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IDENTIFICATION AND CHARACTERIZATION OF GLUCOCORTICOID-REGULATED NUCLEASE(S) IN LYMPHOID CELLS UNDERGOING APOPTOSIS

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Summary—Apoptosis is a physiological process by which selected cells are deleted from a population in response to specific regulatory signals. A hallmark of apoptosis is the internucleosomal degradation of DNA prior to cell death. We are studying glucocorticoid-induced lymphocytolysis as a model system for apoptosis within the immune system. In rat thymocytes, the internucleosomal DNA cleavage which occurs following glucocorticoid treatment is both time- and dose-dependent, and is blocked by the glucocorticoid antagonist RU 486, indicating that this effect is mediated by the glucocorticoid receptor. Similar experiments using glucocorticoid-responsive (wt) and glucocorticoid-resistant (nt^-) S49.1 lymphoma cell lines confirm that internucleosomal DNA degradation and cell death are glucocorticoid receptor-mediated events and thus reflect the direct effects of glucocorticoids on lymphocytes. In an effort to identify the nuclease(s) responsible for the DNA degradation, we have developed two assays to detect nucleases whose activity is altered by glucocorticoid treatment. The first assay involves electrophoresing extracts of nuclear protein from control and glucocorticoid-treated lymphoid cells into SDS–polyacrylamide gels containing [^{32}P]DNA within the gel matrix. This assay is used to estimate the molecular mass of the nuclease, based on the observed *in situ* nuclease activity. The second assay uses HeLa nuclei as a substrate to detect internucleosomal cleavage activity present in nuclear extracts of control and glucocorticoid-treated lymphoid cells. Using these assays we have identified a novel Ca^{2+}, Mg^{2+}-dependent nuclease with an apparent molecular weight of 18 kDa in both S49 wt cells and rat thymocytes treated with glucocorticoids. Furthermore, nuclear extracts of glucocorticoid-treated, but not control, rat thymocytes and S49 wt cells were capable of cleaving HeLa chromatin at internucleosomal sites. In an effort to determine the identity of the nuclease capable of internucleosomal cleavage of DNA, nuclear extracts from dex-treated rat thymocytes were fractionated by gel filtration chromatography under non-denaturing conditions, and the fractions were analyzed using the [^{32}P]DNA SDS–PAGE and HeLa nuclei assays. When analyzed under native conditions, the 18 kDa nuclease described previously appears to exist as a $\simeq$25 kDa protein which may be part of a high molecular weight complex. Interestingly, only the $\simeq$25 kDa form of the protein was associated with internucleosomal DNA cleavage activity where as the high molecular weight form of the enzyme was devoid of this activity.

INTRODUCTION

Apoptosis is a programmed form of cell death, whereby specific cells are deleted from a population in response to discrete regulatory signals. This type of cell death occurs in immune responses, embryonic development and hormone-responsive tissues as well as in many other systems [1]. For example, castration induces apoptotic involution of both the ventral prostate [2] and uterine endometrium [3]. Alternatively, administration of glucocorticoids results in the apoptotic deletion of cortical thymocytes from the thymus (for reviews, see Refs [4, 5]). Although these observations have been exploited clinically for many years in the treatment of lymphoid neoplasms [6, 7], the mechanisms by which apoptosis occurs have yet to be elucidated.

Biochemically, apoptosis is characterized by a reduction in transmembrane transport of glucose [8], amino acids [9] and nucleosides [10], a decrease in protein [11] and nucleic acid synthesis [12] and a decrease in RNA polymerase

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activity [13] and ATP production [14]. Paradoxically, programmed cell death cannot proceed without ATP [15], or RNA and protein synthesis [16], suggesting that the induction of one or more specific proteins is an essential part of the apoptotic mechanism. Another characteristic of the apoptotic process is calcium-dependent degradation of DNA into oligonucleosome-sized fragments [multiples of 180–200 base pairs (bp)], which is denotative of internucleosomal DNA cleavage [17–19].

We are interested in determining what role internucleosomal DNA degradation plays in apoptosis. Glucocorticoid-induced thymocyte death in the rat is a model of apoptosis that we have employed since a large number of apoptotic cells can be isolated from a single animal after 5 h of glucocorticoid treatment. Previously we have found that the induction of DNA degradation is specific for the glucocorticoid class of steroids and is mediated through the glucocorticoid receptor in rats treated with the potent glucocorticoid agonist dexamethasone (dex) *in vivo* [19]. Interestingly, dex has the same effect on rat thymocytes treated *in vitro*, indicating that this is a direct effect of the glucocorticoid on thymocytes and not an effect mediated through an accessory cell type [20]. This finding has been corroborated by the observation that glucocorticoids can also induce apoptosis in certain lymphoid cell lines [5].

