

## Postnatal regulation of glucocorticoid receptor in the liver of chicken

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The specific binding of [<sup>3</sup>H]-dexamethasone to glucocorticoid receptor (GR) and activation of hormone-receptor (H-R) complexes from the liver of chicken at day 0, 5, 10, 30, 60 and 90 were investigated to find out GR regulation during postnatal development. Results showed that GR level (fmol/mg protein) reached a peak by day 5 of postnatal age and was significantly higher (+ 42%) than observed at day-0 (day of hatching), as evidenced also by protein blot experiments and Scatchard analysis of binding data. The GR concentration declined gradually up to day 30, and thereafter, no significant change was observed at day 60 and 90 of postnatal ages. The temperature and salt-dependent activation of GR showed no significant differences in 0 and 30-day old chicken, as determined by DNA-cellulose binding assay. However, nuclear binding of temperature and salt-activated GR complexes was significantly higher in 0-day old chicken. Cross-mixing experiments (wherein nuclei of day-0 were incubated with the H-R complexes of day-30 and *vice-versa*) revealed the role of nuclear specificity in higher binding of temperature and salt-activated H-R complexes at day-0 of postnatal age. DNase I extraction of nuclei bound to activated H-R complexes showed higher extractability at day-0 (70%), compared to day-30 (44%). Above findings suggested that changes in GR concentration as well as chromatin organization might play an important role in glucocorticoid-mediated responses during postnatal development of chicken.

**Keywords:** Glucocorticoid receptor, Postnatal ages, Liver, Chicken, Dexamethasone, Regulation.

Glucocorticoids (GCs) are the key regulators of homeostasis, stress responses and adaptation in developing animals. They have several effects on various animal tissues including liver, where they influence glucose metabolism by enhancing the enzymes involved in gluconeogenesis<sup>1</sup>. The synthesis and secretion of GC hormone from adrenal cortical cells is under the intricate control of corticotropic release hormone (CRH) from the hypothalamus and adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. At cellular level, GCs exert their actions by modulating gene expression through a cascade of regulatory events, initiated by high-affinity interaction with ~94 kDa intracellular protein — the glucocorticoid receptor (GR), belonging to a phylogenetically conserved superfamily of ligand-inducible transcription factors<sup>2</sup>. Members of this family have essential structural and functional features — an amino terminal transactivation domain,

a central zinc-finger DNA-binding domain and a carboxy terminal ligand binding domain<sup>3,4</sup>.

Unliganded GR resides in the cytoplasm as a multi-protein complex comprising a dimer of heat shock protein (hsp90), a monomer of hsp70, hsp56, and a p-23 acidic protein<sup>5,6</sup>. Hsp90 is required for GC binding to GR and may facilitate the proper folding of the GR into an optimal DNA binding conformation<sup>7</sup>. Its decreased level dramatically impairs signal transduction by steroid receptors. On GC binding to GR, it dissociates from such associated proteins (a process known as GR activation or transformation), wherein the nuclear localization signals of GR are unmasked and the receptor is capable of translocating into the nucleus and regulating gene expression<sup>8</sup>.

Numerous mechanisms<sup>3,9,10</sup> have been proposed by which the GC-activated GR can modulate transcription of target genes: (i), a GR homodimer can bind to DNA at specific glucocorticoid response elements (GREs) via their DNA-binding domain and influence transcription<sup>3</sup>, (ii) the GR can modulate transcription by rendering chromatin accessible to other transcription factors<sup>9</sup>, and (iii) the GR can affect other signal transduction cascades through mutual protein-protein interactions with other transcription factors, which mediate the effects of these cascades<sup>10</sup>.

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**Abbreviations:** GCs, glucocorticoids; GR, glucocorticoid receptor; DCC, dextran-coated charcoal; DTT, dithiothreitol; GH, growth hormone; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; PEPCK, phosphoenolpyruvate carboxy-kinase; H-R complex, hormone-receptor complex; Hsp, heat shock protein.

GR is reported to interact with the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), which plays a crucial role in metabolism of glucose, cholesterol, and xenobiotics, as well as in development of central nervous system in fetus<sup>11,12</sup>. It enhances the transcriptional activity of COUP-TFII on the promoter of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis<sup>13</sup>, suggesting that their interaction may be important in the effects of GCs on glucose metabolism.

