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c-*erbA* Encodes Multiple Proteins in Chicken Erythroid Cells

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To identify and characterize the proteins encoded by the *erbA* proto-oncogene, we expressed the C-terminal region of v-*erbA* in a bacterial *trpE* expression vector system and used the fusion protein to prepare antiserum. The anti-*trp-erbA* serum recognized the P75$^{gag\text{-}erbA}$ protein encoded by avian erythroblastosis virus and specifically precipitated six highly related proteins ranging in size from 27 to 46 kilodaltons from chicken embryonic erythroid cells. In vitro translation of a chicken *erbA* cDNA produced essentially the same pattern of proteins. Partial proteolytic maps and antigenicity and kinetic analyses of the in vivo and in vitro proteins indicated that they are related and that the multiple bands are likely to arise from internal initiations within c-*erbA* to generate a nested set of proteins. All of the c-*erbA* proteins are predominantly associated with chicken erythroblast nuclei. However, Nonidet P-40 treatment resulted in extraction of the three smaller proteins, whereas the larger proteins were retained. During differentiation of erythroid cells in chicken embryos, we found maximal levels of c-*erbA* protein synthesis at days 7 to 8 of embryogenesis. By contrast, c-*erbA* mRNA levels remained essentially constant from days 5 to 12. Together, our results indicate that posttranscriptional or translational mechanisms are involved in regulation of c-*erbA* expression and in the complexity of its protein products.

The *erbA* gene was first identified as one of two oncogenes transduced by avian erythroblastosis virus (AEV), an acute leukemia virus which induces erythroid leukemia in chickens and transforms both erythroid and fibroblast cells in vitro (11, 20, 31). Whereas v-*erbA* was shown to lack transforming activity of its own (30), it was nonetheless demonstrated to cooperate in the transformation of erythroid cells by v-*erbB*, the second oncogene encoded by AEV and a truncated homolog of the epidermal growth factor receptor (9, 12, 39). v-*erbA* appeared to function by suppressing the spontaneous differentiation which occurred in erythroblasts expressing *erbB* alone (8, 11, 20). Similar effects were observed with v-*erbA* and other oncogenes (*src*, *fps*, *sea*, and *ras*) in erythroid cells (20). Further studies showed that v-*erbA* allowed erythroblasts to proliferate under an unusually broad range of pHs and salt concentrations (8, 11, 20), suggesting that v-*erbA* could exert profound effects on cell behavior.

Initial characterization of a v-*erbA* molecular clone indicated that the protein encoded by this gene had the same general organization as, but little sequence homology with, the intracellular receptors for steroid hormones (23, 36). Recent isolation of cDNAs corresponding to human, avian, and rat homologs of v-*erbA* has allowed partial characterization of the c-*erbA* protein produced by cell-free translation of in vitro transcripts generated from the cDNAs. These studies have shown that the in vitro translation products of c-*erbA* bind the thyroid hormones 3,5,3′-triiodothyronine (T3) and thyroxine (T4) with high specificity and affinity, thus demonstrating that c-*erbA* encodes a thyroid hormone receptor (29, 33, 37). Thyroid hormone receptor–c-*erbA* cDNA clones have been isolated from cDNA libraries from several species and tissues (2, 29, 33, 37). Most of the divergence between the cDNAs from different species and different tissues of the same species occur at the nontranslated 5′ and 3′ ends of the mRNA. It is possible that the nontranslated regions of the *erbA* mRNAs allow for tissue-specific regulation. Moreover, the mRNAs may be transcribed from different genes, since at least three *erbA* loci exist in rats and humans (19).

It is not clear how the thyroid hormone receptor acts, but there is growing evidence that it may regulate specific gene transcription (34). The receptor has been shown to bind to a 16-base-pair sequence 5′ of the growth hormone gene in rat pituitary tumor cells (13). This sequence conferred T3-inducible transcription on a reporter gene, suggesting that it is a T3 receptor-binding regulatory element. Moreover, the oncogenic form of the thyroid hormone receptor, the v-*erbA* protein, may act at the transcriptional level. In AEV-transformed chicken erythroid cells, v-*erbA* appears to suppress transcription of the anion transporter (band 3) gene, an event which is linked to the terminal differentiation block in these cells (40).

Characterization of c-*erbA* has been performed with in vitro translated *erbA* protein or protein produced in fibroblasts transfected with *erbA* cDNA clones (2, 13, 29, 33, 37). In this paper, we report an analysis of the structure and expression of endogenous c-*erbA* protein. Our evidence suggests regulation of expression at the posttranscriptional and translational levels.

MATERIALS AND METHODS

Cells. HD3 cells (a gift of D. Engel and M. Hayman) are chick erythroblasts infected with tsAEV34, a mutant of AEV which is temperature sensitive for transformation (3). These cells were maintained at the permissive temperature of 35°C. Normal blood cells were isolated from 5- to 12-day-old chicken embryos. The cells were collected in phosphate-buffered saline, washed several times to remove yolk and serum, and then either labeled with [^{35}S]methionine for protein analysis or lysed in guanidinium isothiocyanate for RNA extraction. Since embryonic blood contains mainly erythroid cells (24), it was not necessary to purify the blood cell population obtained from chicken embryos further.