We have hypothesized that DNA degradation is responsible for cell death in apoptosis since this process precedes lymphocytolysis. In support of this hypothesis is the recent finding that an inhibitor of DNA degradation also inhibits cell death [21]; thus we favor the possibility that apoptosis is mediated by a novel nuclease. Using a nuclease assay that detects nuclease proteins based on molecular mass as well as nuclease activity, we have identified two groups of proteins in nuclear extracts from dex-treated rat thymocytes that contain nuclease activity [20, 22]. We report here the characterization of this nuclease activity present in rat thymocyte nuclei following glucocorticoid treatment. In addition, we have analyzed a similar type of glucocorticoid-mediated nuclease activity in mouse lymphoma cell lines: glucocorticoid-sensitive S49.1 G3.5 (wt) cells [23] and glucocorticoid-resistant S49.1 22R.2 (nt^-) cells [24, 25]. The nt^- cell line has a defective glucocorticoid receptor that does not transfer to the nucleus. These cell lines have allowed us to study glucocorticoid-induced DNA degradation in a stable system that permits easy manipulation of the extracellular environment.

We have used two different nuclease assays to study glucocorticoid-induced DNA degradation and nuclease activity in rat thymocytes and S49 cell lines. The first assay, a modification of the Rosenthal and Lacks [26] procedure employs an SDS–polyacrylamide gel that has [^{32}P]DNA incorporated within its matrix. This gel system permits the detection of nuclease activity along with providing an estimate of the molecular mass of the nuclease protein [20, 22, 27]. The second nuclease assay uses HeLa nuclei as a substrate to detect nucleases capable of internucleosomal DNA degradation [22]. Finally, we have examined fractionated nuclear extracts from dex-treated rat thymocytes to identify the nuclease responsible for the internucleosomal cleavage of DNA.

EXPERIMENTAL

Materials

Dexamethasone (dex) was obtained from Steraloids Inc. (Wilton, NH) and RU 486 was kindly provided by Dr Martini, Roussel UCLAF (Romainville, France). [α-^{32}P]dCTP used for nick translations was obtained from ICN Radiochemicals (Irvine, CA) and nick translation DNA reactions were performed using the Bethesda Research Labs nick translation reagent kit (Gaithersburg, MD). Micrococcal nuclease was obtained from Worthington (Freehold, NJ) and all other chemicals were of reagent grade purchased from various commercial sources.

Cell culture

S49.1 G3.5 (wt) cells and S49.1 22R.2 (nt^-) cells were obtained from Ulrich Gehring (University of Heidelberg, Germany) and grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, 75 U/ml streptomycin and Fungizone (250 ng/ml). Stock cultures were passaged every 2–3 days. For experiments, cells were plated at 5.0×10^5 cells/ml in 75 cm^2 plastic flasks (Costar, Cambridge, MA): dex was prepared as a 0.1 mM stock solution in 100% ethanol and stored at −20°C; RU 486 was prepared as a 1.0 mM stock solution in 100% ethanol and stored at −20°C. Ethanol (100%) was used as a vehicle control where appropriate. The final

concentration of ethanol in the media did not exceed 0.1%.

DNA preparation

Prior to DNA isolation, cell viability was determined by trypan blue exclusion and the cells were centrifuged over lymphocyte separation medium (LSM) (Organon Teknika, Durham, NC) at 25°C for 10 min 3000 *g* to remove dead cells. The DNA was isolated by solubilizing the cells in lysis buffer (0.2 M Tris, pH 8.5, 0.1 M Na_2EDTA, 1% SDS) and treated with Proteinase K (0.4 mg/ml) for 1 h at 55°C. The samples were extracted twice with phenol–chloroform (1:1 vol/vol; plus 2% isoamyl alcohol) and precipitated with 2.5 vol 95% ethanol at −70°C. After resuspending the DNA pellet in 10 mM Tris, pH 7.5, 1 mM Na_2EDTA (TE) the samples were treated with RNAse A (0.1 mg/ml) for 4 h at 37°C, followed by Proteinase K (0.4 mg/ml) for 1 h at 55°C and finally phenol–chloroform extracted and ethanol precipitated as before. The DNA was resuspended in TE, the concentration was determined by absorbance at 260 nm and 10 or 15 μg/lane of DNA were electrophoresed on 1.8% agarose gels. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed. Densitometric scans of the photographic negatives were obtained using a GS 300 scanning densitometer (Hoeffer Scientific Instruments).

S49 nuclear extract preparation

S49 cells were treated as described and harvested by centrifugation (4000 *g*, 5 min, 4°C). Nuclei were prepared by hypotonic shock in 1.5 mM $MgCl_2$, 0.25% NP-40 and nuclear proteins were extracted with 0.6 M NaCl, 1 mM Na_2EDTA at 4°C for 1 h. The chromatin was pelleted by centrifugation at 130,000 *g* for 45 min and the supernatant was dialyzed against 800 vol 20 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1 mM Na_2EDTA for 30 min, frozen and lyophilized. The lyophilized samples were resuspended in dH_2O and the protein concentrations were determined by the Bradford [28] method using bovine serum albumin as a standard.

