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STEROID RECEPTOR BINDING TO NUCLEI: EFFECT OF ASSAY CONDITIONS ON THE INTEGRITY OF CHROMATIN

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SUMMARY

Various incubation conditions previously used in cell-free assays of steroid receptor binding to nuclei were evaluated on the basis of their effect on the integrity of the nuclear chromatin. Incubation of nuclei for 60 min in whole cytosol containing no added salt at 25°C resulted in significant DNA degradation and some proteolysis. This deterioration in the integrity of the chromatin increased its capacity to bind the [^{3}H]-progesterone–receptor complex, possibly via exposure of previously "masked" acceptor sites. Decreasing the temperature and/or increasing the salt concentration reduced or eliminated these changes in the chromatin. Incubation of nuclei in an ammonium sulfate-fraction of the cytosol, which contained the partially purified progesterone receptor, resulted in no detectable deterioration of the chromatin integrity irrespective of the temperature ($\leq$25°C) or the added salt concentration (0–0.15 M KCl). These results suggest that some of the conflicting reports on the nature of the nuclear acceptor sites for steroid receptors could be due to the use of inappropriate assay conditions, which permit degradation of the nucleo-protein and result in altered levels of steroid receptor binding. (Steroid/Progesterone/Nuclei/Chromatin.)

INTRODUCTION

Many studies on the mechanism of steroid hormone action have involved investigation of the *in vitro* interaction of steroid–receptor complexes with isolated nuclei [1–11]. Unfortunately, conflicting results of such studies have led to some controversy concerning the chemical identity and tissue specificity of the nuclear acceptor sites for steroid–receptor complexes [see discussions in references 1, 2, 3]. Such discrepancies could result from the use of inadequate methods to quantitate these interactions. Indeed, we have shown recently that the ionic strength used in the assay can have a significant effect on the amount and specificity of binding of the progesterone–receptor complex to isolated nuclear material from the chicken oviduct [1]. This apparently is due to the presence of different classes of nuclear binding sites that have graded affinities for the progesterone–receptor complex [1, 4, 5]. Since the interactions are electrostatic in nature, increasing the ionic strength eliminates receptor interaction with the different classes of binding sites in order of increasing affinity.

Changes in the properties of either or both of the interacting components (steroid receptor and nuclear material) that might occur during the *in vitro* assay also could affect the measured amount of binding. Changes in the properties of steroid–receptor complexes during such incubations have been demonstrated [2, 3, 6, 7]. To our knowledge, however, the effect of such assays on the nuclear material has never been reported. In this report, we show that some assay conditions have no apparent effect on the nuclear chromatin, whereas other conditions result in significant deterioration of the chromatin integrity, which in turn alters the acceptors that bind the progesterone–receptor complex.

EXPERIMENTAL

Animals and tissues. Oviducts from adult laying hens were obtained from a local produce company [4, 5]. Nuclei were isolated from these oviducts by sedimentation through 1.8 M sucrose [12] and stored at −80°C. Oviducts used for the preparation of cytosol were obtained from immature White Leghorn chicks that had been treated with diethylstilbestrol for at least three weeks [4, 5].

Cytosol preparation and fractionation. Cytosol was prepared essentially as described previously [4]. Excised chick oviducts were minced in a Waring blender for 10 sec at full speed in 3 vol. (w/v) of cold 10 mM Tris-HCl, 1 mM EDTA, 12 mM thioglycerol, pH 7.5 (TESH buffer). The minced tissue was homogenized in a Teflon–glass homogenizer with 5 strokes of the motor-driven (Sears "Craftsman" drill) pestle operated at 70 V. The homogenate was centrifuged 10 min at 20,000 *g* and the supernatant liquid then re-centrifuged 60 min at 100,000 *g*. The 100,000 *g* supernatant represented the cytosol. The protein concentration of the cytosol was determined by U.V.

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absorbance at 280 nm and adjusted to 20 mg/ml. Cytosol progesterone receptor was labeled with [^{3}H]-progesterone (final concentration, 21 nM progesterone at 1 μCi/ml) for at least 60 min at 2°C as described previously [4]. The progesterone-labeled cytosol was used either directly in the incubation experiments with isolated nuclei or for the preparation of an ammonium sulfate-fraction (AS-fraction) that contained the progesterone–receptor complex.

When cytosol was used directly in the nuclear incubation experiments, it was pre-incubated for 60 min at 25°C to "activate" the progesterone receptor [2, 8]. The cytosol was then cooled to 2°C and clarified by centrifugation for 10 min at 15,000 *g*. This supernatant represented the unfractionated, whole cytosol and was incubated with purified hen oviduct nuclei under various conditions (see below).

