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Subnuclear Localization of the *trans*-Activating Protein of Human T-Cell Leukemia Virus Type I

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Human T-cell leukemia virus type I is associated with human lymphoid malignancies. The p40$^{x^I}$ protein encoded by the *x* gene of this virus is believed to play some role in virally mediated transformation. This gene is known to encode a transcriptional *trans* activator which previous studies have shown to be a nuclear protein. Further characterization of the intracellular kinetics of this protein showed that it migrated into the nucleus very soon after synthesis. Within the nucleus, p40$^{x^I}$ was distributed almost equally between the nucleoplasm and the nuclear matrix. Given the proposed role of the nuclear matrix in RNA transcription, the association of p40$^{x^I}$ with the matrix places it in an appropriate cellular compartment to exercise an effect on transcription.

Adult T-cell leukemia/lymphoma is a human malignancy endemic to southern Japan, the Caribbean basin, and parts of Africa (3, 41, 54). The etiologic agent for this disease is believed to be human T-cell leukemia virus type I (HTLV-I) (22, 34, 36, 55). The human T-cell leukemia virus genome has been fully characterized (42), and the putative transforming gene, termed *x*, is found between the *env* gene and the 3′ long terminal repeat (21, 42). Considerable attention has been focused on this gene because of its possible role in the induction of a human malignancy. The gene encodes a protein of 40 kilodaltons called p40$^{x^I}$ (48). This protein has also be called *lor*, *x-lor*, and *tat*^{I} (32, 50). We have recently characterized several aspects of the protein, including its half-life (120 min), its relative concentration in infected cells (0.15 to 0.2% of the total cellular protein), and its subcellular localization (nuclear) (47). Other investigators have also shown p40$^{x^I}$ to be a nuclear protein (17). This nuclear localization places the *x* gene product in a class of viral transforming proteins which includes the products of the *myc*, *myb*, *fos*, E1a, simian virus 40 large T, and polyomavirus large T genes (7, 8, 10, 11, 14, 53). Little is known about the exact mechanisms by which these proteins affect the nucleus or its contents to induce or maintain transformation or both. At least four of the proteins, p40$^{x^I}$, E1a, *fos*, and *myc*, have the ability to act in *trans* to regulate the transcription of other genes (2, 18, 29, 43). The molecular level at which this phenomenon occurs is as yet unidentified.

To gain further insight into the function of p40$^{x^I}$, we have undertaken a study to characterize the subnuclear localization of the protein. This approach involved isolation of intact nuclei from HTLV-I-infected human T cells by using a method known to result in nuclei free of cytoplasmic or membrane contaminants with no measurable alteration in nuclear integrity (6). These nuclei were subsequently fractionated into three components, the nucleoplasm, chromatin, and nuclear matrix, and analyzed for their relative content of p40$^{x^I}$. The fidelity of isolation of the three fractions was monitored by using a combination of morphologic and biochemical criteria.