Thymocyte nuclear extract preparation

Thymocytes were prepared essentially as previously described [19]. Male Sprague–Dawley rats [85–100 g body weight (BW)] were bilaterally adrenalectomized 5–14 days before use and were maintained on 0.85% NaCl and rat chow *ad libitum*. Rats were administered dex by i.p. injection of 0.5 ml of the steroid suspended by sonication in phosphate-buffered saline (PBS, 0.01 M sodium phosphate, 0.15 M NaCl) (2 mg/ml). Control animals received PBS. Animals were sacrificed by rapid decapitation and the thymus glands were removed and placed in cold PBS. Thymus tissue was minced with scissors and aliquots were homogenized in a loose fitting Kontes No. 22 glass/glass homogenizer (Kontes Company, Vineland, NJ). The cell suspension was filtered through 202 μm Nitex mesh (Tetko, Elmsford, NY) and centrifuged at 4000 *g* for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in PBS, refiltered and centrifuged again as described above. Nuclei were prepared by a hypotonic shock technique in which the cells were resuspended in cold 10 mM $MgCl_2$, 0.25% Nonidet P-40. The nuclei were pelleted by centrifugation as described above. Nuclear extract was prepared by mixing the nuclei (10^8–10^9 lymphocytes per thymus) with 0.5–1.5 ml cold extraction buffer [0.6 M NaCl, 1 mM Na_2EDTA, 20 mM Tris (pH 7.4)] per thymus and rotating the mixture for 1 h at 4°C. This extract was then centrifuged at 165,000 *g* for 1 h at 4°C in a Beckman 50Ti rotor to pellet the chromatin material. The resulting supernatant was measured for protein concentration by the method of Bradford [28] and then stored at −20°C (freeze storage had no effect on nuclease activity).

HeLa nuclei assay

HeLa S_3 cells were grown in suspension culture at 37°C in Joklik's minimum essential medium (JMEM, Flow Laboratories, McLean, VA) supplemented with 1.5% fetal calf serum, 2 mM glutamine, 75 U/ml penicillin G and 50 U/ml streptomycin sulfate. Cells were harvested by centrifugation (4000 *g*, 5 min, 4°C), the pellet was resuspended in cold PBS, and the cell concentration was determined using a Coulter counter (Coulter Electronics, Hialeah, FL). The cells were repelleted as described above and resuspended in cold 10 mM $MgCl_2$, 0.25% Nonidet P-40 to isolate nuclei. The nuclei were pelleted by centrifugation as described above and then resuspended in digestion buffer [50 mM Tris (pH 7.4), 2 mM $MgCl_2$, 2 mM $CaCl_2$] at a concentration of 10^7 nuclei/ml.

To assay for nuclease activity, 50 μg of thymocyte nuclear extract protein was mixed with 100 μl aliquots (10^6 nuclei) of HeLa nulcei and incubated for 90 min at room temperature with

gentle rotation. HeLa nuclear extracts were prepared as described above for thymocytes and were used in control experiments. Reactions were stopped by adding 55 μl of 5 M NaCl, 25 μl of 0.5 M Na_2 EDTA, 25 μl of 10% SDS and DNA was extracted as described above.

[^{32}P]DNA SDS–PAGE nuclease activity assay

Nuclear extracts were normalized for protein concentration and nuclease activity was assayed by a modification of the Rosenthal and Lacks [26] method. SDS–polyacrylamide gels (15 or 17.5%) were prepared containing 5 μg/ml calf thymus DNA plus 750,000 cpm of a ^{32}P-labeled DNA plasmid (human glucocorticoid receptor DNA was used in the experiments presented here) labeled by nick translation. After the proteins were separated by electrophoresis using the Laemmli [29] gel system, the gels were soaked in 40 mM Tris, pH 7.4, 2 mM $MgCl_2$, 1 mM Na_2EDTA overnight to remove the SDS. Nucleases were activated by adding 2 mM $CaCl_2$ to the above solution and soaking for 2–18 h at room temperature or 3 h at 37°C. Gels containing [^{32}P]DNA were dried and subjected to autoradiography for 6 h at −70°C. Nuclease activity on autoradiographs appeared as a clear area against a black background. Micrococcal nuclease (MN, 0.5 μg), a 17 kDa calcium-dependent nuclease, was used as a positive control.

Gel filtration chromatography

To analyze size fractionated nuclear proteins using the [^{32}P]DNA SDS–PAGE assay, thymocyte nuclear proteins were prepared as previously described with the following extraction buffer: 0.4 M NaCl, 20 mM Tris–HCl, pH 7.8, 1 mM Na_2EDTA. The nuclear proteins (7.5 mg) were separated on a BioGel P-100 column (1.5 × 75 cm) in 0.4 M NaCl, 20 mM Tris–HCl, pH 7.8, 1 mM EDTA at a flow rate of approx. 10 ml/h; 3-ml fractions were collected and 20 μl aliquots were analyzed for nuclease activity using the [^{32}P]DNA SDS–PAGE assay described above.

To analyze size fractionated nuclear proteins by the HeLa nuclei assay, thymocyte nuclear extracts were prepared as previously described and 4.4 mg protein was chromatographed on Sephacryl S-200 (1.5 × 75 cm) in 50 mM NaCl, 20 mM Tris, pH 7.4, 1 mM Na_2EDTA at a flow rate of 12 ml/h: 1-ml fractions were collected and 100 μl aliquots were analyzed using the HeLa nuclei assay.