Preparation of polyclonal antiserum. The bacterial expression vector pTΔE was constructed by inserting the 891-base-pair v-*erbA* *Eco*RV-*Stu*I fragment from pAEV-11 (35) into

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the *Sma*I and *Hin*dIII polylinker sites of pATH10 (kindly provided by T. J. Koerner) after addition of *Hin*dIII linkers at the *Stu*I site. The *trpE-ΔerbA* fusion protein from pTΔE is composed of 323 amino acids derived from *trpE* at the N terminus, followed by 6 amino acids from the polylinker and 219 amino acids from v-*erbA*. Induction of expression of the *trpE-ΔerbA* fusion protein was done in M9CA medium (25) containing 5 μg of indoleacrylic acid for 4 h at 37°C with *Escherichia coli* HB101 as the host bacterium. The insoluble protein fraction was prepared as described by Kleid et al. (21). After solubilization in cracking buffer, the fraction containing the *trpE-ΔerbA* fusion protein was run on a preparative sodium dodecyl sulfate (SDS)-10% polyacrylamide gel. Approximately 500 μg of eluted, SDS-free *trpE-ΔerbA* protein was mixed with Freund complete adjuvant and injected into a 1-year-old New Zealand White rabbit which had previously been bled to provide preimmune serum. Booster injections were repeated at 4-week intervals and contained 250 μg of *trpE-ΔerbA* protein in incomplete Freund adjuvant. Immune serum was obtained by ear bleeds 10 days after each boost.

For affinity purification of anti-*trp-erbA* serum, the electroeluted antigen was bound to Reactigel (HW-65F; Pierce Chemical Co.) at 2 mg/ml of gel in 0.1 M sodium borate buffer (pH 9.5), as suggested by the manufacturer. Ammonium sulfate-precipitated, dialyzed antibodies were bound to the affinity gel by shaking at room temperature overnight. The specific antibodies were eluted with 1 M glycine (pH 2.5) and dialyzed against phosphate-buffered saline. The sera obtained from the two rabbits injected with *trpE-ΔerbA* protein were designated anti-*trp-erbA* 4019 and anti-*trp-erbA* 5794, respectively.

Immunoprecipitation and gel electrophoresis. One day after passage, HD3 cells were labeled with [^{35}S]methionine (10^7 cells per ml, 100 μCi/ml) for 3 to 4 h at 35°C in growth medium lacking methionine. Blood cells were collected from chicken embryos, washed several times with 7 mM Na_2HPO_4–3 mM KH_2PO_4–150 mM NaCl (pH 7.1) to remove egg yolk, and then labeled with [^{35}S]methionine (2×10^7 cells per ml, 200 μCi/ml) for 3 to 4 h at 37°C. Lysis of the cells and immunoprecipitation were performed as previously described (1, 15), with the following modifications. Iodoacetamide was omitted from the lysis buffer, and lysates from erythroid cells were precleared three times with Formalin-fixed *Staphylococcus aureus*. One microgram of affinity-purified anti-*trp-erbA* antibody was used per 100 μl of HD3 lysate (10^7 cells per ml) or 1 ml of erythroid cell lysate (2×10^7 cells per ml). For competitive inhibition (i.e., blocking), 1 μg of anti-*trp-erbA* antibody was incubated with 4 μg of gel-purified *trpE-ΔerbA* protein in 100 μl of lysis buffer for 30 min at 4°C before addition to the lysate.

For V8 digestions, immunoprecipitated *erbA* proteins were separated on an SDS-10% polyacrylamide gel as described above. The gels were dried and exposed to X-ray film for 1 to 3 days. The bands of interest were cut out, and digestion with V8 protease was performed in the gel slices as described by Cleveland et al. (7). The peptides were separated by SDS-20% polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography as described above.

Subcellular fractionation. Labeled HD3 cells or blood cells from chicken embryos were washed once in reticulocyte standard buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$; 38), suspended in the same buffer, and then either disrupted by Dounce homogenization or lysed in the presence of 0.5% Nonidet P-40. The nuclei were recovered by centrifugation at 800 × *g* for 2 min. The nuclear pellet was lysed in 1 ml of lysis buffer (15). The solution was sonicated and centrifuged at 12,000 × *g* for 10 min to remove debris. The cytoplasmic fraction was adjusted to 0.5% Nonidet P-40, 0.5% deoxycholate, and 0.5% SDS, sonicated, and centrifuged as described above to remove debris. Each fraction was analyzed by immunoprecipitation and SDS-PAGE as described above.