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MATERIALS AND METHODS

Cell line and cell fractionation. The human T-cell leukemia virus infected T-cell line SLB-I was used in all studies (48). Whole cells were lysed by exposure to cold (4°C) buffer consisting of 5 mM sodium phosphate (pH 7.4), 50 mM NaCl, 150 mM sucrose, 5 mM potassium chloride, 2 mM dithiothreitol, 1 mM magnesium chloride, 0.5 mM calcium chloride, 0.1 mM phenylmethylsulfonyl floride, and 0.1% Kyro-EOB nonionic detergent (Proctor & Gamble Co.). Intact nuclei were separated from the whole-cell lysate by centrifugation at 1,000 × *g* for 10 min at 4°C onto 300 μl of a 30% sucrose–2.5 mM Tris hydrochloride (pH 7.4)–10 mM NaCl step cushion. Nucleoplasm was removed from the isolated nuclei by suspension of the nuclei at 4°C in Nonidet P-40 (NP-40) buffer consisting of 0.5% NP-40, 10 mM sodium phosphate (pH 7.4), 120 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride at a concentration of 10×10^6 nuclei per ml of buffer. Nuclei were left in this buffer on ice for 30 min, collected by pelleting at 500 × *g* for 7 min, and extracted two more times in NP-40 buffer as described above. The resulting nuclear fraction was treated to remove chromatin by suspension in 100 μl of nuclease digestion buffer consisting of 20 mM Tris hydrochloride (pH 7.4), 100 mM NaCl, 50 mM KCl, 5 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 170 U of micrococcal nuclease (Boehinger Mannheim Biochemicals) per ml. NP-40-extracted nuclei were incubated in this solution for 30 min at 37°C. Nuclease digestion was stopped by the addition of 900 μl of high-salt buffer containing 2 M NaCl, 0.5% NP-40, 5 mM sodium phosphate (pH 7.4), and 10 mM EDTA. The soluble supernatant fraction (chromatin) was separated from the insoluble nuclear matrix fraction by centrifugation onto a 30% sucrose step cushion (same as that described above) at 12,000 × *g* for 15 min.

Analysis of total protein in various nuclear subfractions. Total proteins found in the various subcellular fractions and nuclear subfractions were precipitated with cold (4°C) 20% trichloroacetic acid (TCA). The resulting pellets were washed in absolute acetone; solubilized in sample buffer consisting of 10% glycerol, 10% β-mercaptoethanol, 1.5% sodium dodecyl sulfate (SDS), and 30 mM Tris (pH 6.8); and

electrophoresed on a 7 to 15% linear polyacrylamide gradient gel which was subsequently fixed in a solution of water-methanol-glacial acetic acid (5:5:2 [vol/vol/vol]) with 0.1% Coomassie blue R250.

Labeling and determination of DNA content of various nuclear subfractions. HTLV-I-infected cells (SLB-I) were labeled with ^{3}H-thymidine for 16 and ^{35}S-methionine for 6 h and then fractionated as described above. TCA was added to the various fractions to a final concentration of 10%, and the radioisotope in the TCA-precipitated DNA was counted in a liquid scintillation counter. Duplicate aliquots of individual fractions were treated with TCA (final concentration, 20%), and the radioisotope in the TCA-precipitated protein was also determined by liquid scintillation counting. Relative amounts of ^{3}H-thymidine label and ^{35}S-methionine label in individual fractions were quantitated.

Radiolabeling and liquid-phase RIPA of proteins in various nuclear subfractions. Human T cells infected with HTLV-I were labeled with ^{35}S-methionine for 30 min, 1 h, and 2 h as previously described (47) and lysed as described above. Analysis for the relative content of p53 was performed by a radioimmunoprecipitation assay (RIPA) with anti-p53 monoclonal antibody (20). In similar experiments, cells were labeled for 15 min, 30 min, 1 h, and 2 h with radioactive methionine and chased with a 500-fold excess methionine for 1 h and 2 h after the labeling periods as previously described (47). Intact nuclei were isolated from whole cells and subfractionated as described above. Lysates of each fraction at each time point were assayed by RIPA with anti-p40^{x} polyclonal antisera (47). The amount of lysate used in each fraction was from an equivalent number of cells.

RESULTS

Intact HTLV-I-infected human T-cell nuclei were isolated with a mild nonionic detergent in an isotonic buffer. Nuclei obtained in this manner were compared ultrastructurally with nuclei from HTLV-I-infected whole cells. They showed little or no alteration in nuclear morphology and were found to be devoid of significant cytoplasmic or membrane remnants (Fig. 1A and B). Protein analysis of these nuclei by SDS-polyacrylamide gel electropheresis (PAGE) revealed a pattern consistent with the known major nuclear proteins, i.e., histones H1, H2A, H2B, H3, and H4; lamins A, B, and C; vimentin; and actin (Fig. 2).