Avian growth and development, like in mammals, is under intricate hormonal control, involving endocrine, paracrine and autocrine signaling. The synthesis and secretion of adrenal hormones are initiated during embryogenesis and have been purportedly involved in avian development<sup>14</sup>. Corticosterone, the principal GC, increases during early stages of chicken development and induces several enzymes of carbohydrate metabolism<sup>15,16</sup>. In addition, GCs also have differentiative effects in many eukaryotic embryonic tissues<sup>17,18</sup>. They also induce growth hormone (GH)-producing cell differentiation in rats and chicken<sup>19,20</sup>. The magnitude of action of GCs depends on the level of GR and also on the post-receptor events<sup>21,22</sup>.

Earlier, the age-related changes in GR concentration, activation/transformation and their binding to nuclear chromatin in different mammalian tissues during postnatal development studies have been reported<sup>22-25</sup>. However, studies on changes in the level and physico-chemical properties of GR during avian development are lacking. In the present study, changes in the level of liver GR as well as in magnitude of activation of hormone-receptor (H-R) complexes have been investigated, in order to ascertain the GR regulation during postnatal development. Conformational changes in the chromatin organization during postnatal ages, which may be involved in GC regulation of gene expression in developing chicken have also been studied.

## Materials and Methods

### Materials

Polyclonal rabbit anti-GR-ab, raised against the amino acid (407-423) sequence (SVFSNGYSSPGM-RPDVS) from the N-terminal region of the rat-GR was a gift from Profs N Katunuma and H Kido, Japan. Goat-anti-rabbit-IgG-ALP conjugate was obtained from Bangalore Genei, India.

[1,2,4,6,7-<sup>3</sup>H]-Dexamethasone, a synthetic glucocorticoid (specific activity, 83 Ci/mmol) was obtained from Amersham, England. Unlabeled dexamethasone and other chemicals were from Sigma Chemicals Co., USA. All other chemicals used were of analytical grade. Buffers used in the study were: (A): 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM sodium molybdate, 10% glycerol, 1 mM DTT and 10 mM NaCl; (B): 0.25 M sucrose and 10 mM Tris-HCl (pH 7.6); (C): 0.25 M sucrose, 10 mM Tris-HCl (pH 7.6) and 0.5% Triton X-100, and (D): 0.25 M sucrose, 10 mM Tris-HCl (pH 7.6) and 4.2 mM MgCl<sub>2</sub>.

### Animals

Male chickens (Rhode Island Red, RIR breed) were purchased locally from a veterinary farm. They were maintained at 25 ± 2°C under normal laboratory conditions and fed with a chick mash diet (Premier Hatchery Ltd.) and tap water *ad libitum*. Chickens of various postnatal age groups (0, 5, 10, 30, 60 and 90-day) were used for the experiments.

### Receptor preparation and assay of glucocorticoid receptor

Chicks were sacrificed by cervical dislocation at a fixed time of the day (12:00 h), and the livers were quickly removed, freed of fat and connective tissues, washed in chilled normal saline and blotted dry. A 20% (w/v) homogenate was prepared in ice-cold buffer using a teflon-glass motor driven homogenizer at 4°C. The homogenate was centrifuged at 40,000 × g for 45 min at 2°C in an Allegra™ 64R high-speed refrigerated centrifuge. Aliquots (100 µl) of clear, fat-free cytosol were incubated with 60 nM [<sup>3</sup>H]-dexamethasone alone or with 500-fold excess unlabelled dexamethasone for 4 h at 0°C, to obtain maximum saturable specific binding.

For Scatchard analyses<sup>26</sup>, 5-120 nM [<sup>3</sup>H]-dexamethasone was used along with parallel control tubes containing 500-fold excess unlabeled dexamethasone. The 50 µl of DCC (4% activated charcoal + 0.4% dextran T-70 in buffer A) was added to remove any unbound steroid<sup>27</sup>. After 10 min, charcoal mixture was centrifuged and 100 µl of clear supernatants were transferred to scintillation vials and 4 ml scintillation fluid (Cocktail-W from SRL) added to each vial. The bound radioactivity was measured in a liquid scintillation counter (Wallac 1409) with efficiency of 68% for tritium. Specific saturable binding was calculated by subtracting non-specific binding (radioactivity bound in the presence of

unlabelled dexamethasone) from total binding i.e., when labelled dexamethasone was used alone.

#### Slot blot analyses of GR

Suitability of anti-GR-ab was ascertained by immunoprecipitation of chicken [<sup>3</sup>H]-dexamethasone-receptor complexes in a series of experiments. The blotting was performed on Bio-Rad Bio-Dot® SF Micro-filtration apparatus, following the instructions in the user's manual. Clear fat-free cytosol (adjusted to equal amount of protein from day 0 and 5) was used for the slot blotting experiment as described elsewhere<sup>28,29</sup>.

