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Tyrosine Kinase Activity May Be Necessary but Is Not Sufficient for c-*erbB1*-Mediated Tissue-Specific Tumorigenicity

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Expression of mutant avian c-*erbB1* genes results in tissue-specific transformation in chickens. Site-directed mutagenesis was used to generate kinase-defective mutants of several tissue-specific v-*erbB* transforming mutants by replacement of the ATP-binding lysine residue in the kinase domain with an arginine residue. These kinase-defective v-*erbB* mutants were analyzed for their in vitro and in vivo transforming potentials. Specifically, kinase-defective mutants of erythroleukemogenic, hemangioma-inducing, and sarcomagenic v-*erbB* genes were assessed for their oncogenic potential. In vitro transformation potential was assessed by soft-agar colony formation in primary cultures of chick embryo fibroblasts (CEF). In vivo transformation potential was determined by infection of 1-day-old line 0 chicks with concentrated recombinant retrovirus and then monitoring of birds for tumor formation. These transformation assays demonstrate that kinase activity is absolutely essential for transformation by tissue-specific transforming mutants of the avian c-*erbB1* gene. Since all of the tissue-specific v-*erbB* mutants characterized to date exhibit tyrosine kinase activity in vitro but do not transform all tissues in which they are expressed, we conclude that v-*erbB*-associated tyrosine kinase activity may be necessary but is not sufficient to induce tumor formation.

The avian c-*erbB1* gene encodes a transmembrane growth factor receptor with tyrosine kinase activity; this receptor shares structural homology with the human epidermal growth factor receptor (hEGFR) (10, 22, 23). Members of the c-*erbB* family of proto-oncogenes (i.e., c-*erbB1-4*) have been implicated in the etiology and progression of many human cancers including carcinomas of the breast, ovary, lung, and gastrointestinal tract and tumors of the central nervous system (reviewed in references 33 and 43). Although amplification and overexpression of members of this proto-oncogene family have been correlated with the development of these neoplasias, the identification of specific lesions within these proto-oncogenes that might contribute to tumor formation in humans has been elusive. In contrast, studies of the avian c-*erbB1* gene have demonstrated the identification of specific structural alterations that result in tissue-specific tumorigenicity (3, 14, 15, 30–32, 34–36, 41, 42).

The avian c-*erbB1* gene encodes a 170,000-Da glycoprotein that contains an extracellular NH_2-terminal ligand-binding domain and a COOH-terminal cytoplasmic domain; these domains are separated by a short hydrophobic transmembrane segment (10, 22, 23). The transduced v-*erbB* genes identified in various avian erythroblastosis virus (AEV) isolates contain distinct mutations within multiple regions of the c-*erbB1* coding sequences, including truncation of the ligand-binding domain, as well as COOH-terminal truncations, point mutations, and internal deletions (3, 14, 15, 30–32, 34–36, 41, 42). In addition, the nonacute avian leukosis virus (ALV) has been shown to induce erythroleukemia in chickens by high-frequency proviral insertional mutation of the c-*erbB1* gene (14, 15, 30, 34). Proviral insertion in inbred lines of chickens results in separation of the genomic sequences encoding the ligand-binding domain of the receptor from sequences encoding the transmembrane and cytoplasmic domains (14, 15, 30, 34). Expression of this amino-terminally truncated receptor results in ligand-independent neoplastic transformation of avian erythrocyte precursors. Truncation of the ligand-binding domain in combination with short COOH-terminal deletions and/or internal deletions within the carboxyl-terminal domain further results in the induction of fibrosarcomas, angiosarcomas, renal adenocarcinomas, or hemangiomas (35, 36, 41, 42).

This animal model has, therefore, greatly facilitated the correlation of structural changes in the c-*erbB1* gene with the functional, i.e., oncogenic, properties of this protein product. One obvious question that arises from these studies is whether the intrinsic tyrosine kinase activity of all classes of c-*erbB1* mutants is essential for receptor function, particularly with respect to tissue-specific transformation. This question has become particularly important in light of recent studies demonstrating the signaling properties of apparently defective tyrosine kinases (4, 7, 13, 16, 27, 37, 40, 47).