RNA isolation and analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate procedure as described by Chirgwin et al. (6). The relative amount of *erbA* transcripts was determined by S1 analysis. For this purpose, 5′ and 3′ *erbA* probes from pF1Δ (29) were used. For the 5′ probe, the plasmid was linearized with *Hin*dIII, labeled by replacement synthesis with T4 DNA polymerase as described by Pelham (27), and then digested with *Eco*RV. For the 3′ probe, pF1Δ was digested with *Eco*RV, labeled with T4 DNA polymerase, and finally digested with *Eco*RI. The probe was mixed with 10 μg of total RNA, and hybridization was performed at 53°C. Buffers for hybridization and S1 digestion were as described by Pelham (27). The hybrids were analyzed on 3.5% sequencing gels.

In vitro transcription and in vitro translation. For in vitro transcription, plasmid pF1Δ was linearized with *Eco*RI and transcribed with T7 RNA polymerase (200 U/ml; Boehringer Mannheim Biochemicals) as previously described. In vitro transcribed RNA (0.5 to 1 μg) was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate containing [^{35}S]methionine (New England Nuclear Corp.). For immunoprecipitation, 5 μl of the translation reaction was diluted in 1 ml of lysis buffer containing 0.5 mM methionine.

RESULTS

Specificity of anti-*trp-erbA* antibodies. To produce antibodies to the *erbA* protein, a *trpE*-based bacterial expression vector (26) was constructed comprising the 5′ half of the bacterial *trpE* coding region linked to an *Eco*RV-*Stu*I 891-base-pair fragment from pAEV11 (Fig. 1A). This vector produces the *trpE* protein fused to the 219 C-terminal amino acids of v-*erbA*.

The specificity of the rabbit antiserum (designated anti-*trp-erbA*) raised against the *trpE-ΔerbA* polypeptide was tested in radioimmunoprecipitations from detergent lysates of [^{35}S]methionine-labeled HD3 and a quail cell line coinfected with MC29 and avian leukosis virus (ALV). HD3 is a chicken erythroblast cell line derived by infection with the temperature-sensitive mutant AEV34 (3). It produces the 75-kilodalton (kDa) *gag-erbA* protein (17). MC29 ALV-infected quail cells contain $P110^{gag\text{-}myc}$ (4) and the ALV *gag*-related proteins Pr76 and Pr180 (10). Two anti-*trp-erbA* antisera precipitate only $P75^{gag\text{-}erbA}$, whereas all of the *gag*-related proteins ($P75^{gag\text{-}erbA}$, $P110^{gag\text{-}myc}$, $Pr76^{gag}$, and $Pr180^{gag\text{-}pol}$) were precipitated with anti-*gag* serum (Fig. 1B). Preincubation of the antiserum with *trpE-ΔerbA* protein blocked immunoprecipitation of $P75^{gag\text{-}erbA}$ (see Fig. 6). This indicates that the antiserum raised against *trpE-ΔerbA* does not react with *gag* proteins and recognizes the *erbA* portion of $P75^{gag\text{-}erbA}$.

c-*erbA* in vitro. To determine whether anti-*trp-erbA* also recognizes c-*erbA* protein, we tested its reactivity with in vitro translation products generated from a c-*erbA* cDNA clone, pF1Δ (29). In vitro translation of the mRNA transcribed from pF1Δ in a rabbit reticulocyte lysate produced proteins of approximately 46, 44, 40, 30, 28, 27, and 24.5 kDa (Fig. 2, lane 2). All of these, with the exception of the 28- and 24.5-kDa proteins, were recognized by anti-*trp-erbA* anti-

