MOLECULAR AND CELLULAR BIOLOGY, July 1987, p. 2416–2424
0270-7306/87/072416-09$02.00/0
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Vol. 7, No. 7

# The Chicken c-*erbA* Proto-Oncogene Is Preferentially Expressed in Erythrocytic Cells during Late Stages of Differentiation

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Received 30 December 1986/Accepted 6 April 1987

**We analyzed the expression of the c-*erbA* proto-oncogene in different tissues of chicken embryos. c-*erbA* transcripts were found at low levels in the lung, kidney, liver, and heart and in high amounts in embryonic blood cells. Nuclease mapping assays proved that these transcripts were true c-*erbA* transcripts. In situ hybridization on fractionated embryonic blood cells showed that c-*erbA* transcripts were predominantly found in erythroblasts, particularly during the final step of differentiation. Life span analysis of c-*erbA* mRNAs revealed their relative instability, demonstrating that the high level of c-*erbA* transcripts in embryonic erythroblasts was not the result of passive accumulation. These results suggest that the c-*erbA* genes play some role in erythrocyte differentiation.**

Avian erythroblastosis virus (AEV) induces acute erythroleukemias and sarcomas in vivo and fully transforms erythrocyte progenitors and fibroblasts in vitro (12, 15). Its genome carries two oncogenes, v-*erbA* and v-*erbB*, derived from the two independant proto-oncogenes c-*erbA* and c-*erbB*, respectively (32, 38, 39). The v-*erbB* protein is translated from a subgenomic RNA. The mature gp74$^{erbB}$ protein inserted into the cytoplasmic membrane (19, 27) exhibits kinase activity (13, 20, 22) and shows close similarities with a truncated form of the human epidermal growth factor receptor (9, 37, 43). Genomic viral transcripts are used to translate a *gag-erbA* fusion protein, p75$^{gag\text{-}erbA}$ (1, 18). The predicted amino acid sequence of p75$^{gag\text{-}erbA}$ shows strong homology with steroid receptors (6, 8, 16, 17, 23, 40). During preparation of the present paper, the cloning of human and chicken c-*erbA* cDNAs was reported, demonstrating that the c-*erbA* proto-oncogene encodes the receptor for triiodothyronine (31, 41). Analysis of mutants of AEV (10, 34, 35) and a natural variant, AEV-H, containing only v-*erbB* (42), has shown that v-*erbB* is sufficient for transformation of fibroblasts and erythroid progenitors. Nevertheless, v-*erbA* is necessary to achieve full blockage of erythroid differentiation in the transformed cells. Moreover, we recently provided evidence that v-*erbA* activates the growth of chicken fibroblasts (Gandrillon et al., Cell, in press). The molecular mechanisms by which the gp74$^{erbB}$ and the p75$^{gag\text{-}erbA}$ proteins cooperate to induce erythroleukemia are still unknown. However, the fact that the two viral oncogenes encode proteins related to growth factor and hormone receptor, respectively, suggests that transformation of erythrocytic progenitor cells by AEV results from deregulation of both erythrocytic growth and differentiation signals. In parallel, it was of interest to analyze the function of the respective proto-oncogenes in growth and differentiation of erythrocytic cells. The c-*erbA* proto-oncogene was first identified in chicken DNA (38). The exact number of c-*erbA* copies per haploid chicken genome has not yet been determined. In humans, several c-*erbA*-related genes were identified per haploid genome, but only one, c-*erbA1*, seems to be closely related to the v-*erbA* sequences found in AEV (21, 41). In the human genome, the c-*erbA1* gene is located on chromosome 17 (33). In the chicken genome a c-*erbA* gene is located on a minichromosome (36). Two c-*erbA* transcripts were identified in polysomal RNAs isolated from total chicken embryos (38). However, the tissue distribution of these transcripts was not determined. For humans, several c-*erbA* transcripts were found in the human HeLa and hematopoietic K562 cell lines (22).

In this work we investigated expression of the c-*erbA* genes in different tissues in chicken embryos. We show that c-*erbA* transcripts are preferentially found in normal erythroblasts as at least four different RNA species. A careful survey of c-*erbA* expression along the erythroblast differentiation pathway showed that the c-*erbA* transcripts are most abundant in late erythroblasts. Moreover, few if any c-*erbA* transcripts were detected in chicken erythroleukemic cells representing early erythrocyte progenitor cells. The c-*erbA* mRNAs exhibited a short life span and were actively transcribed in late erythroblasts. These data show that the c-*erbA* proto-oncogene(s) is preferentially expressed during the late stages of erythrocyte differentiation. These results are discussed in terms of the functional relationship between v-*erbA* and c-*erbA* in erythroleukemic transformation by AEV.

## MATERIALS AND METHODS

**Cells and tissues.** All cells and tissues were isolated from white Leghorn embryos and chickens. Blood cells were collected on sodium citrate and washed quickly with phosphate-buffered saline (PBS) before being frozen in liquid nitrogen.

To prepare immature chicken erythrocytes, a 2-month-old chicken was rendered anemic by two subcutaneous injections of phenylhydrazine (6 mg/kg) at a 24-h interval. Two days after the last injection, blood cells were collected and centrifuged at 400 × *g* through a Ficoll-Paque (Pharmacia Fine Chemicals) cushion. The pellet of purified erythrocytes was then washed and frozen. Blood cells of 7-day-old embryos were fractionated on a bovine serum albumin gradient as described by Gazzolo et al. (11). Identification of

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the cells was done by the criteria of Bruns and Ingram (4) and Lucas and Jamroz (24). In the chicken embryo, the earliest differentiation stages identified in blood are basophilic erythroblasts, which mature successively into early polychromatophilic and then late polychromatophilic erythroblasts and finally mature erythrocytes. Late polychromatophilic erythroblasts may be considered homologous to the reticulocytes found in mammals. Erythroleukemic cells (ELC) transformed by AEV-H or E26 virus were isolated as described previously (12, 26). Chicken embryo fibroblasts were prepared and grown as described elsewhere (Gandrillon et al., in press).