In this study, site-directed mutagenesis was employed to generate a mutation in the avian c-*erbB1* gene in which the ATP-binding lysine residue in the kinase domain is replaced with an arginine residue. Structurally analogous mutants of the hEGFR have been demonstrated to lack tyrosine kinase activity (5, 17). This mutation was introduced into two forms of v-*erbB* which have previously been shown to be tumorigenic in three distinct target cells: erythroblasts, fibroblasts, and endothelial cells (31, 35, 36). These kinase-defective v-*erbB* mutants were characterized for in vitro expression and autophosphorylation, and for their in vitro and in vivo transforming abilities. Parallel studies were conducted using equivalent kinase-intact v-*erbB* genes. The results of these studies demonstrate that the intrinsic tyrosine kinase activity of this receptor is absolutely required for tissue-specific transformation mediated by v-*erbB*, both in vitro and in vivo.

Construction of kinase-defective c-*erbB1* mutants. The present study was designed to determine whether the tyrosine kinase activity of mutant c-*erbB1*-encoded receptor proteins is essential for transformation in all three of the target tissues previously identified in our laboratory. In order to address this question, kinase-defective mutants of two previously described

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v-*erbB* genes, 9134 E1 and 9134 S3 (35, 36), were constructed. The E1 v-*erbB* mutant encodes a protein identical in structure to that encoded by the insertionally activated form of the c-*erbB1* gene (14, 30, 34); this mutant lacks all but 42 amino acids of the ligand-binding (extracellular) domain of the receptor and has transmembrane and cytoplasmic domains identical to those of the native receptor. Expression of the v-*erbB* E1 mutant leads to transformation of erythroblasts in vitro and to the development of erythroblastosis in birds (35, 36). A second recently isolated AEV, designated 9134 S3, encodes a v-*erbB* product in which the extracellular domain has also been removed. In addition, this mutant contains an in-frame deletion of 139 amino acids within the carboxyl-terminal domain (35, 36). Unlike the E1 v-*erbB* mutant, the S3 v-*erbB* product cannot transform erythroid cells. Rather, expression of the S3 v-*erbB* product results in the formation of an aggressive malignant tumor designated a hemangioma by avian pathologists (35, 36). The v-*erbB* genes from the 9134 E1 and 9134 S3 AEV isolates were subcloned into a helper-independent retroviral vector (RCAN) (18), and these constructs are designated REBC and REBS3, respectively (reference 32 and this study).

Kinase-defective mutants of these two v-*erbB* proteins were constructed by using the oligonucleotide site-directed mutagenesis technique described by Kunkel et al. (20) to replace the wild-type AAA (lysine) codon, which is the equivalent of the Lys-721 residue of the EGFR, with a CGC codon substituting an arginine residue for the ATP-binding lysine residue. In addition to effecting this amino acid substitution, the oligonucleotide used in this mutagenesis strategy resulted in the incorporation of a unique *Nru*I restriction site. This unique restriction site was subsequently used to confirm maintenance of the point mutation in infected cells and tissues by restriction analysis of amplified genomic DNA sequences.

Specifically, this site-directed mutant oligonucleotide was used to generate a kinase-defective mutant (plasmid pGK$^-$) of the 9134 E1 v-*erbB* gene (32, 36) by replacing a 1,659-bp *Apa*I-to-*Eco*RI fragment of the plasmid pGE+ with a corresponding 1,659-bp *Apa*I-to-*Eco*RI fragment containing the lysine-to-arginine substitution. A second kinase-defective v-*erbB* mutant, the genetic equivalent of the 9134 S3 v-*erbB* gene (36), was constructed by replacement of a 917-bp *Bcl*I-to-*Sph*I restriction fragment of pGK$^-$ with a 500-bp *Bcl*I-*Sph*I fragment from the plasmid pGS3 to generate the plasmid pGS3/K$^-$. Once constructed, kinase-defective v-*erbB* mutants were subcloned from the Bluescript (Stratagene) vector into an adaptor plasmid, Cla12Nco (18), by cloning a purified 1,659-bp *Apa*I-to-*Eco*RI fragment from pGK$^-$ and a 1,242-bp *Apa*I-to-*Eco*RI fragment from pGS3/K$^-$ into pCla12Nco. From each of the adaptor plasmids, a *Cla*I fragment containing the mutant v-*erbB* gene was gel purified and ligated into the unique *Cla*I restriction site of the helper-independent retroviral expression vector RCAN developed by Hughes et al. (18). The resulting recombinant proviral constructs were named pREBC, pREBC/K$^-$ pREBS3, and pREBS3/K$^-$, respectively, and are depicted schematically in Fig. 1. Recombinant proviral constructs, carrying wild-type or kinase-defective v-*erbB* genes, were then used to transfect tissue culture cells.