Cytosol was fractionated by the addition of saturated $(NH_4)_2SO_4$ in TESH buffer to produce 35% saturation and the mixture stirred at least 60 min at 4°C. The suspension was centrifuged 10 min at 15,000 *g* and the pellets stored at −80°C. When needed, the $(NH_4)_2SO_4$-precipitated pellets were placed on ice and dissolved in a volume of TESH buffer equivalent to the original volume of cytosol. The solution was dialyzed against 10 vol. of TESH buffer for 2 h at 4°C and then centrifuged 10 min at 15,000 *g*. The supernatant represented the ammonium sulfate-fraction (AS-fraction) of the cytosol and contained less than 5% of the cytosol protein. The progesterone–receptor complex in this AS-fraction has been shown to be purified about 20-fold in almost 70% yield [13] and in an activated state [2]. This AS-fraction was incubated with purified hen oviduct nuclei under the same conditions as whole cytosol.

Nuclear incubation. The nuclear incubations were set up to simulate various conditions previously used in the assay of steroid receptor binding to isolated nuclei [1–11], but on a larger scale. In each case, about 1.0 mg DNA as hen oviduct nuclei was incubated with approximately 16.0 ml of either whole cytosol, the AS-fraction, or just TESH buffer, all containing 5% (V/V) glycerol. The incubation mixtures contained either no added salt or KCl at a concentration of either 0.05 M or 0.15 M. The mixtures were incubated for 60 min at either 2°C (ice) or 25°C (water bath). At the end of the incubation period, the mixtures were centrifuged 10 min at 1500 *g* at 4°C and the nuclear pellets washed once in TESH buffer containing KCl at the same concentration used in the incubation mixture. The nuclei were subsequently suspended in about 2.0 ml of 25% glycerol (V/V) 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, and stored at −80°C. In some instances, the nuclear preparations were incubated a second time with [^{3}H]-progesterone–receptor (AS-fraction) in 0.15 M KCl to assay for binding as described previously [4].

Chromatin isolation. A portion of the incubated nuclei (0.1–0.5 mg DNA) was used for the isolation of chromatin [11]. Protein was assayed by the method of Lowry *et al.*[14] and DNA (nuclei and chromatin) determined by the method of Burton[15].

DNA Agarose gel electrophoresis. DNA was extracted from incubated nuclei essentially as described by Noll *et al.*[16]. Nuclei (0.5–1.0 mg DNA) were centrifuged and suspended in 1.0 ml cold 1 mM $MgCl_2$. To this suspension the following were added: 0.1 ml 10% (w/v) sodium dodecyl sulfate (SDS), 0.2 ml 5 M $NaClO_4$, 0.2 ml 0.1 M EDTA. After mixing well and warming to 22°C, 1.5 ml chloroform–isoamyl alcohol (24:1, V/V) was added and deproteinization accomplished by vortexing intermittently for several hours at 22°C. The mixture was centrifuged 10 min at 5000 *g* at 22°C. The aqueous phase was removed, cooled to 2°C, and centrifuged in the cold to remove most of the SDS. The supernatant was again deproteinized with chloroform–isoamyl alcohol, but at 2°C. The aqueous phase was dialyzed against 4 changes of 100 volumes of H_2O at 4°C over a period of about 36 h. The retentates (DNA) were frozen and lyophilized. The DNA was then dissolved in H_2O and stored at −20°C. The DNA preparations were analyzed for extent of degradation by electrophoresis of 15 μg samples in 1.4% agarose gels containing ethidium bromide as described by Sharp *et al.*[17] and using the Tris–acetate buffer described by Loening[18]. The DNA in the gels was detected as an orange fluorescent band under long wavelength U.V. light (UVSL-25 Mineralight, Ultra-Violet Products Inc., San Gabriel, CA). The U.V. illuminated gels were photographed with Polaroid type 52 film using a no. 25 red filter.

Histone polyacrylamide gel electrophoresis. Histones were extracted from a portion of the incubated nuclei (0.1–0.2 mg DNA) and analyzed by polyacrylamide gel electrophoresis essentially as described by Panyim and Chalkley[19]. The nuclear suspension was diluted to 2.0 ml with cold 1 mM $MgCl_2$ and made 0.4 N H_2SO_4 by the addition of the 0.2 ml 4.4 N H_2SO_4. Histones were extracted by incubation at 2°C for at least 60 min with intermittent vortexing. The suspension was centrifuged 10 min at 15,000 *g* and the supernatant (histones) saved. The pellet was re-extracted with 0.4 N H_2SO_4, 5% (V/V) glycerol and centrifuged. The two supernatants were combined and dialyzed against 4 changes of 10–20 volumes of 0.9 mM acetic acid at 4°C over a period of about 36 h. The retentates (histones) were frozen and lyophilized. The histones were dissolved in 0.9 M acetic acid, 12 mM thioglycerol, 5% (V/V) glycerol, 10% (w/v) sucrose and stored at −20°C.