RESULTS

To determine what role DNA degradation might play in apoptosis, we have focused our efforts on studying nuclease activity that is present subsequent to glucocorticoid treatment of rat thymocytes. Since we and others shown that glucocorticoid treatment of rat thymocytes results in internucleosomal DNA degradation [17–19, 22, 30, 31] we were interested to know if glucocorticoid treatment of S49 cells would produce a similar result. S49 wt and nt$^-$ cells were treated with dex or vehicle, and the DNA was subsequently isolated and analyzed by agarose gel electrophoresis. The densitometric scans of the ethidium bromide-stained agarose gels show that dex treatment results in the formation of oligonucleosome-sized (multiples of 180–200 bp) DNA fragments in S49 wt but not nt$^-$ cells (Fig. 1). Treatment of S49 wt cells

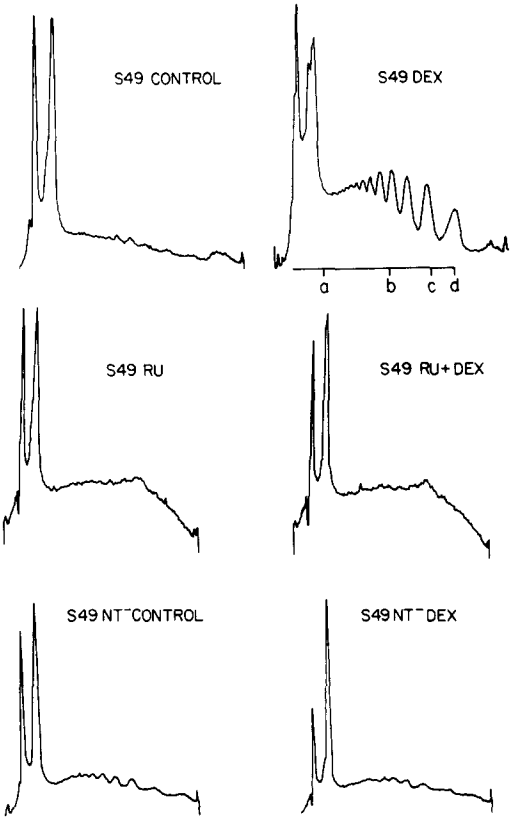

Fig. 1. The effect of dex on DNA degradation in wt and nt$^-$ cells. S49 wt cells and S49 nt$^-$ cells were treated with control vehicle or dex (10^{-7} M) for 18 h. wt Cells were also treated with RU 486 (RU, 10^{-6} M) or RU$^+$ dex (10^{-6} and 10^{-7} M, respectively) for 18 h. The DNA was isolated and electrophoresed on 1.8% agarose gels (10 μg/lane). The gels were stained with ethidium bromide (0.5 μg/ml), photographed and the negatives were scanned with a scanning densitometer. Molecular weight marker positons are indicated below the scan of DNA from dex-treated wt cells: (a) 23,000 bp; (b) 871 bp; (c) 310 bp; (d) 194 bp.

with RU 486, a glucocorticoid antagonist [32], did not result in DNA degradation and completely inhibited dex-induced internucleosomal DNA degradation. These results indicate that glucocorticoid-induced internucleosomal DNA degradation in S49 cells is glucocorticoid receptor-mediated and are in agreement with previous reports by Distelhorst [33] and others [34]. These data also show that glucocorticoid-induced DNA degradation in S49 wt cells is similar to that observed in rat thymocytes with respect to the pattern of internucleosomal DNA degradation and glucocorticoid receptor specificity. Furthermore, these studies indicate that S49 wt cells and rat thymocytes respond to glucocorticoids in an analogous fashion. Finally, it is important to note from the S49 wt cell studies that glucocorticoids are acting directly on the lymphoid cells rather than through a secondary indirect mechanism.

We next wanted to establish if a specific nuclease or nucleases could be identified in nuclear extracts of dex-treated lymphoid cells; thus we have developed two assay systems to analyze nuclear extracts for nuclease activity (Fig. 2). To determine the approximate molecular mass of nucleases that are present and active in response to glucocorticoid treatment, we have used the [^{32}P]DNA SDS–PAGE nuclease assay [27] illustrated in Fig. 2. This assay is a modification of the Rosenthal and Lacks [26] procedure which we have used previously [20, 22]. For this assay, calf thymus DNA and [^{32}P]DNA are incorporated within the matrix of an SDS–polyacrylamide gel. After electrophoresis of nuclear extracts through the gel, the SDS is removed and nucleases are activated by adding Ca^{2+}. Upon autoradiography, the autoradiograph appears black due to the presence of the [^{32}P]DNA, whereas clear zones are indicative of nuclease activity that has degraded the [^{32}P]DNA in the gel matrix. The position of this clear zone in relation to molecular weight marker proteins provides an estimate of the molecular mass of the nuclease.

