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A Novel Cell-Cycle-Dependent 350-kDa Nuclear Protein: C-Terminal Domain Sufficient for Nuclear Localization

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We have screened human scleroderma patients for immunoreactivity with the components of the nucleus and the mitotic apparatus. We announce the identification of a novel cell-cycle-dependent nuclear protein using serum from a CREST patient AH. AH protein first appears at the nucleus of G2-phase and associates with the centrosome throughout the cell cycle. As chromosomes condense during the prophase, AH protein becomes enriched at the kinetochores. During mitosis, AH protein progressively disperses from the kinetochore and becomes diffusely localized in the cytoplasm and in telophase; it appears to be enriched within the intracellular bridge. Molecular cloning and transfection studies reveal that the 350-kDa AH protein contains a coiled-coil and a globular domain at the C-terminus that is sufficient for nuclear localization.

Many important proteins were identified using autoimmune sera from patients with systemic sclerosis and Raynaud's phenomenon. Among them are both nuclear antigens, such as the snRNPs and kinetochores, and the cytoplasmic organelle such as the centrosome. Therefore, these patient sera continue to be valuable reagents for the molecular dissection of the cellular structures they identify. For example, most of the molecular components of the mammalian nuclear proteins, CENP A-D, were identified by this approach (1,2). In addition several centrosomal antigens are beginning to be revealed by these human autoantibodies. The most prominent among these include pericentrin, NuMA / SP-H antigen / SPN antigen and PCM-1 (3-8).

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Abbreviations: Demecolcine/Colcemid, N-Deacetyl-N-methylcolchicine; DAPI, 4', 6-Diamidino-2-phenylindole.

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To identify novel nuclear/mitotic proteins, we have screened scleroderma patients for cell cycle dependent immunoreactivity with the components of the nucleus and the mitotic apparatus. Here, we announce the identification of a novel cell cycle dependent 350 KDa nuclear and centrosomal coiled coil protein.

METHODS

Isolation of cDNA Clones. A λZap Hela cDNA expression library was screened (2.3 X 10^5 pfu's) using AH human CREST autoimmune serum. Two positive clones were identified, purified, and the cDNA inserts was sequenced.

Expression of Recombinant Proteins and Antibody Generation. To generate fusion proteins, two independent EcoR I fragments of C1 (nt. 794-1574 and nt. 249-794) were subcloned into pGEX-3X vector (Pharmacia). Fusion proteins were purified using glutathione agarose beads and used for immunization of BALB/c (H-2^d) inbred mice (Jackson Laboratories) (9). Antibodies generated were depleted of GST antibody and used for immunofluorescence microscopy and immunoblot analysis.

Immunoblot and Immunofluorescence Analysis. Mammalian cells were enriched in prophase and prometaphase by modifying the method described by Johnson et al. (10). Briefly, Hela cells were arrested in the S phase by incubation for 16-20 hours in 2.5 mM thymidine. The cells were then released from the S phase by changing to a fresh medium for 5-6 hours. This was followed by application of a 10 minutes 70ng/ml Colcemid. Mammalian cell lysates and recombinant proteins were resolved on SDS-PAGE (11) and transferred to nitrocellulose for western blot analysis using the alkaline phosphatase color developing system. Hela cells and COS-7 cells were cultured on sterile glass coverslips and fixed in cold methanol and processed for immunofluorescence as previously described (12).