Total histones were quantitated by a modification of the method of Bramhall *et al.*[20]. Aliquots containing 0–50 μg of either extracted histones, standard histones, or bovine serum albumin in the acetic acid–thioglycerol–glycerol–sucrose solution (above) were spotted on Whatman 3MM filter paper circles (2.3 cm diameter). The volume on each filter was made up to 50 μl with the same solution, and the filters were dried for 10 min under a heat lamp. The protein was

fixed on the filters by placing them in 5% (w/v) sulfosalicylic acid (2 ml per filter) at 2°C and then heating the solution to 55°C. The filters were rinsed with 7% (V/V) acetic acid and then stained with 1.0% (w/v) xylene brilliant cyanin G (Coomassie Blue G) in 7% acetic acid for 30 min at 55°C. The filters were destained in 7% acetic acid at 55°C and then dried overnight at room temperature. The protein-bound stain was eluted from the filters with 2.0 ml 66% (V/V) ethanol, 1% (V/V) NH_4OH at 55°C and the absorbance at 610 nm of the eluates determined.

The extracted nuclear histones were analyzed for degradation by electrophoresis in 15% polyacrylamide gels containing 2.5 M urea and 0.9 M acetic acid [19]. The 11.5 cm gels were pre-electrophoresed overnight at 1 mA/gel in 0.9 M acetic acid. The extracted histones (15–20 μg) were layered on the gels and separated by electrophoresis at 1.5 mA/gel until a pyronine Y marker just ran off the end of one gel (about 5.5 h). The gels were stained in 0.1% (w/v) Coomassie Blue R-250 in 7% (V/V) acetic acid for at least 36 h (to allow the stain to diffuse completely through the gel) and destained electrophoretically. The gels were scanned (within a week after destaining) at 560 nm with a Beckman Acta CII spectrophotometer equipped with a gel scanner. The relative amount of stain in each protein band was determined during the scan by a Hewlett-Packard 3380A Integrator-recorder, which quantitated the area under each peak of a record.

RESULTS AND DISCUSSION

Effect of nuclear incubation on chromatin DNA integrity

Since many nucleases have been described [21], it might be expected that a cytosol preparation would contain some deoxyribonuclease activity. The results shown in Fig. 1 indicate that this was probably the case. When nuclei were incubated in whole cytosol (Panel A) containing no added salt at 25°C, the recovery of chromatin DNA was less than one-half that

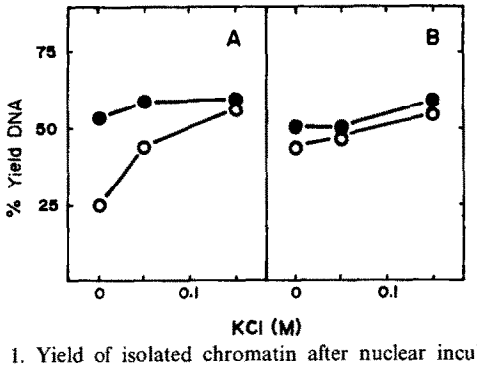

Fig. 1. Yield of isolated chromatin after nuclear incubation. Nuclei were incubated at 2°C (●) or 25°C (○) in either whole cytosol (A) or the cytosol AS-fraction (B) containing various concentrations of KCl. Chromatin was isolated from the nuclei and the percent yield of DNA determined. Each point represents the average of four experiments.

of nuclei incubated at 2°C. As the salt concentration was increased, the yield of chromatin DNA from nuclei incubated at 25°C increased, until at 0.15 M KCl it was the same as that from nuclei incubated at 2°C. Essentially no change in the yield of chromatin DNA from nuclei incubated in whole cytosol at 2°C occurred as the KCl concentration was increased. This was also the case for nuclei incubated in the cytosol AS-fraction (Panel B) or TESH buffer alone (data not shown) at either 2°C or 25°C. The yields of chromatin DNA in these experiments were only about 50% because of the small amounts of nuclei used in the assays.

A reasonable explanation for the results presented in Fig. 1 is that a salt and temperature-sensitive deoxyribonuclease and/or protease was present in whole cytosol, but not in the AS-fraction, and that this enzyme(s) was capable of acting on the deoxyribonucleoprotein of nuclei during the incubation in cytosol. Evidence for nuclease activity is shown in Fig. 2 for two replicate experiments, wherein after incubation of nuclei in whole cytosol, the nuclear DNA was isolated and electrophoresed in agarose gels. Only the DNA from nuclei incubated in cytosol without added salt at 25°C (gel b) exhibited significant degradation (as evidenced by increased mobility in the gels) compared to the DNA from nuclei incubated without added salt at 2°C (gel a) or with 0.15 M KCl at 2°C or 25°C (gels c and d, respectively). The DNA from unincubated control nuclei had the same mobility (gels not shown) as the DNA in gels c and d in each experiment, indicating that no apparent DNA breakdown occurred under those conditions. In Experiment 2, additional gels were run (not shown) with DNA from nuclei incubated in whole cytosol containing 0.05 M KCl at 2°C and 25°C, and under both conditions the DNA migrated as in gel a. Incubation of nuclei in the cytosol AS-fraction caused no detectable DNA degradation (gels not shown) under any of the conditions tested (0 or 0.15 M KCl at 2°C or 25°C). Comparison of the gels from Experiments 1 and 2 shows that some variability occurred in the amount of chromatin DNA breakdown with different cytosol and/or nuclear preparations. Thus, DNA degradation probably explains the loss of chromatin DNA (Fig. 1) during incubation of nuclei in whole cytosol. It is also possible, however, that degradation of chromatin proteins could result in solubilization of DNA, and that this could account for part of the loss.