The original Rosenthal and Lacks [26] assay relied on the ability of ethidium bromide to bind to the DNA within the gel matrix to detect the presence or absence of DNA, and thus a loss of ethidium bromide staining was indicative of nuclease activity. Recent reports have suggested that histones and calcium-binding proteins

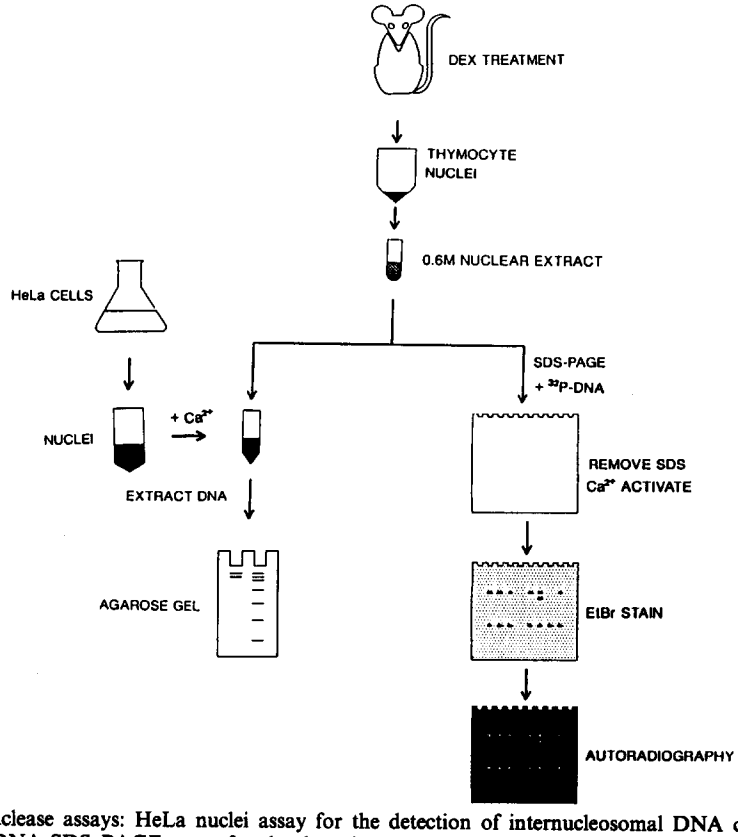

Fig. 2. Nuclease assays: HeLa nuclei assay for the detection of internucleosomal DNA cleavage and [^{32}P]DNA SDS–PAGE assay for the detection and size approximation of nuclease proteins.

could produce an apparent loss of DNA due to an inhibition of the DNA–ethidium interaction [35, 36] and hence, produce apparent false positive signals. The use of radiolabeled DNA within the gel matrix obviates this problem by relying on the presence or absence of the [^{32}P]DNA to provide the signal, not on the ability of the ethidium bromide to interact with the DNA.

Although the [^{32}P]DNA SDS–PAGE assay is a very useful technique for the study of nucleases, it was necessary to develop another assay that could identify internucleosomal cleavage activity in order to ensure that nuclease activity present in the [^{32}P]DNA SDS–PAGE system corresponded to the glucocorticoid-regulated DNA degradation that occurs during apoptosis. We have developed the HeLa nuclei assay illustrated in Fig. 2 for this purpose [22]. This assay takes advantage of the fact that HeLa cells do not undergo apoptosis in response to glucocorticoid treatment (data not shown) [37]. Nuclei from HeLa cells are isolated and nuclear extracts from rat thymocytes or S49 cells are added to the nuclei suspension along with calcium and magnesium. After incubation, the DNA is extracted from the HeLa nuclei and analyzed by agarose gel electrophoresis. If the nuclear extract contains a nuclease possessing internucleosomal cleavage activity, the HeLa DNA will be degraded into DNA fragments that are multiples of 180–200 bp.

The [^{32}P]DNA SDS–PAGE assay was used to determine the approximate size of glucocorticoid-dependent nucleases in nuclear extracts from S49 cells (Fig. 3). wt And nt$^-$ S49 cells were treated with or without dex and nuclear extracts were prepared and assayed as described above. MN, a calcium-dependent, 17 kDa protein possessing internucleosomal DNA cleavage activity, was included in the assay as a positive control and produced a clear zone in the autoradiograph of the [^{32}P]DNA-labeled polyacrylamide gel. A similar clear zone was apparent in the nuclear extract from dex-treated wt cells and had an approximate molecular mass of 18 kDa. There was no apparent nuclease activity in the nuclear extract from control, RU 486 or RU 486 plus dex-treated cells. Similarly, dex treatment did not alter nuclease activity in S49 nt$^-$ cells, although some nucleases were present in both control and dex-treated cells. These results indicate that there is a glucocorticoid-dependent nuclease present in S49 cells similar in size to one that we have previously detected in