Tissues were quickly dissected from 11-day-old chicken embryos, washed in PBS, and then frozen in liquid nitrogen.

**Preparation and labeling of probes.** The various probes used are shown in Fig. 3D. Probe E4 was amplified after cloning in a plasmid and then released by digestion of plasmid DNA with *PstI* and isolated by electrophoresis. It was labeled by nick translation with [$^{32}$P]dCTP (26).

Probe E1 was cloned in a plasmid as an *XhoI-SalI* fragment. After being cut with *SalI*, the plasmid DNA was labeled with $^{32}$P at the 5′ ends with T4 polynucleotide kinase (25). The labeled E1 fragment was then released by digestion with *XhoI* and isolated by electroelution.

Probe E2 was first isolated as a *SalI-SacI* fragment labeled at the 5′ ends with $^{32}$P by using T4 polynucleotide kinase. It was then digested with *AvaI*, and the specific E2 fragment was purified by electroelution.

Probe E3 was cloned as a *PvuI-SacI* fragment in the Bluescribe plasmid (Genofit) containing bacteriophage T3 and T7 transcriptional promoters. The appropriate promoter was chosen to synthesize the complementary strand able to hybridize with the c-*erbA* mRNAs. The synthesis was performed at 37°C for 30 min in a final volume of 25 μl of a solution containing 40 mM Tris, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 30 mM dithiothreitol, 0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rGTP, 0.02 mM rUTP, 25 μl of [$^{35}$S]rUTP or [$^{32}$P]rUTP (3,000 Ci/mmol; Amersham Corp.), 25 U of RNasin (Promega Biotec), 10 U of T7 RNA polymerase, and 1 μg of *Bam*HI-restricted DNA template. After 30 min of incubation at 37°C, the reaction was stopped by adding 10 μg of tRNA and 1 U of RNase-free DNase (Boehringer Mannheim) for 15 min at 37°C, followed by phenol-chloroform (1:1, vol/vol) extraction and ethanol precipitation. The size of the RNA probe synthesized was controlled by electrophoresis on a 5% acrylamide–7 M urea denaturing gel.

The v-*erbB* probe was a *Bam*HI-*Eco*RI fragment (634 base pairs [bp]) of the pAEV11 DNA clone containing the AEV genome (39).

The chicken beta-globin probe, kindly provided by K. Sherrer and T. Imaizumi, was a *TaqI* restriction fragment of 620 bp of recombinant plasmid DNA carrying cDNA of chicken beta-globin (29).

**In situ hybridization.** Cells ($2 \times 10^5$ per slide) were centrifuged in a cytofuge (Shandon) at room temperature onto acid-washed and gelatine-chrome alum-coated slides at 700 rpm for 10 min and then fixed in 1% paraformaldehyde (Merck, Darmstadt) and stored at −70°C. Just before hybridization, the slides were rinsed in PBS–5 mM $MgCl_2$ for 10 to 15 min. To permeabilize the cell membrane, the slides were treated by incubation with proteinase K (Boehringer) at a final concentration of 5 μg/ml in PBS for 15 min and then immediately immersed in PBS containing glycine at 2 mg/ml (3) for 30 s. The slides were prehybridized in 50% formamide–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution for 1 h. Hybridization was performed with $5 \times 10^5$ cpm of $^{35}$S- or $^{32}$P-labeled RNA probe per slide as described (2). The slides were then coated with Ilford K-5 liquid emulsion for 1 to 3 days, developed with Kodak D19, and stained with toluidine blue.

**RNA preparation.** Total RNAs were extracted from whole chicken embryos or isolated from embryonic yolk sac, blood, liver, heart, lung, or kidney by homogenization in a solution containing 1% sodium dodecyl sulfate (SDS)–200 μg of proteinase K per ml–20 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–200 mM EDTA. The homogenate was centrifuged overnight through a CsCl gradient at 20°C at 25,000 rpm in an SW27 rotor (Beckman) as described (14). To check the quality of the RNAs, 5 μg from each sample was electrophoresed in a 1% agarose gel and then stained with ethidium bromide (0.5 μg/ml in 150 mM Tris hydrochloride buffer, pH 7.4). For some experiments polyadenylated RNAs were isolated from total RNAs on oligo(dT)-cellulose (Collaborative Research Inc.) as described by Maniatis et al. (25).

**RNA blotting and hybridization.** For the dot-blot assay, 1 μg of each RNA preparation was dissolved in 80 μl of solution containing 50% deionized formamide, 6% formaldehyde, and 1× MOPS buffer (1× MOPS buffer is 20 mM MOPS [3-morpholinopropanesulfonic acid, pH 7], 50 mM sodium acetate, 10 mM EDTA). After incubation for 15 min at 60°C and cooling on ice, the samples were dot-blotted onto nitrocellulose paper preequilibrated in 20× SSC with a Hybrid-Dot manifold apparatus (Bethesda Research Laboratories). The blots were then baked at 80°C for 2 h.

For Northern blots, 5 to 10 μg of polyadenylated RNAs or 25 μg of total RNAs were electrophoresed in 1.2% agarose gel containing 1× MOPS buffer and 3% formaldehyde and then transfered to nitrocellulose paper preequilibrated in 20× SSC as described (25). rRNAs electrophoresed in parallel were used as size markers (28, 23, 18, and 16S RNAs were estimated as 5.1, 3.3, 2.1, and 1.7 kilobases [kb], respectively). Hybridization of dot-blots and Northern blots was performed for 48 h at 42°C with $1 \times 10^6$ to $2 \times 10^6$ cpm of labeled probe per ml of final hybridization solution containing 50% formamide, 3× SSC, 50 mM Tris hydrochloride (pH 7.5), 20 μg of tRNA and 20 μg of denatured salmon sperm DNA per ml, 1 mM EDTA, and 1× Denhardt solution. After hybridization, the nitrocellulose blots were washed for 1 h in 1× SSC at 42°C and twice for 30 min each in 0.1 × SSC–0.1% SDS at 50°C, then rinsed with 0.1 × SSC, dried, and exposed to Kodak X-ray film.

