Formation of Nuclear Bodies in Hepatocytes of Estrogen-treated Roosters

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As a model for cellular growth and stimulation without accompanying proliferation, we have examined the induction and formation of nuclear bodies (NBs) in hepatocytes of estrogen-treated roosters. Four-week-old roosters were injected with a single intramuscular dose of estradiol and then killed at time points of 8 h, 48 h, and 4 wk post-injection. For immunofluorescence analyses, livers were excised and isolated hepatocyte nuclei were fixed and then labeled with antibody to the coiled body-specific protein, p80-coilin. In control animals (no estradiol) or in animals 8 h post-injection, each hepatocyte nucleus contained an average of 1.0 coiled body (CB), which appeared randomly distributed in the nucleoplasm. At 48 h post-injection, there were an average of 2.7 CBs/nucleus and many of these appeared to be in contact with the nucleolus. Pairs of CBs were also observed. By 4 wk post-injection an average of 1.5 CBs/nucleus were detected, with no apparent relationship to the nucleolus observed. By serial-section electron microscopy of intact livers, two different types of round NBs were observed, sometimes in close proximity to each other and to the expanded interchromatin granule region in maximally stimulated cells. One type of NB was a classical CB that averaged 0.35 μm in diameter and the other NB type was ring shaped, averaged 0.25 μm in diameter, was composed of a fibrous shell surrounding a hollow interior, and appeared as a simple NB when sectioned tangentially through its outer shell. Immunoelectron microscopy revealed that CBs were the only class of NBs that contained p80-coilin. From these data, we conclude that CBs proliferate in response to estrogen stimulation, possibly arising from the nucleolar surface and then increasing in number by replicative division.

INTRODUCTION

Primarily because of major advances in their detection by means of specific antibody probes and RNA hybridization, there has been a recent resurgence of interest in nuclear coiled bodies (CBs)¹ (see Brasch and Ochs, 1992 and Lamond and Carmo-Fonseca, 1993 for review). A recent report by Andrade *et al.* (1993) on the

nature of CBs in cultured cells indicated that the number and size of CBs varied with the different stages of the cell cycle and with the proliferative state of the cell. In mitotic cells, few CBs were detected, whereas numerous small CBs appeared in middle to late G₁ and then as larger, but fewer, CBs in S and G₂ phase. When induced to proliferate from quiescent states because of serum starvation or hormone deprivation, CBs correspondingly increased in number and size.

CBs have been studied primarily in actively cycling/proliferating cells in culture, so we were interested in examining CB expression in a model system for cell growth without accompanying cell division. Such a model of cell stimulation without proliferation

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¹ Abbreviations used: CB, coiled body; NB, nuclear body.

has been described in hepatocytes of young roosters after injection with estradiol (Brasch and Peters, 1985; Brasch *et al.*, 1989). Although not typically exposed to estrogens, roosters do, in fact, have estrogen receptors and liver hepatocytes respond to estradiol by synthesizing the yolk-specific vitellogenins and very-low-density-lipoproteins (VLDL). Vitellogenesis, in particular, is a well-defined model of gene regulation in egg-bearing vertebrates (Shapiro, 1982). This complex process is under direct hormonal control and involves activation of major target genes in the liver (Jost *et al.*, 1986; Evans *et al.*, 1987). Although naturally confined to females, vitellogenesis can be experimentally induced in males and juveniles. This constitutes a true primary response to estrogen, and liver nuclei are totally reprogrammed and remodeled to accommodate enhanced transcription of all major classes of RNA (Shapiro, 1982; Brasch and Peters, 1985; Evans *et al.*, 1987). A particular advantage of this model system is the fact that hepatocytes are reprogrammed for vitellogenesis without the need for cell division and, consequently, they are unaffected by cell cycle-related changes.

In addition to *de novo* synthesis of yolk proteins (Brasch and Peters, 1985), an estrogen-induced increase in various types of nuclear bodies (NBs) has also been noted (Brasch and Peters, 1985; Brasch *et al.*, 1989; Brasch and Ochs, 1992). A peak effect was observed at 24–48 h after a single injected dose of estradiol, followed by a regression to normal by 2–4 wk. A partial isolation of NBs was attempted to analyze their composition by gel electrophoresis (Brasch *et al.*, 1989). The resulting gel patterns were very complex and because the preparation was not totally pure, the identification of potential NB proteins was not possible. In addition to gel analysis, NB numbers and appearance were examined by electron microscopy. NBs first appeared at 6 h post-injection as compact spheroids with ring-like profiles and later as more complex morphotypes of 15–17 NBs/hepatocyte nucleus at peak induction of 24–48 h. NBs were occasionally found in pairs and no special relationship to the nucleolus was apparent. In this well characterized system for gene regulation by a steroid hormone (Shapiro, 1982; Jost *et al.*, 1986; Evans *et al.*, 1987), NBs were proposed as potential sites for estrogen receptors (Padykula and Clark, 1981) or as loci for amplified gene activity (Brasch *et al.*, 1989).

In a preliminary investigation of this model system with antibodies to the coiled body-specific protein p80-coilin (Brasch and Ochs, 1992), we noted that estradiol induced the formation of coilin-containing NBs as observed by indirect immunofluorescence. In the present study, we describe experiments to further characterize the nature of these NBs and their mechanism of formation. This model is particularly attractive for the study of CB formation and function for

several reasons. First, the system is physiological and not the result of artificially manipulating cells in culture. Second, it is well characterized morphologically and biochemically (Brasch and Peters, 1985; Brasch *et al.*, 1989; Brasch, 1990) and has been used as a model for gene regulation, because at peak induction the primary hormone target genes, vitellogenin and VLDL, account for approximately 50% of all the mRNA synthesized (Shapiro, 1982; Brasch, 1990). Third, the system is inducible from a resting state and the effects are totally reversible, eventually returning to baseline (Burch and Evans, 1986). Fourth, and perhaps most important for this paper, there is no cell cycling or division upon activation of resting cells. Stimulated hepatocytes simply increase in size and metabolic rate without dividing (Brasch and Peters, 1985). The rooster system, then, affords the opportunity to relate the expression of CBs to transcriptional events without the additional complexity of replication-related processes. Studies with this *in vivo* model might provide important new insights into developmental and gene regulatory aspects of CB function, as well as basic information regarding structural changes that occur in nuclei in response to steroid hormone action (see Brasch and Ochs, 1995 for review).