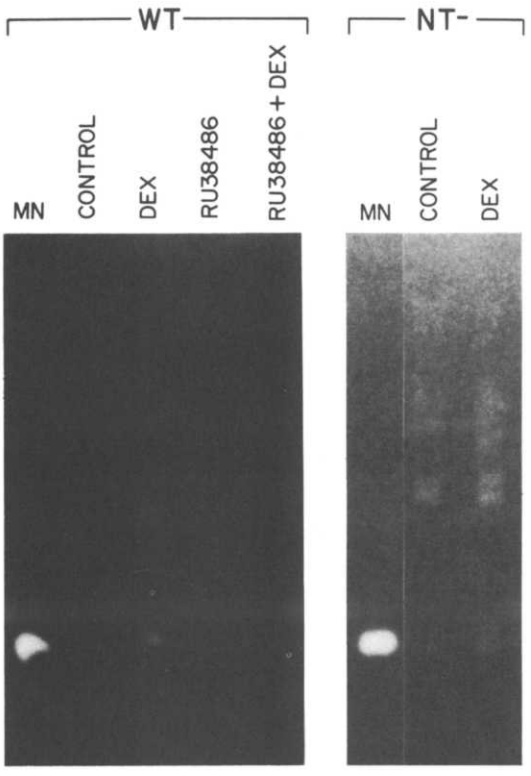

Fig. 3. The effect on dex on nuclease activity in nuclear extracts of S49 wt and nt$^-$ cells. wt Cells were treated with control vehicle, dex (10^{-7} M), RU 486 (RU 38486, 10^{-6} M) or RU +dex (10^{-6} and 10^{-7} M, respectively) for 18 h and harvested. nt$^-$ cells were treated with control vehicle or dex (10^{-7} M) for 18 h. Nuclear extracts (2 μg protein/lane) were analyzed after electrophoresis through 17.5% polyacryalmide gels. MN was used as a positive control (0.5 μg). Autoradiograph of [^{32}P] DNA-containing gels, exposed to Ca^{2+} for 2 h.

nuclear extracts from dex-treated rat thymocytes [20, 22].

We next examined nuclear extracts from rat thymocytes treated with or without dex *in vivo* and S49 wt cells treated with or without dex *in vitro* in the HeLa nuclei assay to determine if these extracts contained nucleases that are able to degrade DNA at internucleosomal sites (Fig. 4). In both cell types, nuclear extracts from glucocorticoid-treated cells activated internucleosomal degradation of HeLa DNA while extracts from control cells did not. Hence, nuclear extracts of both rat thymocytes and S49 wt cells possess an endonuclease whose internucleosomal cleavage activity is glucocorticoid-dependent.

In order to further characterize the nuclease in nuclear extracts of dex-treated lymphoid cells it was necessary to separate nucleases whose activity was altered by glucocorticoid treatment from other nucleases in the cell. The nuclear extracts of dex-treated rat thymocytes were

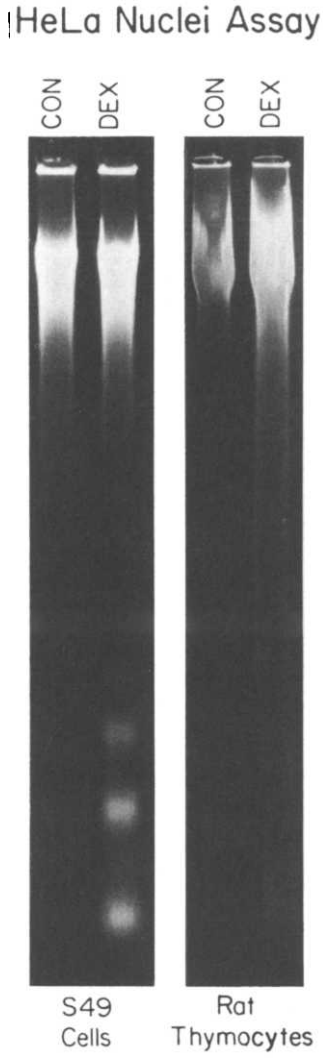

Fig. 4. Detection of internucleosomal DNA cleavage activity in extracts from glucocorticoid-treated rat thymocytes and S49 wt cells. Adrenalectomized rats were treated with vehicle or dex (5 mg/kg BW) and sacrificed 5 h later. S49 wt cells were treated with dex (10^{-7} M) or vehicle for 18 h. Nuclear extracts were prepared and 50 μg protein tested in the HeLa nuclei assay. HeLa DNA was isolated and electrophoresed on 1.8% agarose gels (15 μg/lane). The gels were stained with ethidium bromide and photographed.

fractionated by gel filtration chromatography and the fractions were analyzed using the [^{32}P]DNA SDS–PAGE assay (Fig. 5). The 18 kDa nuclease previously observed by SDS–PAGE eluted in two peaks. The first peak corresponded to the void volume (>100 kDa) (fractions 20–21) and the second peak migrated at a position equivalent to a molecular weight of approx. 25 kDa (fractions 34–36). Thus, the nuclease appears to be present both as a discrete $\simeq$25 kDa protein (18 kDa by SDS–PAGE) and as part of a much larger complex. Gel filtration chromatography determines the mass of a protein in the native state and is dependent upon the Stokes radius of the protein while SDS–PAGE involves denatured proteins, hence it is reasonable to expect that the different sizes reported here are descriptive of the same protein. The native protein in its globular form may have a molecular mass of $\simeq$25 kDa, whereas the denatured form of the same protein may have a molecular mass of 18 kDa.