**Nuclease protection assay.** The DNA probes were mixed with total RNA in a solution containing 80% formamide, 0.3 M NaCl, 0.1 mM EDTA, and 20 mM Tris hydrochloride, pH 7.4. The mixture was heated at 85°C for 10 min and then hybridization was run for 12 h at a temperature 3°C above the melting point of the respective probes. Hybridization was performed at 49°C with probe E2 and 56°C with probe E1. The hybrids were then digested for 3 h at 15°C in a solution containing 30 mM sodium acetate (pH 4.5), 3 mM $ZnSO_4$, 300 mM NaCl, 10 μg of salmon sperm DNA per ml, and 100 U of S1 nuclease (Boehringer) per 10 μg of RNA. *Escherichia coli* tRNA was used as the hybridization control. The digested samples were electrophoresed on a 4 or 6% acrylamide–bisacrylamide denaturing gel containing 50% urea. The gel was then dried and autoradiographed.

RNA probe E3 was mixed with total RNA in a solution containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid], pH 6.7, 400 mM NaCl, and 1 mM EDTA. The mixture was heated at 85°C for 10 min, and then

hybridization was run for 12 h at 50°C. The hybrids were then digested for 1 h at 25°C in a solution containing 10 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 300 mM NaCl, 300 U of T1 RNase, and 12.5 μg of RNase A. The digested samples were electrophoresed as described above.

**Determination of c-*erbA* transcript stability.** Blood cells were sterilely collected from a 15-day-old embryo and washed twice in alpha-MEM (alpha modification of Eagle medium) containing 10% fetal calf serum. Samples of $5 \times 10^9$ cells were incubated in the same medium containing actinomycin D (5 μg/ml) (Sigma Chemical Co.) at 37°C in an incubator flushed with 2.5% $CO_2$. At regular intervals portions were used for immediate extraction. A standard fraction of each RNA preparation was either hybridized with v-*erbA* probe E1 for the S1 protection assay or dot-blotted onto nitrocellulose paper and hybridized with the beta-globin probe.

## RESULTS

**Detection of c-*erbA* transcripts in chicken embryo tissues.** To identify the tissues which preferentially express c-*erbA* genes in chicken embryos, RNAs were extracted from various tissues from 11-day-old embryos and the c-*erbA* transcripts were analyzed in a dot-blot assay with a v-*erbA*-specific probe (probe E4; see Fig. 3D). A positive signal was observed mainly in yolk sac, lung, kidney, heart, and blood (Fig. 1a). As controls, similar RNAs were probed with a beta-globin and a v-*erbB* probe. As expected, the globin probe gave a strong signal with blood RNAs and only a faint signal with yolk sac RNAs. The v-*erbB* probe showed up mainly the liver RNAs and to a smaller extent heart, lung, and kidney RNAs. No signal was observed with this probe on blood RNAs.

These results show that c-*erbA*-related transcripts are found at different levels in various embryonic tissues. The v-*erbA* probe gave a stronger signal with RNAs isolated from late embryos (Fig. 1b).

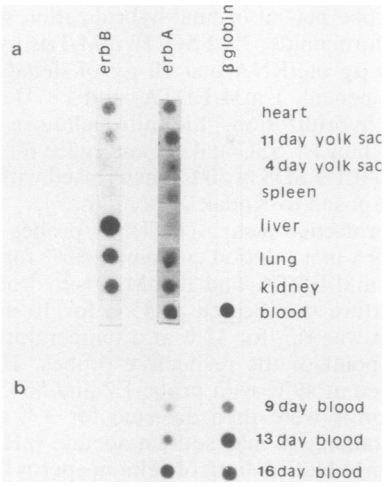


FIG. 1. Assay of (a) c-*erbA*, c-*erbB*, and beta-globin transcripts in tissues of 11-day-old chicken embryos and (b) c-*erbA* and beta-globin transcripts in blood cells at different stages of embryogenesis. Total RNAs were dot-blotted onto nitrocellulose and probed with the labeled probe E4 (v-*erbA*), the *Bam*HI-*Eco*RI fragment of v-*erbB*, and the *Taq*I restriction fragment of beta-globin cDNA (see Materials and Methods and Fig. 3D). The blot hybridized with the v-*erbA* probe in panel a was exposed for 5 days; all other blots were exposed for 2 days.

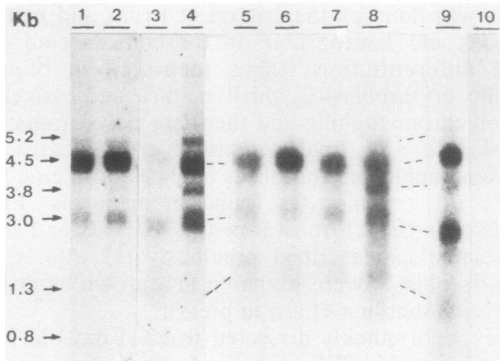


FIG. 2. Northern blot analysis of c-*erbA* transcripts isolated from whole embryos and chicken tissues. Polyadenylated RNAs were electrophoresed in a 1.2% agarose–formaldehyde gel, blotted onto nitrocellulose, and hybridized to probe E4 (described in Materials and Methods and Fig. 3D). Unless indicated otherwise, embryonic tissues were collected from 11-day-old embryos. As the picture was obtained from different experiments, quantitative comparison between the samples was not possible. Lanes: 1, whole 2-day-old embryo; 2, whole 4-day-old embryo; 3, yolk sac; 4, embryonic blood; 5, embryonic kidney; 6, embryonic lung; 7, embryonic heart; 8, blood from 16-day-old embryo; 9, erythrocytes from 3-week-old anemic chicken; 10, nonpolyadenylated RNA from chicken embryo fibroblasts.