RESULTS

Using immunofluorescence technique, the distribution of AH antigen was followed in relation to the microtubules and the chromosomes as the mammalian Hela cells traverse the cell cycle. AH antigen was localized as a distinct spot at the perinuclear centrosome in G1 and as two spots in S-G2 interphase, and at the poles of the spindle in mitosis (Fig. 1). In addition, AH serum strongly stained a subpopulation of nuclei homogeneously except the region occupied by the nucleolus. The staining nuclei had two dots of centrosomal staining, suggesting AH serum recognized the G2-phase nuclei (Fig. 1 A-C). During prometaphase, general strong nuclear staining of AH serum was replaced by restricted paired foci throughout the nucleus, reminiscent of kinetochore region of the chromosome (Fig. 1 D-F). During metaphase, weak puntate staining was occasionally observed at the mitotic midplate where mitotic sister chromosomes locate (Fig. 1 G-I). Furthermore, preparations of isolated mitotic Hela chromosomes confirmed the location of AH antigen at the kinetochore (Data not shown). As cells progressed through anaphase and telophase, only diffuse cytoplasmic staining was observed (Fig. 1 J-R). Thus, the staining pattern of AH antigen consisted of two components: one cell-cycle independent centrosome staining, and the other cell-cycle dependent nuclear staining in the G2 phase of the cell cycle.

We used the AH autoimmune serum as a probe to screen a λZAP human Hela cDNA expression library. We screened 2.3 X 10^5 plaques and identified 2 positive plaques. Western blot analysis using the cell lysate prepared from R1090 host bacteria infected with

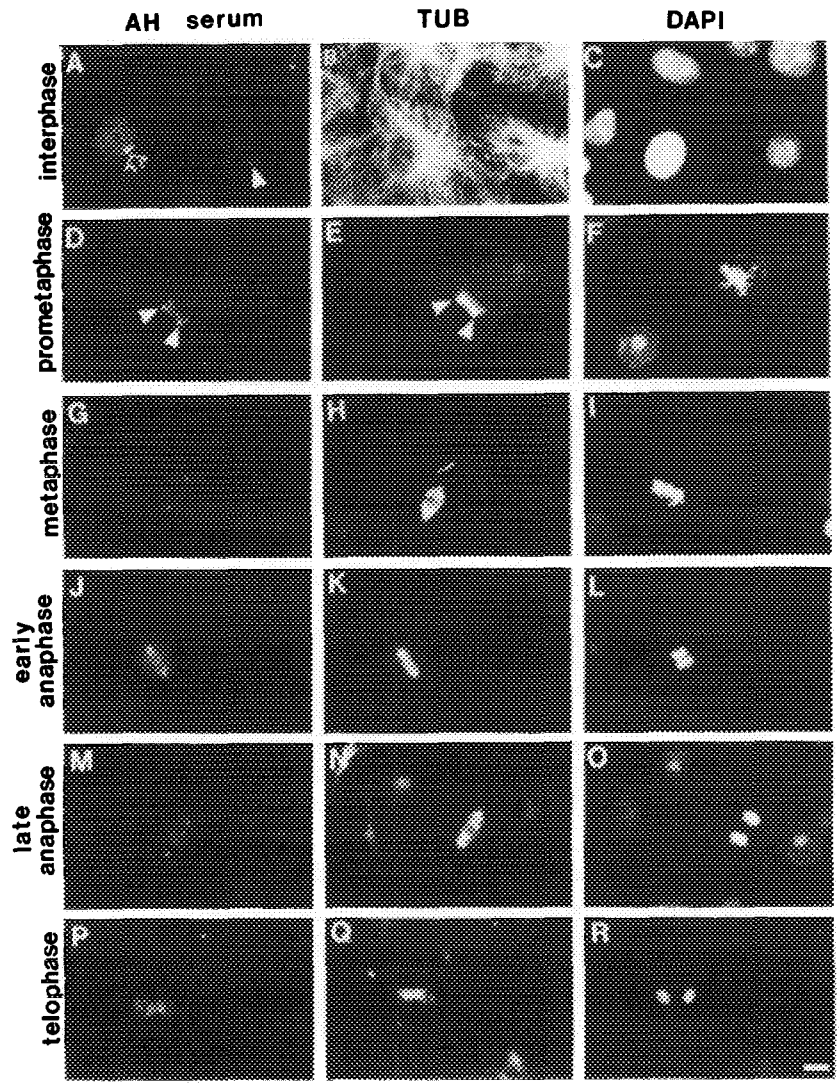

Fig. 1. Immunofluorescence Localization of AH Antigen in COS-7 cells. COS-7 cells were fixed, stained with AH serum (A, D, G, J, M, P), anti-β-tubulin (B, E, H, K, N, Q) and DAPI (C, F, I, L, O, R). Cells are shown in interphase (A, B, C, open arrow for G2 duplicated centrosomes and closed arrow for G1 centrosome), prometaphase (D, E, F), metaphase (G, H, I), early anaphase (J, K, L), late anaphase (M, N, O) and telophase (P, Q, R). Bar, 8 μm.

