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Transformation of Chicken Embryo Fibroblasts by Direct DNA Transfection of Single Oncogenes: Comparative Analyses of *src*, *erbB*, *myc*, and *ras*

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Chicken embryo fibroblasts (CEF) have been used extensively to study the transformation parameters of a number of avian sarcoma-leukemia viruses. Previously, oncogene transformation of CEF has been conducted almost exclusively with replicating viruses, because of perceived difficulties with direct DNA transfection. Here, we show that CEF can be efficiently and stably transfected by selection for the neomycin resistance gene (*neo*). Cotransfection of *neo* with various oncogenes resulted in CEF transformation in vitro and, in several instances, sarcoma formation in vivo. Transfection of *src*, *myc*, *erbB*, and *ras*, either singly or in combination, resulted in soft-agar colonies with unique morphologies. Transfection of a family of v-*src*, c-*src*, and v/c-*src* chimeric constructs demonstrated the ability of the assay to discriminate between transforming and nontransforming genes. Transfection of a number of *erbB* variants showed that internal mutations, primarily in the kinase domain, contribute significantly to the ability to transform fibroblasts. The tumorigenic potential detected by transfection of oncogenes faithfully reproduced those previously reported by using viral infections. Our studies establish the utility of CEF transformation by direct DNA transfection. This method should prove useful in analyzing oncogenes (e.g., *myc*) that do not readily transform rodent cell lines and in studying host-range mutants of oncogenes, such as those recently identified for *src* and *erbB*.

A number of in vitro and in vivo systems have been developed to characterize the transforming potential of oncogenes (for example, see references 3, 4, 6, 17, and 43). Assays for tumorigenesis are frequently conducted through the inoculation of whole animals with either viruses carrying putative oncogenes or cells transformed by oncogenes. Direct injection of animals with oncogene DNA has also proven useful in some instances (14).

The most widely used in vitro assays for transformation utilize oncogenes introduced into cells by infection with viral vectors or by transfection with DNA. These in vitro assays frequently employ established rodent cell lines (NIH 3T3 or RAT-1) and score for the transformed state on the basis of the loss of contact inhibition (44) or anchorage-dependent growth (23). These assays have been extended to primary or secondary rat embryo or kidney cells (25, 38), which usually require the cotransfection of two oncogenes to detect transformation. This apparent requirement for multiple oncogenes has been taken as evidence for a multistep pathway of transformation. A single oncogene, however, can sometimes transform primary embryo cells when cotransfected with the neomycin resistance gene (*neo*) (24, 46). Land et al. (24) suggested that G418 selection might facilitate the growth of transformants by removing an inhibitory environment presented by the normal cells in the cultures.

A number of important sarcoma-leukemia viruses are of avian origin, and chicken embryo fibroblasts (CEF) have been used extensively to study the transformation parameters of these viruses (48). Thus far, transformation studies in CEF have been conducted primarily through the use of infectious viruses. In CEF, unlike rodent cells, direct transfection of oncogene DNA has previously met with limited success. Cooper and Okenquist (7) showed that calcium phosphate-mediated transfection of genomic DNA carrying Rous sarcoma virus produced transformants in CEF inefficiently, unless infectious virus was generated and allowed to spread throughout the culture. Similarly, Kawai and Nishizawa (22) showed that Polybrene-mediated direct DNA transfection yielded higher but still marginal frequencies of transformation, despite the fairly wide range of doses tested. As a result of these studies, it was thought that CEF were refractory to DNA transfection or integration.

In our analysis of CEF transfection, we found that the *neo* gene (pSV2neo) (45) could be efficiently introduced into CEF cells to yield G418-resistant cells in a dose-dependent fashion. Transfection frequencies on the order of one drug-resistant cell per thousand cells have been achieved routinely throughout the course of our studies (see below). Furthermore, other avian cells, QT6 (a chemically transformed quail cell line), are noted for successful transfection (31). These findings prompted us to reexamine oncogene transformation of CEF using standard calcium phosphate transfection protocols. We report here that CEF can be transformed efficiently by a number of oncogenes (*src*, *myc*, *erbB*, *ras*) when cotransfected with and selected for *neo*. The specificities of transformation based on studies of oncogenic and nononcogenic variants faithfully reproduced those generated by virus infections.

This transformation assay for oncogene activity in avian embryo fibroblasts provides an important complementary and/or alternative approach to the use of rat embryo cells. It provides a more suitable host for avian oncogenes, especially those which do not transform established rodent cell lines efficiently (e.g., *myc* and *erbB*) (8), and in conjunction with these systems it provides an opportunity to study the

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underlying basis of the species-specific transformation of certain oncogenes.