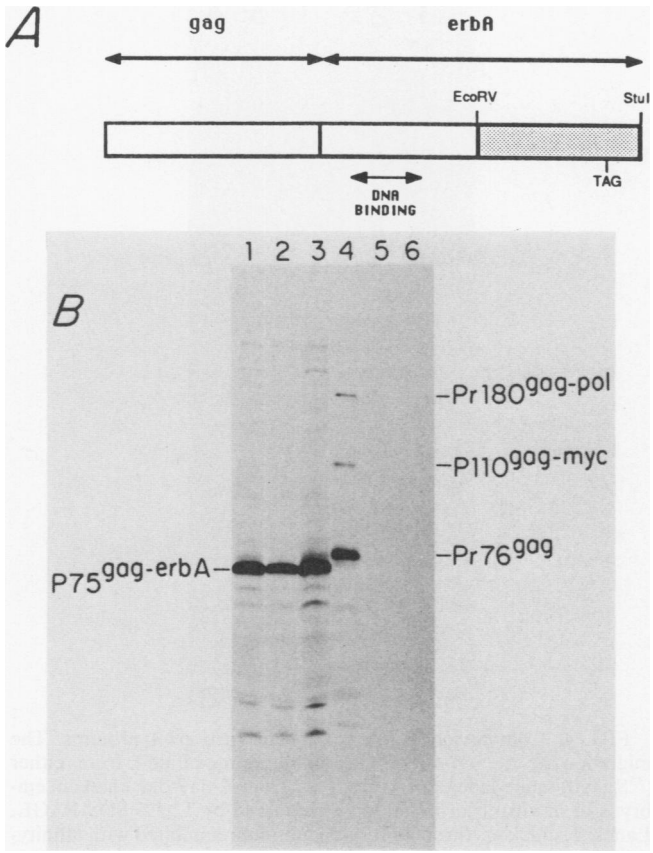

FIG. 1. Specificity of anti-*trp-erbA* serum. (A) $P75^{gag\text{-}erbA}$; the v-*erbA* portion represented by the hatched box was linked to the bacterial *trpE* protein and the gel-purified fusion protein used to raise anti-*trp-erbA* serum. (B) $P75^{gag\text{-}erbA}$ and $Pr76^{gag}$ were immunoprecipitated from [^{35}S]methionine-labeled HD3 and MC29 ALV-infected quail cells, respectively, by using crude anti-*trp-erbA* and anti-*gag* sera, and the immunoprecipitated proteins were separated by 10% SDS-PAGE as described in Materials and Methods. Lanes: 1 to 3, HD3 lysates; 4 to 6, ALV-infected quail cell Q8 (MC29) lysates. Antisera: anti-*gag*, lanes 1 and 4; anti-*trp-erbA* 4019, lanes 2 and 5; anti-*trp-erbA* 5794, lanes 3 and 6.

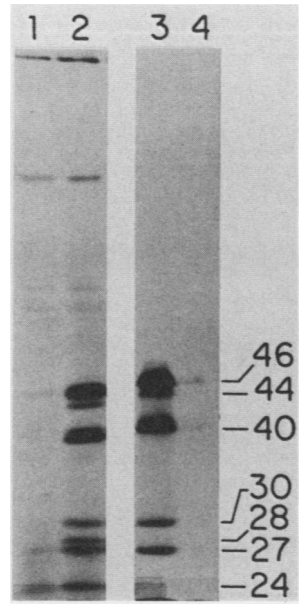

FIG. 2. In vitro translation of transcripts from chicken cDNA clone pF1Δ and immunoprecipitation of the protein products with crude anti-*trp-erbA* 5794. RNA transcribed from pF1Δ was translated in a rabbit reticulocyte lysate as described in Materials and Methods. The total or immunoprecipitated protein products were separated by 10% SDS-PAGE. Lanes: 1, no RNA; 2, total protein translated from *erbA* RNA; 3, immunoprecipitation with crude anti-*trp-erbA* 5794; 4, immunoprecipitation after incubation of the antibody with a 10-fold molar excess of gel-purified *trpE-ΔerbA* protein. The numbers on the right indicate molecular weight (10^3).

body (Fig. 2, lane 3). Precipitation of the c-*erbA* translation products by anti-*trp-erbA* was blocked by preincubation of the antiserum with excess *trpE-ΔerbA* protein, indicating that recognition of the proteins by the antibodies occurs through determinants shared with the fusion protein (Fig. 2, lanes 3 and 4). Since the antibody was raised against the C-terminal half of v-*erbA*, its failure to precipitate the 28- and the 24.5-kDa proteins suggests that they consist of only the N-terminal portion of c-*erbA*.

One result of the in vitro translation of pF1Δ was the fact that multiple polypeptides were produced in significant amounts. A similarly complex pattern of c-*erbA* in vitro products had been noted previously by Sap et al. (29). To determine whether the multiple proteins were related, we digested the individual bands with V8 protease and separated the partial proteolytic fragments on a high-percentage polyacrylamide gel. All of the proteins synthesized in vitro share two diagnostic peptides of 14 and 12.5 kDa (Fig. 3). In addition, there are peptides unique to each band. That the multiple proteins were not generated by posttranslational modifications was shown by pulse-chase experiments which failed to demonstrate any precursor-product relationship between the bands (data not shown). Our results from in vitro translation and immunoprecipitation experiments showed that the pF1Δ c-*erbA* cDNA-encoded proteins are likely to represent a C-terminally nested set.

Identification of c-*erbA* proteins in vivo. We chose chicken erythroid cells in which to examine the c-*erbA* protein in vivo because these cells have been reported to have the highest levels of *erbA* transcripts (18). We used circulating blood of chicken embryos (obtained by vein puncture), which contain almost exclusively erythrocytes and a few (<1%) thrombocytes (24). Erythrocytes from chicken embryos were labeled with [^{35}S]methionine, lysed as described in Materials and Methods, and immunoprecipitated with affinity-purified anti-*trp-erbA* antibodies. Essentially the same pattern of *erbA* proteins was obtained from erythrocytes and in vitro translated cDNA, with the exception of an additional 33-kDa band in the erythrocytes (Fig. 4). Immunoprecipitation of all of the protein bands from chicken erythrocytes could be blocked by preincubation of the antibody with the purified *trpE-ΔerbA* fusion protein (Fig. 4). We also observed a protein of approximately 150 kDa in chicken erythrocytes (see Fig. 6 and 8). Precipitation of the 150-kDa protein was blocked by incubation of anti-*trp-erbA* with *trpE-ΔerbA* fusion protein. This protein is likely either to possess *erbA*-related determinants or to be associated with a c-*erbA* protein(s).