Extraction of the nucleoplasm from isolated nuclei was accomplished by three successive washes in 0.5% NP-40 buffer (Fig. 1). This buffer extracts soluble proteins from nuclei by permeabilizing the nuclear membrane (6). The resulting nuclei demonstrated the preservation of overall nuclear and nucleolar architecture, with the depletion of some of the electron-dense nucleoplasm (Fig. 1C). Analysis of the nucleoplasm from three successive washes by SDS-PAGE revealed no detectable proteins related to structural components of chromatin or nuclear matrix (Fig. 2), indicating no significant contamination of the nucleoplasmic fraction with chromatin-associated histone proteins or nuclear matrix-associated structural proteins.

Extraction of the chromatin fraction was accomplished by digestion of the NP-40-treated nuclei with micrococcal nuclease and subsequent washing in high-salt buffer (6, 28) (Fig. 1). SDS-PAGE analysis of this fraction revealed all of the major histone proteins found in intact nuclei (Fig. 2). Morphologic examination of nuclei treated in this fashion revealed a residual structure with a network of fibers extending to and from a surrounding membrane (Fig. 1D). Embedded in this network is the nucleolus. SDS-PAGE analysis of this structure revealed proteins known to be structural components of the nuclear matrix, i.e., actin; vimentin; and lamins A, B, and C (Fig. 2). Thus, this structure (Fig. 1D) corresponds both morphologically and biochemically to what has previously been defined as the nuclear matrix (16, 44).

As an additional control, cells were labeled with ^{3}H-thymidine prior to isolation of nuclei and subsequent subnuclear fractionation. Fractions were then analyzed for relative content of incorporated ^{3}H-thymidine (Fig. 3). The bulk (>95%) of the incorporated nuclear ^{3}H-thymidine was associated with the chromatin fraction, with less than 2% in the nucleoplasm (NP-40 washes), and the remaining 2 to 5% was associated with the matrix fraction. This amount of matrix-associated DNA is consistent with that reported in previous studies (4).

Since all fractions were to be analyzed for relative amounts of p40^{x} by liquid-phase RIPA, a final control was performed with an antibody to a protein with a known subnuclear localization. Cellular protein p53 was chosen for two reasons. First, it has been shown to be associated with chromatin in non-simian virus 40-infected, chemically transformed mammalian cells (39). Second, it is expressed at relatively high levels in HTLV-I-infected human T cells (30). RIPA was performed with a monoclonal antibody to p53 (20) on the same nuclear subfractions subsequently used for p40^{x} localization. This assay revealed that the majority of p53 was in the chromatin fraction (Fig. 4, lanes f and g), consistent with data obtained in transformed murine cells. Smaller amounts of the protein were found in the nucleoplasm (Fig. 4, lanes a and b), and virtually no p53 was found associated with the nuclear matrix fraction (Fig. 4, lanes h to j).

RIPA analysis of the various nuclear subfractions with anti-p40^{x} antibody is shown in Fig. 5A. The experiment was done as a pulse-chase labeling study, and several features of the p40^{x} protein were revealed. First, the bulk of the intranuclear p40^{x} protein was found in the nucleoplasm and matrix fraction; very little was associated with the chromatin fraction (Fig. 5A). The relative amount in each fraction was quantitated by counting radioactivity associated with the p40^{x} band in the various fractions (Fig. 5B) (47). Approximately 53% of the intranuclear p40^{x} was found in the nucleoplasm, less than 1% was found in the chromatin, and 47% was found in the matrix. These relative amounts are particularly significant when viewed in the context of distribution of the total labeled proteins found in the nucleus after the 6-h incubation period (Fig. 3). Approximately 75% of the labeled intranuclear proteins were extracted with the nucleoplasm (Fig. 3, washes 1 and 2). An additional 22% were removed with the chromatin (Fig. 3, chromatin), leaving less than 3% in the matrix (Fig. 3, matrix), yet half of p40^{x} was found in this fraction (Fig. 5A and B). This result indicates that the association of p40^{x} with the nuclear matrix is unlikely to be spurious and is stable, withstanding the initial NP-40 treatment and subsequent micrococcal nuclease high-salt extraction procedures. Second, the synthesis of p40^{x} in HTLV-I-infected cells could be detected as early as 15 min after the initiation of labeling. Within the same time frame, the protein was found in the nucleus and was associated with the matrix fraction, making the time between cytoplasmic synthesis, migration into the nucleus, and matrix association very rapid. Third, the kinetics of p40^{x} turnover were the same in whole cells and all nuclear fractions examined. We have previously shown the protein to have a half-life of 120