MATERIALS AND METHODS

Plasmids. Plasmids were kindly provided by J. M. Bishop (pMC38), L. Sealy (pAEV 11-3), T. Yamamoto (pAE7.7), R. Ellis (pH1), A. Zelenetz and G. Cooper (psrc11), H. Hanafusa (pN4, pB5, pBB4, pHB5, and TT105), and R. Pelley (pAEV-R/C and pAEV-C). psrc11 (36) represents a nonpermuted SR-D Rous sarcoma virus genome with portions of the *gag-env* sequences deleted. pN4 (see Fig. 2 and reference 20) carries the cloned v-*src* gene of NY-SRA strain of Rous sarcoma virus. The *src* genes of pTT105 and pHB5 (20) are derived from c-*src* sequences. pB5 and pBB4 are chimeric constructs of v-*src* and c-*src*, exchanged at the C-terminal *Pst*I and *Bgl*II sites, respectively (20). pH1 (12) and pMC39 (51) are plasmids carrying, respectively, the permuted genomes of Harvey sarcoma virus and avian leukemia virus strain MC29. In both plasmids, the viral inserts are bounded by *Eco*RI sites; digestion with *Eco*RI followed by ligation restores the functional viral transcriptional units. pAEV-R (avian erythroblastosis virus vector) is the same as pAEV 11-3 described by Sealy et al. (40), which carries an entire AEV-R genome in a nonpermuted form. To replace the v-*erbB* sequence of pAEV 11-3 by insertionally activated c-*erbB* (IA-c-*erbB*), an *Xba*I site was introduced to replace the *Xmn*I site located at the 3′ untranslated region of pAEV 11-3 (R. Pelley et al., manuscript in preparation). pAEV-C was generated by excising the *Apa*I (located at the very 5′ end of v-*erbB*) to *Xba*I fragment of pAEV 11-3 and replacing it with a corresponding fragment from IA-c-*erbB* (33). The 3′ *Xba*I site of IA-c-*erbB* was generated by placing an *Xba*I linker into the *Nsi*I site (located at the 3′ untranslated region of IA-c-*erbB* cDNA). pAEV-R/C was similarly constructed, except an *Eco*RI (located at the 3′ end of v-*erbB*) to *Xba*I fragment was exchanged with a corresponding fragment of IA-c-*erbB*. pAEV-ΔB is identical to pAEV 11-3, except a *Bam*HI fragment within the *erbB* gene is removed to generate a nonfunctional *erbB* gene (40).

CEF culture. CEF cells were prepared from the skin of 10- to 11-day-old embryos following removal and collagenase digestion (100 U/ml; type IV; Worthington Diagnostics). Good tissue dispersal was generally achieved after 3 h of digestion at 37°C with intermittent pipetting action to mechanically dissociate large tissue fragments. Collagenase solutions were made up in complete medium.

CEF cells were grown in medium consisting of 50% medium M199 (GIBCO Laboratories) and 50% Dulbecco modified Eagle medium (low glucose; GIBCO), supplemented with 3 to 5% fetal calf serum (GIBCO), 2% heat-inactivated chicken serum (GIBCO) (incubated at 55°C for 30 min), and antibiotics (penicillin-streptomycin; GIBCO). The sodium bicarbonate level of this medium was 2.95 g/liter. Cultures were maintained at 37°C in 5% CO_2 environments and fed every other day with fresh growth medium.

CEF transfection. For transfection experiments, early (4th- to 8th-) -passage CEF cells were used because of the limited number of population doublings prior to senescence (25 to 30 in our experiments). Primary cultures were not employed because other contaminating cell types represent an appreciable fraction of their total cell population. At the outset, four to five 100-mm-diameter plates containing approximately 0.5×10^6 cells were plated for each construct or set of constructs to be analyzed. At 16 to 20 h after seeding or 4 to 8 h prior to addition of the transfection cocktails, all cultures were refed with fresh growth medium. The transfection cocktails were prepared as follows. For solution A, to 2.5 ml of 2× HEPES-buffered saline (42 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.1], 270 mM NaCl) was added 50 μl of 100× phosphate solution (1:1 mixture of 70 mM Na_2HPO_4 and NaH_2PO_4). The resulting solution was mixed thoroughly and then set aside. For solution B, a final volume of 2.5 ml was achieved through the addition of water, DNA solutions (in 10 mM Tris–1 mM EDTA, pH 8.0), and 300 μl of 2 M $CaCl_2$ (in that order), followed by vigorous agitation. The initial concentrations of DNA solutions were maintained sufficiently high so that their volumes did not account for more than 15% of solution B. The transfection cocktails were completed by mixing solutions A and B together, vigorously agitating, and allowing the precipitate to form for 20 to 30 min at room temperature. The cocktails were then forcefully expelled from a pipette four to five times, in an attempt to create as fine a particulate as possible. A 1.0-ml volume of the transfection cocktail was then delivered to each 100-mm-diameter plate and allowed to remain for 20 h. After the treatment period, the medium containing the calcium phosphate precipitate was removed and replaced with fresh growth medium. The cultures were allowed to recover for 24 h before the onset of selection, the addition of 400 μg of G418 per ml (100% activity; GIBCO). Selective conditions were maintained sufficiently long (about 10 days) to ensure the elimination of all nonresistant cells. During this time, cultures were fed on every third day or as needed when maximal cell killing occurred, generally at 4 to 7 days. Following selection, cells were grown 1 to 2 days in standard growth medium or further expanded prior to soft-agar or tumorigenic analysis.