positive λ phages showed immuno reactive fusion proteins (data not shown). Since we used for screening the whole AH serum which may contain antibodies to more than one cellular component, it was necessary to confirm that the positive phages actually corresponded to our antigen. To do this, we affinity purified the antibody against the expressed fusion proteins and the purified antibodies retained immunoreactivity to nucleus and the kinetochores (data not shown). The cDNAs from these two phages were autoexcised and

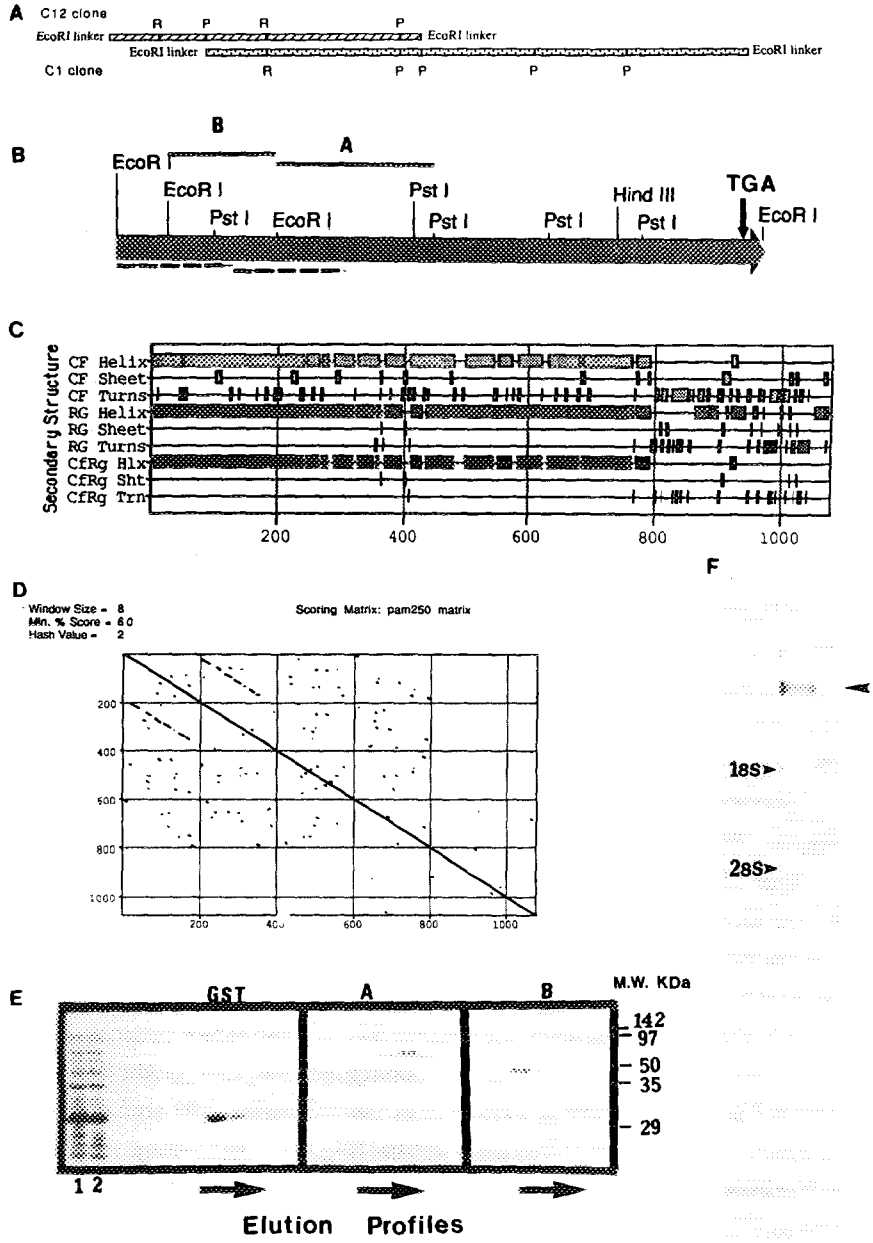

Fig. 2. Molecular Cloning of AH Antigen and Partial Primary Sequence Structure Analysis. (A) Two independent clones C1 and C12 with overlapping region and the restriction sites are shown. R for EcoR I and P for Pst I. (B) Combined sequence of C1 and C12. Stop codon TGA is indicated. Solid lines A and B show the regions for GST fusion protein generation; dashed lines show the internal repeats within the protein sequence. The partial protein sequence can be obtained in the GenBank with the accession number of U25725. (C) Secondary structure of the protein is predicted a helical coiled-coil domain. (D) Comparison of AH protein sequence with itself and an pair of internal repeat is revealed at the N-terminus. (E) Purification of GST-Fusion proteins GST-AH-A, GST-AH-B and GST only. Total cell lysate (lane 1) and flow through (lane 2) and the elution profiles are shown for GST only, GST-AH-A, GST-AH-B respectively. (F) Northern blot analysis reveals a mRNA of 10-12 kb.

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  1 VEKGEFALRL SSTQEEVHQL RRGIEKLRVR IEADEKKQLH IAEKLKERDG ENDSLKDKVE