The comigration of the c-*erbA* cDNA cell-free translation products with the proteins specifically immunoprecipitated from erythrocytes with anti-*trp-erbA* strongly suggests that the in vivo proteins are encoded by c-*erbA*. To further document this relationship, we compared the V8 protease digestion products of the in vivo and in vitro synthesized proteins. The major V8 digestion products of the 46-, 44-,

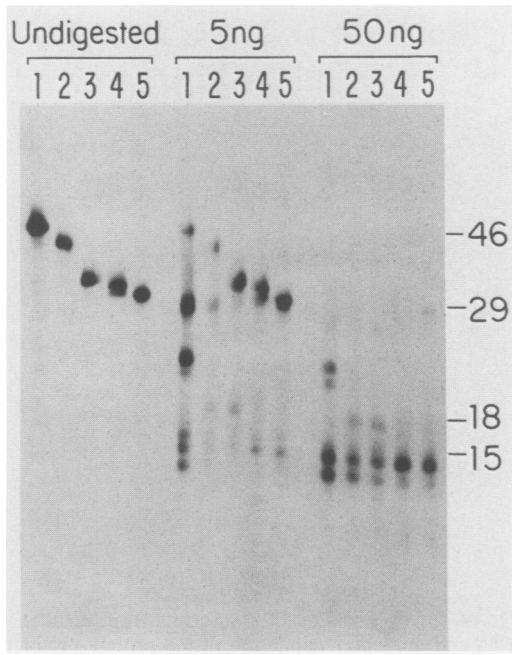

FIG. 3. Digestion of in vitro translated *erbA* proteins with V8 protease. The digestions were done as described in Materials and Methods, and the peptides were separated by 20% SDS-PAGE. Lanes: 1, 46-kDa protein; 2, 40-kDa protein; 3, 30-kDa protein; 4, 28-kDa protein; 5, 27-kDa protein. The numbers on the right represent molecular weight markers (10^3).

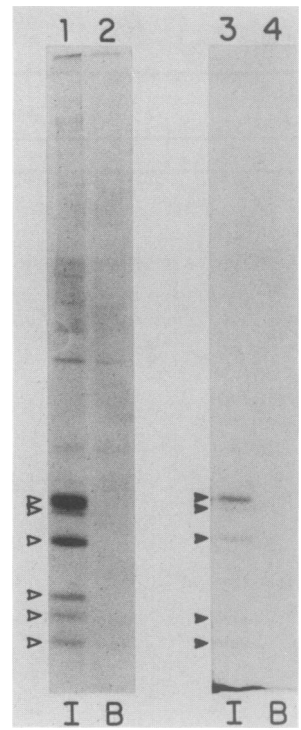

FIG. 4. Comparison of in vivo and in vitro *erbA* proteins. The migration of the *erbA* proteins immunoprecipitated from either [^{35}S]methionine-labeled erythrocytes from 7-day-old chicken embryos or in vitro translation were compared by 12.5% SDS-PAGE. Lanes: 1, chick erythrocyte lysate immunoprecipitated with affinity-purified anti-*trp-erbA* 5794; 2, chick erythrocyte lysate immunoprecipitated with anti-*trp-erbA* 5794 blocked with *trpE-ΔerbA*; 3, in vitro translated c-*erbA* proteins immunoprecipitated with affinity-purified anti-*trp-erbA* 5794; 4, in vitro translated c-*erbA* proteins immunoprecipitated with anti-*trp-erbA* 5794 blocked with *trpE-ΔerbA*. Open arrowheads indicate in vivo *erbA*-specific proteins, and filled arrowheads indicate in vitro *erbA*-specific proteins. I, Immunoprecipitation; B, immunoprecipitation with antibody preincubated with purified *trpE-ΔerbA* protein.

and 40-kDa proteins are related to each other and are the same for the in vivo and in vitro generated proteins (Fig. 5). The 27- to 33-kDa proteins were obtained from blood cells in amounts too low for us to be able to use them for protease digestion.

Subcellular localization of c-*erbA* protein. To determine the subcellular localization of the endogenous *erbA* proteins precipitated with anti-*trp-erbA*, we divided the labeled cells into cytoplasmic and nuclear fractions by either Dounce homogenization in the absence of detergents or lysis in the presence of 0.5% Nonidet P-40 (see Materials and Methods). The localization of v-*erbA* was not affected by the two different methods of cell disruption (data not shown). More than half of the v-*erbA* was found in the cytoplasm; the rest was tightly bound in the nucleus (Fig. 6). A different result was observed with c-*erbA* in blood cells. After the cells were dounced, all of the *erbA* proteins were found in the nuclei, although a fraction of the 27- to 33-kDa proteins could be detected in the cytoplasm (Fig. 6, lanes 5 to 8). In the presence of Nonidet P-40 however, the 27- to 33-kDa bands were found exclusively in the cytoplasmic fraction, whereas the 40- to 46-kDa bands remained in the nucleus. We noted that low-molecular-weight background proteins were retained in nuclei under our extraction conditions, suggesting that the *erbA* proteins are not extracted solely on the basis of size. This difference in extractability may be due to sequence differences and may reflect different functional properties.