Soft-agar assay. For analysis in soft agar, 0.5×10^6 cells in 4.0 ml of 0.35% agar (Bacto-Agar or Noble agar; Difco Laboratories) were plated into 60-mm-diameter dishes containing 4.0 ml of prehardened 0.5% agar. After 1 to 3 days, an additional 3.0 ml of 0.35% agar was added to immobilize cells floating freely in the film of fluid on the surface of the previous 0.35% layer. All agar suspensions were prepared in complete growth medium (described above). Approximately 1 week later, 5 drops of complete growth medium (about 300 μl) were added to each dish and again as needed to prevent desiccation. Results were determined after approximately 21 days, and only colonies containing 50 or more cells were counted.

Tumorigenesis assay. To assay the tumorigenic potential of transfected CEF cells, the wing-webs of young chicks (6 days old or less) were inoculated subdermally with 10^7 $G418^r$ cells in a volume of 0.1 ml. The transfected cells were delivered in growth medium supplemented only with gentamicin at 100 μg/ml. The birds were examined at 2- to 3-day intervals, and the final result was determined 2 weeks postinjection.

There are three important criteria which in large part determine the success and reliability of this assay: (i) using very young chicks, 6 days old or less; (ii) introducing sufficient numbers of transformed cells as concentrated inoculum, on the order of 10^7 cells in 0.1 ml; and (iii) utilizing populations of cells selected for $G418^r$ alone and not further selected for anchorage-independent growth as the source of cells for this assay.

TABLE 1. CEF transfection by pSV2neo DNA

Amt of pSV2neo/plate[a]	No. of Neo^r colonies/10^6 cells[b]
0 ng	0
1 ng	17 ± 34
10 ng	57 ± 73
100 ng	350 ± 184
1 μg	930 ± 188
10 μg	3,300 ± 587
10 μg + 20 μg of SS DNA	4,400 ± 601
10 μg + 50 μg of SS DNA	3,400 ± 340

[a] Except where indicated, all included additions of salmon sperm (SS) DNA to maintain the total input level at 20 μg per 100-mm-diameter plate.

[b] Corrected for cloning efficiency and cytotoxicity; results shown are the average for four dishes plus or minus standard deviation of experimental results.

RESULTS

***neo* dose-response and transfection efficiency.** We initially observed that calcium phosphate-mediated transfection of CEF cells with pSV2neo (45) resulted in the generation of a substantial number of G418-resistant cells. This suggested that, contrary to previous belief, CEF could rather efficiently acquire and stably integrate foreign DNA into their genomes. To fully document this phenomenon and optimize conditions for CEF transfection, we conducted the following detailed analysis with the *neo* gene.

The *neo* gene was introduced at levels ranging from 1 ng to 10 μg/100-mm-diameter plate of cells. The total input DNA level was maintained constant at 20 μg per plate through the introduction of salmon sperm DNA. Two sets of dishes were utilized for each dose of the *neo* gene examined; one set was selected with G418 to determine the number of G418^r cells in the transfected population, and the other set was grown in the absence of G418. This second set provided correction factors for cellular toxicity incurred as a result of the transfection protocol and made it possible to express each final result as the number of G418^r cells per 100% surviving cells. Untreated CEF grown in the absence of G418 were used to determine the intrinsic cloning ability of these cells.

The results of a representative Neo^r dose-response experiment are shown in Table 1. In general, a 10-fold increase in the input level of the *neo* gene resulted in about a 3-fold increase in the number of G418^r cells. At a level of 10 μg of pSV2neo per 100-mm-diameter plate, the transfection efficiency reached approximately 1 in 1,000 cells. Increasing the level of carrier DNA to 50 μg per plate had little influence on the number of G418-resistant cells which developed.

Toxicity induced by the calcium phosphate transfection protocol varied significantly between experiments, with 20 to 30% killing being typical. Within a given experiment, however, cell killing was relatively constant and appeared little affected by the identity or amount of plasmid utilized. The toxicity appeared to be primarily a consequence of exposure to the solution containing the calcium phosphate precipitate. Untreated CEF had cloning efficiencies of 20 to 30%.

These experiments indicated that CEF could be transfected by the calcium phosphate method and, under the appropriate conditions, with an efficiency comparable to that of NIH 3T3 cells.

v-*src* dose-response curve. We next proceeded to develop an oncogene transformation assay in CEF by direct DNA transfection. We chose to study the prototypic oncogene, v-*src*, in psrc11, a construct in which the oncogene is flanked by Rous sarcoma virus long terminal repeats (LTRs) (36). psrc11 (20 μg/100-mm-diameter plate) and pSV2neo (6 μg/100-mm-diameter plate) were cointroduced into CEF by calcium phosphate precipitation. The transformation of CEF was monitored by anchorage-independent growth in soft agar. Without G418 selection, 73 colonies per 10^6 cells were detected. The colonies displayed typical *src* morphology