#### Preparation of activated of GR complexes

A 20% (w/v) homogenate of the pooled liver tissues from two ages (0 and 30-day) was prepared in buffer B and centrifuged at 2000 × g for 10 min at 2°C to sediment the nuclei. Thereafter, the supernatant was centrifuged at 40,000 × g for 45 min at 2°C and [<sup>3</sup>H]-dexamethasone was added to the clear cytosol, to a final concentration of 60 nM; bound hormone-receptor (H-R) complexes were separated by DCC (in buffer B) treatment. Aliquots of these complexes were then subjected to heat (25°C) and salt (20 mM Ca<sup>2+</sup>) activation for 45 min to obtain activated complexes<sup>23,28,29</sup>. Aliquots of cytosols were also kept at 0°C for 45 min to provide the unactivated receptor complexes as controls.

#### DNA-cellulose binding assay

Commercially available DNA-cellulose was suspended overnight in buffer B at 2°C. From the slurry, aliquots containing 50 µg DNA were transferred to microfuge tubes and 1 ml of chilled buffer B was added to each tube and cellulose was pelleted by centrifuging at 2000 × g for 10 min at 2°C. Aliquots of activated H-R complexes were added to the cellulose pellets and mixed well. After 1 h, the reaction was stopped by adding 1 ml of buffer B and centrifuged at 2000 × g for 10 min at 2°C<sup>27,28</sup>. The cellulose pellets were washed twice with buffer B, and the final pellet was suspended in cocktail-W and transferred to a scintillation vial. The radioactivity bound in pellets was counted and expressed as CPM/100 µg DNA.

#### Nuclear binding assay

The crude nuclear pellets obtained as above were further processed<sup>29,32</sup>. To the pellet was added chilled buffer C, and the contents were gently homogenized and centrifuged at 2000 × g for 10 min at 2°C. The

pellet was then washed twice with buffer B, followed by centrifugation as earlier, and the final pellet thus obtained was suspended in buffer B to yield homogenous slurry. Aliquots of this containing 100 µg DNA were pipetted into microfuge tubes and washed in buffer B to obtain purified nuclear pellets. Aliquots of activated H-R complexes were added to it and after 1 h the reaction was stopped by adding 1 ml of buffer B. The pellets were subsequently processed as above, and the bound radioactivity was counted as described for the DNA-cellulose binding assay. In cross-mixing experiments, purified nuclei of day 0 were incubated with the H-R complexes of day 30 and *vice-versa* to ascertain the change, if any, in either the nuclei and/or receptors at these respective ages.

#### DNase I digestion studies

DNase I digestion studies were performed on purified nuclei<sup>30-32</sup>. Heat activated H-R complexes were allowed to interact with their respective purified nuclei as described above. After washing off the unbound complexes, the pellets were incubated with DNase I in a total volume of 100 µl at 2°C for 45 min. The DNase I was dissolved in buffer D and used at a concentration of 150 units/100 µg DNA. The control tubes received 100 µl of buffer only<sup>31</sup>. The nuclear pellets were mixed properly, and the reaction was stopped by adding 1.0 ml buffer B, followed by centrifugation at 2000 × g for 10 min at 2°C. The pellets were processed and the bound radioactivity determined as described above. The results were expressed as % [<sup>3</sup>H]-dexamethasone-receptor complexes bound to nuclei. Controls were taken as 100% bound.

#### Protein and DNA estimation

Protein content of the receptor preparation was measured according to the dye-binding method of Bradford<sup>33</sup>, using bovine serum albumin (BSA) as standard. The concentration of DNA in the purified nuclear suspension was determined by the method of Burton<sup>34</sup>.

Data obtained from different sets of experiments were analyzed statistically. The level of significance (*P*-value) between two sets of data was calculated according to Student's *t*-test.

## Results

#### Developmental changes in GR level

The receptor assay in chicken liver showed a peak value of GR level by day 5 of postnatal age, which was significantly higher than the values observed for

other age groups (Fig. 1a). Scatchard analyses revealed a higher level of GR at day-5 (435 fmol/mg protein), compared to that at day-0 (326 fmol/mg protein) of postnatal age (Fig. 1b). From the slope of the curves, apparent dissociation constant ( $K_d$ ) was found to be similar (2.84 nM for day 0 and 2.98 nM for day 5), indicating that affinity of hormone for the receptor remained unchanged at day-0 and 5 of postnatal age. The slot blot analyses of receptor preparation also confirmed the increased level of GR at day-5, as compared to day-0 of postnatal age (Fig. 2).