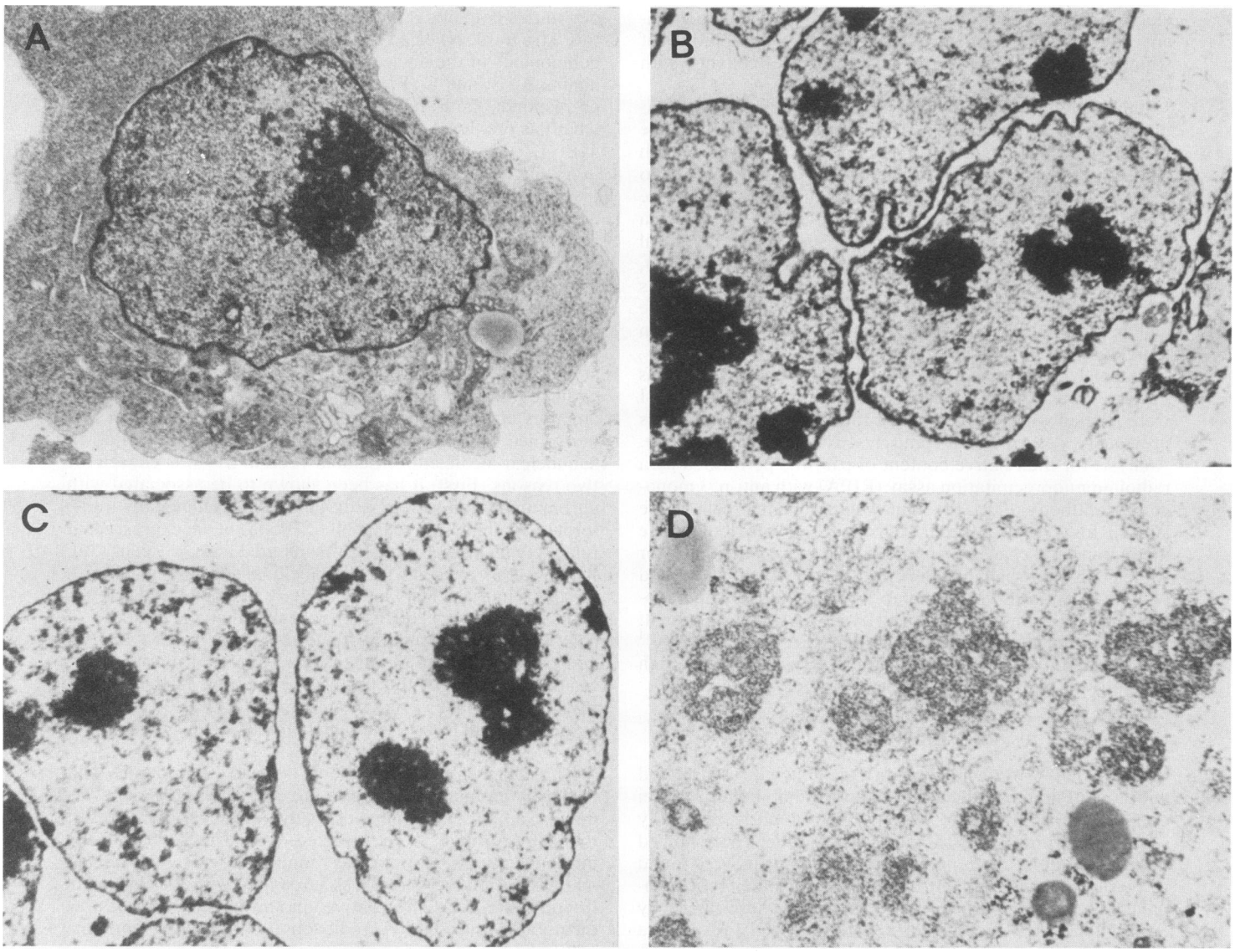

FIG. 1. Ultrastructural evaluation of nuclei and various nuclear subfractions. (A) HTLV-I-infected whole cells with intact nuclear morphology prior to any fractionation procedure. (B) Nuclei isolated with isotonic detergent buffer. Whole cells shown in panel A were lysed as described in Materials and Methods. (C) Nuclei isolated after treatment to remove the nucleoplasm. Nuclei shown in panel B were treated as described in Materials and Methods. (D) Nuclear matrix remaining after extraction of the chromatin. The nuclear fraction shown in panel C was treated to remove the chromatin as described in Materials and Methods. All cells and subcellular fractions shown were fixed and sectioned for electron microscopy as previously described (37). Magnification in all panels, ca. 8, 190.

min (47). This same half-life was seen in the nucleoplasm and matrix, indicating that the association of the protein with these fractions does not significantly alter its turnover.

DISCUSSION

The subnuclear localization of the $p40^{xI}$ protein in the nucleoplasm and the matrix may have implications regarding its function. In the nucleoplasm, the protein is in an environment where it may exert influence over a number of nuclear enzymes or proteins or both involved in the regulation of transcription. Alternatively, the nucleoplasm fraction may