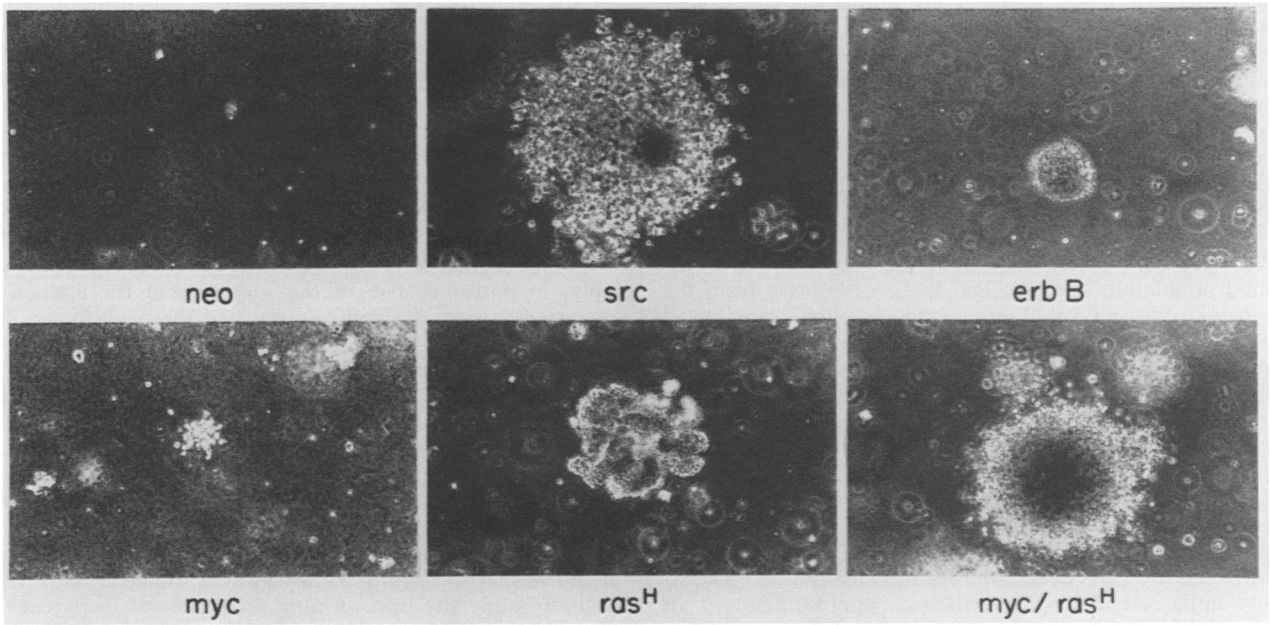

FIG. 1. Morphologies of oncogene-transformed CEF soft-agar colonies. The colonies were derived from cells transfected with the following DNAs in the presence of 6 μg of pSV2neo per 100-mm-diameter plate: 20 μg of salmon sperm DNA (panel *neo*), 20 μg of psrc11 (panel *src*), 20 μg of pAEV-R (panel *erbB*), 20 μg of pMC38 (panel *myc*), 20 μg of pH1 (panel *ras*^{H}), and 10 μg each of pMC38 and pH1 (panel *myc*/*ras*^{H}).

TABLE 2. CEF transformation by psrc11 DNA

Expt and amt of psrc11/plate[a]	No. of soft-agar colonies/10^6 cells[b]
A	
20 μg[c]	73 ± 13
20 μg	20,680 ± 2,992
B[d]	
0 ng	0
2 ng	0
20 ng	53 ± 5
200 ng	38,360 ± 3,207
20 μg	56,600 ± 2,394
50 μg	12,680 ± 2,081

[a] In the presence of 6 μg of pSV2neo per plate.
[b] Plus or minus standard deviation of experimental results.
[c] G418 was added to all other plates.
[d] Sufficient amounts of salmon sperm DNA were added to maintain total DNA input levels at approximately 65 μg per 100-mm-diameter plate.

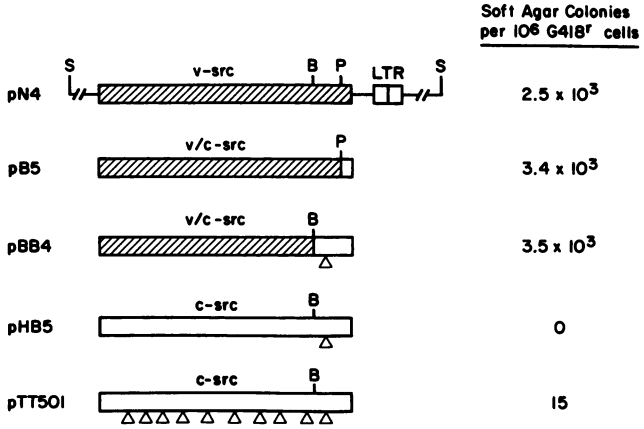

FIG. 2. CEF transformation by v-*src* and c-*src* and their chimeras. All the *src* constructs are contained within a *Sal*I fragment, as shown for pN4 (20). The constructs were first digested with *Sal*I to liberate the inserts and ligated to restore a functional 5′ LTR promoter. The *src* fragments were not purified prior to ligation. All except pN4 were introduced at a level of 5 μg per plate in the presence of 1.7 μg of pSV2neo per plate. pN4 was introduced at 8 μg per plate with 2.6 μg of pSV2neo. v-*src* (strain NY-SRA) sequences which carry the v-*src*-specific C-terminal 12 amino acids and eight internal mutations (▨), c-*src* sequences (▭), and locations of the c-*src* introns (△) are shown. S, *Sal*I; P, *Pst*I; B, *Bgl*II. Only those sites involved in the construction of chimeras are shown. The LTR of avian sarcoma virus is indicated.