Results from studies of the protein content of chromatin isolated from incubated nuclei are shown in Fig. 3. Again the incubation in whole cytosol (Panel A) without added salt at 25°C caused the greatest change: in this case the chromatin protein:DNA ratio was more than twice that of chromatin from nuclei incubated at 2°C. As the salt concentration was increased, the protein:DNA ratio of nuclei incubated at 25°C decreased, until at 0.15 M KCl it was about the same as that of nuclei incubated at 2°C. Changing the KCl concentration had a much smaller effect on

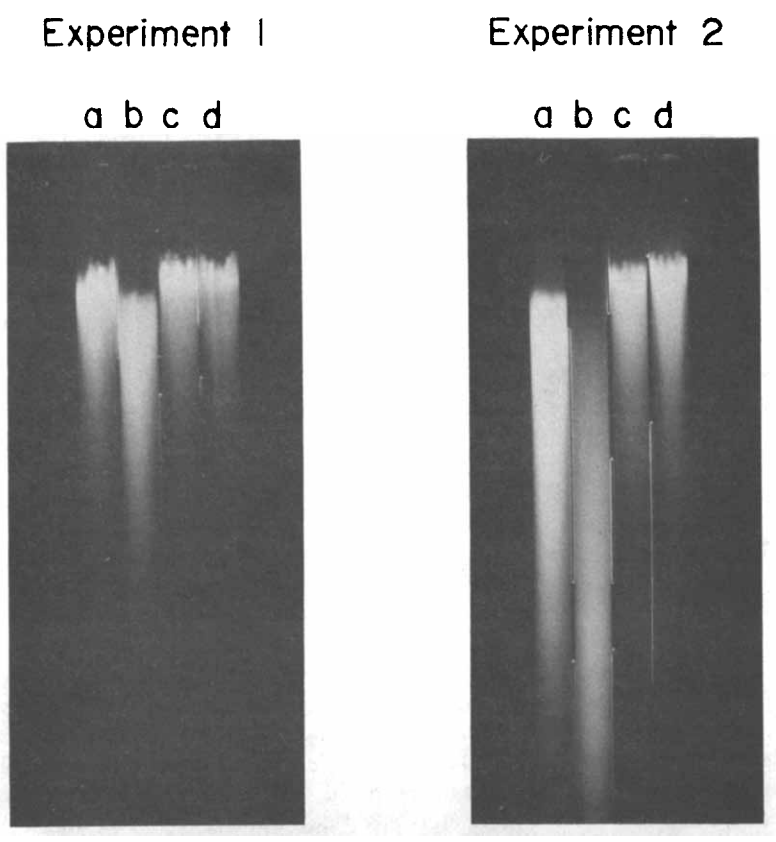

Fig. 2. Agarose gel electrophoresis of DNA after nuclear incubation. In two replicate experiments, nuclei were incubated in whole cytosol containing no KCl at 2 C (a) or 25 C (b) or containing 0.15 M KCl at 2 C (c) or 25 C (d). DNA was isolated from the nuclei and electrophoresed in agarose gels containing ethidium bromide. The gels were photographed under ultraviolet illumination.

the chromatin protein:DNA ratio of nuclei incubated in whole cytosol at 2 C. This was also true for nuclei incubated in the cytosol AS-fraction (Panel B) or TESH buffer alone (data not shown) at either 2°C or 25°C. The chromatin protein:DNA ratios (3–5) in these experiments were somewhat high. When such small amounts of chromatin are prepared, however, it is not possible to wash the pellets as thoroughly as usual without significantly decreasing the yield.

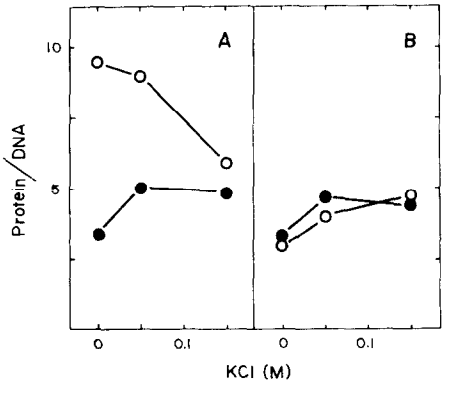

Fig. 3. Protein:DNA ratio of isolated chromatin after nuclear incubation. Nuclei were incubated at 2 C (●) or 25°C (○) in either whole cytosol (A) or the cytosol AS-fraction (B) containing various concentrations of KCl. Chromatin was isolated from the nuclei and the protein and DNA content determined. Each point represents the average of two experiments.

