Differential expression of the cellular oncogenes
c-src and c-yes in embryonal and adult chicken tissues

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The cellular onc-genes c-src and c-yes are expressed very differently during chicken embryonic development. The c-src mRNA and its translational product are detectable at high levels in brain extracts of chicken embryos and adult chickens, whereas muscle extracts show an age-dependent decrease in the amounts of c-src-specific mRNA and $pp60^{c-src}$ kinase activity. In contrast, the levels of c-yes mRNA in brain, heart, and muscle are relatively low in early embryonic stages and increase later on to values comparable to those found for liver, while in adult animals the pattern of c-yes expression is similar to that of the c-src gene. From the close correlation between the levels of $pp60^{c-src}$, its enzymatic activity, and its corresponding mRNA at a given stage of development and in given tissues, it appears that the expression of $pp60^{c-src}$ is primarily controlled at the level of transcription. It is suggested that because of the different patterns of expression, the two cellular oncogenes, c-src and c-yes, play different roles in cell proliferation during early embryonic stages as well as in ensuing differentiation processes.

The genomes of acutely transforming retroviruses contain sequences which are responsible for the transformation of culture cells and for the induction of tumors in animals (for review see Bishop, 1983). These viral onc-genes (v-onc) are derived from cellular DNA sequences, so-called c-onc genes (Stehelin et al., 1976), which are highly conserved during evolution (Spector et al., 1978a; Takeya & Hanafusa, 1982; Barnekow & Schartl, 1984). There is evidence in several cases that c-onc genes, when activated by point mutations or DNA rearrangements, may be involved in tumor induction (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982; Alitalo et al., 1983). Their physiological role, however, still remains unclear. The highly conserved nature of the cellular genes homologous to the retrovirus onc-genes and their protein products suggests that they are involved in the control of fundamental processes, essential to all metazoan organisms (Spector et al., 1978b; Shilo & Weinberg, 1981;

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Schartl & Barnekow, 1982; Barnekow & Schartl, 1984). As their viral counterparts are known to play the essential role in cell proliferation and differentiation during malignant cell transformation, one is tempted to speculate that the c-onc genes could exhibit similar functions in normal cells (Graf & Beug, 1978; Bauer & Yoshikawa, 1980; Ellis et al., 1981; Bishop & Varmus, 1982). In some cases a correlation of c-onc gene expression with differentiation processes has been reported (Westin et al., 1982a,b; Sorge et al., 1984). Other cellular onc-genes have been shown to be expressed differentially during mouse embryogenesis (Müller et al., 1982, 1983).

Recently, we have reported on the expression of the cellular counterpart of the src-gene of Rous sarcoma virus, the c-src gene, in different tissues and cells of chicken embryos and adult chickens (Barnekow & Bauer, 1984). The results showed age- and tissue-dependent differences in the c-src gene product expression. To check for a possible control mechanism at the transcriptional level, we performed a systematic study on polyadenylic-acid-containing RNA from brain, muscle, heart, and liver of chicken embryos of different ages as well as adult chickens, in order to determine the amount of c-src-specific mRNA by dot-blot analysis and Northern blot hybridization. In addition, we followed during chicken development the mRNA pattern of c-yes, whose viral counterpart codes for a protein that is highly homologous to $pp60^{c-src}$ (Kitamura et al., 1982; Takeya & Hanafusa, 1983).

Materials and Methods

Antisera

Antisera from RSV-tumor-bearing rabbits (TBR-sera) were prepared by simultaneous injection of SR-D-RSV and Prague-C strain RSV into newborn rabbits in a modification (Ziemiecki & Friis, 1980) of the procedure described by Brugge and Erikson (1977). A total of three different sera were used throughout the experiments, all of which yielded similar results.