 61 NLERELQMSE ENQELVILDA ENSKAEVETL KTQIEEMARS LKVFELDLVT LRSEKENLTK
121 QIQEKQGQLS ELDKLLSSFK SQLEEKEQAE IQIKEESKTA VEMLQNQLKE LNEAVAALCG
181 DQEIMKATEQ SLDPPIEEEH QLRNSIEKLR ARLEADEKKQ LCVLQQLKES EHHADLLKGR
241 VENLERELEI ARTNQEHAAL EAENSKGEVE TLKAKIEGMT QSLRGLELDV VTIRSEKEDL
301 TNELQKEQER ISELEIINSS FENILQEKEQ EKVQMKEKSS TAMEMLQTQL KSSMREWQPC
361 IMTKKPVSAK EQNLSSQVEC LELEKAQLLQ GLDEAKNNYI VLQSSVNGLI QEVEDGKQKL
421 EKKDEEISRL KNQIQDQEQL VSKLSQVEGE HQLWKEQNLE LRNLTVELEQ KIQVLQSKNA
481 SLQDTLEVLQ SSYKNLENEL ELTKMDKMSF VEKVNKMTAK ETELQREMHE MAQKTAELQE
541 ELSGEKNRLA GELQLLLEEI KSSKDQLKEL TLENSELKKS LDCMHKDQVE KEGKVREEIA
601 EYQLRLHEAE KKHQALLLDT NKQYEVEIQT YREKLTSKEE CLSSQKLEID LLKSSKEELN
661 NSLKATTQIL EELKKTKMDN LKYVNQLKKE NERAQGKMKL LIKSCKQLEE EKEILQKELS
721 QLQAAQEKQK TGTVMDTKVD ELTTEIKELK ETLEEKTKEA DEYLDKYCSL LISHEKLEKA
781 KEMLETQVAH LCSQQSKQDS RGSPLLGPVV PGPSPIPSVT EKRLSSGQNK ASGKRQRSSG
841 IWENGGGPTP ATPESFSKKS KKAVMSGIHP AEDTEGTEFE PEGLPEVVKK GFADIPTGKT
901 SPYILRRTTM ATRTSPRLAA QKLALSPLSL GKENLAESSK PTAGGSRSQK VKVAQRSPVD
961 SGTILREPTT KSVPVNNLPE RSPTDSPREG LRVKRGRLVP SPKAGLESKG SENCKVQ*
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Fig. 3. Partial Protein Sequence of AH Protein. DNA sequence was analyzed using MacVector 4.1 and AssemblyLIGN.

designated pBluescriptSK-C1 and pBluescript-C12 containing a 1.6 kb and a 2.8 kb insert respectively (Fig.2 A). Moreover, the antibodies purified against each of the fusion proteins cross reacted (data not shown), suggesting that cDNA clones C1 and C12 were related and might in fact encode the same protein.