represent a pool of unbound $p40^{xI}$, and the matrix fraction may represent the actual level at which the protein exerts its influence. One current model holds that the matrix complex may be the nuclear counterpart to the cytoskeleton, which is known to be critical in the structural organization of cytoplasmic functions (23). Several studies have indicated that the nuclear matrix may play an important role in both DNA replication and RNA transcription (reviewed in references 23 and 35). Evidence indicating the role of the matrix in transcription initially came from studies showing that nascent RNA was associated with it. In some cells, DNA associated with the matrix is greatly enriched in sequences which are being transcribed in those cells (26). More direct evidence has come from recent studies demonstrating that all three components of the transcriptional complex, i.e., nascent RNA transcripts, active RNA polymerase II, and transcribed DNA sequences, are associated with the nuclear matrix (1, 25–27).

$p40^{xI}$ and $p37^{xII}$ (found in human T-cell leukemia virus type II) are *trans*-acting regulators of the transcription of

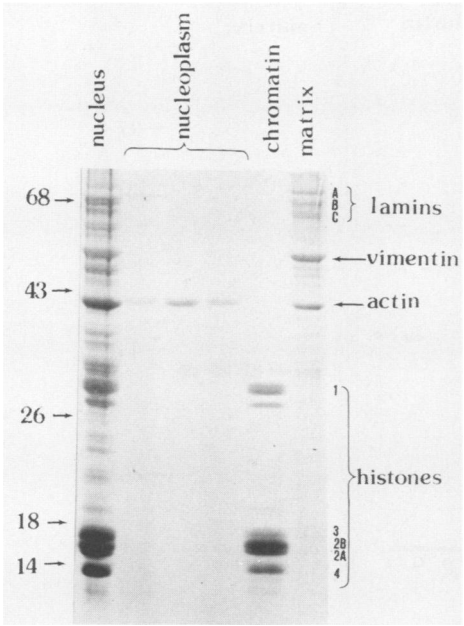

FIG. 2. Total proteins found in various subcellular fractions and nuclear subfractions. Specific fractions are labeled above the lanes. Molecular weight markers are on the left (in thousands).