Transfection and analysis of v-*erbB* expression and kinase activity in vitro. The chemically transformed quail fibroblast line, QT6 (28), was obtained from C. Moscovici (Gainesville, Fla.). QT6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g of glucose per liter and supplemented with 5% fetal calf serum (FCS), 1% chick serum, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 250 ng of amphotericin B per ml (all from Gibco Laboratories, Grand Island, N.Y.).

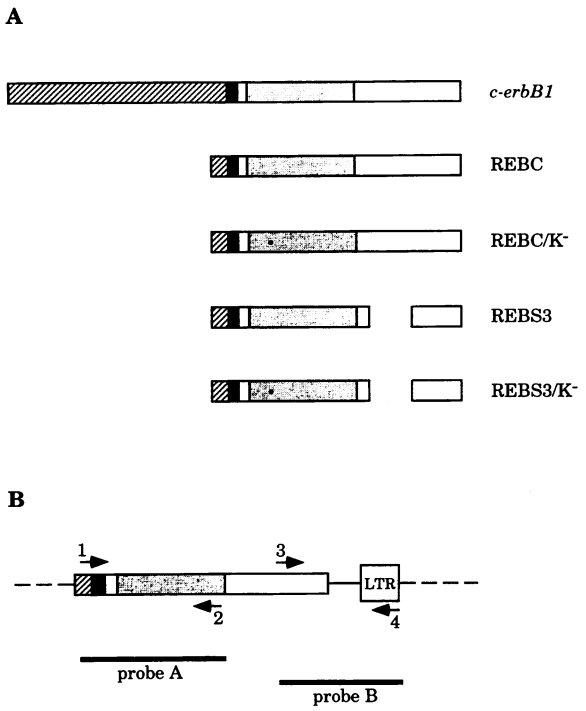

FIG. 1. Schematic diagram of mutant c-*erbB1* constructs and PCR primers. (A) The c-*erbB1* protein contains approximately equivalent extracellular and intracellular domains (~600 amino acids [aa] each) separated by a 23-amino-acid transmembrane domain (solid black box). The hatched region represents the extracellular ligand-binding portion of the molecule. The intracellular portion of the protein is further subdivided into a catalytic kinase domain (grey box) and a carboxyl-terminal regulatory domain (white box). The mutants of c-*erbB1* analyzed in this study all contain an amino-terminal truncation which removes most of the ligand-binding domain. In addition to amino-terminal truncation, the REBS3 mutant contains a 139-aa in-frame deletion within the carboxyl-terminal regulatory domain (dashed line). The REBC/K$^-$ and REBS3/K$^-$ constructs contain a point mutation in the catalytic kinase domain changing the ATP-binding lysine residue to an arginine (black dot) (see Materials and Methods). The mutation also resulted in the formation of a unique *Nru*I site at this position. (B) The presence of integrated provirus in infected chickens was confirmed by amplification of specific viral sequences by PCR. Primers were designed to amplify two distinct segments of the integrated proviral sequence. Primers 1 and 2 amplify a segment of DNA which encodes a short portion of the extracellular domain, the transmembrane domain, and most of the catalytic kinase domain, including the region encoding the point mutation in the kinase-defective mutants. The second segment, amplified by primers 3 and 4, encompasses a portion of the carboxyl-terminal regulatory domain and some of the 3′ LTR of the retroviral vector RCAN. Amplification products of genomic DNA isolated from infected chicken tissues were hybridized with probes A and B, which correspond to the specific sequences amplified by primers 1 and 2 and 3 and 4, respectively.

QT6 fibroblasts were cotransfected by calcium phosphate precipitation (45), using the selectable marker pSV2neo (39). Stable QT6 transfectants were selected by resistance to geneticin, and mass transfectants were screened for expression of the mutant v-*erbB* proteins by immunoprecipitation. Approximately 0.5×10^6 to 1×10^7 stably transfected QT6 cells were incubated for 2 to 16 h in DMEM lacking methionine (Spe-