***erbA* expression in avian erythropoiesis.** In chicken embryos, mature erythrocytes of the primitive lineage can be observed at around day 5 of embryogenesis. Between days 6 and 7, erythroblasts of the first definitive lineage begin proliferating (24). By day 12, the erythrocytes of the definitive lineage are fully differentiated (14). Since v-*erbA* has been shown to affect erythroid differentiation negatively (11, 40), it seemed possible that c-*erbA* also plays a role in differentiation. Hence, we attempted to ascertain whether the levels of c-*erbA* mRNA and protein were modulated during early development of erythrocytes. Blood cells from 5- to 12-day-old chicken embryos were isolated. A portion of the cells was labeled with [^{35}S]methionine, and total RNA was extracted from the rest. *erbA* mRNA was analyzed by S1 analysis after total RNA levels were normalized from the different time points. During the time interval tested, total RNA levels per cell dropped 10- to 20-fold and protein synthesis decreased about 5-fold (data not shown).

When equal amounts of total RNA from cells at different stages were compared, no difference in steady-state *erbA* mRNA could be observed by S1 analysis with 5′ and 3′ *erbA* probes (Fig. 7). Thus, the ratio of total RNA to steady-state c-*erbA* mRNA remained constant throughout differentiation.

c-*erbA* protein was precipitated from labeled cells with anti-*trp-erbA* after the volumes of lysate were adjusted for equal incorporation of [^{35}S]methionine label. A three- to fivefold increase in c-*erbA* protein synthesis, as determined by densitometry, was observed from day 5 to day 7 or 8 (Fig. 8), after which levels remained the same (data not shown). The onset of the increase of c-*erbA* protein synthesis occurred concomitantly with the start of proliferation of the

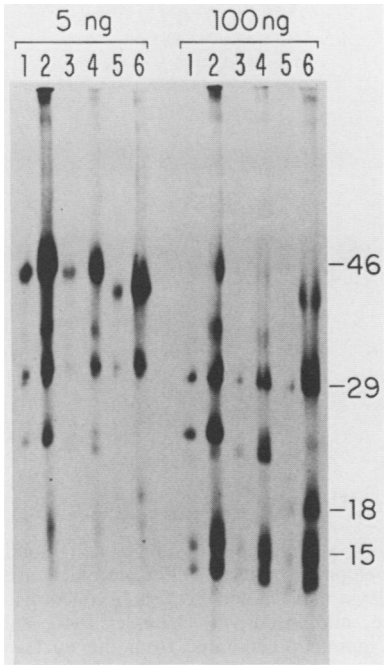

FIG. 5. Digestion with V8 protease of in vivo and in vitro *erbA* proteins. *erbA* proteins were immunoprecipitated from [^{35}S]methionine-labeled blood cells from 7-day-old chicken embryos. The peptides generated by digestion with V8 protease from the 46-, 44-, and 40-kDa proteins were compared with those obtained from in vitro translated *erbA* proteins of the same size. Lanes: 1, blood cells, 46 kDa; 2, in vitro, 46 kDa; 3, blood cells, 44 kDa; 4, in vitro, 44 kDa; 5, blood cells, 40 kDa; 6, in vitro, 40 kDa. The numbers on the right represent molecular weight markers (10^3).