Preparation of cell extracts and immunoprecipitation

Chicken embryo-tissue extracts were prepared from fertile white-leghorn pathogen-free eggs (Lohmann-Tierzucht GmbH., Cuxhaven, Federal Republic of Germany), as was described recently (Barnekow & Bauer, 1984). These tissues were also prepared from 3-month-old chickens. The samples were washed twice with ice-cold phosphate-buffered saline (PBS) and then mixed with 10 ml of extraction buffer, pH 7.0 (10-mM sodium phosphate, 40-mM NaF, 10-mM EDTA, 1% Triton X-100, and 5% Trasylol (Bayer, Leverkusen, FRG) as protease inhibitor). The mixture was homogenized at 4°C in a Sorvall Omnimixer for 2-5 min and centrifuged at 20 000 g for 60 min. Clarified supernatants were used for immunoprecipitation experiments as described recently (Barnekow & Bauer, 1984).

Radioactive labeling of cells

Muscle and brain tissue from 5-day-old chicken embryos was trypsinized, and then transferred to a 90-mm petri dish. Subconfluent cultures were incubated at 37°C for 4 h with phosphate-free

Dulbecco's modified Eagle's medium containing 1 mCi of [^{32}P]-orthophosphate/ml of medium. The cells were washed with PBS and lysed with 1 ml of extraction buffer. The lysate was clarified and the supernatant incubated with 10 µl of heat-inactivated TBR-serum (56°C; 30 min) on ice for 60 min. A 10% suspension of formalin-fixed *Staphylococcus aureus* Cowan strain I was added to absorb immune-complexes. After 30 min the labeled immunecomplexes were washed four times with kinase washing buffer and once with H$_2$O, and the pellet was then resuspended in 30 µl of sample buffer, boiled for 2 min, and centrifuged for 4 min at 10 000 g. The supernatant was loaded onto an 11% polyacrylamide slab gel with 2.5% acrylamide stacking gel (Laemmli, 1970). The labeled proteins were detected by autoradiography after Coomassie Blue staining, destaining, and drying of the gels. The molecular weights of the proteins on the gels were calibrated using ^{14}C-labeled protein markers (Amersham Ltd., United Kingdom) and ^{32}P-labeled heavy chain of IgG.

Protein kinase assay

The protein kinase assay was carried out by a modification (Barnekow & Bauer, 1984) of the method of Collett and Erikson (1978). For quantitation, the radioactive gel bands were cut out and solubilized, and their radioactivity was determined by liquid-scintillation counting.

Phosphotyrosine phosphatase

Measurement of the phosphotyrosine phosphatase reaction was performed according to the method of Foulkes et al. (1981). Organ extracts were diluted to 1.3 mg of protein/ml in 50-mM Tris/HCl, pH 7.0, at 25°C, containing 30-mM 2-mercaptoethanol. The heavy chain of IgG specifically radiolabeled with ^{32}P at tyrosine was used as a substrate.

Isolation of cellular RNA

Total RNA was extracted from embryonic and adult chicken organs in a modification of a procedure described by Adams et al. (1977). Freshly prepared organs (0.5-1.5 g) were cut into small pieces and homogenized in 13 ml of 8-M guanidine hydrochloride (BRL, Neu-Isenburg, FRG), 0.1-M sodium acetate (pH 5.0), followed by mincing in a Sorvall Omnimix for 5 min at full speed to reduce the viscosity.

The homogenates were centrifuged for 10 min at 10 000 g and the clarified supernatants were mixed with 0.5 vol. of absolute ethanol and stored at -20°C overnight to precipitate nucleic acids. After centrifugation at -10°C and 6000 g for 20 min, the pellets were resuspended in 6.5 ml of 7-M guanidine hydrochloride, 25-mM EDTA (pH 7.5). The solutions were made 50 mM in sodium acetate, and after adding 0.5 vol. of absolute ethanol, the RNA was again precipitated for at least 2 h at -20°C.

The RNA was sedimented at -10°C and 6000 g for 20 min. Precipitation from 7-M guanidine hydrochloride solution was repeated twice, and the final pellets were dissolved in 4 ml of 25-mM EDTA (pH 7.5) and extracted once with chloroform/i-butanol (4:1). The organic phase was back-extracted with 2 ml of 25-mM EDTA (pH 7.5)

and the RNA precipitated from the combined aqueous phases overnight by adding 2 vol. of 3-M sodium acetate (pH 5.6). After centrifugation for 20 min at 4°C and 20 000 g, the RNA pellets were resuspended in 1 ml of sterile water and the purity of the preparations was checked by measuring the OD ratio of 260 nm/280 nm, which was always above 1.9:1. Furthermore, the amounts of 27S and 18S rRNA were compared by PAGE of aliquots and scanning of the gels at 260 nm.