MATERIALS AND METHODS

Animals

Four-week-old White Leghorn roosters were treated with a single intramuscular injection of 17- β -estradiol (Sigma Chemical, St. Louis, MO) in propylene glycol as carrier at 20 mg/kg body weight. Controls were given an equivalent dose of carrier only. Injected animals were killed at time points 8 h, 48 h, and 4 wk post-injection. For continuous stimulation, some roosters were injected once every 3 days for 3 wk.

Isolation of Hepatocyte Nuclei

Livers were excised, rinsed in cold saline to remove blood and connective tissue, and then blotted and weighed. For each gram of liver, 1 ml of isolation buffer (IB) was added (0.25 M sucrose, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4) and the tissue was minced with scissors (all solutions were used at 4°C and contained 1 mM of fresh phenylmethylsulfonyl fluoride to prevent proteolysis). After decanting the liquid, fresh IB was added and the tissue was homogenized in a rotary blade mixer three times for 15 s at maximum speed on ice. The resulting homogenate was poured into a clean beaker and 1.2 volumes of high sucrose buffer was added (2.2 M sucrose, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4). This solution was further homogenized in a Dounce homogenizer until the mixture was a smooth fluid. The homogenate was filtered through sterile gauze into a fresh beaker on ice. This homogenate was then gently poured into 35-ml cellulose nitrate tubes filled halfway with high sucrose buffer. The resulting interface was stirred with a spatula and the tubes centrifuged at 16,000 $\times g$ for 60 min in a SW28 swinging bucket rotor precooled to 4°C. After centrifugation, the tubes were decanted and drained to prevent the supernatant from contaminating the nuclear pellet. The resulting nuclear pellet was resuspended in 5 ml of IB and used immediately for immunofluorescence or stored frozen in 10% dimethyl sulfoxide for later Western blotting analysis.

In subsequent experiments, proteolysis was controlled by the use of the following protease inhibitor "cocktail" in place of phenyl-

methylsulfonyl fluoride: 5 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 5 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 mM *p*-chloromercuriphenylsulfonic acid, 0.1 mM tosyl-L-lysine chloromethyl ketone, 1 mM *N*-ethylmaleimide, 1 $\mu\text{g}/\text{ml}$ α -2-macroglobulin, and 0.8 mM Pefabloc SC. All inhibitors were purchased from Sigma Chemical, except for Pefabloc SC, which was purchased from Boehringer Mannheim (Indianapolis, IN).

Immunofluorescence

For immunofluorescence, nuclei from each time point were cyto-centrifuged onto polylysine-coated microscope slides, fixed with cold methanol/acetone, and labeled with either rabbit antibody R288 to p80-coilin (Andrade *et al.*, 1993) or human anti-coilin autoantibody from patient Sh (Andrade *et al.*, 1991).

Electron Microscopy

For conventional electron microscopy, small pieces of liver were fixed overnight at 4°C in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2), postfixed in 2% OsO_4 , dehydrated in ethanol, and embedded in Polybed 812 (Polysciences, Warrington, PA). Thin sections were stained with lead citrate and uranyl acetate. For serial-section analyses, ribbons were collected on slotted grids.

Immunoelectron Microscopy

For post-embedding immunoelectron microscopy using Unicryl (BioCell, Ted Pella, Redding, CA) hydrophilic acrylic resin, small pieces of chicken liver were fixed for 1 h at room temperature (RT) in 3% formaldehyde/0.1% glutaraldehyde in Hanks' balanced salt solution (HBSS). After fixation, tissue was dehydrated in microcentrifuge tubes in a sequence of 30% EtOH at 4°C, 50% EtOH at -20°C, 75% EtOH at -20°C, 50% EtOH/50% Unicryl at -20°C, 100% Unicryl at -20°C, and then polymerized in a UV cryo-chamber (Ted Pella) for 2 days at -20°C and 1 day at RT. Thin sections were collected on nickel grids, blocked 30 min with 1% bovine serum albumin (BSA)/HBSS, incubated for 1 h at RT with rabbit R288 anti-coilin diluted 1/100 in 1% BSA/HBSS, rinsed in HBSS, and blocked in BSA/HBSS; the sections were then incubated for 30 min at RT with biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) diluted 1/100 in BSA/HBSS, rinsed in HBSS, blocked with BSA/HBSS, and incubated for 30 min at RT with 10 nm avidin-gold (BioCell, Ted Pella) diluted 1/20 in BSA/HBSS. Finally, tissue sections were rinsed in HBSS, fixed with 1% glutaraldehyde/HBSS for 15 min at RT, rinsed with water, and stained for 15 min at RT with 1% uranyl acetate.