Since both dex-treated S49 wt cells and rat thymocytes possess an internucleosomal endonuclease as determined by the HeLa nuclei assay and both cell types possess an 18 kDa nuclease when treated with dex, we hypothesized that the 18 kDa protein is the endonuclease that has internucleosomal cleavage activity. To determine the identity of the internucleosomally active nuclease, nuclear extracts from dex-treated rat thymocytes were fractionated by gel filtration chromatography and the fractions were assayed with the HeLa nuclei assay (Fig. 6). One peak of internucleosomal nuclease activity was evident, migrating at a position equivalent to $\simeq$25 kDa (fractions 43–55), a size similar to the position of the 18 kDa nuclease as determined by gel filtration in Fig. 5. However, no internucleosomal nuclease activity was present in the high molecular weight regions although the [^{32}P]DNA SDS–PAGE assay indicated that nucleases are present. These data suggest that the 18 kDa nuclease may be the internucleosomally active protein.

DISCUSSION

In this report we have used two lymphoid cell systems, rat thymocytes treated with glucocorticoids *in vivo* and a glucocorticoid-sensitive S49 lymphoma cell line, to show that glucocorticoids mediate a nuclease capable of internucleosomal cleavage of DNA as detected by the HeLa nuclei assay. In addition, we have examined the nucleases present in these cell types subsequent to glucocorticoid treatment with the [^{32}P]DNA SDS–PAGE assay and determined that an 18 kDa nuclease is present in both S49 wt cells and rat thymocytes. Finally, we have found that the 18 kDa nuclease from rat thymocytes has an apparent molecular weight of $\simeq$25 kDa by gel filtration chromatography and that a protein of similar mass to this nuclease has inherent internucleosomal cleavage activity.

The [^{32}P]DNA SDS–PAGE assay described here provides a very useful technique to analyze the relative activity of a nuclease along with the relative molecular mass of the nuclease.

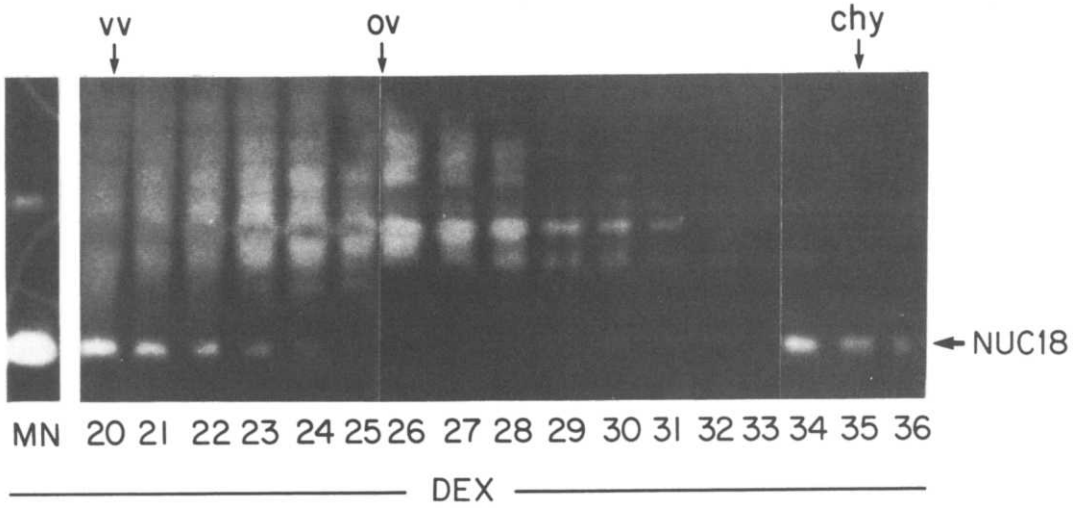

Fig. 5. Gel filtration analysis of nuclear extracts from dex-treated rats assayed with the [^{32}P]DNA SDS–PAGE assay. Nuclear extracts were prepared from thymocytes of rats treated with dex (see Experimental) and fractionated by gel filtration. Aliquots (20 μl) from each fraction were analyzed for nuclease activity with the [^{32}P]DNA SDS–PAGE (15% polyacrylamide) nuclease assay. Lane 1 contains 0.3 μg MN. Lanes 2–36 contain aliquots of the column fractions as labeled. Gel filtration markers positioned above the corresponding fractions: blue dextran (void volume, VV), ovalbumin (43 kDa, ov) and chymotrysinogen (14.4 kDa, chy).

Furthermore, the ionic requirements and activity of various nuclease inhibitors can be easily analyzed [27]. Recent reports have suggested that histones and calcium-binding proteins can produce artifactual nuclease activity in the Rosenthal and Lacks assay [35, 36], the assay from which the [^{32}P]DNA SDS–PAGE assay was derived. However, when histones are removed from nuclear extracts of dex-treated rat thymocytes by cation-exchange chromotography, the nuclease activity remains in the nuclear extract (M. L. Gaido and J. A. Cidlowski, unpublished observation). Hence, the 18 kDa nuclease detected in nuclear extracts of glucocorticoid-treated lymphoid cells is not a histone.

The HeLa nuclei assay described here is also a very powerful tool in the study of nucleases since it is possible to distinguish internucleosomal cleavage activity from other nuclease activities. Moreover, this assay can be used to determine the specific ion, pH or cofactor requirements of an endonuclease (R. A. Schwartzman and J. A. Cidlowski, submitted for publication). Since this assay provides a more physiologically relevant substrate (HeLa chromatin) than assays that use plasmid of λDNA as a substrate [38–41] it has the advantage of being able to predict what effect a nuclease may have on chromatin within the cell.