Poly(A)$^+$ selection of RNA

Total RNA was enriched for poly(A)-containing RNA by one cycle of affinity chromatography on oligo(dT)-cellulose (Aviv & Leder, 1972). Oligo(dT)-cellulose (BRL, Neu-Isenburg, FRG) was suspended in loading buffer (0.5-M NaCl, 50-mM Tris, pH 7.3, 10-mM EDTA), washed with several column volumes of 0.5-M NaOH and sterile water, and equilibrated with loading buffer.

Total RNA was pelleted and dissolved in loading buffer at a concn. of 0.5 to 1 mg/ml (1 OD_{260} was assumed to be 20 µg/ml), heated to 65°C for 5 min, and quickly chilled on ice before application to the column at the natural flow rate. The eluate was reapplied and the column was washed with loading buffer until the OD_{260} of the eluate was less than 0.02. Bound RNA was eluted with 2 ml of 60°C sterile water and precipitated at -70°C after adding 0.1 vol. of 3-M sodium acetate (pH 5.6) and 2.2 vol. of absolute ethanol. Enrichment for poly(A)+ RNA was approx. 10- to 15-fold.

Agarose-gel electrophoresis and Northern blotting

Samples of 5 µg of poly(A)+ RNA were analyzed by formaldehyde agarose-gel electrophoresis (Lehrach et al., 1977). RNA was denatured for 10 min at 65°C in electrophoresis buffer (20 mM morpholinopropanesulfonic acid (MOPS) (pH 7.0), 5-mM sodium acetate, 1-mM EDTA) containing 50% formamide, and 2.2-M formaldehyde. Denatured RNA was separated on horizontal gels containing 1.2% agarose and 2.2-M formaldehyde in electrophoresis buffer. Transfer of the RNA onto gene screen membranes (NEN, Dreieich, FRG) was carried out as capillary blot according to the instructions of the supplier. The filters were baked for 3 h at 90°C to bind the RNA irreversibly.

Dot-blot analysis

Poly(A)$^+$ RNA was dissolved in sterile water, heated to 80°C for 5 min, and chilled on ice. Serial dilutions were prepared and 2 µl of each sample was spotted onto sheets of nitrocellulose, which had been equilibrated with 20 x SSC (Thomas, 1980) and air dried before use. The RNA was immobilized by baking for 3 h at 80°C.

Filter hybridization

Filters were prehybridized for at least 6 h at 42°C in hybridization buffer containing 5 x SSC, 50-mM Tris (pH 7.5), 0.1% sodium pyrophosphate, 0.1% SDS, 5x Denhardt reagent (1x Denhardt reagent: 0.02% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 0.1 mg of denatured calf thymus DNA per ml, and 50% formamide. Subsequently the blots were hybridized for three days with 5 x 10^6

c.p.m. of nick-translated probe per ml of hybridization buffer. The 0.4-kb PstI-G and 0.35-kb PstI-H fragments of pSRA-2 (DeLorbe et al., 1980) served as *src*-specific probe and the 0.6-kb PstI/EcoRI insert from p73EcoP10 (obtained from K. Toyoshima) was taken as a probe for *c-yes*. These fragments, prepared from restriction-enzyme digests of the plasmids by polyacrylamide-gel electrophoresis (Maxam & Gilbert, 1977), were nick-translated in the presence of [$-^{32}$P]dCTP (3000 Ci/mmol) to specific radioactivities of 4-8 x 10^8 c.p.m. per µg of DNA. After hybridization, the blots were washed twice in 1 x SSC, 0.1% SDS at room temperature and 4 x 25 min at 50°C. A final wash with 1 x SSC was carried out to remove the SDS. The blots were exposed to X-ray films (Kodak XAR-5, Siemens, Frankfurt/M., FRG) with intensifying screens (Dupont, Frankfurt/M.,FRG) at -70°C for 1 to 5 days. Signal strength was quantitated by visual comparison and densitometer scanning of the autoradiography dots and bands.