**Characterization of c-*erbA* transcripts in embryonic tissues.** To characterize the c-*erbA*-related transcripts revealed in the various embryonic tissues, polyadenylated RNAs were isolated from total embryos and separated tissues and analyzed on Northern blots after hybridization with the E4 probe. Polyadenylated RNAs extracted from total 2- or 4-day-old embryos (Fig. 2, lanes 1 and 2) revealed two major c-*erbA*-related transcripts of 4.5 and 3.0 kb. These transcripts are probably the same ones described by Vennstrom and Bishop (38). Two additional minor bands at 5.2 and 3.8 kb were visible. The two major bands were seen in yolk sac RNAs (lane 3). In embryonic blood cells (lanes 4 and 8), four c-*erbA* transcripts were identified, the two major transcripts being of nearly the same abundance. In nonhematopoietic tissues such as kidney (lane 5), lung (lane 6), and heart (lane 7), only the 4.5- and 3.0-kb transcripts were revealed, with a predominance of the larger one. In some RNA preparations faint bands at 1.3 and 0.8 kb were shown by the v-*erbA* probe. As these bands were not repeatedly found in different RNA preparations from the same tissue, we cannot be sure whether they represent true transcripts or degradation products.

As the v-*erbA* probe used in these experiments enclosed sequences shared by several related genes of the steroid receptor family (6, 16, 17, 23, 40), it was necessary to check whether the RNA transcripts found in embryonic tissues were derived from the c-*erbA* genes. The structure of the c-*erbA*-related transcripts was analyzed in a nuclease protection assay with various probes covering different domains of the v-*erbA* gene (Fig. 3D). Probe E1 contained mainly v-*erbA* sequences of domain 1 (680 nucleotides) linked to 506 nucleotides of upstream *gag* sequences. Probe E2 covered 366 nucleotides at the 3′ side of v-*erbA*. Probe E3 covered 855 nucleotides over domains 1 and 2. Probes E1 and E2 were used under DNA forms labeled specifically at the 5′ end of their noncoding strands. Probe E3 was used as an RNA chain (RNA probe) complementary to the coding v-*erbA* DNA strand, labeled over its whole length by in vitro synthesis from a recombinant plasmid (see Materials and