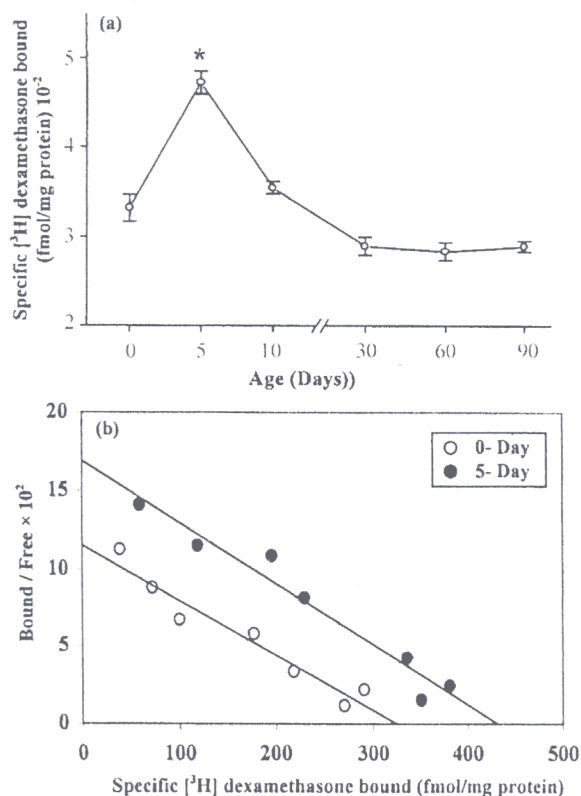


Fig. 1—(a) Specific saturable binding of [<sup>3</sup>H]-dexamethasone in the liver of chicken at different postnatal ages [Tissue fractionation and receptor assay conditions are described in 'Materials and Methods'. Values are mean ± S.D. from 4-5 chicken of each age group. \*Statistically significant as compared to other age groups]; and (b) Scatchard plot of the specific binding of [<sup>3</sup>H]-dexamethasone to its receptor in the liver of 0 and 5-day old chicken. [Ratio of bound to free hormone concentrations is plotted against specific bound hormone receptor/mg protein. Curves depict the mean values for 3 separate assays. Intercepts on X-axis gave the number of specific receptor binding sites 326 ± 14 and 435 ± 15 fmol/mg protein at 0- and 5-day of age, respectively. Slopes indicate dissociation constant ( $k_d$ ) values of 2.84 ± 0.10 and 2.98 ± 0.38 nM for receptor at 0 and 5-day of postnatal age]

#### Activation studies of the GR

Temperature (25°C for 45 min) and salt (25 mM Ca<sup>2+</sup> at 0°C for 45 min)- dependent activation of the GR was studied at day-0 and 30 using DNA-cellulose and nuclear-binding assays. These ages were selected, as the level of GR was similar, hence any change in activation binding was not attributed to the GR level, but rather to its activation properties. DNA-cellulose binding assays in liver revealed that the magnitude of activation of [<sup>3</sup>H]-dexamethasone-receptor complexes by temperature and salt was significantly higher (2-2.5 fold), as compared to the unactivated receptor complexes incubated at 0°C for 45 min. However, degree of activation was similar for hepatic GR obtained from 0 and 30-day old chicken (Fig. 3). No postnatal difference was observed in the *in vitro* activation of hormone-receptor complexes under the conditions mentioned above.

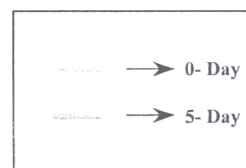


Fig. 2—Slot blot analysis of liver GR from 0- and 5-day old chicken [An equal amount of liver cytosol protein containing GR from 0 and 5-day old chicken was applied on to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-ALP conjugate. Arrows indicate the position and content of GR from 0 and 5-day old chicken]