Western Blotting

Gel electrophoresis and Western blotting was done according to the procedure of Chan and Pollard (1992). Equal numbers of nuclei from each time point were mixed with electrophoresis sample buffer (63 mM Tris-HCl, 10% glycerol, 2.3% sodium dodecyl sulfate, 2% β -mercaptoethanol, 0.005% bromophenol blue, pH 6.8) and heated at 95°C for 5 min before separation on a 10% sodium dodecyl sulfate resolving gel with a 4% stacking gel as described by Laemmli (1970). The gel was calibrated with prestained molecular weight markers (Bio-Rad, Richmond, CA) and protein separation was verified by staining with 0.1% Coomassie blue. For immunoblotting, proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) using 50 V for 2 h at 4°C as described by Towbin *et al.* (1979). The transfer efficiency was confirmed by Ponceau S staining. Before application of antibody, the nitrocellulose membranes were cut into strips and saturated with 5% nonfat dry milk in phosphate-buffered saline (M-PBS) for 1 h at room temperature. Nitrocellulose strips were then incubated for 45 min at RT with either rabbit R288 polyclonal antibody to p80-coilin or anti-coilin human serum Sh, both diluted 1/100 in 5% M-PBS. Strips were then washed three times for 30 min with PBS-0.2%

Tween 20 to remove unbound antibody and then incubated with either affinity-purified goat anti-rabbit IgG or goat anti-human IgG conjugated to horseradish peroxidase (Cappel, West Chester, PA) diluted 1/2,000 in 5% M-PBS. After three 30-min rinses with PBS-0.2% Tween 20, immunoreactive bands were detected by chemiluminescence using the ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's directions. Autoradiograms were exposed to Kodak XAR-5 film for 1–15 min before development.

RESULTS

Estradiol Induced the Formation of Coiled Bodies

In control roosters (no estradiol), most hepatocyte nuclei contained a single prominent coilin-containing nuclear body (hereafter referred to as a CB) that had no particular positional relationship to the nucleolus (arrowheads in Figure 1, a and b). An occasional nucleus was observed with multiple CBs. The most dramatic results were apparent 48 h after hormone injection (Figure 1, c and d). At this time point, there was a noticeable increase in the number of CBs/nucleus, many of which were in contact with the nucleolus (single arrowheads in Figure 1c) or occurred in pairs (double arrowheads in Figure 1d).

Figure 2 is a graphical representation of the frequency distribution of CBs in 500 nuclei at the same control and 48 h time points illustrated in Figure 1. Four percent of control nuclei had no CBs, 64% had one, 24% had two, 5% had three, 1% had four, and <1% had five or six CBs/nucleus. The average was 1.4 CBs/nucleus. By 8 h, the numbers had shifted slightly so that <1% of the nuclei had no coiled body, 85% had one, 10% had two, 2% had three or four, and <1% had five or six CBs/nucleus. The average was 1.2 CBs/nucleus. At 48 h there was a dramatic shift in the CB distribution (see Figure 2), with 2% of the nuclei having no CB, 27% with one, 34% with two, 26% with three, 9% with four, 2% with five, and 1% with six CBs/nucleus. The average number of CBs/nucleus at this time point was 2.2. By 4 wk, the numbers of CBs had subsided to near control levels. At this time point, 0% of the nuclei had no CB, 67% had one, 23% had two, 9% had three, and 1% had four CBs/nucleus. The average was 1.5 CBs/nucleus. Interestingly, the numbers of CBs closely mirrored the known hepatocyte metabolic activity at each time point (Brasch and Peters, 1985; Brasch *et al.*, 1989; Brasch, 1990), with 48 h being maximum stimulation.

Nuclear Bodies Consisted of Only Two Types: Coiled Bodies and Ring-shaped Bodies

Figure 3 is a composite of electron micrographs showing representative hepatocyte nuclei from livers of roosters injected with estradiol. In control animals (Figure 3a), typically no NBs were observed in any random thin section. This was perhaps not too surprising considering there were an average of only 1.4

Figure 1. Combination phase and immunofluorescence (a and c) or immunofluorescence alone (b and d) for the localization of p80-coilin in hepatocyte nuclei of sham-injected control roosters (a and b) or roosters 48 h after a single injection of estradiol (c and d). Single arrowheads in a and b denote coiled bodies. Single arrowheads in c and d indicate coiled bodies that are in close proximity to nucleoli and double arrowheads indicate pairs of coiled bodies. Bar, 10 μ m.

CBs/nucleus from our immunofluorescence studies (Figures 1 and 2). This situation changed dramatically, however, 48 h after a single injection of estradiol (Figure 3b). At this time point, numerous NBs could be observed that were distributed more or less throughout the nucleoplasm (arrowheads in Figure 3b).

In general, only three different types of NBs were observed by random thin section electron microscopy: simple NBs (single arrowheads in Figure 3b), CBs (double arrowheads in Figure 3, b and c), and ring-shaped NBs (single arrowheads in Figure 3, c and d). Simple NBs appeared as solid "fibrous" structures of variable size, but always smaller than CBs or ring-shaped bodies. Ring-shaped NBs averaged 0.25 μ m in diameter and appeared hollow with a fibrous shell, whereas CBs averaged 0.35 μ m in diameter and were composed of coiled fibrils that sometimes appeared as small granules in cross section. All three types of NBs were round in shape and usually appeared singly in the nucleoplasm. In some thin sections at 48 h post-

injection, however, pairs of CBs (double arrowheads in Figure 3b) could be observed. This observation is in line with our immunofluorescence data from Figure 1d where pairs of CBs were evident at this time point using a specific antibody to p80-coilin as a probe. A single dose of estradiol resulted in the production of numerous NBs at 48 h, so we were curious as to the effect of continuous stimulation produced by administration of estradiol once every 3 days for 3 wk (Figure 3c). As with the 48-h time point, many NBs were observed in the nucleoplasm (arrowheads). Note the close association between the ring-shaped body and the CB in Figure 3c and the close proximity of these NBs to the highly elaborated interchromatin granule region. Four weeks after a single injection of estradiol (Figure 3d), few NBs were observed, and those present were generally of the ring-shaped variety (arrowhead in Figure 3d).