DNA sequence analysis of both C1 and C12 revealed a common open reading frame encoding a polypeptide of 1017 amino acids with a stop codon at the nucleotide 3056 (Fig. 2B and Fig. 3). This partial sequence can be obtained from GenBank with accession number U25725. Search of the EMBL DNA data base and pir protein data base revealed that these sequences are novel and the N-terminal 780 amino acids share weak homology to myosin heavy chain B, D, II and beta, tropomyosin alpha chain, kinesin-related protein CENP-E and KAR3, neurofilament triplet M and L protein, lamin and paramyosin. The homology between our protein and those known protein is primarily due to a secondary structure motif of the N-terminal 780 amino acids. MacVector 4.1 computer program predicted the secondary structure of the protein as a strong helix within the N-terminal 780 amino acids suggesting that these amino acids form a coiled-coil domain (Fig. 2C). Interestingly, when the protein sequence is compared to itself, the sequence of amino acids 15 to 160 is strikingly similar to the downstream sequence amino acids 200-340 (Fig. 2D). The significance of these repeats awaits further studies. The sequence contains three potential cAMP kinase sites (KRLSS at a.a. 826-822; KRQRS at a.a. 838-834; and RRTT at a.a. 909-906), one potential Ca Kinase II site (RNLTV at a.a. 465-462), seven potential N-glycosylation sits, eight potential protein kinase C sites and eight potential cdc2 kinase sites. This raises an interesting possibility of the phosphorylation of this protein being one of the key regulatory element during the cell cycle.

To estimate the size of the messenger RNA encoding this protein, total RNA from Hela cells was prepared and 30 μg was used for Northern blot analysis. A EcoR I 2.5 kb fragment from C1 clone was used as a template for rediprime DNA labeling using [^{32}P]dCTP. Using this probe, a mRNA of size 10-12 kb (Fig. 2F) was revealed, implying a

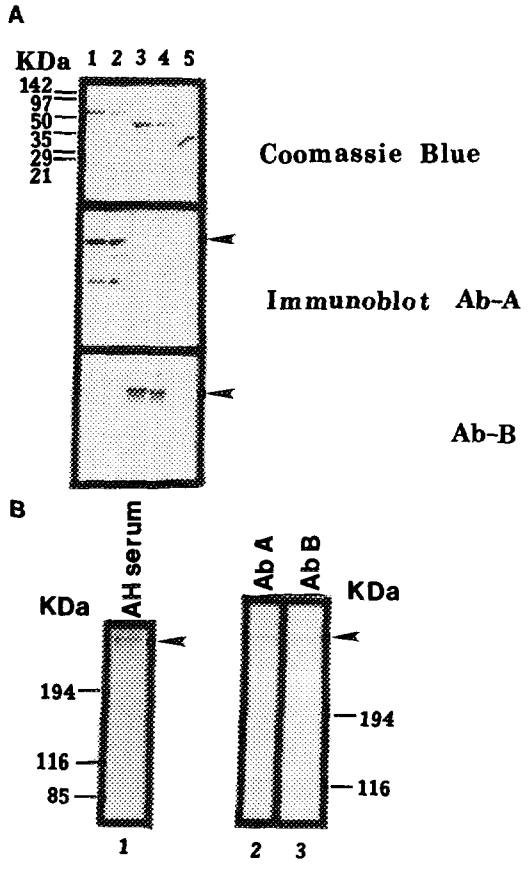

Fig. 4. AH serum as well as polyclonal antibodies A and B generated against fusion protein recognize a single protein of ~350 kDa. (A) The first row shows the coomassie blue staining of GST-AH-A (lane 1 and 2), GST-AH-B (lane 3 and 4) and GST only (lane 5). The second row shows the immunoblot using polyclonal antibody A, while the third row shows the immunoblot using polyclonal antibody B. (B) Immunoblot of Hela cells extract using AH serum (lane 1), antibody A (lane 2) and antibody B (lane 3).