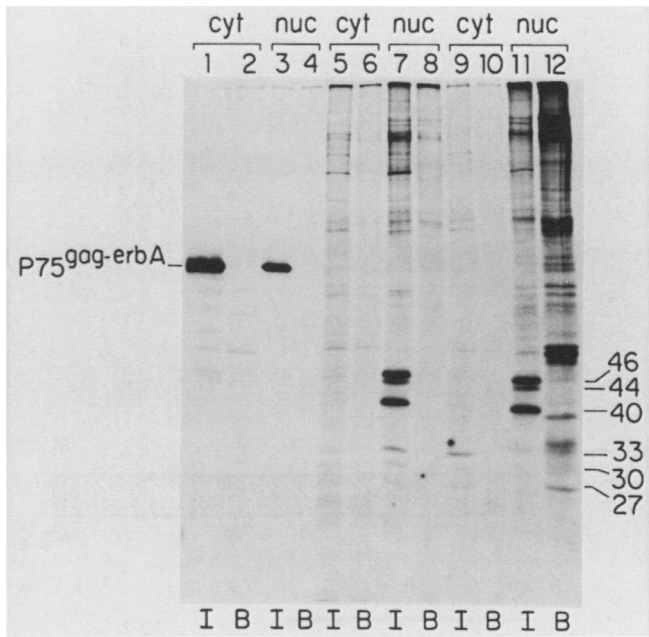

FIG. 6. Subcellular localization of v-*erbA* and c-*erbA*. v-*erbA* was immunoprecipitated with affinity-purified anti-*trp-erbA* 5794 from [^{35}S]methionine-labeled HD3 cells which were lysed in the presence of Nonidet P-40. c-*erbA* proteins were immunoprecipitated from [^{35}S]methionine-labeled blood cells from 7-day-old chicken embryos. The cells were either disrupted by Dounce homogenization or lysed in the presence of Nonidet P-40. Lanes: 1 and 2, HD3 cells, cytoplasmic fraction (cyt); 3 and 4, HD3 cells, nuclear fraction (nuc); 5 and 6, embryonic blood cells, cytoplasmic fraction after Dounce homogenization; 7 and 8, embryonic blood cells, nuclear fraction after Dounce homogenization; 9 and 10, embryonic blood cells, cytoplasmic fraction after lysis with Nonidet P-40; 11 and 12, embryonic blood cells, nuclear fraction after lysis with Nonidet P-40. I, Immunoprecipitation; B, immunoprecipitation with antibody preincubated with purified *trpE-ΔerbA* protein. The numbers on the right indicate the molecular weights of the c-*erbA* proteins (10^3).

erythroid cells of the first definitive lineage (24). Note that only the 40- to 46-kDa *erbA* bands were detected here since the lysate was made from nuclei prepared in the presence of Nonidet P-40. In contrast to c-*erbA*, the level of synthesis of the 150-kDa protein specifically recognized by our anti-*trp-erbA* serum decreased over the same period of time.

DISCUSSION

Previous studies on the protein encoded by c-*erbA* have relied on in vitro translation of RNAs generated from cDNA clones (2, 13, 29, 33, 37). To identify and characterize the c-*erbA* protein in vivo, we prepared an antiserum against a bacterial fusion protein consisting of *trpE* linked to the C-terminal half of the *erbA* portion of v-*erbA*, a region which comprises the putative ligand-binding domain. Antibodies raised against the bacterially expressed protein specifically immunoprecipitated the *gag-erbA* fusion protein from AEV-transformed erythroblasts, c-*erbA* proteins produced by in vitro translation, and a set of proteins from avian embryonic erythrocytes. We showed that the multiple in vitro generated c-*erbA* proteins are related to the proteins precipitated from avian erythroid cells in terms of both size and partial proteolytic fragment maps. Our results showed that anti-*trp-erbA* serum specifically recognized *erbA*-encoded proteins.

Expression of *erbA*-thyroid hormone receptor RNAs has been previously detected in different cell types (2, 18, 33, 37). To examine c-*erbA* protein synthesis, we chose avian erythroid cells, since these had been shown to have the highest levels of *erbA* transcripts in chicken embryos (18) and we were unable to detect specific *erbA* proteins in other cell types. In immunoprecipitations from chicken embryonic blood cells, we detected a complex protein pattern similar to the one obtained in in vitro translation (Fig. 4). Multiple protein species had been previously observed upon cell-free translation of c-*erbA* cDNAs (2, 29, 33, 37). Digestion with V8 protease showed that the same distinct peptide patterns were generated from the equivalent in vitro and in vivo bands, suggesting that the proteins are related and can be produced from a single RNA (Fig. 5). The fact that they are recognized by an antibody against the C-terminal region and do not appear to arise from posttranslational processing is consistent with the notion of multiple initiation sites. We surmise that the different *erbA* proteins originate from translational initiations at different in-frame AUG codons to produce a C-terminally nested set. This view is supported by recent experiments showing that removal of the 5′ AUG codons results exclusively in translation of the lower-molecular-weight forms (J. Bigler, unpublished data). Alternative translational initiations have been shown to increase the polypeptide complexity of several other genes (16, 22, 32).

We have not excluded the possibility that synthesis of the multiple proteins is specific for erythrocytes, since both in vivo and in vitro proteins have been translated in erythroid cells. Another point to consider is that the c-*erbA* cDNA clone used here has had 250 base pairs 5′ of the coding region