Results

Expression of c-src gene product during chicken development in different organs

Previous studies in our laboratory showed that the *c-src* gene product is expressed in an organ-specific manner (Bauer et al., 1982; Barnekow et al., 1982; Schartl & Barnekow, 1982). In this investigation we have extended our experiments and report on an age- and organ-specific expression of the *c-src* gene product in chicken embryos as well as in adult chickens. High amounts of the *c-src* gene product as measured by the kinase assay or by immuneprecipitating metabolically labeled [^{32}]pp60^{c-src} are always found in brain and muscle cells of early embryos (Figs. 1,5A). In muscle tissue of adult animals a pp60^{c-src}-specific kinase activity is barely detectable, whereas in brain cells, the activity remains high (Fig. 5A).

Determination of phosphotyrosine-specific phosphatase activity

It appeared possible that the organ-specific differences in pp60^{c-src} kinase activity were due to differential activity of a phosphotyrosine phosphatase, which might interfere in vivo and in the in vitro assays with the cell extracts. Therefore, we have tested the various extracts for the presence of a phosphotyrosine-specific phosphatase. As shown in Table 1, no significant amounts of tyrosine phosphatase can be observed in brain and muscle extracts of adult chickens. Liver extracts, which exhibit the highest amount of pp60^{c-src} kinase activity, show slightly higher phosphatase activity. These data indicate that the different activities of the pp60^{c-src} kinase are directly correlated with the *c-src* gene expression and are not the result of different kinase/phosphatase interactions.

Levels of c-src- and c-yes-specific mRNA during chicken development

The differences observed in the kinase activity of pp60^{c-src} and the amount of [^{32}P]pp60^{c-src} could be due to a regulation either on the transcriptional or on the posttranscriptional level. Therefore, we analyzed poly(A)+ from corresponding organs by dot-blot analysis and Northern blot hybridization using *src*-specific DNA probes. In parallel
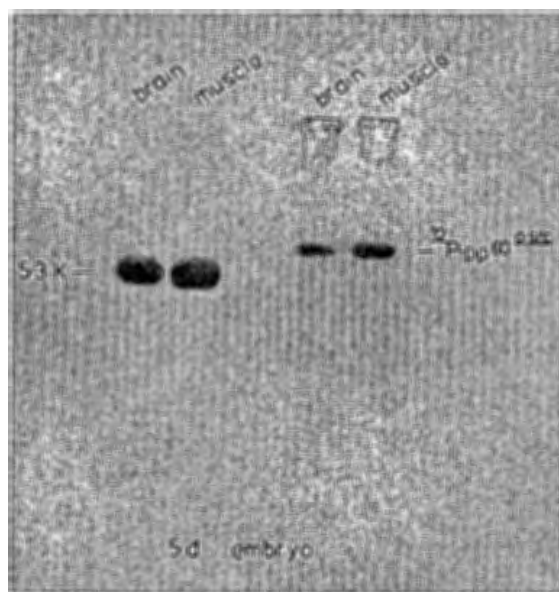
Fig. 1.   Demonstration of ^{32}P-pp60^{c-src} and its kinase activity in 5-day-old chicken muscle and brain cells. Equal amounts of metabolically labeled ^{32}P-protein were immunoprecipitated with TBR-serum, the immunecomplexes washed four times, and the proteins separated on an 11% polyacrylamide gel. The molecular weight of proteins on the gels was calibrated using ^{14}C-labeled protein markers and the heavy chain of IgG (53 K).

experiments, i.e. with samples of the same poly(A)+ RNA preparations, the expression of c-yes was followed by molecular hybridization. In most cases two or three independent preparations were analyzed at least twice to assure the significance of the data obtained.