(Fig. 1, panel *src*). This level of transformation is comparable to that previously reported by Kawai and Nishizawa (22), who used a Polybrene-mediated transfection protocol. When transfected cells were first grown in the presence of G418, however, a substantial yield of soft-agar colonies (approximately 20,000 per 10^6 cells) was generated. G418 selection increases the sensitivity of detection by as much as 3 orders of magnitude. We believe this is accomplished by removing the background of unsuccessfully transfected cells from the population.

We then measured the response of CEF to various doses of oncogene, to document that the transformation was *src* mediated. The amount of the psrc11 plasmid was varied from 2 ng to 50 μg per plate, but the amount of pSV2neo was kept constant, 6 μg per plate, and the total amount of DNA was held constant at 65 μg per plate through the use of salmon sperm DNA. At 20 ng per plate, psrc11 gave a low but significant number of transformed colonies (Table 2). Increasing psrc11 to 200 ng per plate brought about a near-maximal response (4.0×10^4 colonies per 10^6 G418-resistant cells). At a psrc11 dose of 50 μg per plate, a decrease in colony number was seen; the colonies were markedly reduced in size and aberrant in appearance. These results suggest the existence of both a critical threshold level and a tolerable level of *src* gene expression in the transformation of CEF cells. The apparent deleterious effect of high levels of psrc11 is presumably due to the toxic effect of overexpression of v-*src* noted by others (47).

We were next concerned about the origin of the transformed population of cells. Did these cells arise from the expansion of a very small number of *src*-transformed cells, or did they develop from a sizeable number of cells independently transformed by the *src* gene, representing an appreciable portion of the total G418^r population? Cells were cotransfected with 20 μg of psrc11 and 6 μg of pSV2neo per 100-mm-diameter plate. The cells were then selected with G418 and allowed to form colonies in the original dishes without replating steps. In this way the fate of each G418-resistant cell could be monitored. Only frankly transformed colonies with distinct *src* morphologies were scored as positive. As a consequence, the result is likely to be considerably underestimated. Nevertheless, approximately 1 of every 10 G418^r clones displayed a frankly transformed phenotype (data not shown). This frequency is roughly comparable to that for soft-agar colony development at an equivalent v-*src* input level. These results demonstrated that, under the conditions of the analysis, the transformed population always represented an appreciable fraction of the total G418^r population.

Transforming potential of v-*src* and c-*src* and their chimeras. To test the ability of this transformation assay to discriminate between transforming and nontransforming genes, a family of v-*src*, c-*src*, and v/c-*src* constructs were used (Fig. 2). These constructs were derived from permuted viral clones that required ligation prior to transfection to restore the 5′ LTR promoter (20, 21). The v/c-*src* chimera consisted of the 5′ end of v-*src* and the 3′ end of c-*src* fused at the *Pst*I or *Bgl*II site (pB5 and pBB4, respectively). The v-*src* and v/c-*src* constructs were transforming, whereas the c-*src* constructs scored negatively. The presence or absence of intron sequences (Fig. 2, triangles) had no effect on the transforming ability of c-*src*. These results are in good agreement with infection assays using viruses harboring these genes (20, 21). The transformation efficiencies in this experiment were lower than we oberved for psrc11, presumably, in part, because of the requirement for ligation to generate expressible configurations of these plasmids. The v/c-*src* chimeras that retain tyrosine 527 were previously reported to be incapable of transforming NIH 3T3 cells (36) yet as virus were capable of transforming CEF (20). This led to the postulate that there is chicken versus mouse species specificity in transformation by these constructs. Our data support this thesis, ruling out the possibility that the previous disparity (20, 36) was due to differences in the approaches used (infection versus transfection).

Tumorigenic potentials of *src*-transformed CEF. Having demonstrated the utility of using direct transfection of CEF cells to study the transforming properties of oncogenes in vitro, we wished to test the tumorigenic potential of the transfected cells. While tumorigenic assays in nude mice have frequently been used for in vitro-transformed rodent cell lines, we knew at the outset that similar efforts in the

TABLE 3. Tumorigenesis assay of transfected CEF

Gene or oncogene and plasmid(s) used for transfection[a]	No. of birds with tumors/ no. inoculated	Tumor characteristics	
		Growth[b]	Dimensions[c] (cm)
neo			
pSV2neo	0/5	—[d]	
src			
psrc11	6/9	+, ++	0.5–>2.3
	5/6[d]	+	<0.5
myc, *ras*			
pMC38	0/8	—	
pH1	2/7	+	≤0.5
pMC38 + pH1	7/7	+, ++	0.5–2.0
erbB			
pAEV-7.7	1/4	+	≤0.5
pAEV-R	4/4	+	≤0.5
pAEV-R/C[e]	5/5	+, ++	0.5–2.5

[a] pSV2neo plasmid was present as a cotransfectant in all experiments. Except where indicated, transfected G418[r] cells were injected into birds without prior selection through soft agar.