An interesting observation that we report here is that the 18 kDa nuclease is also present as a high molecular weight complex in nuclear extracts from dex-treated rat thymocytes. This larger complex may represent nuclease bound to oligonucleosomes which dissociates from the substrate DNA through SDS–PAGE or an inactive form of the nuclease which is part of a high molecular weight complex. Perhaps, upon dex treatment, the 18 kDa nuclease is released and becomes active. Alternatively, the large complex may include a protein possessing target specificity for internucleosomal regions of DNA. Once the protein complex is at the target site, the nuclease cleaves the DNA and is released along with the DNA fragments. The actual form of the nuclease within the cell and the significance of the high molecular weight complex remains to be determined.

Since protein and RNA synthesis are necessary for glucocorticoid-induced apoptosis to occur, we have previously proposed that the nuclease responsible for internucleosomal DNA degradation was a product of glucocorticoid-dependent *de novo* protein synthesis [22]. In this report we have shown that a candidate nuclease which maybe responsible for the internucleosomal cleavage appears to be part of a high molecular weight complex in addition to existing as a discrete low molecular weight nuclease. This suggests that the internucleosomally active nuclease need not be synthesized *de novo* but may be activated, either enzymatically or through some other mechanism. If this is the case, the synthesis of an enzyme, enzymatic

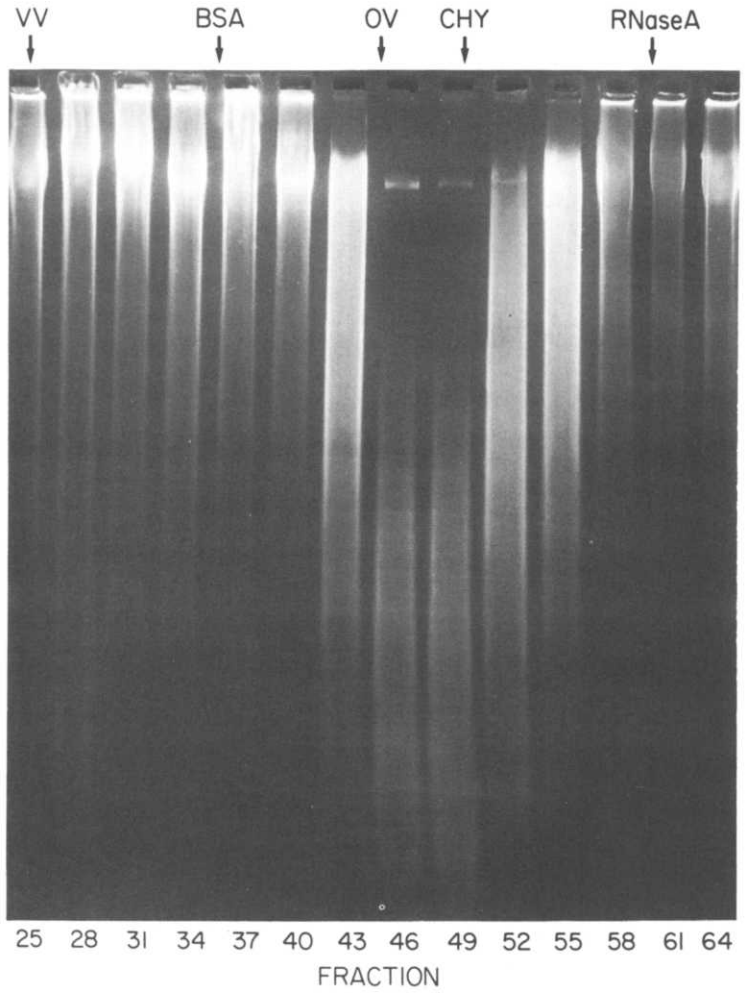

Fig. 6. Gel filtration analysis of nuclear extracts from dex-treated rats assayed with the HeLa nuclei assay. Lanes 1–14 contain HeLa DNA from the HeLa nuclei assays of gel filtration fractions as labeled. Gel filtration markers positioned above the corresponding fractions: blue dextran (void volume, VV), bovine serum albumin (67 kDa, BSA), ovalbumin (43 kDa, ov), chymotrysinogen (14.4 kDa, chy), RNAse A (13.7 kDa).

cofactor or a compound that relieves the inhibition of an enzyme may be the protein whose synthesis is necessary for apoptosis. This certainly does not discount the possibility, however, that some *de novo* synthesis of the nuclease may occur.

Apoptosis has been implicated in such diverse systems as metamorphosis in amphibians [1], regression of limb buds [42], cyclical endocrine-regulated maintenance of reproductive tissues [3, 43] and immunological responses [45, 45]. It is possible to envision a central mechanism that can be regulated in a variety of ways by many different stimuli. The activation of the nuclease responsible for internucleosomal cleavage of DNA may represent that step in apoptosis where the various signals converge into a common pathway of programmed suicide. Purification of the internucleosomally active nuclease will aid in deciphering what mechanisms are involved in its activation and what role this nuclease plays in the apoptotic process.

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