Figure 4 is a high magnification composite summary of the various NB profiles observed in hepatocytes of

Figure 2. Quantitative distribution of coiled bodies in rooster hepatocyte nuclei from a sham-injected control compared with 48 h after a single injection of estradiol.

estrogen-treated roosters. NBs were observed singly (Figure 4, a-d) or in pairs (Figure 4, e-l). Paired NBs were either of the homogeneous type, consisting of pairs of ring-shaped NBs (Figure 4, e-h) or CBs (Figure 4i), or of the heterogeneous type, consisting of ring-shaped bodies paired with CBs (Figure 4, i-k). Consistent with our previous immunofluorescence data, NB pairs were only observed in the 24–48 h and continuous stimulation samples. Occasionally, what appeared to be hollow ring-shaped bodies were filled with electron-dense material of unknown origin (Figure 4, c and d).

NBs were small and infrequent in number, so we felt that a more rigorous serial-section analysis was essential to determine their distribution and numbers, as well as their possible associations with each other and with the nucleolus. Figure 5 illustrates three different series of serial sections. To our surprise, we discovered that ring-shaped NBs (Figure 5, a and c) could be converted to simple nuclear bodies in subsequent serial sections. Obviously this meant that many, if not all, simple NBs in our samples were actually “grazing” sections through the edge of hollow ring-shaped NBs. This conclusion is consistent with the observation that simple NBs were always smaller in diameter than ring-shaped bodies. Furthermore, it is now apparent that

together, the simple NB and the ring-shaped NB constitute a three-dimensional hollow sphere consisting of a fibrous exterior “shell”. A thin section through the middle of this hollow sphere would produce a ring-shaped body and a section through the fibrous shell would result in a simple NB profile. Additional information was obtained from the serial-section series in Figure 5, e-h, which indicated that a hollow ring-shaped NB may also be completely filled with electron-dense material. The significance of this observation is unknown at the present time, but if NBs function in the transport of material through the nucleoplasm then this image may represent a “charged” hollow NB in the process of transporting the enclosed contents for “discharge” at some specified location.

Figure 6 is a serial-section series of a single 48-h post-injection hepatocyte nucleus showing the close association between a ring-shaped NB and a CB. This figure serves to illustrate the importance of serial-section electron microscopy to the current study. Without serial sectioning, an accurate account of NB distribution would not have been possible in this nucleus because Figure 6d is the only one of the four serial sections shown here that accurately depicts the presence and close association of both a CB and a ring-shaped NB, which together form a heterogeneous NB pair.

Only Coiled Bodies Contained p80-Coilin

Based on the observation that two major types of NBs (CBs and ring-shaped bodies) existed in estradiol-stimulated hepatocytes, we performed immunoelectron microscopy to determine which type stained with antibodies to p80-coilin. Figure 7 shows that CBs (Figure 7a), but not ring-shaped NBs (Figure 7b), stained for coilin by postembedding immunoelectron microscopy.

The Amount of p80-Coilin Increased in Response to Estradiol Stimulation

Considering that the numbers of CBs varied according to the administration of estradiol, peaking at 24–48 h and returning to near control levels after 4 wk, it was of considerable interest whether or not the levels of p80-coilin protein would also reflect these differences. Figure 8 (lanes 1–4) illustrates the results of Western blotting of equal numbers of nuclei from control, 48 h, continuous stimulation, and 4-wk post-injection samples. Levels of coilin were minimal in control and 4-wk samples (lanes 1 and 4, respectively) and increased in the 48 h and continuous stimulation lanes (lanes 2 and 3, respectively).

A second experiment (represented by Figure 8, lanes 5–7, and Figure 2) was designed to control for what appeared to be proteolysis and to examine earlier time

Figure 3. Electron microscopy of hepatocytes from livers of estrogen-treated roosters. (a) negative control; (b) 48 h post-injection; (c) continuous stimulation; (d) 4 wk post-injection. N, nucleoli; single arrowheads, ring-shaped nuclear bodies; double arrowheads, coiled bodies. Bar, 1 μ m.

points of stimulation. When a protease inhibitor cocktail (see MATERIALS AND METHODS for details) was used in the isolation buffer in place of phenylmethylsulfonyl fluoride alone, most of the lower molecular weight bands observed in lanes 1–4 disap-

peared, leaving just the 80 kDa band (arrowhead in Figure 8) for coilin. In this experiment (represented graphically in Figure 2), coilin was barely detectable in control nuclei (lane 5, Figure 8), increased to easily detected levels at 8 h after estradiol administration

Figure 4. Summary of various nuclear body profiles (types) observed in hepatocytes of estrogen-treated roosters. In general, three types of classic nuclear bodies were observed: simple nuclear bodies (a), ring-shaped nuclear bodies (b), and coiled bodies (l). Nuclear bodies were observed singly (a-d) or in pairs (e-l). Some ring-shaped nuclear bodies appeared to be filled with electron-dense material (c and d). Of the paired NBs, some consisted of pairs of ring-shaped NBs (e-h), some consisted of both a ring-shaped NB and a CB (i-k), and some were pairs of CBs (l). Bar, 0.25 μ m.

(lane 6), and greatly increased in amount in the 48 h time point (lane 7).

DISCUSSION

Nuclear Bodies in Hepatocytes of Estrogen-treated Roosters

The results presented in this paper extend, and in some aspects differ from, those reported by Brasch *et al.* (1985, 1989). The previous studies, based on electron microscopy and stereology, reported a gradual increase in NBs after injection of estradiol, up to 15–17 NBs/nucleus at peak induction. In contrast, we did not detect more than five CBs in any single whole-cell nucleus in the present study using immunofluorescence microscopy and p80-coilin staining. A number of explanations could account for these differences. First, it is likely that only a portion of the NBs detected

by Brasch *et al.* (1989) contained p80-coilin, whereas we detected CBs only. Second, the resolution of immunofluorescence microscopy utilized in our study may not have been adequate to detect some of the smaller CBs that would be easily detected by electron microscopy, resulting in our underestimation of smaller structures. Third, we arrived at CB numbers by direct light microscopic immunofluorescence observation, whereas Brasch *et al.* (1989) estimated NB numbers by statistical means, assuming a random distribution of NBs throughout the nucleus and from our immunofluorescence and serial-section electron microscopy observations, it was apparent that NBs were seldom found in the nuclear periphery.