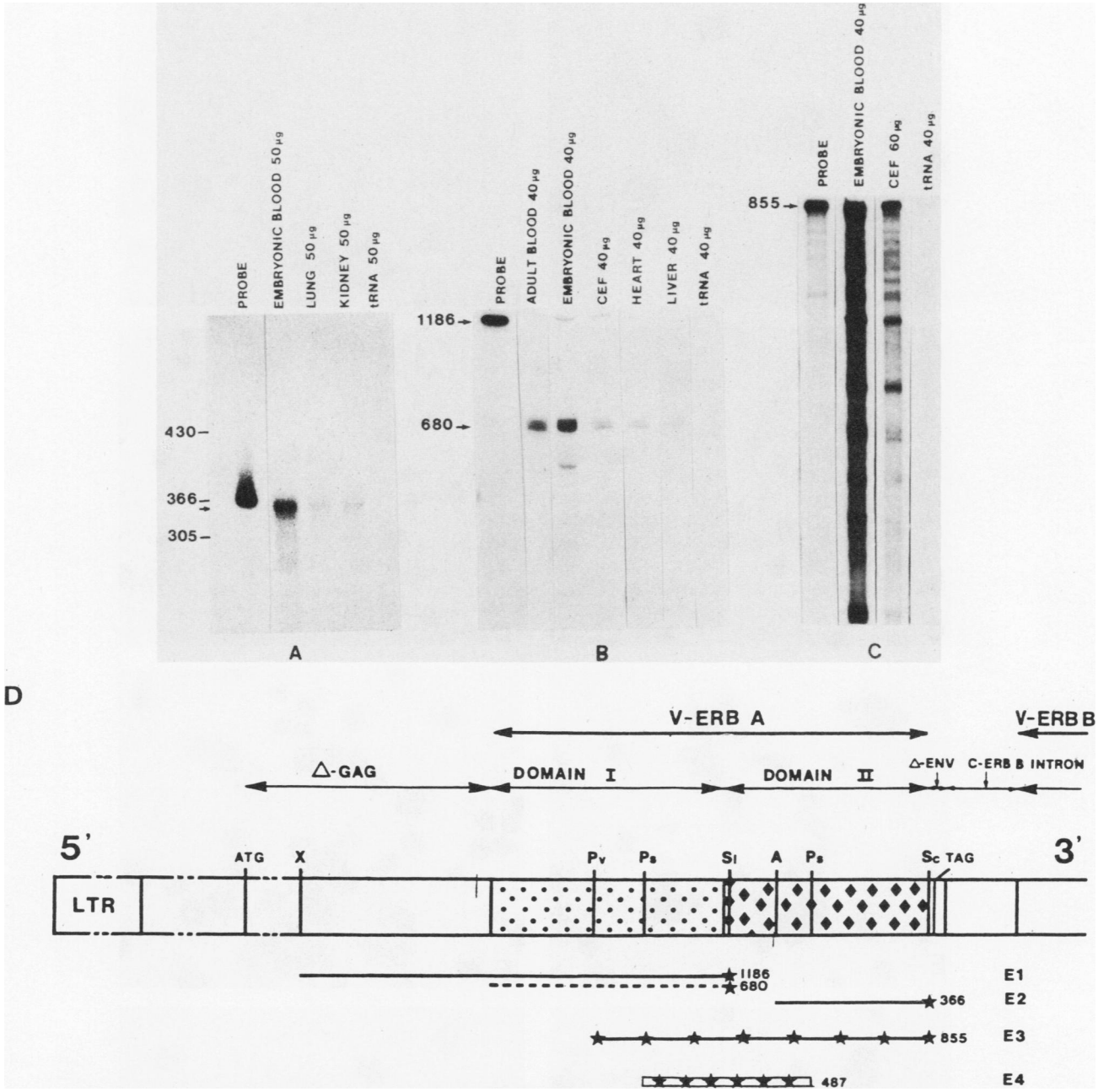


FIG. 3. Structural analysis and quantitation of c-*erbA* transcripts by the nuclease protection assay. (A) S1 nuclease analysis of c-*erbA* transcripts with the E2 probe. From left to right: 5'-end-labeled E2 probe without digestion, RNAs from 15-day-old embryo blood, and 11-day-old embryo organs. (B) S1 nuclease analysis of c-*erbA* transcripts with the E1 probe. From left to right: 5'-end-labeled E1 probe without digestion, RNAs from anemic adult blood, 15-day-old embryo blood, and 11-day-old embryo organs. (C) RNase protection assay with RNA probe E3. From left to right: E3 probe without digestion, 15-day-old embryo blood, and CEF from 10-day-old chicken embryo. For panels A to C, sizes are indicated in nucleotides. (D) Structure of the *gag-erbA* region of AEV proviral DNA and probes used to analyze c-*erbA* mRNAs. The dotted line in probe E1 corresponds to the 680 *erbA*-specific nucleotides. Abbreviations: X, *Xho*I; Pv, *Pvu*II; Ps, *Pst*I; Sl, *Sal*I; A, *Ava*I; Sc, *Sac*I; LTR, long terminal repeat. ATG and TAG indicate the initiation and termination codons, respectively, of $p75^{gag\text{-}erbA}$. Probes are E1 (*Xho*I-*Sal*I), E2 (*Ava*I-*Sac*I), E3 (*Pvu*II-*Sac*I), and E4 (*Pst*I-*Pst*I). Stars indicate radioactive nucleotides.

Methods). Total RNAs were isolated from various embryonic tissues and chicken embryo fibroblasts (CEF) grown in culture, hybridized in a liquid reaction mixture with each of these probes, and then digested with nuclease. The protected labeled probe fragments were then analyzed by polyacrylamide gel electrophoresis.

Probe E2 was protected by RNAs isolated from embryonic blood, lung, kidney (Fig. 3A). A slight reduction in the size of the probe suggested that some mismatches occurred within the 10 to 15 nucleotides at the 3' end of the protected probe. Probe E1 gave a double band of about 680 nucleotides with RNAs isolated from embryonic blood, heart, liver, fibroblasts, and anemic adult erythrocytes (Fig. 3B). The length of the protected fragments corresponded exactly to the size of the v-*erbA*-specific sequences in the probe. Probe E3 was revealed to be the most sensitive due to its high

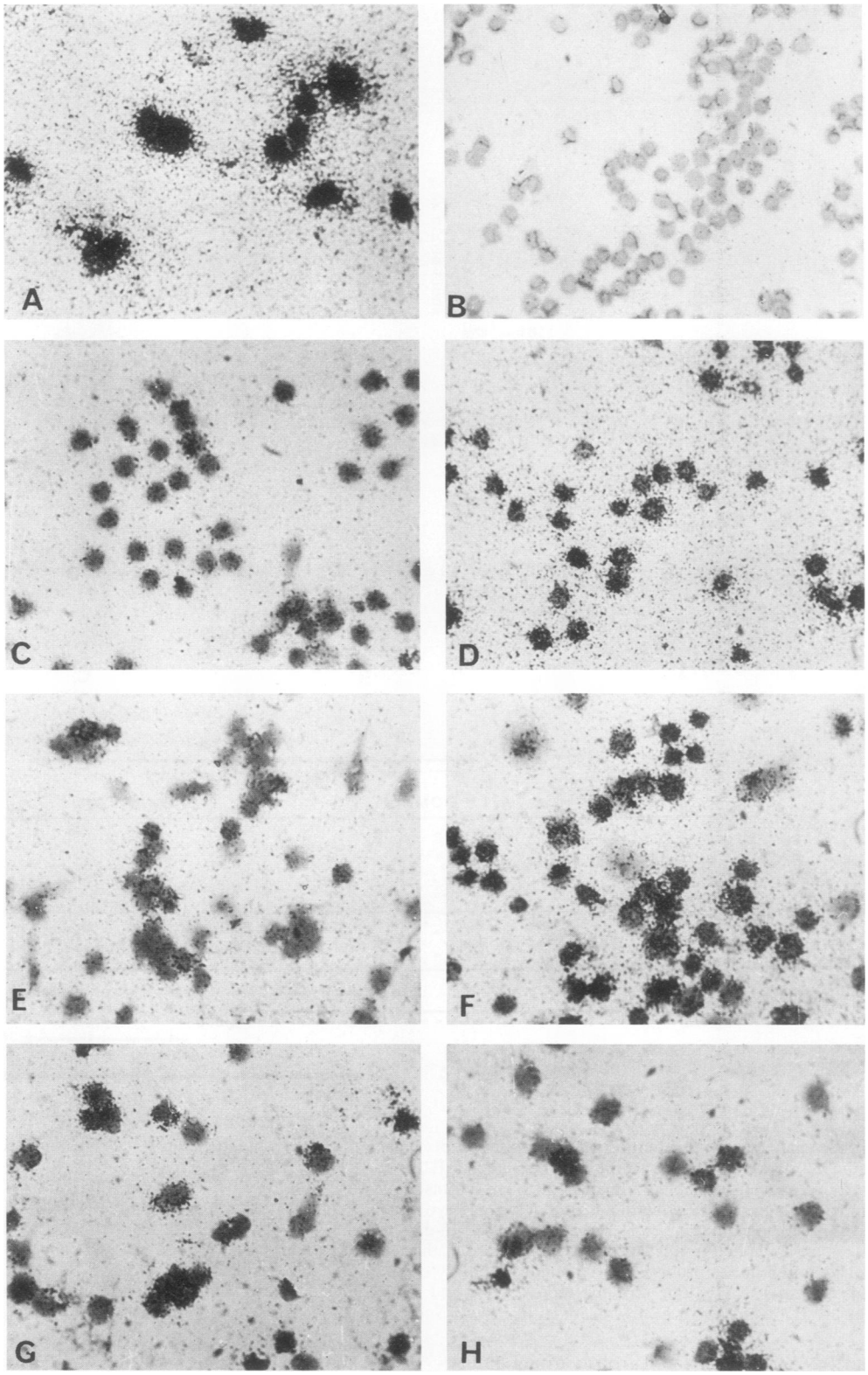


FIG. 4. Detection of c-*erbA* transcripts by in situ hybridization. (A) ELC transformed by AEV. (B) Blood cells of normal adult chicken. (C) Blood cells of 7-day-old embryo. (D) Blood cells of 15-day-old embryo. (E, F, G, and H) 7-day-old embryo blood cells fractionated on bovine serum albumin density gradient. (E) Cells retained at density 1.060 g/cm$^3$; (F) cells retained at density 1.070 g/cm$^3$; (G) cells retained at density 1.080 g/cm$^3$; (H) cells recovered in the pellet (density higher then 1.080 g/cm$^3$).