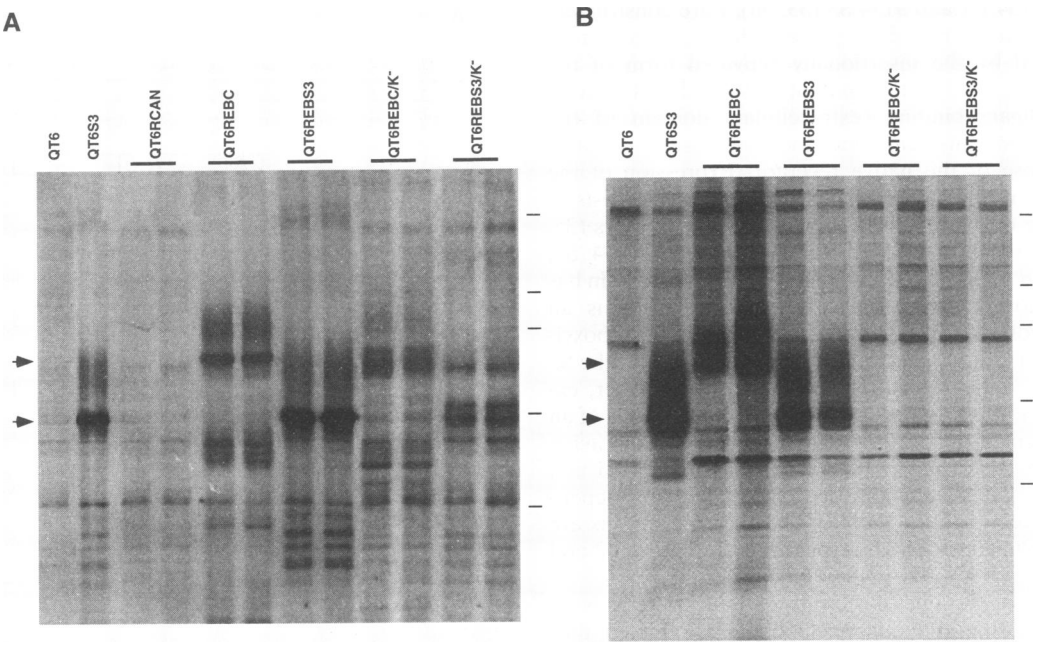

FIG. 2. Analysis of v-*erbB* expression and kinase activity in transfected fibroblasts. (A) Immunoprecipitation of v-*erbB* proteins from transfected QT6 cells. Transfected QT6 cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Cells were subsequently lysed and immunoprecipitated with a polyclonal c-*erbB1*-specific antibody (25). Immunoprecipitated proteins were analyzed by SDS-PAGE followed by autoradiography. QT6 cells represent the untransfected host cell line used as a negative control. QT6S3 cells, a positive control, are a stably transfected cell line and express the v-*erbB* S3 product. This protein migrates as two diffuse bands with M_rs of 62,000 to 64,000. Protein products of the predicted 82,000-to-84,000 M_r (unglycosylated and terminally glycosylated [25]) were immunoprecipitated from REBC- and REBC/K$^-$-transfected QT6 cells (upper arrowhead), and products with M_rs of 62,000 to 64,000 were precipitated from REBS3- and REBS3/K$^-$-transfected QT6 cells (lower arrowhead). No c-*erbB1*-specific products were precipitated from QT6 cells transfected with the RCAN vector alone (QT6RCAN). (B) In vitro kinase activity of v-*erbB1* products. Immune complex kinase assays were performed to assess the intrinsic protein tyrosine kinase activity of each transfected viral construct. Assays were performed by immunoprecipitating v-*erbB* products from unlabeled cell lysates with a polyclonal c-*erbB1*-specific antiserum (25) followed by the addition of $MnCl_2$ and [γ-^{32}P]ATP in the presence of the phosphatase inhibitor Na_3VO_4 (see text). Phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. Immune complex kinase assays on cell lysates from untransfected QT6 cells or QT6S3 cells were performed as negative and positive controls, respectively. Kinase-intact mutant proteins REBC and REBS3 are autophosphorylated, as demonstrated by the ^{32}P-labeled protein bands at M_rs of 82,000 to 84,000 and 62,000 to 64,000, respectively (arrowheads). However, the corresponding kinase-defective mutants REBC/K$^-$ and REBS3/K$^-$ are not capable of autophosphorylation, as evidenced by the lack of ^{32}P-labeled proteins in the correct molecular size ranges for these proteins (QT6REBC/K$^-$ and QT6REBS3/K$^-$). The relative positions of molecular size markers are indicated by dashes (205, 116, 97.4, 66, and 45 kDa from top to bottom).

cialty Media Inc., Lavallette, N.J.) supplemented with 1% dialyzed FCS and 150 μCi of [^{35}S]methionine (Du Pont/NEN). The radiolabeled cells were immunoprecipitated as described previously (24). Analysis of immunoprecipitated protein products from cells transfected with all four recombinant retroviral vectors demonstrates the expression of v-*erbB*-specific protein products of the appropriate molecular weights (Fig. 2A).