By combining phase and immunofluorescence microscopy in the same image, we were able to conclude that a number of CBs were in contact with nucleoli at the 48-h time point. This relationship between nucleoli

Figure 5. Serial-section analysis of ring-shaped nuclear bodies. Two different sets of serial sections (a,b and c,d) revealed that ring-shaped nuclear body profiles (a and c) can appear as simple nuclear body profiles in subsequent serial sections (b and d). The serial-section series e-h demonstrates that some ring-shaped nuclear bodies are filled with electron-dense material of unknown origin. Bar, 0.25 μ m.

Figure 6. Serial-section analysis of a 48-h post-injection hepatocyte showing the close association between a ring-shaped nuclear body and a coiled body (arrowheads). Bar, 1 μm ; (inset) bar, 0.25 μm .

and CBs was not observed in previous studies (Brasch and Peters, 1985; Brasch *et al.*, 1989), possibly because of the lack of serial sectioning or because CBs are difficult to identify solely by electron microscopy. In the present study, then, immunofluorescence microscopy has a number of key advantages compared with electron microscopy, not the least of which is the ability to examine the nucleus as a whole and to analyze the distribution of a specific molecule and/or structure. On the other hand, immunofluorescence microscopy suffers from two major drawbacks that necessitated the use of both conventional electron microscopy and immunoelectron microscopy in the present study. First, immunofluorescence lacks the resolution of electron microscopy and may not detect very small structures. Second, with immunofluorescence microscopy the identity of the structure being labeled is often only assumed. Therefore, only a combined approach utilizing the features of both immunofluorescence and immunoelectron microscopy can overcome the inherent deficiencies of either technique when used alone.

In a previous study of CB distribution throughout the cell cycle in synchronized cultured cells (Andrade *et*

al., 1993), it was observed that the numbers of CBs varied with the stage of the cell cycle, peaking in late G_1 or S-phase, even though the total amount of coilin remained constant throughout the cell cycle. In our study, however, both the number of CBs and the amount of coilin protein increased in response to estradiol stimulation. At the other end of the scale, fewer CBs and decreased amounts of coilin were observed in the control and 4-wk samples compared with the elevated levels evident at 48 h and in continuously stimulated livers. The differences in p80-coilin protein expression observed in the hepatocytes of estrogen-treated roosters and the previous studies done with cultured cells (Andrade *et al.*, 1993) may be reconciled by taking into account that cultured cells are continuously growing and dividing (and in this sense they are continuously in an active state of proliferation), whereas the estradiol-treated hepatocytes begin as quiescent, differentiated cells that are activated by estradiol to increase in size and metabolic activity, and then eventually return to their former baseline level of activity without dividing. Thus, the demand for CBs is continuously high and the expression of p80-coilin may already be near maximal level in cycling cells,

Figure 7. Postembedding colloidal gold immunoelectron microscopy with anti-coilin on thin sections of Unicryl-embedded liver tissue from roosters 48 h after a single injection of estradiol. Coiled bodies (arrowhead and inset to a), but not ring-shaped bodies (arrowhead and inset to b), were labeled with antibodies to p80-coilin. Bar, 1 μm ; (inset) bar, 0.25 μm .

whereas CB and p80-coilin expression is transiently "up-regulated" from a minimal baseline to high levels in hormone-stimulated cells. This latter effect represents a primary estrogenic response involving new gene expression and subsequent "nuclear remodeling" manifested by an enlarged nucleolus, expanded ribonucleoprotein network, and increased numbers of NBs (Brasch and Peters, 1985; Brasch *et al.*, 1989; Brasch and Ochs, 1995).

Formation of Nuclear Bodies

At the present time, the process of formation of NBs remains enigmatic. In the estrogen-stimulated rooster hepatocyte model, the appearance of dumbbell-shaped pairs of identical NBs (homogeneous pairs) could imply a process of replication by division or "budding". Along these lines, we noted previously in cultured cells that as numbers of CBs increased in a particular nucleus, there was a corresponding decrease in their size (Brasch and Ochs, 1992; Andrade *et al.*, 1993). This same observation was also apparent for the immunofluorescence localization of p80-coilin in the 48-h time point after administration of a single injection of estradiol, in which we observed both pairs of CBs and additional smaller, but more numerous, CBs. Thus, our data suggest a number of intriguing possibilities to explain the origins and mechanism of CB proliferation. Some CBs could be derived from the nucleolus

and others may form *de novo* in the nucleoplasm (Andrade *et al.*, 1993; Carmo-Fonseca *et al.*, 1993). Once formed, CBs may multiply by replicative division (hence "dumbbell-like" structures) or by the conversion of one nuclear body type into another (perhaps as represented by the close association of NBs of two different types). However, the finding that only CBs contained p80-coilin (this paper) would argue against interconversion of one nuclear body type into another. We anticipate that additional serial-section electron microscopy at different time points will enable us to more accurately reconstruct the process of NB formation in this physiological animal model system.