These increased protein:DNA ratios of chromatin from nuclei incubated in cytosol without added salt at 25°C could result from two causes. One would be the adsorption of cytosol protein to the chromatin during nuclear incubation. Such adsorption can occur during the isolation of chromatin by some procedures and can be eliminated by a high salt wash [22, 23]. The procedure used here, however, included such a wash. This wash may have been inadequate, however, due to the limitations on washing such small amounts of chromatin, as discussed above. The most probable cause of increased protein:DNA ratios, however, would be the degradation and loss of chromatin DNA during nuclear incubation, as discussed above, without the concomitant loss of chromatin protein. The proteins from chromatin regions where the DNA was degraded may have aggregated with one another or with intact regions of the chromatin, resulting in high protein:DNA ratios for the isolated material.

Effect of nuclear incubation on chromatin protein integrity

The above studies still did not answer the question of whether or not proteases were acting on the

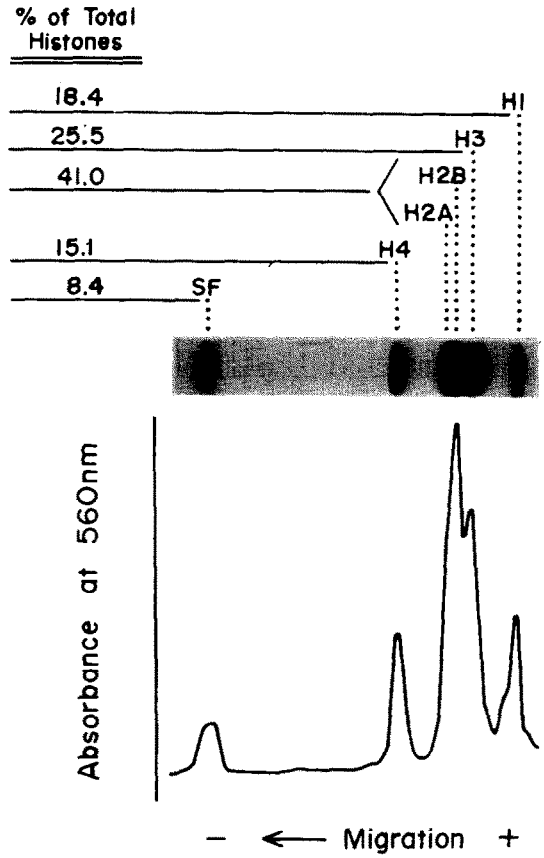

Fig. 4. Polyacrylamide gel electrophoresis of histones after nuclear incubation. Nuclei were incubated in whole cytosol containing 0.05 M KCl at 25°C. The histones were extracted and electrophoresed in polyacrylamide gels. The gels were stained with Coomassie Blue R, destained, and scanned at 560 nm. During the scan, the area under each peak was determined by a Hewlett-Packard 3380A Integrator-recorder, and this data is presented as the percent of total histones. H = histone; SF = small fragment (of protein).

nuclear chromatin. We felt that a straightforward way to measure proteolytic activity would be to analyze the pattern of histone migration after polyacrylamide gel electrophoresis, since the histones are quite susceptible to most proteases and the different species (H1-H4) exhibit differential sensitivity to proteolytic attack [16]. An example of this type of analysis is presented in Fig. 4, which shows the histone pattern for nuclei incubated in whole cytosol containing 0.05 M KCl at 25°C. The pattern of the histone bands (H1-H4) and the peak areas for nuclei under these incubation conditions were essentially the same as those for unincubated control nuclei (data not shown). The nuclei incubated in the cytosol, however, had a much greater amount (8.4%) of a small fragment of protein (SF), that migrated ahead of the histones, than the unincubated nuclei (2.7%). On the basis of its migration relative to that of the histones, this small fragment was estimated to contain 30–40 amino acids.

The type of data shown in Fig. 4 was compared for nuclei incubated in either whole cytosol or the AS-fraction containing 0.05 M or 0.15 M KCl at 2°C or 25°C, and this is presented for two replicate experiments in Fig. 5. A most surprising aspect of this data is the absence of any significant effect on the histone patterns of any of the incubation conditions, although incubation in whole cytosol resulted in a small (1–5%) but consistent decrease in the amount of H2 (A + B) histone relative to incubation in the AS-fraction. Even when nuclei were incubated in whole cytosol without added salt and at 25°C (conditions resulting in the greatest breakdown of chromatin DNA), the histone pattern (data not shown) was essentially the same as those in Fig. 5. The same histone pattern was also observed with nuclei incubated in TESH buffer alone and with unincubated nuclei (data not shown).