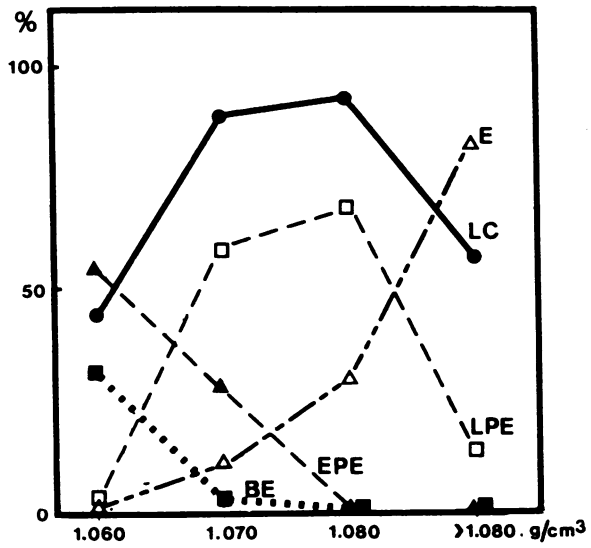


FIG. 5. Identification of cells that express c-*erbA* transcripts in 7-day-old embryo blood. Blood cells from 7-day-old embryos were centrifuged through a bovine serum albumin density gradient. Cells recovered in each fraction were assayed for c-*erbA* transcripts by in situ hybridization on smears. Separate smears were used for cell identification after staining with Wright-Giemsa. Abscissa, Density of the fraction; ordinate, percentage of each cell type in the fraction. Abbreviations: LC, labeled cells; BE, basophilic erythroblasts; EPE, early polychromatophilic erythroblasts; LPE, late polychromatophilic erythroblasts; E, mature erythrocytes (in 7-day-old embryos these mature erythrocytes belong to the primitive erythrocyte lineage). Unidentified blast cells were observed only in fraction 1.060 g/cm$^3$, where they represented 10% of the cells (these cells are not depicted in the figure).

radioactive specific activity. It gave one major protected band of about 855 nucleotides with RNAs extracted from embryonic blood and fibroblasts (Fig. 3C). Other minor protected bands were observed.

All these results demonstrate that embryonic blood, lung, heart, liver, kidney, and fibroblasts contain RNAs highly homologous to the v-*erbA* sequences, which probably represent transcripts of the c-*erbA* genes.

**c-*erbA* transcripts found preferentially in erythrocytic cells.** The preliminary detection of c-*erbA* transcripts in embryonic tissues by the dot blot assay showed that these transcripts were most abundant in blood cells (Fig. 1a and b). Direct measurement of the relative amounts of c-*erbA* transcripts in various tissues was provided by the nuclease protection assay with probes E1, E2, and E3. As an internal standard between the various experiments, we used RNAs isolated from 15-day-old embryo blood cells. For the same amount of total RNA, c-*erbA* transcripts were nearly 10 times more abundant in blood cells of 15-day-old embryo and adult anemic chickens than in nonhematopoietic tissues, including embryonic liver, lung, heart, kidney, and fibroblasts (Fig. 3). All these nonhematopoietic cells expressed nearly the same amount of c-*erbA* transcripts.

Embryonic blood is mainly populated with erythrocytic cells. However, it contains a few thrombocytes and granulocytes (24). To identify the embryonic blood cells which expressed c-*erbA* transcripts, we performed in situ hybridization on blood smears made from embryos at different developmental stages. The E3 RNA probe labeled with [$^{35}$S]uridine was used for these experiments. Typical hybridization patterns are shown in Fig. 4. A strong hybridization signal was obtained on AEV-transformed erythroleukemic cells used as positive controls (Fig. 4A). This signal was due to hybridization of the probe with viral transcripts containing v-*erbA*. At the same time fully mature erythrocytes from an adult normal chicken containing few RNAs did not reveal any significant in situ hybridization with the *erbA* probe (Fig. 4B). Also, no c-*erbA* transcripts could be detected in these cells by Northern blot analysis (data not shown). Figures 4C and D show strongly positive cells in blood smears made from 7 and 15-day-old embryos, respectively. In 7-day-old embryo blood, virtually no leukocytes were detected, and more than 60% of the cells were labeled. In 15-day-old embryo blood, nonerythrocytic cells, mainly thrombocytes, accounted for less than 1% of the total, whereas 90% of the cells were labeled. No significant signal was obtained after in situ hybridization of 7- or 15-day-old embryo blood smears with a control riboprobe containing an insect nucleotide sequence not represented in the vertebrate genome (not shown). These results unambiguously demonstrate that c-*erbA* transcripts are found in erythrocytic cells.

As confirmation of the expression of c-*erbA* transcripts in erythrocytes, we analyzed RNAs in purified erythrocytes. As a source of immature cells, we used blood from chickens rendered anemic by phenylhydrazine injections. Immature erythrocytes were purified by separating out leukocytes on a cushion of Ficoll-Paque. The four c-*erbA* transcripts were found in these erythrocytes (Fig. 2, lane 9).