[b] +, Tumors decreased in size with time; ++, tumors continued to enlarge throughout the course of the experiment.

[c] Maximum dimensions determined 2 to 6 weeks postinoculation.

[d] —, No tumor formed.

[e] Injected cells were derived from pooled soft-agar colonies which were grown on plastic plates for several passages prior to inoculation.

chicken system would be compromised by several factors. First, the single-oncogene-transformed CEF are not immortalized and so will have limited population doublings in vivo. Second, no immunocompromised chicken line similar to nude mice exists. To avoid possible complications with viral rescue of the transfected constructs, we chose to use CEF obtained from the line 0 embryos, a line free of endogenous viral loci (1). Line 0 is not sufficiently inbred to be completely histocompatible with the injected cells. Nevertheless, taking several precautions (discussed in Materials and Methods), including the use of young recipient birds (6 days or less) and an inoculum consisting of cells selected only for G418[r] (but not for anchorage-independent growth), we were able to demonstrate the tumorigenic potential of v-*src*-transformed cells. At 2 weeks postinjection, psrc11-transfected cells (10^7 G418[r] cells) induced wing-web sarcomas in six of nine birds, whereas the pSV2neo-transfected cells were completely nontumorigenic (Table 3). Several of the *src*-induced tumors grew progressively and reached a size of 2.0 cm. As a comparison, in another experiment v-*src*-transformed cells were selected for both G418 resistance and anchorage-independent growth prior to injection; these cells induced sarcomas in five of six birds. These tumors, however, in general, were of smaller size and regressed.

Transforming potential of v-*myc* and v-*ras*^{H}. v-*myc* and v-*ras*^{H} (oncogene of Harvey sarcoma virus) (18) represent two extensively studied oncogenes. When introduced into CEF via avian retroviral vectors, each gene can independently induce transformation (19, 26, 37). However, in transfection experiments, cooperation between *ras* and *myc* DNA is required for transformation of rat embryo cells in the absence of a selectable marker (24). We have tested the transforming potential of v-*ras*^{H} and v-*myc* in our assay system. Results from these experiments are shown in Table 4. When individual oncogenes were cointroduced with *neo* and grown under selective conditions, v-*ras*^{H} and v-*myc* transformed CEF at moderate (6,000/10^6 cells) and high (47,000/10^6 cells) frequencies, respectively. In CEF, without G418 selection, transfected populations failed to produce soft-agar colonies. The morphologies of soft-agar colonies induced by v-*ras*^{H} and v-*myc* were distinct. The v-*ras*^{H} colonies were of medium to large size, irregularly shaped, lobulated, and consisted of closely associated cells (Fig. 1, panel *ras*^{H}). v-*myc*-derived colonies were small and irregularly shaped and consisted of loosely associated cells (Fig. 1, panel *myc*). When both v-*ras*^{H} and v-*myc* were introduced simultaneously (each at half the level of that used singularly) and selected for G418 resistance, the colonies displayed novel morphologies. They were generally large and spherical and consisted of small cells in loose association (Fig. 1, panel *myc*/*ras*^{H}). The distinct phenotype of v-*myc*/v-*ras*^{H}-transformed cells was further manifested by heightened tumorigenicity (seven of seven birds) and more aggressive growth properties in vivo (Table 3). In contrast, cells transfected by v-*ras*^{H} alone induced only small, regressive tumor nodules in two of seven birds tested. v-*myc*-transfected cells were nontumorigenic. The lack of tumorigenicity of MC29-infected CEF has been previously noted (37) and is consistent with the present finding.

TABLE 4. CEF transformation by v-*myc* and v-*ras*^{H} plasmids

Plasmid[a]	μg of DNA/plate	Soft-agar colonies	
		Morphology	No./10^6 G418[r] cells
pMC38 (v-*myc*)	20	Small, loose association, irregularly shaped	4.7×10^4
pH1	20	Medium to large, tight association, lobulated	6×10^3
pMC38 + pH1 (v-*myc* + v-*ras*^{H})	10 each	Medium to large, loose association, irregularly shaped	2.7×10^4

[a] In the presence of 6 μg of pSV2neo per plate.

Transforming potential of v-*erbB* and IA-c-*erbB* and their chimeras. A major impetus for this work was our search for an efficient method with which to study the transformation properties of the *erbB* gene. Viruses carrying different forms of *erbB* often display different tissue specificities; some are leukemogenic, while others sarcomagenic (2, 16, 30, 33, 53). These tissue specificities are manifested only in chicken cells, thus precluding their analysis in established rodent cell lines. The *erbB* gene encodes the epidermal growth factor receptor (11). We have previously shown that truncation of the ligand-binding domain of the receptor by retroviral insertion leads to the activation of its leukemogenic potential (32, 34). This IA-c-*erbB* represents the 3′ half of the receptor and is strictly leukemogenic. Transducing viruses carrying variant forms of IA-c-*erbB* have been isolated and shown to have expanded disease tropisms (2, 15, 26, 35, 49, 53). These viruses are additionally able to transform fibroblasts and induce sarcomas. The *erbB* genes present in these viruses invariably carry structural alterations at their C termini (to include at least one of the three major autophosphorylation sites), but they also carry other scattered point mutations internally. We wished to confirm and extend this analysis by using direct DNA transfection to avoid the possible complications encountered in previous studies due to the potentially different replicative abilities and tropisms of the various virus isolates. We took advantage of pAEV-R vector DNA (41, 50), which carries a v-*erbB*^{R} gene that is both fibroblast and erythroblast transforming. This particular avian erythroblastosis virus isolate also carries the v-*erbA*