some genes (9, 19, 51). If, as postulated, the matrix is important in RNA transcription, the association of $p40^{xI}$ with it places the protein in an appropriate subnuclear compartment to exert such an effect. Alternatively, if $p40^{xI}$ is a DNA-binding protein, critical DNA-protein interactions may occur at the level of matrix-associated rather than chromatin-associated DNA. A final possibility is that direct DNA binding is unimportant and that $p40^{xI}$ mediates its effects on transcription directly by modulation of a non-DNA component of the transcriptional complex or indirectly through a factor important in regulating the complex. The E1A protein of adenovirus is involved in the oncogenic transformation of mammalian cells infected with the virus (40). This protein bears several striking similarities to $p40^{xI}$. Like $p40^{xI}$, E1A is a transcriptional *trans* activator which is important in viral gene expression and replication (2, 5, 18), is localized in the nucleus (14), has a short half-life (52) and, within the nucleus, is associated with the nuclear matrix (14). Recent studies have shown that recombinantly produced E1A maintains its nuclear localization and its effect on transcription while lacking the ability to directly bind DNA (15, 31). This observation supports the theory that transcriptional *trans* activation by some proteins may occur at sites other than the DNA. The subnuclear localization of E1A again implicates the nuclear matrix as such a site.

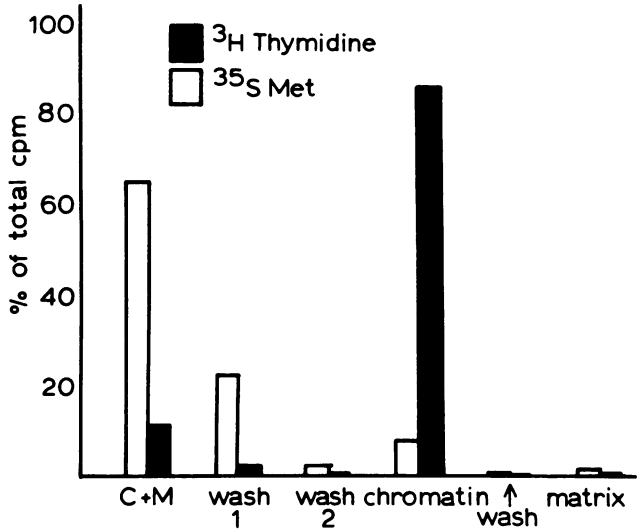

FIG. 3. Evaluation of ^{3}H-thymidine-labeled DNA content of various subcellular fractions and nuclear subfractions. Relative amounts of ^{3}H-thymidine label and ^{35}S-methionine (^{35}S Met) label in individual fractions are shown. Fractions labeled: C+M, cytoplasm and membrane from initial nuclear isolation; wash 1 and wash 2, nucleoplasm extracted by NP-40 washing (only two nucleoplasm washes are shown, as there was no measurable ^{3}H-thymidine or ^{35}S-methionine in nucleoplasm wash 3); chromatin, chromatin fraction; wash, second high-salt wash (see Materials and Methods); and matrix, remaining pellet.