Both genes, c-src and c-yes, show transcripts of about 3.9 kb (Fig. 2). The c-src mRNA levels show a strong correlation with the c-src gene product expression. We find a constantly high amount of c-src-specific transcripts in the brain throughout embryonic development as well as in adult chickens, whereas in muscle an age-dependent decrease of c-src mRNA with hardly detectable hybridization in adult muscle can be observed (Figs. 4,5A).   Liver from adult chickens, displaying high amounts of pp60^{c-src} kinase activity (Fig. 5A), also have increased levels of c-src transcripts compared to the moderate amounts found during embryogenesis (Fig. 5A).   In heart extracts no significant variation in c-src gene expression could be observed in any stage of chicken development (Figs. 3,5A).

Interestingly, the pattern of the c-yes gene expression is totally different from that of c-src.   The amounts of the 3.9-kb c-yes transcripts in brain, muscle, and heart increase from day 8 to day 14 of embryogenesis, reaching values comparable to those found for liver from all stages tested (Figs. 2,3,5B).   In the adult chicken a decrease

Table 1. Time course of phosphotyrosine phosphatase activity
in various organs of adult chicken

The phosphotyrosine phosphatase activity is expressed
as percentage of ^{32}P counts released from ^{32}P-IgG heavy
chain.

Min	Liver	Brain	Muscle
0	0.75	0.45	0.45
2	2.00	0.45	0.45
4	2.40	0.60	0.45
8	3.00	0.60	0.45
15	3.50	0.60	0.45
25	4.20	0.80	0.45

of *c-yes* mRNS can be observed in heart and especially in muscle
extracts (Fig. 5B), while the amounts in brain and liver are compar-
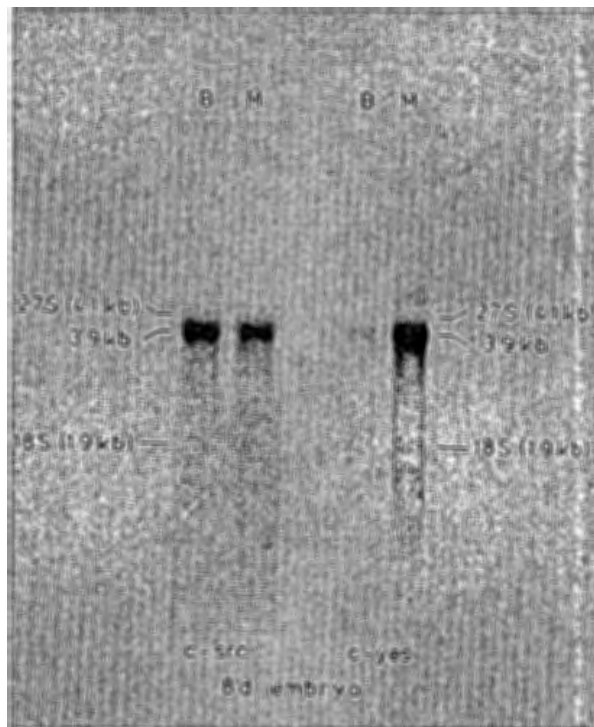able to those in the late embryogenic stage.

Fig. 2. Comparison of *c-src* and *c-yes* expression in
brain (B) and muscle (M) from 8-day-old chicken
embryos. Autoradiograms showing the hybridization
of equal amounts of poly(A)$^{+}$ RNA with the *v-onc-*
specific probes. Molecular weights were determined
using RSV and Y73 viral RNA and chicken ribosomal
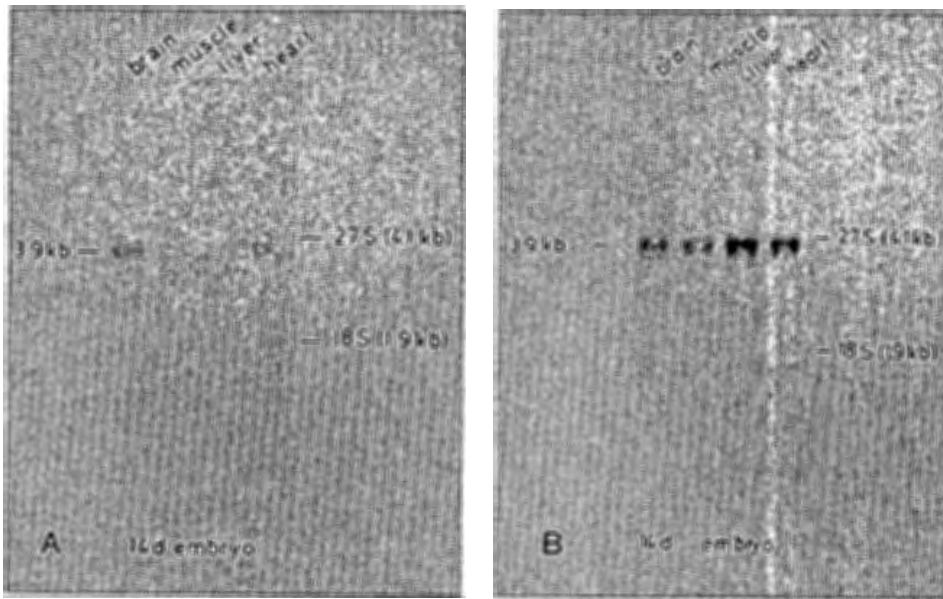RNA as size standards.