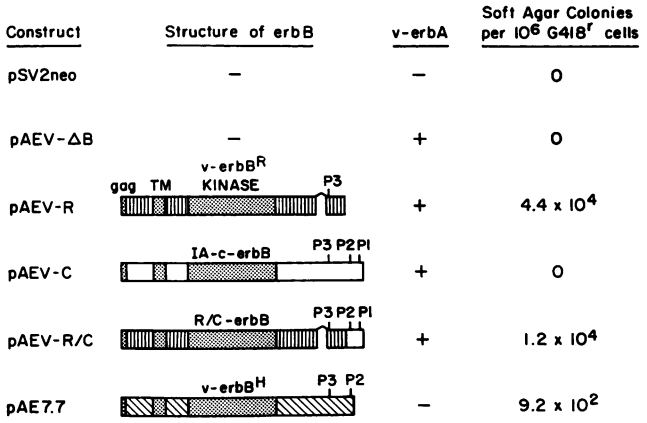

FIG. 3. CEF transformation by v-*erbB* and IA-c-*erbB* and their chimeras. In each case, CEF were transfected with 20 μg of each of the following plasmids and 6 μg of pSV2neo per 100-mm-diameter plate. pAEV-ΔB, pAEV-R, pAEV-C, and pAEV-R/C are all based on pAEV 11-3 (40, 41), a plasmid carrying a nonpermuted AEV-R viral genome. pAE7.7 carries a permuted AEV-H genome (53) and rquires *Hin*dIII digestion followed by ligation to recreate a functional form. P1, P2, and P3 indicate the approximate locations of three C-terminal tyrosines, corresponding to the major autophosphorylation sites (10). TM, Transmembrane domain; *gag*, the first six amino acids of the *gag* coding sequence of avian sarcoma-leukosis virus. This *gag-erbB* fusion, found in virtually all *erbB* transducing viruses and in the cDNA of IA-c-*erbB*, is generated via splicing (32, 39). The AEV-R-derived (▥), AEV-H-derived (▨), and IA-c-*erbB*-derived (□) sequences are shown.

gene, a gene which is related to the thyroid hormone receptor and does not have transforming potential on its own (13, 40). To test other *erbB* genes via this vector, we removed the v-*erbB*^{R} gene and replaced it with IA-c-*erbB* or a chimeric construct between v-*erbB*^{R} and IA-c-*erbB*. The structures of these constructs and the results are summarized in Fig. 3.

pAEV-ΔB, which carries a functional v-*erbA* but a defective v-*erbB*, was not transforming, consistent with previous work which showed that v-*erbB* is the oncogenic determinant (40, 41). pAEV-R is the DNA of the transforming virus (41, 50), which transformed CEF and induced sarcomas in vivo as expected (Table 3). The sarcomas induced by inoculation with these pAEV-R-transfected CEF, however, were small and regressed after approximately 2 weeks. pAEV-C was created by removing the v-*erbB* gene in pAEV-R and replacing it with IA-c-*erbB* (Fig. 3). This construct was not transforming. Inspection of the structural differences between the *erbB* gene of pAEV-R and pAEV-C revealed that the former carries multiple lesions: a 72-amino-acid truncation at the C terminus, a 21-amino-acid internal deletion, and eight point mutations (5, 32). It is not yet clear which of these mutations activate the fibroblast-transforming potential, although the C-terminal truncation is a likely candidate, since this lesion which affects the autophosphorylation sites is commonly found in *erbB*-transducing viruses, including another avian erythroblastosis virus isolate, AEV-H (53). Indeed, AEV-H DNA, in the form of pAE7.7, when tested in our CEF assay was transforming, albeit with a reduced efficiency compared with that of AEV-R. The lower efficiency is again likely to be in part due to the requirement of prior ligation to make this permuted plasmid (pAE7.7) functional. To test whether the C terminus of *erbB* is inhibitory to fibroblast transformation, pAEV-R/C DNA was constructed. In this construct, the C terminus of v-*erbB* was restored, while the internal lesions remained. To our surprise, pAEV-R/C was very transforming in CEF and in several instances induced progressively growing sarcomas in vivo (Table 3). This provided evidence that truncation of the C terminus of *erbB* is not obligatory for fibroblast transformation and drew our attention to the importance of internal mutations. In a more detailed study to be published elsewhere (32a), we have confirmed these observations and uncovered single point mutations in the kinase domain that can activate the fibroblast-transforming and sarcomagenic potentials of *erbB*. These analyses fully established the sensitivity and reliability of studying tissue-specific transformation of CEF by direct oncogene transfection.