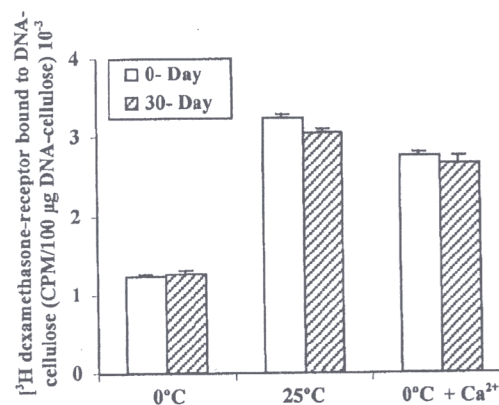


Fig. 3—Specific binding of liver [<sup>3</sup>H]-dexamethasone-receptor complexes to DNA-cellulose upon heat and calcium activation in 0 and 30-day old chicken [Activation conditions are described in 'Materials and Methods'. The results are mean ± S.D. of 3 experiments, each set done in triplicate with pooled tissues from 3-4 chicken of same age group]

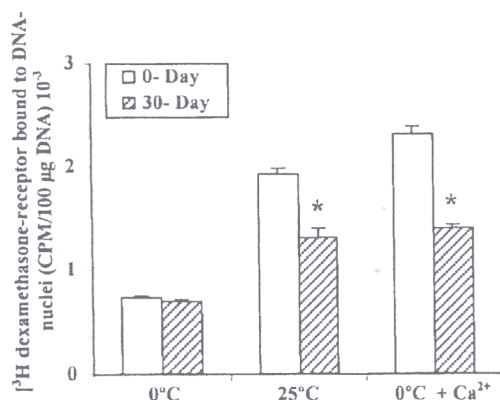


Fig. 4—Specific binding of liver [<sup>3</sup>H]-dexamethasone-receptor complexes to purified nuclei upon heat and calcium activation in 0 and 30-day old chicken [Preparation of purified nuclei and activation conditions are described in ‘Materials and Methods’]. The results are mean ± S.D. of 3 experiments, each set done in triplicate with pooled tissues from 3-4 chicken of same age group. \*Statistically significant as compared to 0-day old.

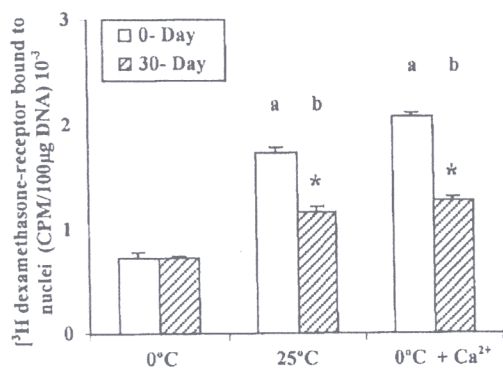


Fig. 5—Specific binding of liver [<sup>3</sup>H]-dexamethasone-receptor complexes to purified nuclei upon heat and calcium activation in 0 and 30-day old chicken under cross-mixing conditions [Experimental procedures are same as for Fig. 4. In cross-mixing experiments, nuclei from 0-day and activated receptor complexes of 30-day old chicken (a) and *vice versa* (b) were used. The results are mean ± S.D. of 3 experiments, each set done in triplicate with pooled tissues from 3-4 chicken of same age group. \*Statistically significant as compared to 0-day old]

In contrast to DNA-cellulose binding assay, nuclear binding of thermal and salt-activated glucocorticoid-receptor complexes was significantly higher in immature (0-day), compared to mature (30-day) chicken (Fig. 4). Nuclear exchange assays, wherein both thermally and salt-activated receptors of 30-day old were allowed to interact with the nuclei of 0-day and *vice-versa* showed significantly higher binding by the hepatic nuclei of 0-day old, as compared to those from 30-day old chicken (Fig. 5). These findings

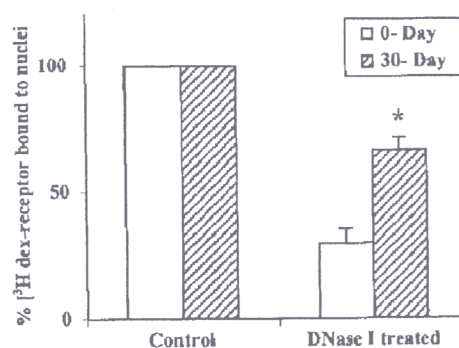


Fig. 6—DNase I extractability of bound [<sup>3</sup>H]-dexamethasone-receptor complexes from the liver nuclei of 0 and 30-day old chicken [DNase I digestion procedures are given in the ‘Materials and Methods’]. The values are mean ± S.D. of 3 separate experiments. \*Statistically significant as compared to 0-day old]

indicated higher binding capacity of immature nuclei to activated H-R complexes than those of mature ones.