Fig. 3. Hybridization of *c-src* (A) and *c-yes* (B) mRNA in four organs of 14-day-old chicken embryos. 5 μg of poly(A)$^+$ RNA were analyzed by the Northern blotting technique as described in Materials and Methods.
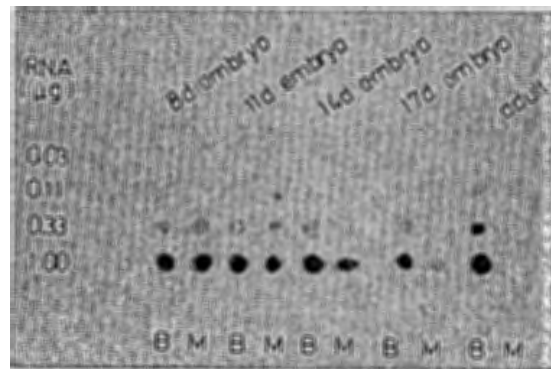

Fig. 4. Dot-blot analysis of *c-src* expression at various developmental stages of chickens in brain (B) and muscle (M).

Discussion

Cellular interaction and proliferation play an important role in the interaction between cells and retroviruses (for review see Friis, 1978). If the cellular DNA sequences from which the viral *onc*-genes were derived play a role in similar processes in normal cells, a specific pattern of gene expression could be expected during embryonic development of organisms.

Previous studies had shown a differential expression of the cellular *src*-gene product pp60^{src} and its phosphokinase activity in chicken embryo-muscle cells during development (Barnekow et al., 1981). An organ-specific expression of the *c-src* gene product in chicken and also other species has also been reported, demonstrating the highest activity of pp60^{c-src} kinase in nervous tissue (Bauer et al., 1982; Barnekow et al., 1982; Schartl & Barnekow, 1982; Barnekow & Bauer, 1984). Similar results have also been described by Cotton and Brugge (1983). An elevated expression of *c-src* gene product in brain cells can be followed from mammals down to insects (Schartl & Barnekow, 1982; Jacobs & Rübsamen, 1983; Anders et al., 1984), and increased expression has recently also been reported in neural retina by Sorge et al. (1984). In contrast to the pp60^{c-src} expression in neural cells, the expression in muscle tissue shows an age-dependent decrease, with barely detectable amounts of pp60^{c-src} kinase activity in adult chickens (Barnekow et al., 1981).

The mechanisms of regulation responsible for the different levels of pp60^{c-src} in various organs of embryos of different ages and of adult animals is still unknown. To test for a control on the transcriptional level, we have made a systematic study on polyadenylic-acid-containing RNA of embryonic tissues and tissue extracts of adult chickens showing that *c-src* transcripts can be detected in significantly different amounts in brain, muscle, heart, and liver cells of chicken embryos of different ages and adult animals. The results demonstrate a close correlation between *c-src*-specific mRNA levels in the organ extracts tested and amounts of the *c-src* gene product.