The relative amount of the small protein fragment (SF), however, did vary considerably with the different incubation conditions as shown in Fig. 5. The largest amount of SF was extracted from nuclei incubated in whole cytosol containing 0.05 M KCl at 25°C. Even more SF was observed when the incubation mixture contained no added salt (data not shown). As shown in Figure 5A, reducing the temperature to 2°C resulted in a slight decrease in the amount of SF extracted from nuclei incubated in whole cytosol containing 0.05 M KCl. It is also evident from Fig. 5A that nuclei incubated in the AS-fraction containing 0.05 M KCl had significantly less SF than nuclei incubated in whole cytosol under the same conditions. In fact, the amount of SF extracted from nuclei incubated in the AS-fraction plus 0.05 M KCl was about the same as that from nuclei incubated in TESH buffer plus 0.05 M KCl and from unincubated nuclei (data not shown). Irrespective of which nuclear proteins SF was derived from, these results suggest that measurable protein breakdown occurred in nuclei during incubation in whole cytosol under certain conditions, whereas little or none occurred with the AS-fraction. This explanation is complicated, however, by the fact that in nuclei incubated in either whole cytosol or the AS-fraction containing 0.15 M KCl, little or no SF was observed (Fig. 5B). This was also true for nuclei incubated in TESH buffer plus 0.15 M KCl (data not shown). It seems likely that at this higher salt concentration, the SF was extracted during incubation, irrespective of the temperature or cytosol composition. The presence of SF in unincubated nuclei (data not shown) also suggests that some protein degradation occurred in nuclei *in vivo* and/or during isolation.

When cytosol alone was extracted and analyzed for the presence of SF, none was found. In fact, all acid-extractable proteins in the cytosol gels were significantly larger than the H1 histone (not shown). It should be noted that larger proteins migrating behind the H1 histone were also extracted from nuclei (not shown), but these proteins were not included in the analyses reported here.

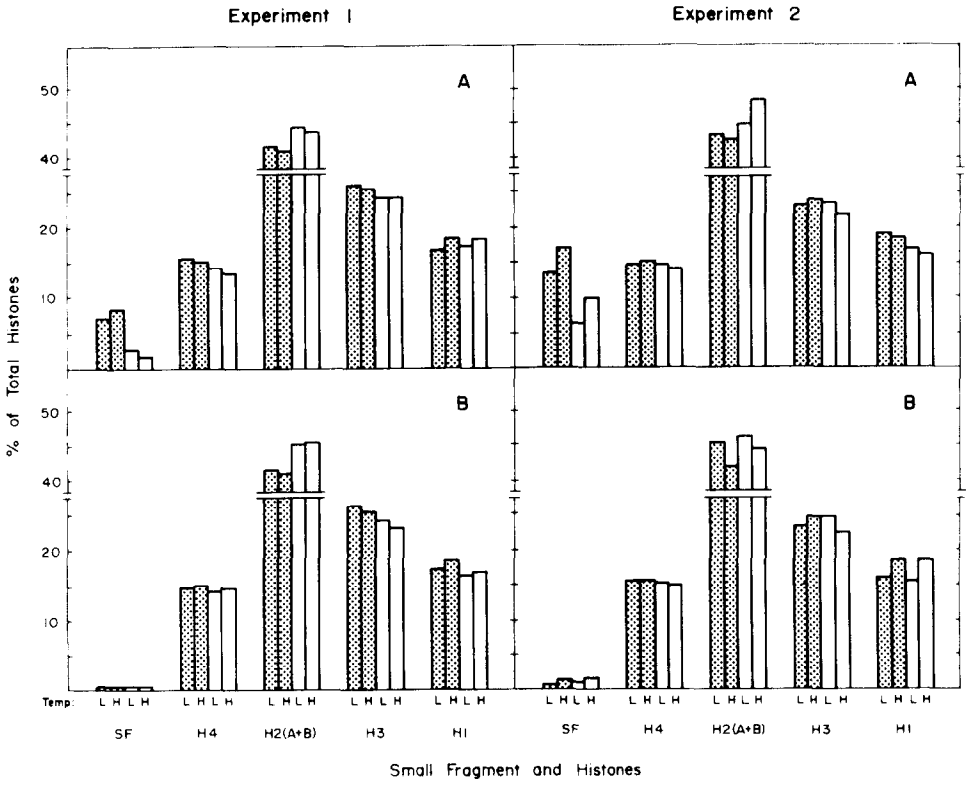

Fig. 5. Relative amounts of the small fragment of protein and the individual histones after nuclear incubation. In two replicate experiments, nuclei were incubated in either whole cytosol (stippled bars) or the cytosol AS-fraction (white bars) containing 0.05 M KCl (A) or 0.15 M KCl (B) at 2°C (L: low temperature) or 25°C (H: high temperature). The histones were extracted and electrophoresed in polyacrylamide gels. The gels were stained, destained, and scanned. The relative intensity of each stained protein band in each gel was determined as described in the legend of Fig. 4 and the "Experimental" section and is expressed as the percent of total histones quantitated.