The results of Western blotting (Figure 8) compared with the numbers of CBs/nucleus (Figure 2) were very interesting. As expected, the amount of coilin was greater in the 48 h and continuous stimulation samples compared with the controls. What was perhaps unexpected was the increase in coilin at 8 h, despite the fact that the average number of CBs/nucleus actually decreased from 1.4 in the controls to a value of 1.2 CBs/nucleus at the 8-h time point. Several explanations could account for this apparent discrepancy. Early in stimulation, the amount of coilin may increase without a corresponding increase in numbers of CBs/nucleus. The newly synthesized coilin may be present as "soluble" protein in the nucleoplasm or it may be added

Figure 8. Western blotting of isolated chicken liver hepatocyte nuclei with antibody to p80-coilin. Lane 1, control; lane 2, 48 h after a single injection of estradiol; lane 3, continuous injection with estradiol over a 3-wk period; lane 4, 4 wk after a single injection of estradiol. Lanes 5–7 represent a second experiment designed to examine an earlier time point and to address the problem of proteolysis. Lane 5, control; lane 6, 8 h after a single injection of estradiol; lane 7, 48 h after a single estradiol injection. For each experiment, equal numbers of nuclei were loaded in each lane. Arrowhead indicates the major band for p80-coilin.

to already existing CBs to increase their size, without an increase in number. The decrease at 8 h in average number of CBs/nucleus compared with the controls, 1.2 vs. 1.4, was apparently due to an increase at 8 h in nuclei with 1 vs. 2 CBs/nucleus. In control nuclei, 64% had one CB/nucleus and 24% had two CBs/nucleus whereas at 8 h, 85% of nuclei had one CB and only 10% had two CBs/nucleus. This increase in % of nuclei with one rather than two CBs at 8 h could be explained if CBs were to fuse together upon stimulation with estradiol. Such a fusion of CBs would be analogous to what occurs during nucleogenesis with the fusion of pre-nucleolar bodies after mitosis to form the interphase nucleolus at the site of active nucleolar organizer regions (Jiménez-García *et al.*, 1994). Therefore, the behavior of CBs is very similar to that of nucleoli, and a relationship between the two has already been noted by us and others (Ramón y Cajal, 1903; Raska *et al.*, 1990; Lafarga *et al.*, 1991; Brasch and Ochs, 1992; Malatesta *et al.*, 1994; Meier and Blobel, 1994; Ochs *et al.*, 1994; Wu *et al.*, 1994). Further experimentation will be necessary to confirm or deny these various possibilities but at the very least the data support a dynamic view of CBs (and other NBs as well), with an ability to increase or decrease in number depending upon the demands of the cell.

Function of Coiled Bodies and Relationship to Other Nuclear Structures

Many suggestions have been made regarding the putative function(s) of CBs (Raska *et al.*, 1991; Brasch and Ochs, 1992; Carmo-Fonseca *et al.*, 1992; Lamond and Carmo-Fonseca, 1993). Recent papers by Wu *et al.* (1993, 1994), Bauer *et al.* (1994), and Jiménez-García *et al.* (1994) have pointed out that CBs contain components involved in both pre-mRNA and pre-rRNA processing, suggesting that they may be sites for assembly, storage, or sorting of these components. In line with these speculations, a previous association has been noted between CBs and nucleoli (this paper and Ramón y Cajal, 1903; Raska *et al.*, 1990; Malatesta *et al.*, 1994; Meier and Blobel, 1994; Ochs *et al.*, 1994) and between CBs and the interchromatin granule regions (this paper and Ferreira *et al.*, 1994) that may serve as “splicing islands” or as storage sites for splicing components (Huang *et al.*, 1994). Our data are also compatible with an assembly, storage, or “sorting” function for CBs in the pathway leading to synthesis of either pre-mRNA and/or pre-rRNA. More recent papers indicating that CBs have no poly(A)⁺ RNA (Matera and Ward, 1993; Huang *et al.*, 1994) and that they reform relatively late after mitosis at a time when active pre-mRNA synthesis and splicing are already occurring (Ferreira *et al.*, 1994), would further argue against a direct role for CBs in pol II transcription or splicing events.

Although CBs have no detectable DNA (Raska *et al.*, 1991; Thiry, 1994), their reported increase during the cell cycle in late G₁ or S-phase (Andrade *et al.*, 1993) could be interpreted to mean that they participate in DNA replication. However, our results showing an increase in CBs in response to estradiol in a model system in which no DNA replication occurs, would seem to all but rule out a function for CBs in DNA replicative events.

Nuclear Bodies and Hormones

Various types of NBs are a prominent feature of hormone-responsive cells (Bouteille *et al.*, 1974; Padykula and Clark, 1981; Vagner-Capodano *et al.*, 1982; Brasch and Ochs, 1992, 1995). In particular, CBs have been observed in cells responsive to estrogen (this paper; Brasch and Ochs, 1992; Ochs *et al.*, 1994) and thyrotropin (Andrade *et al.*, 1993). The other major type of NB encountered in our study was the ring-shaped NB. It, too, has been observed in estrogen-responsive cells and tissues (this paper and Padykula and Clark, 1981; Brasch and Peters, 1985; Brasch *et al.*, 1989; Brasch and Ochs, 1992). Interestingly, a ring-shaped NB of similar size and description, and containing the PML protein, has been recently described and is responsive to the effects of the hormone-like morphogen, retinoic acid, in promoting differentiation of acute promyelocytic

leukemia cells into mature granulocytes (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). However, antibodies to these, and similar NBs (Ascoli and Maul, 1991), would not react with chicken hepatocyte nuclei. Whether the PML-containing ring-shaped NBs referred to above are similar to those observed in estrogen-sensitive cells must await definitive antibody labeling studies at the EM level.

Like the steroid hormone family, retinoic acid exerts its effects in the nucleus by means of nuclear receptors that in turn directly affect transcription of specific genes by means of hormone-responsive elements (Evans, 1988). Therefore, as evidenced in hormone-responsive cells, NBs as a group (including CBs) may play a key role as signal-transducing structures in the nucleus, linking the action of hormones to ensuing transcriptional or post-transcriptional pathways.