**Differential expression of c-*erbA* genes along erythrocyte differentiation pathway.** The results presented above show that c-*erbA* transcripts are most abundant in the erythrocytic cells of old embryos. In the blood of 15-day-old embryos as in anemic chickens, erythrocytic cells are mainly late polychromatophilic erythroblasts and mature erythrocytes of the definitive lineage. In 7-day-old embryos, erythrocytic cells are composed mostly of mature erythrocytes of primitive lineage and a few basophilic and polychromatophilic erythroblasts of definitive lineage. In situ hybridization analysis showed that the average number of grains over blood cells was smaller for the 7-day-old embryo (Fig. 4C) than for the 15-day-old embryo (Fig. 4D). This observation suggested that the amount of c-*erbA* transcripts in erythrocytes might differ according to the differentiation stage. We therefore compared the amount of c-*erbA* RNAs in isolated erythrocytic cells of different differentiation stages. Two approaches were used. First, we fractionated 7-day-old embryo blood cells through a buoyant density gradient of bovine serum albumin (Fig. 5). The cells retained in the lightest fraction (density of 1.060 g/ml) were composed mainly of basophilic and early polychromatophilic erythroblasts. Cells within the intermediate fractions (density 1.070 to 1.080 g/ml) were mainly late polychromatophilic erythroblasts. Cells in the pellet (density higher than 1.080 g/ml) were almost exclusively erythrocytes of the primitive lineage. In situ hybridization with the E3 probe on each cell fraction provided two main observations. First, the highest frequency of labeled cells was found in the two intermediate fractions. Second, labeling intensity as judged from the average number of grains per cell was higher in these two fractions (Fig. 4E, F, G, and H). These observations suggest that c-*erbA* transcripts might be more abundant in late polychromatophilic erythroblasts than in early polychromatophilic and basophilic erythroblasts and mature erythrocytes. The second approach consisted in searching c-*erbA* transcripts in erythroleukemic cells, which are supposed to represent early erythrocytic progenitor cells blocked in differentiation. Erythroleukemic cells of two different origins

were used. ELC E26 were freshly isolated from blastoderms transformed by E26 virus in vitro. These cells were mostly immature erythrocytes at a differentiation stage earlier than basophilic erythroblasts. ELC AEV-H were derived from chicken bone marrow cells transformed by AEV-H in vitro. These cells contain cells blocked at the CFU-erythrocytic stage together with differentiated basophilic and polychromatophilic erythroblasts resulting from spontaneous differentiation (10). c-*erbA* transcripts were assayed in RNAs isolated from these ELC by the S1 protection assay with the E1 probe (Fig. 6). No protected band corresponding to c-*erbA* transcripts was detected in E26 ELC. A faint signal corresponding to c-*erbA* RNAs was observed in AEV-H-transformed ELC. This signal was about 10 times lower than that in 15-day-old embryo erythrocytes.

**Life span of the c-*erbA* transcripts in erythrocytic cells.** The results presented above show that c-*erbA* transcripts are more abundant in erythrocytic cells at late differentiation stages. One might assume that these mRNAs accumulate during differentiation and are no longer synthesized in the late cells. We therefore analyzed the half-life of the c-*erbA* mRNAs in the erythrocytes of 15-day-old embryos. Globin mRNAs were used as an internal control. Erythrocytes were maintained in culture medium containing actinomycin D for 6 h at 37°C. At different times, samples were taken for RNA extraction. Total RNAs were analyzed for globin mRNAs in the dot-blot assay and c-*erbA* mRNAs in the S1 protection assay with probe E1 (Fig. 7). Whereas the half-life of globin mRNAs appeared to be longer than 6 h in these conditions, the half-life of the c-*erbA* mRNAs was only between 1 and 2 h. Moreover, comparison of the amount of c-*erbA* transcripts after 4 h with and without actinomycin D clearly showed that c-*erbA* transcripts were newly synthesized in the cells maintained without drug.

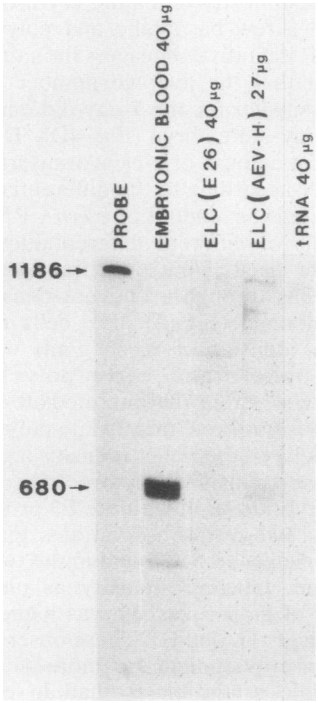


FIG. 6. Assay of c-*erbA* transcripts in ELC. c-*erbA* transcripts were assayed by S1 nuclease protection with the E1 probe. From left to right: labeled E1 probe without nuclease digestion, 15-day-old embryo blood, ELC, and *E. coli* tRNA control. Sizes are indicated in nucleotides.

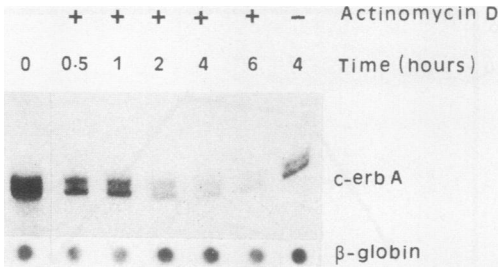


FIG. 7. Life span of c-*erbA* transcripts in erythrocytes from 15-day-old embryos. Amounts of total RNA isolated from constant numbers of cells were assayed by the S1 protection assay with the E1 probe or dot-blotted onto nitrocellulose and hybridized to the beta-globin probe. Time, Time of exposure to the drug.