When Kitamura et al. (1982) predicted the amino acid sequence of the *v-yes* product pp90$^{gag-yes}$ from the nucleotide sequence of the *onc*-gene of the Y73 virus, they found strong homology between the *yes* part and pp60^{v-src}. From this observation and on the basis that the gene products of the viral and cellular *src* genes are structurally very similar (Sefton et al., 1980), though not identical (Takeya & Hanafusa, 1983), Kitamura et al. (1982) suggest that the cellular counterparts of the two cellular *onc*-genes, *c-src* and *c-yes*, originate from a common prototype sequence.

We compared the tissue-specific and age-dependent expression pattern of *c-src* with that of *c-yes*. The results obtained from the hybridization studies demonstrated that the two cellular *onc*-genes are regulated differently and that they are not coordinately expressed. The function of each gene may be required only in certain tissues, or during definite stages of embryonic development.

A quantitative difference in the transcription of *c-src* and *c-yes* can also be observed. Using equal amounts of poly(A)+ RNA, one obtains a stronger hybridization signal with the *yes*-specific DNA fragment than with the *src*-specific probe during embryogenesis of chickens and in adult animals, suggesting that there are more *c-yes* mRNA molecules per cell than *c-src* transcripts in late embryonic stages (14d and 17d) as well as in adult chickens. When Shibuya et al. (1982) compared the amount of *c-src* and *c-yes*-specific mRNA in chicken and quail embryo cells, they found 4 copies of *c-src* mRNA and 16 copies of *c-yes* transcripts per chicken cell, and 2 copies of *c-src* mRNA and 12 copies of *c-yes* mRNA per cell in quails. Thus, our results are in agreement with their observations.

c-src-specific kinase activity
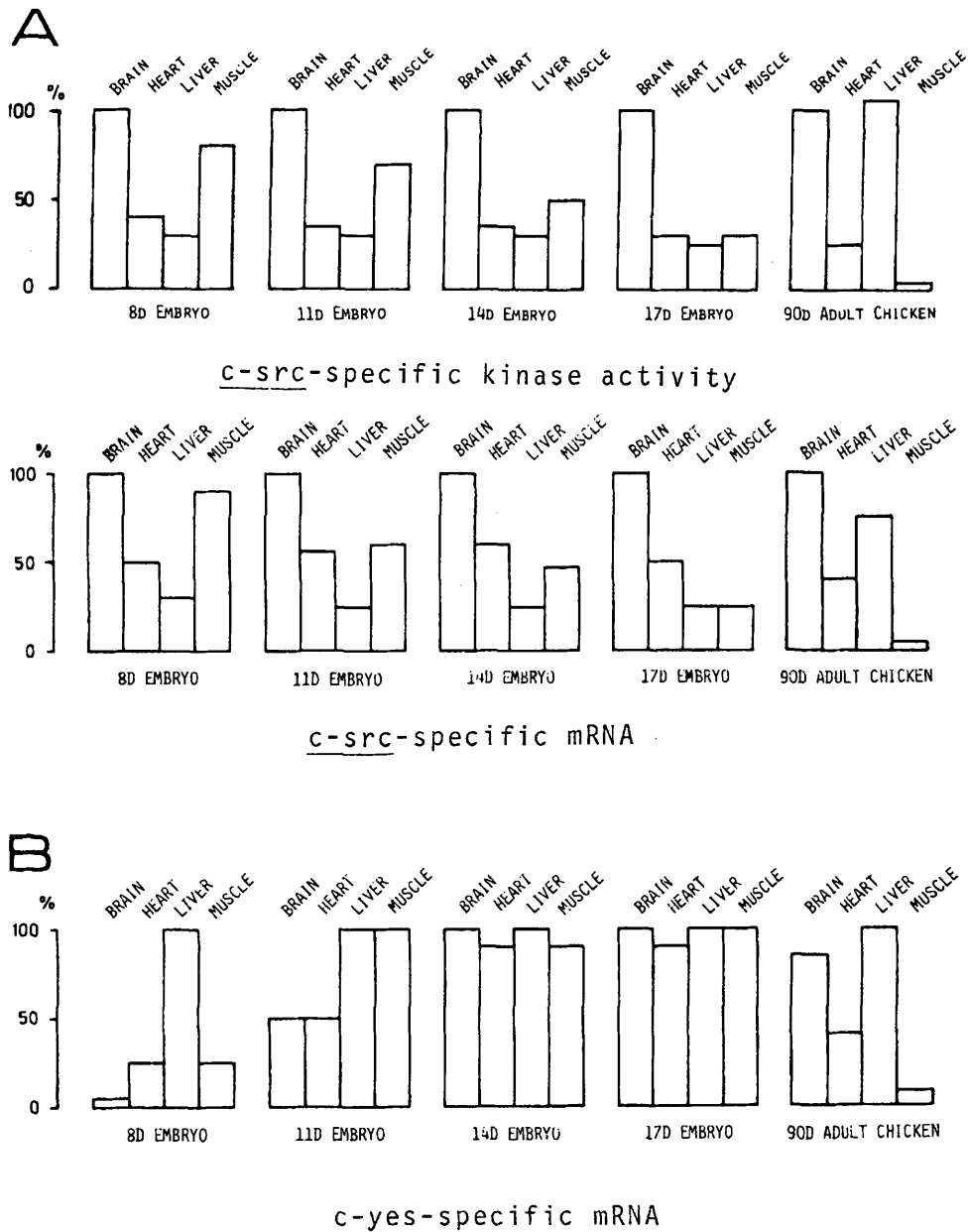
c-src-specific mRNA