Implications for Environmental Estrogens

Much interest has focused recently on the realization that certain environmental organochlorines, including DDT and its derivatives, can mimic the effects of estrogen (the so-called "environmental estrogens") and may, in fact, contribute to the rising incidence of female breast cancer (Bulger and Kupfer, 1983; Robison *et al.*, 1985; Soto *et al.*, 1991; Falck *et al.*, 1992; Wolff *et al.*, 1993) and the reduction in male sperm count and fertility (Carlsen *et al.*, 1992). The observation that a female hormone like estrogen can exert a physiological response in juvenile male animals (this paper; Brasch and Peters, 1985; Brasch *et al.*, 1989; Colborn *et al.*, 1993) also has profound implications for the possible harmful effects of environmental estrogen-like compounds on the "feminization" and sexual development of young males (Colborn *et al.*, 1993; Sharpe and Skakkebaek, 1993), and possible long-term effects on the fertility and sterility of adults as well (Sharpe and Skakkebaek, 1993). How damaging these environmental estrogens are to the reproductive status of humans is an important topic for future investigation, and the vitellogenic response in young roosters may provide a useful animal model to assay for estrogenic activity.

The present study is among the first to document the dynamic behavior of coiled bodies in an *in vivo* animal model that is based upon a physiological response to estrogen. Most of the previous studies on CBs have been confined to cultured cell systems. As in any rapidly advancing field of study, more questions than answers have been raised about this previously ignored organelle (Brasch and Ochs, 1992; Lamond and Carmo-Fonseca, 1993). Whether CBs occur universally, the circumstances under which they form, and their precise functional role(s), will all be topics of continuing interest in our understanding of this specialized nuclear domain (Brasch and Ochs, 1995).

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REFERENCES

- Andrade, L.E.C., Chan, E.K.L., Raska, I., Peebles, C.L., Roos, G., and Tan, E.M. (1991). Human autoantibody to a novel protein of the nuclear coiled body: immunological characterization and cDNA cloning of p80-coilin. *J. Exp. Med.* 173, 1407-1419.
- Andrade, L.E.C., Tan, E.M., and Chan, E.K.L. (1993). Immunocytochemical analysis of the coiled body in the cell cycle and during cell proliferation. *Proc. Natl. Acad. Sci. USA* 90, 1947-1951.
- Ascoli, C.A., and Maul, G.G. (1991). Identification of a novel nuclear domain. *J. Cell Biol.* 112, 785-795.
- Bauer, D.W., Murphy, C., Wu, Z., Wu, C.-H.H., and Gall, J.G. (1994). *In vitro* assembly of coiled bodies in *Xenopus* egg extract. *Mol. Biol. Cell* 5, 633-644.
- Bouteille, M., Laval, M., and Dupuy-Coin, A.M. (1974). Localization of nuclear functions as revealed by ultrastructural autoradiography and cytochemistry. In: *The Cell Nucleus*, vol. 1, ed. H. Busch, New York: Academic Press, 5-64.
- Brasch, K. (1990). Nuclear protein modifications in vitellogenic rooster liver. *Cell. Mol. Biol.* 36, 659-671.
- Brasch, K., Harrington, S., and Blake, H. (1989). Isolation and analysis of nuclear bodies from estrogen-stimulated chick liver. *Exp. Cell Res.* 182, 425-435.
- Brasch, K., and Ochs, R.L. (1992). Nuclear bodies (NBs): a newly "rediscovered" organelle. *Exp. Cell Res.* 202, 211-223.
- Brasch, K., and Ochs, R.L. (1995). Nuclear remodeling in response to steroid hormone action. *Int. Rev. Cytol.* 159, 161-194.
- Brasch, K., and Peters, K.E. (1985). Nuclear protein, matrix and structural changes in rooster liver after estrogenic induction of vitellogenesis. *Biol. Cell* 54, 109-121.
- Bulger, W.H., and Kupfer, D. (1983). Estrogenic action of DDT analogs. *Am. J. Ind. Med.* 4, 163-173.
- Burch, J.B.E., and Evans, M.I. (1986). Chromatin structural transitions and the phenomenon of vitellogenin gene memory in chickens. *Mol. Cell. Biol.* 6, 1886-1893.
- Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N.E. (1992). Evidence for decreasing quality of semen during past 50 years. *Br. Med. J.* 305, 609-613.
- Carmo-Fonseca, M., Ferreira, J., and Lamond, A.I. (1993). Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis: evidence that the coiled body is a kinetic nuclear structure. *J. Cell Biol.* 120, 841-852.
- Carmo-Fonseca, M., Pepperkok, R., Carvalho, M.T., and Lamond, A.I. (1992). Transcription-dependent colocalization of the U1, U2, U4/U6 and U5 snRNPs in coiled bodies. *J. Cell Biol.* 117, 1-14.
- Chan, E.K.L., and Pollard, K.M. (1992). Autoantibodies to ribonucleoprotein particles by immunoblotting. In: *Manual of Clinical Laboratory Immunology*, 4th ed., ed. N.R. Rose, E.C. de Macario, J.L. Fahey, H. Friedman, and G.M. Penn, Washington, DC: American Society for Microbiology, 755-762.
- Colborn, T., vom Saal, F.S., and Soto, A.M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* 101, 378-384.
- Dyck, J.A., Maul, G.G., Miller, W.H., Jr., Chen, J.D., Kakizuka, A., and Evans, R.M. (1994). A novel macromolecular structure is the

