding properties of NFIV [1], it is suggested that NFIV may play a role in DNA replication.

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