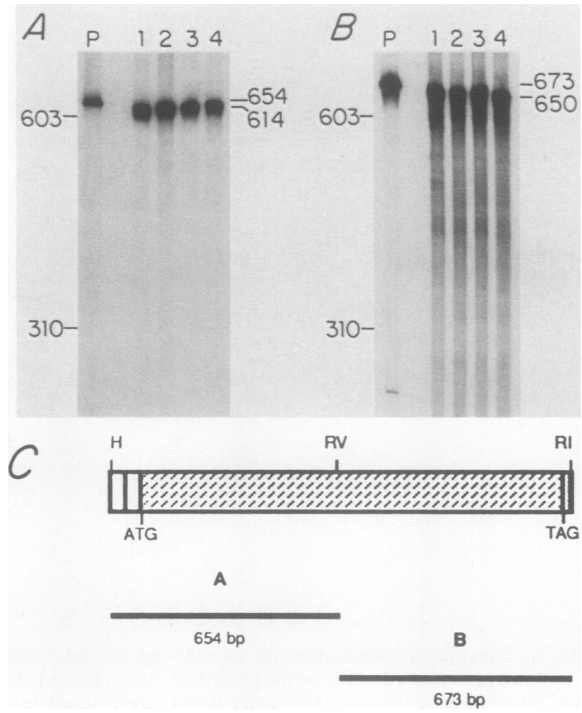

FIG. 7. Determination of c-*erbA* mRNA content in chicken blood cells during embryonic development with S1 nuclease protection. RNA was isolated from blood cells of 5-, 7-, 9-, and 12-day-old chicken embryos. Panels: A, S1 analysis of total RNA with probe A; B, S1 analysis of total RNA with probe B. Lanes: 1, blood cells from 5-day-old embryos; 2, blood cells from 7-day-old embryos; 3, blood cells from 9-day-old embryos; 4, blood cells from 12-day-old embryos. P, Probe. The numbers to the left and right of the panels indicate molecular size in base pairs. (C) c-*erbA* insert of pF1Δ. Blank box, Polylinker sequences of pTZ19R; dotted boxes, 5′ and 3′ untranslated *erbA* cDNA sequences; hatched box, c-*erbA* coding region (29). Restriction sites: H, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV.

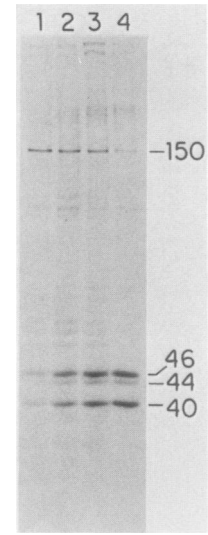

FIG. 8. c-*erbA* protein content in chicken blood cells during embryonic development. Blood cells from chicken embryos were collected on days 5, 6, 7, and 8 of embryogenesis, labeled with [^{35}S]methionine, and lysed with Nonidet P-40, and the c-*erbA* proteins were immunoprecipitated from the nuclear lysates with affinity-purified anti-*trp-erbA* 5794. Lanes 1 to 4 contained blood cells from 5-, 6-, 7-, and 8-day-old embryos, respectively. The numbers on the right indicate the molecular weights (10^3) of the proteins specifically precipitated with affinity-purified anti-*erbA* serum.

removed to increase translational efficiency (29). Whereas this cannot account for the multiple proteins we detected in vivo, it raises the possibility that the full-length *erbA* mRNA contains translational regulatory sequences.

The high-affinity, low-capacity thyroid hormone receptor is a nuclear protein (28, 29). In our analysis, we found at least half of the v-*erbA* protein in the cytoplasm (Fig. 6), in agreement with several previous reports (1, 5) but in contrast to one report (29) claiming that v-*erbA* is exclusively nuclear. The reason for this discrepancy is not clear. When we analyzed subcellular fractions of chicken erythroid cells for c-*erbA*, we found that the 46-, 44-, and 40-kDa proteins were exclusively nuclear, whether nuclei were prepared by Dounce homogenization or lysis in the presence of Nonidet P-40. The 27- to 33-kDa bands, however, are apparently less tightly nucleus associated, since a fraction was found in the cytoplasm after dounce homogenization and they were completely extracted from nuclei in the presence of Nonidet P-40. This result suggests different properties for the set of larger bands compared with the set of smaller bands. One explanation is that the smaller proteins lack the putative DNA-binding region—consistent with initiation from methionines further downstream—and are therefore not as tightly bound as *erbA* proteins that contain the putative DNA-binding region. A population of thyroid hormone receptors capable of binding ligand but not interacting with DNA might function to titrate the levels of free hormone, or it might serve a function distinct from gene regulation. Experiments are in progress to determine more accurately the initiation sites of the various *erbA* proteins.

We were interested in finding out whether expression of c-*erbA* proteins changes when mature erythrocytes appear. Hentzen et al. (18) found a correlation between the number of late polychromatic erythroblasts of the adult lineage and the number of cells labeled by in situ hybridization with a v-*erbA* probe. Although it is not possible to compare their results directly with ours, our protein data suggest increased levels of c-*erbA* protein synthesis around days 7 and 8 of embryogenesis, when the blood consists mainly of late polychromatic erythroblasts (24). However, our S1 analyses indicate that levels of *erbA* mRNA are essentially constant throughout differentiation, in contrast to a previous report (18). The discrepancy could be due to the fact that these researchers used dot blot hybridization with total RNA. In our hands, rRNA cross-hybridized with *erbA* probes.

Our results show that multiple forms of the c-*erbA* protein can be generated both in vitro and in vivo and that these forms have different degrees of nuclear retention. In addition, we found an increase in c-*erbA* protein levels in erythroid differentiation and an apparent uncoupling between c-*erbA* mRNA and protein. Taken together, these results raise the possibility that posttranscriptional or translational mechanisms are involved in the regulation of c-*erbA* expression.

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