- target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* 76, 333–343.
- Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* 240, 889–895.
- Evans, M.L., O'Malley, P.J., Krust, A., and Burch, J.B.E. (1987). Developmental regulation of the estrogen receptor and the estrogen responsiveness of five yolk protein genes in the avian liver. *Proc. Natl. Acad. Sci. USA* 84, 8493–8497.
- Falck, F., Jr., Ricci, A., Jr., Wolff, M.S., Godbold, J., and Deckers, P. (1992). Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. *Arch. Environ. Health* 47, 143–146.
- Ferreira, J.A., Carmo-Fonseca, M., and Lamond, A.I. (1994). Differential interaction of splicing snRNPs with coiled bodies and interchromatin granules during mitosis and assembly of daughter cell nuclei. *J. Cell Biol.* 126, 11–23.
- Huang, S., Deerinck, T.J., Ellisman, M.H., and Spector, D.L. (1994). In vivo analysis of the stability and transport of nuclear poly(A)⁺ RNA. *J. Cell Biol.* 126, 877–899.
- Jiménez-García, L.F., Segura-Valdez, M. de L., Ochs, R.L., Rothblum, L.I., Hannan, R., and Spector, D.L. (1994). Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. *Mol. Biol. Cell* 5, 955–966.
- Jost, J.P., Moncharmont, B., Jiricny, J., Salvz, H., and Hertner, T. (1986). In vitro secondary activation (memory effect) of avian vitellogenin II gene in isolated liver nuclei. *Proc. Natl. Acad. Sci. USA* 83, 43–47.
- Koken, M.H.M., Puvion-Dutilleul, F., Guillemin, M.C., Viron, A., Linares-Cruz, G., Stuurman, N., de Jong, L., Szosteki, C., Calvo, F., Chomienne, C., Degos, L., Puvion, E., and de Thé, H. (1994). The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *EMBO J.* 13, 1073–1083.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lafarga, M., Andres, M.A., Berciano, M.T., and Maquiera, E. (1991). Organization of nucleoli and nuclear bodies in osmotically stimulated supraoptic neurons of the rat. *J. Comp. Neurol.* 308, 329–339.
- Lamond, A.I., and Carmo-Fonseca, M. (1993). The coiled body. *Trends Cell Biol.* 3, 198–204.
- Malatesta, M., Zancanaro, C., Martin, T.E., Chan, E.K.L., Amalric, F., Lüthmann, R., Vogel, P., and Fakan, S. (1994). Is the coiled body involved in nucleolar functions? *Exp. Cell Res.* 211, 415–419.
- Matera, A.G., and Ward, D.C. (1993). Nucleoplasmic organization of small nuclear ribonucleoproteins in cultured cells. *J. Cell Biol.* 121, 715–727.
- Meier, U.T., and Blobel, G. (1994). NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *J. Cell Biol.* 127, 1505–1514.
- Ochs, R.L., Stein, T.W., Jr., and Tan, E.M. (1994). Coiled bodies in the nucleolus of breast cancer cells. *J. Cell Sci.* 107, 385–399.
- Padykula, H.A., and Clark, J.H. (1981). Nuclear bodies as functional indicators in the target cells of sex steroid hormones. In: *The Cell Nucleus*, vol. 9, ed. H. Busch, New York: Academic Press, 309–339.
- Ramón y Cajal, S. (1903). Un sencillo metodo de coloracion del reticulo protoplasmico y sus efectos en los diversos organos nerviosos. *Trab. Lab. Invest. Biol.* 2, 129–221.
- Raska, I., Andrade, L.E.C., Ochs, R.L., Chan, E.K.L., Chang, C.M., Roos, G., and Tan, E.M. (1991). Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. *Exp. Cell Res.* 195, 27–37.
- Raska, I., Ochs, R.L., Andrade, L.E.C., Chan, E.K.L., Burlingame, R., Peebles, C., Gruol, D., and Tan, E.M. (1990). Association between the nucleolus and the coiled body. *J. Struct. Biol.* 104, 120–127.
- Robison, A.K., Sirbasku, D.A., and Stancel, G.M. (1985). DDT supports the growth of an estrogen-responsive tumor. *Toxicol. Lett.* 27, 109–113.
- Shapiro, D. (1982). Steroid hormone regulation of vitellogenin gene expression. *CRC Crit. Rev. Biochem.* 12, 187–203.
- Sharpe, R.M., and Skakkebaek, N.E. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 341, 1392–1395.
- Soto, A.M., Justicia, H., Wray, J.W., and Sonnenschein, C. (1991). *p*-Nonyl-phenol: an estrogenic xenobiotic released from “modified” polystyrene. *Environ. Health Perspect.* 92, 167–173.
- Thiry, M. (1994). Cytochemical and immunocytochemical study of coiled bodies in different cultured cell lines. *Chromosoma* 103, 268–276.
- Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.
- Vagner-Capodano, A.M., Bouteille, M., Stahl, A., and Lissitzky, S. (1982). Nucleolar ribonucleoprotein release in the nucleoplasm as nuclear bodies in cultured thyrotropin-stimulated thyroid cells: autoradiographic kinetics. *J. Ultrastruct. Res.* 78, 13–25.
- Weis, K., Rambaudo, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A., and Dejean, A. (1994). Retinoic acid regulates aberrant nuclear localization of PML-RAR in acute promyelocytic leukemia cells. *Cell* 76, 345–356.
- Wolff, M.S., Toniolo, P.G., Lee, E.W., Rivera, M., and Dubin, N. (1993). Blood levels of organochlorine residues and risk of breast cancer. *J. Natl. Cancer Inst.* 85, 648–652.
- Wu, Z., Murphy, C., and Gall, J.G. (1994). Human p80-coilin is targeted to sphere organelles in the amphibian germinal vesicle. *Mol. Biol. Cell* 5, 1119–1127.
- Wu, Z., Murphy, C., Wu, C.-H.H., Tsvetkov, A., and Gall, J.G. (1993). Snurposomes and coiled bodies. *Cold Spring Harbor Symp. Quant. Biol.* 58, 747–754.