## DISCUSSION

This work analyzed the expression of the c-*erbA* genes in various tissues of chicken embryos. c-*erbA* transcripts were found in several tissues, and the main expression occurred in erythrocytic cells. Identification of the c-*erbA* transcripts relied on three different approaches. In Northern blots the transcripts were revealed by hybridization at high stringency with a v-*erbA*-specific probe. In liquid hybridization, they were revealed by protection with single-stranded probes covering various v-*erbA* domains. This assay showed that all the tested tissues contained c-*erbA* transcripts with a structure colinear with that of the v-*erbA* gene. However, we cannot rule out that some transcripts revealed with the v-*erbA* probe in the Northern blot analysis were not protected by the probes used in the nuclease assay. If such transcripts exist, they would derive from c-*erbA*-related genes. Each of the probes used in the nuclease assay, especially probe E3, gave several protected fragments. The doublet and minor bands might result from either partial mispairing between the c-*erbA* transcripts and the probe or the occurrence of several species of c-*erbA* transcripts with differences in structure. The third approach to detect c-*erbA* transcripts in isolated normal cells was in situ hybridization. Confirmation that this assay revealed c-*erbA* transcripts was made by comparing results from in situ hybridization with those from hybridization with RNA extracted from the same cells. In AEV ELC, which contain several hundred v-*erbA* RNA copies per cell, we observed a tremendous signal by in situ hybridization. In contrast, in mature adult erythrocytes no signal was seen by in situ hybridization and no c-*erbA* could be detected after RNA extraction. Moreover, the intensity of the hybridization signal with the 7- and 15-day-old embryonic blood cells closely correlated with the amount of c-*erbA* transcripts revealed in these cells by the dot-blot or S1 nuclease assay. On smears of chicken bone marrow, which contains erythrocytic cells at various stages of differentiation together with many nonerythrocytic cells, in situ hybridization with the E3 probe revealed only a fraction of the cells as positive (data not shown), showing that the labeling did not result from nonspecific attachment of the probe to cell components.

c-*erbA* transcripts were found in many different tissues of chicken embryos, including liver, heart, lung, kidney, and blood. It is likely that the transcripts revealed in the solid tissues did not result from circulating-blood contamination.

First, blood contamination was low, as assessed by hybridization with a globin probe. Second, the relative amounts of the different c-*erbA* transcript species were different between blood and other tissues. Third, c-*erbA* transcripts were detected in chicken embyro fibroblasts passaged several times in culture and virtually free of immature erythrocytes. In situ hybridization showed unambiguously that erythrocytic cells contain c-*erbA* transcripts. Erythrocytic and nonerythrocytic cells exhibit two major differences in expressing c-*erbA* transcripts. First, erythrocytic cells expressed four c-*erbA* mRNA species, whereas only two of them were detected in nonerythrocytic cells. We do not know yet whether these different mRNAs are transcribed from different c-*erbA* genes or result from differential transcription strategies for the same gene. Second, all nonerythrocytic cells had nearly the same low amount of c-*erbA* transcripts, nearly 10 times less than in late erythrocytic cells. To estimate the number of c-*erbA* RNA copies in normal erythrocytes, we compared the intensities of the c-*erbA* protected bands in 15-day-old embryo erythrocytes with those of v-*erbA* protected bands in serial dilutions of RNAs isolated from AEV-transformed ELC containing a known number of viral transcripts (5). We could roughly estimate that late polychromatophilic erythroblasts contained an average of 7 to 10 copies of c-*erbA* transcripts per cell (data not shown).

The c-*erbA* genes appear to be differentially expressed along the erythrocyte differentiation pathway. The highest level of transcripts was found in late polychromatophilic erythroblasts, which represent the last differentiation step before fully mature erythrocytes. That c-*erbA* transcripts are actually synthesized in these cells was demonstrated in two ways. First, from experiments in which blood cells were incubated in vitro with and without actinomycin D, it was concluded that c-*erbA* transcripts were synthesized in circulating polychromatophilic erythroblasts. Second, the half-life of the c-*erbA* transcripts, between 1 and 2 h, was extremely short with regard to the time required for maturation of polychromatophilic erythroblasts from early erythroblasts stages (24 to 48 h) (4). In these conditions the occurrence of high amounts of c-*erbA* transcripts in polychromatophilic erythroblasts could not result from an accumulation process. The identification of cells that expressed high levels of c-*erbA* RNAs in the blood of 7-day-old embryos relied on cell fractionation with a density gradient and in situ hybridization. Identification of the cells that expressed c-*erbA* was based on the number of cells positive versus distribution of the different types of cells within each fraction. The data suggest that expression is higher in late polychromatophilic erythroblasts than in earlier erythroblasts. However, a definitive conclusion would need simultaneous detection of both c-*erbA* transcripts and differentiation markers within the same cells. ELC transformed by either E26 or AEV were considered erythrocyte progenitor cells blocked in their differentiation (15, 26). In particular ELC transformed by AEV are blocked at the CFU-E stage, which precedes the basophilic erythroblast stage (30). No c-*erbA* transcripts were detected in E26 ELC. In ELC transformed by AEV-H, low amounts of c-*erbA* transcripts were detected. As these cells exhibit a high rate of spontaneous differentiation (10), most of the c-*erbA* transcripts detected were presumably expressed in the differentiated cells. These results suggest that c-*erbA* genes are poorly expressed, if expressed at all, in erythrocyte progenitor cells. However, we cannot rule out that c-*erbA* transcription is repressed in these cells as a result of leukemic transformation. Therefore, all these results demonstrate that the c-*erbA* genes are preferentially expressed during late stages of erythrocyte differentiation. It was recently shown that c-*erbA* genes encode a receptor for triiodothyronine (31, 41). This hormone has been shown to potentiate human erythropoiesis in vitro (7). Our results suggest that this hormone might also play some role in differentiation of chicken erythrocytes.

The expression of the v-*erbA* product in AEV-transformed ELC is necessary for maintaining both blockage of differentiation and cell multiplication (10). We therefore assume that these effects result from functional interference between the v-*erbA* and c-*erbA* genes or their respective products in erythrocyte progenitor cells infected by AEV.

## ACKNOWLEDGMENTS

We thank A. Sergeant for help with the S1 nuclease protection assay and fruitful discussions, B. Bloch for introducing us to in vitro hybridization, and P. Couble for providing us the *Bombyx mori* riboprobe.

This work was supported by the Association pour la Recherche contre le Cancer, the Centre National de la Recherche Scientifique (grant 06933), the Institut National de la Recherche Agronomique, the Institut National de la Santé et de la Recherche Médicale (grant 86204), and the Fédération Nationale des Centres de Lutte contre le Cancer.

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