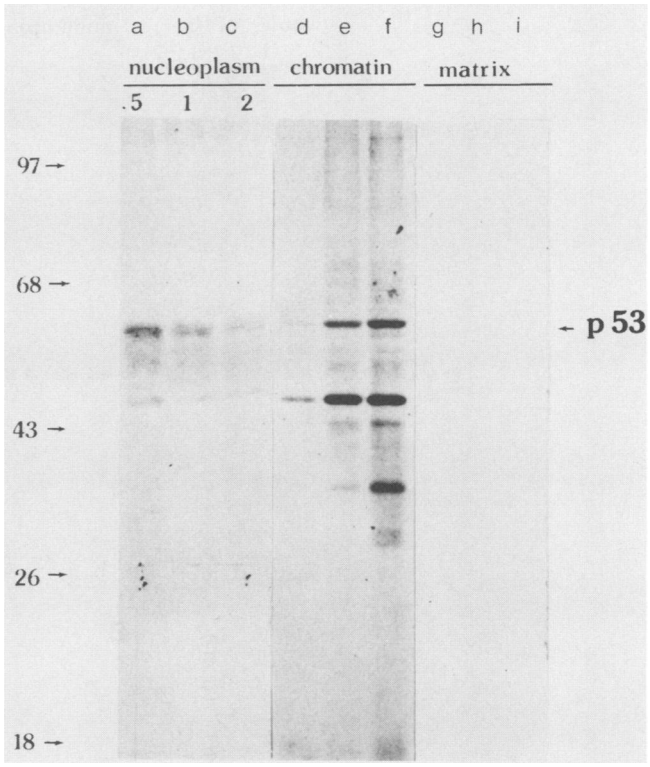

FIG. 4. Liquid-phase RIPA with anti-p53 monoclonal antibody and ^{35}S-methionine-labeled, HTLV-I-infected human T cells (SLB-I). RIPA was performed with various lysates, mouse monoclonal antibody as the primary antibody, rabbit monoclonal antibody as the secondary antibody, and staphylococcal protein A as described previously (48). The majority of the p53 was found in the chromatin fraction at 1 and 2 h of labeling, and smaller amounts were found in the nucleoplasm early during labeling. Note that no p53 was found in matrix fraction. The three lanes found under each subfraction represent metabolic labeling for 30 min (.5), 1 h (1), and 2 h (2). Numbers at left represent molecular weights in thousands.

Other gene products known to be associated with the nuclear matrix include the large T simian virus antigen of 40 (8), the viral and cellular *myb* gene products (6, 45), the viral and cellular *myc* gene products (12), and the N-*myc* cellular gene product (46). At least two of these proteins (large T and *myc*) are thought to exert influence over the transcription of other genes (29, 33). One recent report suggests that the