Effect of nuclear incubation on [^{3}H]-progesterone-receptor binding

Since nuclei incubated in whole cytosol with no added salt at 25°C exhibited significant DNA degradation and some protein breakdown, we were most interested in knowing whether or not this deterioration in the integrity of the chromatin altered its capacity to bind the [^{3}H]-labeled progesterone-receptor complex. This interest arose from the fact that many investigators studying steroid receptor binding to nuclei have used these conditions in their assays and have often obtained conflicting results [see discussions in references 1, 2, 3]. In previous studies of [^{3}H]-progesterone-receptor binding to nuclei and chromatin, we have shown that the ionic strength of the assay mixtures can have a significant effect on the amount of binding measured [1]. Consequently, we have usually used the AS-fraction with 0.15 M KCl at 2°C in our assays. We felt that these conditions gave the most consistent and physiologically significant results [1, 4, 5]. Indeed, as we have shown above, it is just these conditions which result in the least deterioration of the chromatin integrity.

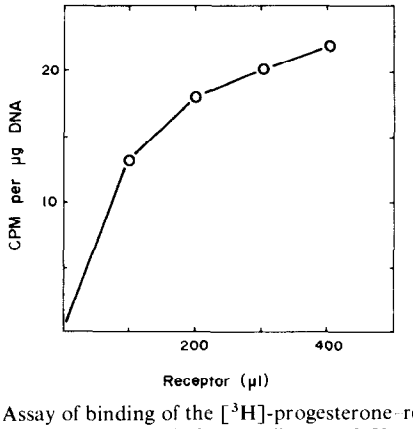

Fig. 6. Assay of binding of the [^{3}H]-progesterone-receptor complex to nuclei. Nuclei (four replicates of 50 µg DNA per point) were incubated in 0.18 M KCl with increasing amounts of the cytosol AS-fraction (prepared in January) containing the [^{3}H]-labeled progesterone-receptor complex. After 90 min at 2°C, the nuclei were collected by centrifugation and washed, and the radioactivity and DNA determined, all as described previously [4].

Figure 6 shows a typical binding assay with increasing amounts of the [^{3}H]-progesterone-receptor (cytosol AS-fraction) carried out as described previously [4]. At about 200 µl of receptor, saturation of nuclear binding sites began to occur. Using 400 µl of receptor, therefore, the binding capacity was deter-

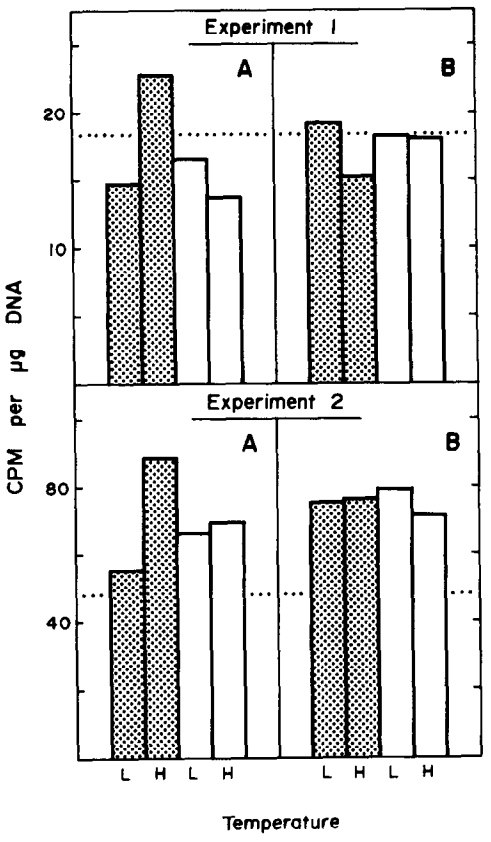

Fig. 7. Binding of the [^{3}H]-progesterone–receptor complex to nuclei after incubation. In two experiments, nuclei were incubated in either whole cytosol (stippled bars) or the cytosol AS-fraction (white bars) containing no KCl (A) or 0.15 M KCl (B) at 2°C (L: low temperature) or 25°C (H: high temperature). In these experiments, neither the whole cytosols nor the AS-fractions were exposed to progesterone, so that any nuclear binding of the progesterone–receptor complex during the incubation was eliminated [2, 4]. After incubation, the capacities of the nuclear preparations (quadruplicate portions containing 50 µg DNA) for binding the [^{3}H]-labeled progesterone–receptor complex (400 µl) were assayed as described in the legend of Fig. 6, except that in Experiment 2 the assay mixtures contained 0.15 M KCl and the AS-fraction was prepared in July. The dotted lines represent the binding capacities of unincubated control nuclei in each experiment.

mined for nuclei that had been incubated as described above, either in whole cytosol or the AS-fraction containing no added salt or 0.15 M KCl at 2°C or 25°C. As shown in Fig. 7 for two experiments, the whole cytosol–no salt–25°C incubation conditions, which resulted in DNA degradation and some protein breakdown, also caused an increase in nuclear binding capacity relative to all the other incubation conditions. Although this increase was observed in most experiments, it was not observed all the time. This variability is similar to that found in the degree of DNA degradation (Fig. 2) and is probably attributable to variation in cytosol and/or nuclear preparations. A similar increase in steroid receptor binding, however, has been shown by others to occur when *both* whole cytosol and nuclei or chromatin are incubated at room temperature (21–25°C) [2, 9]. This increased binding could result from the concerted nuclease and protease activity described above, which could expose previously repressed acceptor sites. Such "masked" acceptor sites for the progesterone–receptor have been identified in large numbers in the nuclear chromatin from different hen tissues [5, 10].