c-yes-specific mRNA

Fig. 5. Expression of *c-src* (A) and *c-yes* (B) in brain, heart, liver, and muscle extracts of 8-day-old (8 D), 11-day-old (11 D), 14-day-old (14 D), and 17-day-old (17 D) chicken embryos and 90-day-old (90 D) adult chickens.

Demonstration of the age- and tissue-specific distribution of the pp60^{c-src} kinase activity. (A, upper panel): Equal amounts of protein were immunoprecipitated with a pp60^{c-src} reactive tumor-bearing rabbit (TBR) serum, the kinase assay performed, and the proteins separated on an 11% polyacrylamide gel. The continously high expression of pp60^{c-src} kinase activity in brain extracts was taken as 100%.

(Fig. 5, cont'd.) Expression of *c-src* mRNA (A, lower panel), and *c-yes* m RNA (B) during embryonic development of chickens and in adult animals: The amount of mRNA hybridizing with the *v-onc*-specific probes was quantitated by density scanning of autoradiograms from dot-blot and Northern blot experiments. The continously high levels of *c-src* mRNA in brain and of *c-yes* mRNA in liver were taken as 100%. Furthermore, the differences between *c-src* and *c-yes* expression were confirmed by rehybridization of the blots with the second *v-onc* probe.

Tissue-specific expression of cellular *onc*-genes have also been reported by Gonda et al. (1982), who investigated the expression of *c-myc*, *c-src*, *c-myb*, and *c-erb* in chickens. Each of these *onc*-genes is described in a variety of tissues and appears to be independently controlled from one tissue to another (Chen, 1980). Other cellular *onc*-genes, especially *c-fos* and *c-fms*, have been studied in certain tissues during mouse embryogenesis (Muller et al., 1982, 1983). Both genes are expressed tissue-specifically, but whereas *c-fms* expression increases during embryonic development, *c-fos* does not show a stage-specific expression in the organs tested.

The data described in this report show significant differences in the expression patterns of two cellular *onc*-genes, *c-src* and *c-yes*, at various developmental stages of the chicken. Since the *v-yes* gene product is structurally related to the $pp60^{v-src}$ and both proteins exhibit a tyrosine-specific kinase activity, one could argue that $pp60^{src}$-specific antibodies may react with the *yes*-gene product, with the viral gene product, and, due to the structural similarities, also with the cellular gene product. However, the finding of very low amounts of $pp60^{c-src}$ kinase activity in certain tissues, which on the other hand contain high levels of *c-yes*-specific mRNA and thus presumably high amounts of *c-yes* gene product, suggests that there is little, if any, immunological relationship between the gene products of the *c-src* and the *c-yes* gene.

Acknowledgements

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