In order to confirm the kinase-defective status of the v-*erbB* products encoded by the REBC/K$^-$ and REBS3/K$^-$ constructs, in vitro immune complex kinase assays were performed as follows. Approximately 0.5 × 10^6 cells were grown in DMEM containing 5% FCS, washed twice in ice-cold phosphate-buffered saline, and lysed for 5 min on ice in a buffer containing 50 μM sodium orthovanadate [20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]), pH 7.3, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol (kinase lysis buffer). Lysates were incubated on a rocking platform with a polyclonal anti-*erbB* antibody (24) at 4°C for 15 min followed by the addition of Immunopure A/G and continued incubation at 4°C for 10 min. Samples were washed five times in kinase lysis buffer. Pellets were resuspended in kinase assay buffer (20 mM HEPES, pH 7.3; 10% glycerol; 0.1% Triton X-100; 700 μM sodium orthovanadate; 5 mM $MnCl_2$) containing 10 μCi of [γ-^{32}P]ATP, followed by incubation for 10 min on ice. In vitro phosphorylation reactions were stopped by addition of 2× sodium dodecyl sulfate (SDS) sample buffer, and reaction products were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (21) and detected by autoradiography. The results of these experiments are illustrated in Fig. 2B and indicate that the kinase-intact REBC and REBS3 mutant proteins become phosphorylated in vitro, whereas the kinase-defective REBC/K$^-$ and REBS3/K$^-$ mutant proteins, as predicted, display no in vitro kinase activity.

Viral infection, in vitro transformation assays, and tumorigenicity assays. Once expression and kinase activity, or inactivity, of these v-*erbB* protein products were confirmed by using transfected QT6 cultures, filtered conditioned culture supernatants were used to infect primary cultures of CEF which were prepared from day-10 line 0 chicken embryos obtained from the USDA Avian Disease and Oncology Laboratory, East Lansing, Mich. These cells were fully infected after approximately 2 weeks of passaging and treatment with conditioned culture supernatants. Expression of v-*erbB* products and the intrinsic kinase activity of these products in infected CEF were both analyzed by the methods described above, and these results paralleled our observations for the QT6 transfected

TABLE 1. Transformation potential of kinase-defective v-*erbB* products[a]

Virus	No. of soft-agar colonies in CEF (per 0.5×10^6 cells)[b]	No. of chicks with indicated tumor/ no. of chicks injected		
		Erythro-blastosis	Heman-gioma	Sarcoma
None	0	0/4	0/4	0/4
RCAN	0	0/6	0/6	0/6
REBC	5	14/16	0/16	0/16
REBC/K$^-$	0	0/14	0/14	0/14
REBS3	506	0/34	32/34	9/34
REBS3/K$^-$	0	0/14	0/14	0/14

[a] See text for methods and discussion.
[b] Average values from four assays.

cultures (data not shown). Infectious recombinant retrovirus was then collected from these CEF cultures by filtration and concentration of conditioned culture supernatants as previously described (32). The integrity of each infectious retrovirus sample was confirmed by extraction of viral genomic RNA and dot blot analysis, as described by Pelley et al. (32), by using DNA probes that were vector (ALV long terminal repeat [LTR]; 250-bp *Bam*HI fragment) or insert (v-*erbB*; 560-bp *Bam*HI fragment) specific. Dot blot analyses were performed by using the Genius Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. Each aliquot of concentrated infectious recombinant retrovirus was analyzed in this manner to ensure the integrity of the retrovirus, including maintenance of the cloned insert, and to quantitate the levels of retrovirus used to infect birds.

In order to determine whether kinase activity is essential for the observed tissue-specific transformation associated with expression of these v-*erbB* mutants, both in vitro and in vivo transformation assays were performed. Transformation of cultured primary CEF infected with recombinant retrovirus was assessed by using a standard soft-agar colony assay as described elsewhere (1). We have shown previously that the v-*erbB* product expressed by the REBC virus is not capable of transforming CEF in vitro, whereas the 9134 S3 virus does transform CEF in vitro (31, 32, 36). In this study, we have extended these results by analyzing the in vitro transforming capacity of the REBS3, REBC/K$^-$, and REBS3/K$^-$ recombinant retroviruses. The results of these experiments are summarized in Table 1. Uninfected CEF and CEF infected with the viral vector alone were negative for soft-agar colony formation. CEF infected with REBC produced no colonies in soft agar, consistent with the previous findings that REBC cannot transform fibroblasts in vitro or induce fibrosarcomas in vivo (31, 32, 36). The REBS3 virus is capable of transforming CEF, as indicated by the large number (>500) of colonies formed by REBS3-infected CEF in soft agar (Table 1). However, neither of the kinase-defective viral constructs (REBC/K$^-$ or REBS3/K$^-$) is capable of inducing anchorage-independent growth of CEF in vitro (Table 1).