The *erbB*-induced soft-agar colonies were characteristically small, spherical, and compact; they are typified by colonies derived from pAEV-R (Fig. 1, panel *erbB*). These colonies are similar to those induced by pAEV-R viruses but very different from those induced by Rous sarcoma virus or MC29. All the data generated from our direct transfection assays are thus in complete agreement with viral infection data. This approach should prove very useful in delineating the region of *erbB* critical for fibroblast transformation, without resorting to more tedious and complicated viral assays.

DISCUSSION

Avian acute retroviruses have been a rich source for studying and identifying oncogenes. At least 15 such oncogenes have been isolated, with the most recent additions being v-*jun* (28) and v-*crk* (29). Most of these viruses can induce sarcomas in vivo and transform CEF in vitro. Due to the previously perceived difficulty of stable transfection of CEF, the characterization of oncogenes in CEF was carried out almost exclusively in the viral form. While there are obvious advantages to using viruses to study oncogenes (namely, in vivo analysis and assay on target cells refractory to transfection in vitro), the dependence on helper virus for replication, the rapidity of generation of mutations during viral propagation, and the generally more time-consuming cloning steps are some of the drawbacks of this approach. In this study, we have defined conditions for efficient transfection of CEF and demonstrated its utility with the characterization of several oncogenes, both of avian and rodent origin and under the control of avian and murine LTRs.

Under our conditions, using a conventional calcium phosphate transfection method and the *neo* gene as a selection marker, the transfection efficiency of CEF reached one in a thousand. In retrospect, the inability of previous workers to demonstrate high-efficiency CEF transfection was likely a consequence of their source of DNA (i.e., cellular DNA from transformed cells) and the now generally accepted notion that for primary cells, in the absence of selection markers, cooperation of two oncogenes is required for efficient transformation (25, 38). In our studies, the use of cloned oncogene DNA and the *neo* gene helped overcome these difficulties. In our experiments, single oncogene fragments, in the absence of the *neo* gene for selection, were essentially incapable of transforming CEF. The only exception was the v-*src* gene (psrc11), which yielded a low number of colonies, consistent with the previous report (22). The pronounced enhancement of colony formation brought on by the inclusion of the *neo* gene and selection for G418 resistance can be explained in at least two ways. First, selection for *neo* increases the sensitivity of detection by eliminating unsuccessfully transfected cells. Second, as previously sug-

gested in rat embryo cell tansformation (24), the imposition of G418 selection is likely to remove the inhibitory environment provided by the surrounding normal cells and allow the effects of single oncogenes to become manifested. Efficient infections with acute retroviruses that transform virtually all normal cells can be viewed as producing a similar effect. This may explain the apparent paradox that viral infections can lead to CEF transformation with single oncogenes in the absence of selection markers. The recent report (27) that the phenotypes of v-*myc*-transformed cells can be suppressed by coculturing with normal cells lends further support to this hypothesis.

With the present assay, we have examined the effects of individual oncogenes on CEF more closely. Due to the various constructs and promoters used, it is difficult to compare the transforming potentials of all tested oncogenes. Among similar constructs, however, the relative strengths of the individual oncogenes were reproducible. CEF transformed by individual oncogenes have distinct morphologies and tumorigenicities; for instance, *myc* colonies are characteristically loosely associated, whereas *ras* colonies are more compact in nature. CEF transformed by *myc* or *ras* individually have no to low tumorigenicity when injected into young birds. A combination of the *myc* and *ras* genes, on the other hand, yields a novel in vitro phenotype and significantly enhanced tumorigenicity. Since the transfected cells used for in vivo tumorigenic analysis were not preselected for anchorage-independent growth, the in vivo tumorigenesis and the in vitro soft-agar colony assays offer two independent measures of the degree of transformation. We noted an apparent correlation between in vitro colony size and the tumorigenic potential, but there were exceptions.

The transformation specificities of our in vitro soft-agar data completely parallel those obtained by means of infectious viruses. We show by direct transfection that v-*src* and v/c-*src* chimeras were transforming, yet c-*src* was not. An additional rigorous test came from the tissue-specific transformation by the *erbB* gene. Previously, on the basis of viral infection analysis, we and others (2, 30, 32, 33) have demonstrated that an N-terminally truncated but otherwise unmutated *erbB* was strictly leukemogenic. Upon additional mutations within the truncated molecule, viral *erbB* genes acquired fibroblast-transforming potential. This tissue-specific transformation pattern was reproduced in our transfection assay, which conclusively rules out the possibility that the observed difference is due to viral tropisms. Instead, our studies implicate the importance of the conformation of the catalytic domain of *erbB* in determining the accessibility or the kinase activity toward tissue-specific substrates (this study and reference 32a).

In summary, we have optimized conditions for CEF transformation by individual oncogenes via direct DNA transfection. This assay provides an important alternative or complementary system to the use of rodent cells. When employed in parallel, assays with avian and rodent cells may facilitate the characterization of host-range mutants of oncogenes. Recently, a number of *src* mutants which transform only rat cells but not chicken cells have been isolated (9, 52). These mutants are of potential value in the identification of cellular proteins involved in transformation. Our assay should greatly facilitate these efforts.

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