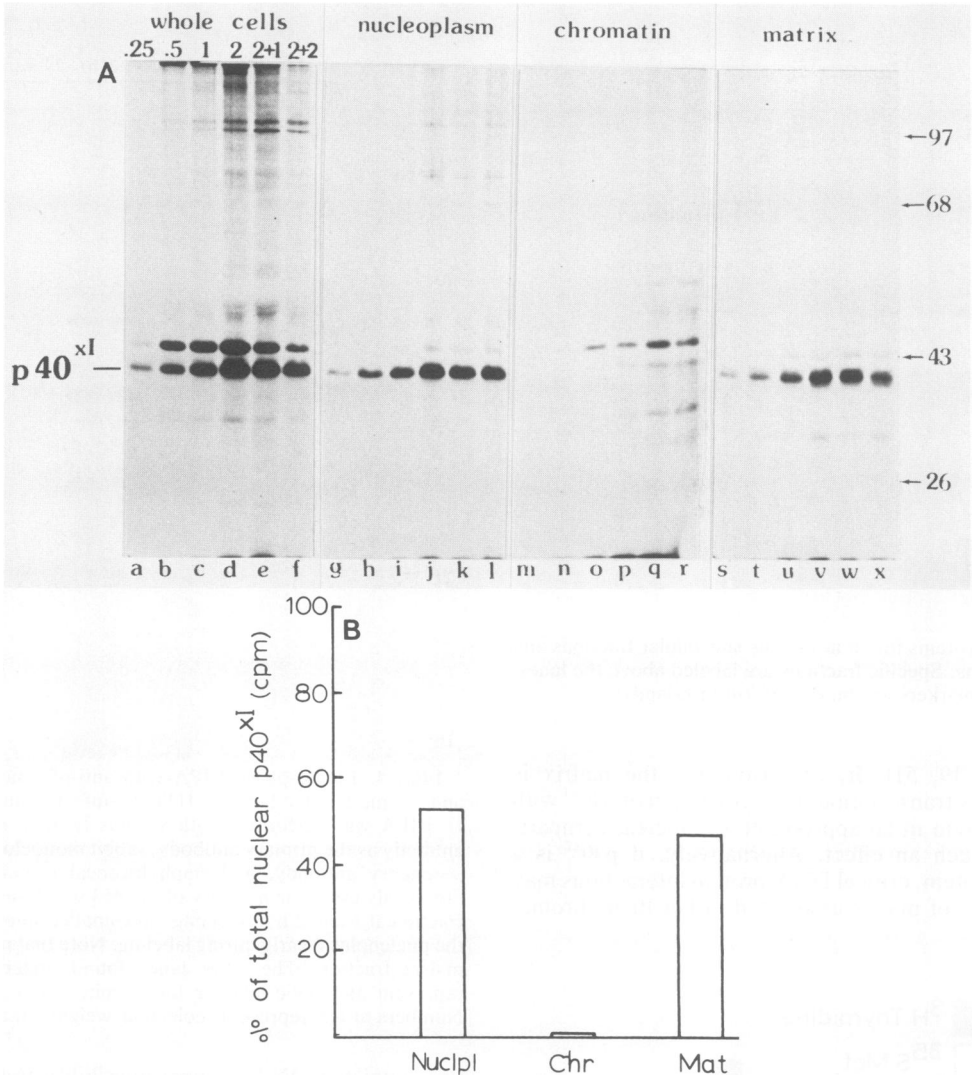

FIG. 5. (A) Liquid-phase RIPA with anti-p40^{xI} polyclonal antisera (47) and ^{35}S-methionine-labeled, HTLV-I-infected human T cells (SLB-I). Cells were labeled for 15 min (.25), 30 min (.5), 1 h, and 2 h with radioactive methionine and chased with excess cold methionine as described in the text (2+1 and 2+2 represent 2 h of methionine labeling followed by 1 and 2 h of methionine chasing, respectively). Lysates of each fraction at each time point were assayed by RIPA. The amount of lysate used in each fraction was from an equivalent number of cells. Lanes: a to f, whole-cell lysates; g to l, nucleoplasm extracted from nuclei at each time point; m to r, chromatin fraction at each time point; s to x, residual nuclear matrix fraction at each time point. Numbers at right represent molecular weights in thousands. (B) Relative amounts of ^{35}S-methionine-labeled p40^{xI} found in the various nuclear subfractions. Amounts were determined by quantitating the radioactivity associated with the p40^{xI} band at 2 h of labeling in the nuclear subfractions shown in panel A. Counts were quantitated as previously described (47). NuclpI, Nucleoplasm (sum of all three nucleoplasm washes); Chr, chromatin; Mat, matrix. The prominent band seen migrating at 43 kilodaltons above the p40^{xI} protein in panel A is actin, which has been seen by us (47, 48) and others (32) in immunoprecipitations of the p40^{xI} protein.

association of at least some of these gene products with the nuclear matrix is an artifact of the fractionation procedure (13). That study, however, used a hypotonic buffer solution for the initial preparation of isolated nuclei, and no data on the relative content of known proteins in the various subfractions were presented. Considerable evidence exists that hypotonic buffers disrupt not only normal nuclear structures (9, 23, 24, 38, 49) but also certain matrix-protein interactions (24, 49). These data underscore the importance of mild initial isolation procedures (6) and the use of careful controls documenting the morphologic and biochemical integrity of the various fractions obtained (44), as well as the demonstration that significant cross-contamination of one fraction with another does not occur.

Further understanding of the exact role of p40^{xI} in HTLV-I-mediated viral replication and cellular transformation could be greatly facilitated by the isolation of biologically active protein. This should allow for testing of direct DNA binding by the protein as well as protein association with various nuclear substructures.

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