#### DNase I extraction of bound GR from nuclei

The sensitivity of hepatic nuclear chromatin digestion by DNase I was compared at two ages (0 and 30-day). The data showed higher extractability of bound H-R complexes from nuclei of 0-day (70%) than that of 30-day (44%) old chicken (Fig. 6). This in turn, confirmed our observation of greater binding of activated GR in 0-day old chicken.

#### Discussion

GCs control several metabolic processes and regulate basal and stress-related homeostasis<sup>35</sup>. They play a pivotal role in immune responses, inflammatory reactions, development and aging processes<sup>36,37</sup> and also influence emotions and cognitive processes like learning and memory<sup>38,39</sup>. Their actions are mediated by the GR, an ubiquitous intracellular protein, which functions as a hormone-activated transcription factor of many target genes. Response to GCs requires a critical threshold of transcriptionally active GR molecules in the cells<sup>40</sup>. Transcription of target genes would not occur, in case the GR level is below the threshold or some essential steps for receptor function, such as activation are inhibited.

Timed changes in the activity of transcription factors, including the GR are required for orchestrated expression of gene networks during development<sup>41</sup>. Modulation of their activity is an important mechanism for control of gene function. Such

modulation may involve changes in synthesis of a particular transcription factor, translational modifications and/or induction of antagonist that specifically represses the activity of transcription factor. Development and aging may partially be characterized by changes in the responsiveness of tissue and cells to certain hormonal modulators<sup>42</sup>. The magnitude of cell response to GCs depends on the level of receptor and efficiency of GR-mediated signal transduction<sup>10</sup>. In many cases, changes in receptor function appear to be closely associated with altered cell responsiveness to hormone during lifespan<sup>24</sup>. Earlier, quantitative changes in receptor molecules have been demonstrated during postnatal development and aging of mouse<sup>22,29</sup>.

Our findings demonstrate quantitative changes in GR level in liver of chicken during postnatal development and the maximum GR level at day 5 of postnatal age, probably associated with changing dietary and metabolic adjustments at this phase of lifespan. The yolk sac absorbed during hatching can supplement nutritional requirements for a limited period only, thus by day 5 of postnatal age, there is total dependency on external diet. Earlier, higher hepatic GR concentration was found in weaning, as compared to mature rats<sup>23</sup>. Thus, our studies show similar correlations between dietary requirements and GR concentration. Scatchard and slot blot analyses confirm a higher level of GR at day-5, without any change in the affinity towards the hormone, as evident from similar  $K_d$  values at day-0 and day-5. Earlier studies<sup>23,24,32</sup> have shown that the affinity of GR for hormone does not change as function of age in different tissues of animals.

In the present study, activation studies of GR have been carried out from the liver of 0 and 30-day old chicken to determine the physicochemical changes in receptor molecule at activation/translocation level. No age-related changes have been observed in the magnitude of activation as revealed by DNA-cellulose binding assay. However, using purified nuclei as a binding medium, significantly lower binding of heat and salt-activated H-R complexes is observed in 30-day old chicken, as compared to day-5. Nuclear exchange studies suggest that higher binding in 0-day old chicken is because of nuclear-binding properties, irrespective of the H-R complexes used from either age group. These findings indicate that an alteration in nuclear chromatin organization might have contributed to the higher binding of H-R complexes in

the liver of 0-day old chicken. Therefore, DNase I digestion of the hepatic nuclei bound to activated H-R complexes from both the age groups, was done to determine the chromatin organization.

The supercoiling of DNA double helix around histone core confers the specificity of digestion of DNA in chromatin by pancreatic DNase I<sup>43</sup>. It makes single-stranded cuts in the double-stranded DNA at 10-bp intervals and its multiples and is widely used to identify chromatin organization in animals<sup>44</sup>. DNase I cuts the DNA, where it is maximally exposed and hence depicts the degree of chromatin compaction. The higher digestion of chromatin in day-0 chicken in our study suggests a less condensed chromatin organization at this stage of postnatal development, resulting probably in a higher *in vitro* binding of activated H-R complexes. However, at day 30, chromatin might have acquired a more compact organization, resulting in a lower binding of activated H-R complexes. Our findings corroborate with earlier reports<sup>22,45</sup> that digestibility of chromatin by DNase I is decreased with the development in mice and rats.

In conclusion, the present study suggests that changes in the level and activation binding of GR, as well as chromatin organization in the liver of chicken contribute to functional alterations in glucocorticoid action, which in turn may influence response of liver to glucocorticoids during development.

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