large polypeptide may be encoded. In fact, both polyclonal antibodies A and B specific for fusion proteins within the coiled-coil domain (Fig. 2B (solid lines), 2E and Fig. 4A) and AH serum recognized a single protein band of molecular weight 350 kDa (Fig. 4B) in prometaphase enriched cells. Furthermore, immunocytochemical studies using both A and B antibodies revealed strong staining in a subpopulation of the nuclei (Fig. 5 A, B, C, D). While the interphase centrosome was inaccessible using A and B antibodies, the mitotic centrosomes were accessible only by antibody B, and not with antibody A which recognizes an epitope on the AH protein adjacent to the epitope of the antibody B (Fig. 5 E, F). Nevertheless, we conclude that the cDNA clones C1 and C12 encode the AH protein. Moreover, the cell-cycle independent centrosome staining and the cell-cycle dependent nuclear staining revealed by the original serum is indeed due to the reactivity to the same protein.

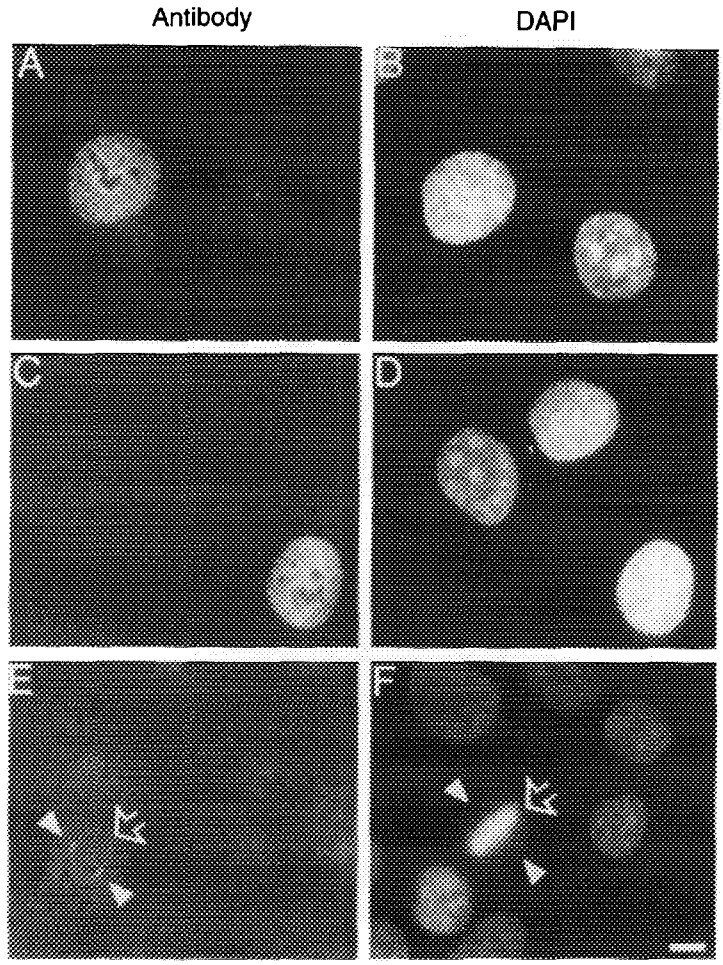

Fig. 5. Immunofluorescence Localization of AH Antigen in Hela cells using antibodies A and B. Hela cells were fixed, stained with antibody A (A), antibody B (C, E), respectively, and DAPI (B, D, F). Cells are shown in interphase (A, B, C, D) and metaphase (E, F, closed arrow for spindle poles and open arrow for spindle midzone). Bar, 5 μm.