The higher binding under all conditions in Experiment 2 in Fig. 7 (compared to Experiment 1) is probably the result of two factors which increase receptor binding: (1) the lower salt concentration, due to the electrostatic nature of the interaction [1], and (2) the AS-fraction being prepared during the summer, due to the circannual rhythm in the receptor's capacity to bind to deoxyribonucleoprotein [24]. The lower receptor binding of unincubated nuclei relative to nuclei incubated under all conditions in Experiment 2, suggests that some changes which affect binding may occur even when no degradation occurs. Such changes must be minor when using the AS-fraction at 2°C, however, because with these conditions the *in vitro* pattern of receptor binding to multiple acceptor sites [4] is basically the same as that observed *in vivo*, when chicks are injected with increasing amounts of radiolabeled progesterone and the quantity of steroid bound to isolated nuclei is determined [25]. This *in vivo–in vitro* correlation also suggests that the methods of isolation and storage of nuclei used in this study do not significantly alter the integrity of the chromatin acceptor sites. One can never be certain, however, that subtle changes in a cell organelle are not occurring during isolation and are just not detected. This method of isolation, however, does yield nuclei that closely resemble those of intact tissue on a morphological basis (as determined by electron microscopy), and the conditions of storage prevent the loss of nuclear RNA polymerase activity that is observed with other storage conditions [12]. Thus, the chromatin degradation and increased receptor binding observed with the whole cytosol–no salt–25°C incubation conditions indicate that that combination is least favourable for studying steroid receptor binding to nuclei, and that, by implication, the conditions of choice for such studies would probably be the AS-fraction with 0.15 M KCl at 2°C.

CONCLUSIONS

The results reported here demonstrate that some incubation conditions used for the assay of steroid receptor binding to nuclei can alter the chromatin integrity. The whole cytosol–no salt–25°C conditions resulted in significant DNA degradation and some protein breakdown, which in turn usually caused an increase in the capacity of the chromatin to bind the [^{3}H]-progesterone–receptor complex. This could result from the derepression of formerly "masked" acceptor sites [5, 10]. We would, therefore, recommend either avoiding the use of these incubation con-

ditions when studying *in vitro* interactions of nuclei and chromatin or including nuclease and protease inhibitors in the incubation mixtures. When possible, a fraction of the cytosol (in this case, a partially purified receptor preparation) should be used for such studies. As we have shown here, incubation of nuclei in the cytosol AS-fraction resulted in no detectable deterioration of the chromatin integrity.

One variable which could have influenced the integrity and binding capacity of the chromatin, but which was not examined in this study, was the difference in the pH of the whole cytosol and the AS-fraction. The pH of the whole cytosol (~6.7 at 22°C) was about 0.5 units less than that of the AS-fraction (~7.2 at 22°C). Although increasing the Tris concentration (in the TESH buffer) to 50 mM could have eliminated this discrepancy, we chose not to do so, because other investigations of steroid receptor binding to nuclei have often utilized cytosols prepared in buffers containing 10 mM Tris [see discussions in references 1, 2, 3]. This pH difference probably had little effect, however, as judged by the findings of Dounce and Umaña[26, 27]. Although they used different isolation conditions, they observed only small differences in the amount of proteolytic activity associated with cytosol and nuclear fractions between pH 6.5 and 7.0. Similar studies of nuclease activity have not been reported to our knowledge.

Attempts were also made to monitor changes in the integrity of chromatin by assaying its ability to serve as a template for DNA-dependent RNA synthesis *in vitro* [11] after nuclear incubation. These attempts were unsuccessful, however, due to variability within and between experiments. We also considered examining the nonhistone chromatin proteins (NHCP's) for degradation as a means of checking chromatin integrity, but we rejected the idea for several reasons. The extensive heterogeneity of the NHCP's [23, 28] would have made quantitative analysis (such as that shown in Figs 4 and 5 for the histones) difficult, and minor changes might easily have gone undetected. In addition, Sellwood *et al.*[29] observed no degradation of NHCP's even when nuclei were incubated at 37°C. The limited heterogeneity and documented proteolytic susceptibility of the histones [16], on the other hand, suggested they would be good candidates for the type of quantitative electrophoretic analysis to be used in this study.

The results described in this report, in addition to their direct relevance to studies of steroid receptor binding to nuclei, should also provide a note of caution for all investigations of the *in vitro* interaction between two cellular components, for example, peptide hormone binding to membrane-receptor preparations or cytosol activation of nuclear RNA polymerase activity. We suggest that, in all such studies, the investigator check the integrity of both components for changes that might be occurring during the incubation. If such changes are occurring, then steps can be taken to eliminate them. When this is not possible, an awareness of the changes can at least temper conclusions drawn from the results.

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