Eventhough data search did not reveal any nuclear localization signal, we subcloned a ~1.6 kb 3' cDNA fragment that contained part of the coiled-coil domain into a mammalian protein expression vector under the control of SV40 early promoter (Fig. 6A). The resulting construct pSVK-Flag-AHc was used to transfect Hela and COS-7 cells. As early as 10 hours after transfection, flag-tagged AHc antigen can be observed in the nucleus as strong bright staining (Fig. 6 B and C, transfected cells (arrow)), while the untransfected cells in the same field were not stained. These results suggest that the C-terminus of the protein is sufficient to target the protein to the nucleus and might in fact define a novel mechanism of nuclear localization.

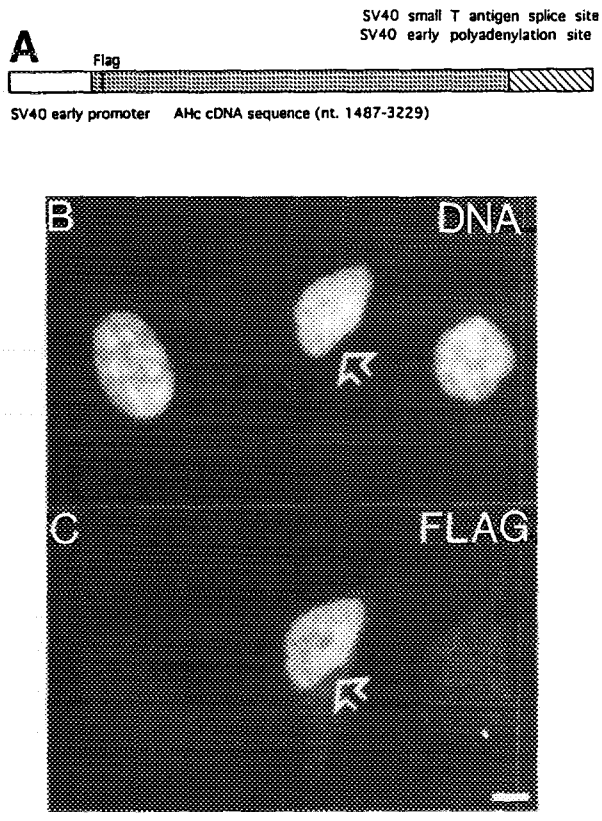

Fig. 6. AHc Protein Enters Nucleus in Hela Cells. Plasmids with ~500 amino acids of C-terminal AH protein downstream of SV40 early promoter (A) were transfected into Hela cells. Cells were fixed 10 hours after transfection and processed for immunofluorescence using anti-FLAG antibody (C) and DAPI (B). Bar, 4 μm.

DISCUSSION

Using autoimmune serum AH we isolated partial cDNA clones and generated independent polyclonal antibodies A & B to different regions of the encoded protein. These antibodies and the original serum identified the same single protein band at 350 kDa from the mammalian cell extracts (Fig 4 B). Thus, we conclude that the serum staining is due to the distribution of a single protein, AH. Individually, antibody A and B stained a subset of structures stained by the whole serum. While antibody B stains both the centrosomal AH and the nuclear AH, antibody A detects only the nuclear form of the AH protein. How could this discrepancy of different staining by two antibodies to the same protein be reconciled? One possibility is that the epitope reactive to antibody A is hidden at the centrosome and revealed within the nucleus. In that event, it would be likely that the A-epitope defines the precise boundary of inaccessibility at the centrosome, because the adjacent B-epitope is accessible. Thus, it is possible that the hidden A epitope at the centrosome may in fact be

part of the domain that targets AH protein to the centrosome. The nuclear but not centrosomal targeting of the truncated protein AHc, which excludes the A epitope region, supports this hypothesis. Although the precise mapping of the targeting domains will require further studies, it would be worthwhile to dissect how AH molecule is targeted to two organelles. It is possible that AH protein may play distinct roles at the centrosome and within the nucleus.

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