To test whether kinase activity is required for v-*erbB*-mediated tumorigenicity, we injected 1-day-old line 0 chicks intraperitoneally with purified, concentrated recombinant retrovirus (RCAN, REBC, REBS3, REBC/K$^-$, and REBS3/K$^-$). Birds were monitored daily, and animals were either necropsied after they succumbed to disease or sacrificed at a point exceeding the latency for tumor formation (31, 36). In control experiments (chicks uninjected or injected with the RCAN vector), none of the birds succumbed to disease and all tissues and organs examined appeared normal (Table 1). Consistent with previous reports (31, 32, 36), infection of birds with the REBC virus resulted in the induction of erythroblastosis within a 3- to 6-week period postinfection. There was no evidence of any other tumor type (fibrosarcoma or hemangioma) developing in birds infected with the REBC virus (Table 1), in agreement with previous findings (31, 32, 36). Birds infected with the REBS3 virus rapidly (in many cases within 2 weeks) succumbed from hemangiomas, a diffuse tumor of endothelial origin; these observations with REBS3 are consistent with the previously described course of infection associated with the 9134 S3 virus (35, 36). In addition, the observation that the S3 v-*erbB* mutant has the same in vivo transformation potential in the RCAN retroviral vector as the original 9134 S3 viral isolate provides further evidence that the tissue specificity of transformation associated with the S3 v-*erbB* oncogene is not simply a function of viral tropism. In no case were tumors of any tissue observed in birds which were infected with either of the kinase-defective mutants, REBC/K$^-$ or REBS3/K$^-$ (Table 1). All tissues and organs of birds infected with these kinase-defective viruses appeared normal.

Analysis of genomic DNA from virally infected tissues. In order to establish that chickens injected with kinase-defective viruses did not fail to develop neoplasms because of lack of viral infection, the presence of integrated proviral DNA in infected chicken tissues was confirmed by analysis of genomic DNA. Genomic DNA was isolated from tissues of all infected and control groups, and DNA was amplified by PCR. Genomic DNA was isolated from cultured cells or animal tissues according to previously described methods (2). Two segments of proviral DNA were amplified and are illustrated in Fig. 1. To amplify the DNA segment encoding the transmembrane domain and most of the kinase domain, an oligonucleotide primer (5′-CCGCTGGGGTCCTGGGTGAGA) corresponding to a region just upstream of the sequences encoding the transmembrane domain and a primer to the complementary strand of DNA near sequences encoding the carboxyl-terminal end of the kinase domain (5′-TCGGATGTAGTCAAGGAG GCA) were used. These primers are predicted to amplify a segment of DNA that is 619 bp in length. A second segment of DNA, which encompasses a short portion of sequence encoding the COOH terminus of the v-*erbB* gene and downstream sequences of retroviral vector origin including sequences encoding the ALV 3′ LTR, was amplified by using an oligonucleotide primer corresponding to the carboxyl terminus of the v-*erbB* gene (5′-ATCCAATCAGGCAATCACCAA) along with a second primer corresponding to the complementary strand of DNA in the 3′ LTR of the retroviral vector RCAN (5′-ACCCGTCTGTTGCCTTCCTAA) downstream of the v-*erbB* gene. This primer set is predicted to amplify a segment of DNA approximately 600 bp in length. Optimum amplification conditions were determined by using the PCR Optimizer (Invitrogen). The PCR-amplified DNA fragments were analyzed by Southern hybridization (38) for the presence of both v-*erbB*- and retrovirus-specific sequences. Hybridization probes A and B (Fig. 1) were made by using Geneclean (Bio 101)-purified fragments of amplified DNA from parental plasmids. Chemiluminescent detection was performed according to the manufacturer's (Boehringer Mannheim) instructions, and signals were detected by autoradiography.

Samples from birds infected with the recombinant v-*erbB*-bearing retroviruses hybridized both with the probe corresponding to the kinase domain and with the probe encompassing both 3′ v-*erbB* and retroviral 3′ LTR sequences, and the sizes of the bands detected were consistent with the size predicted for each product (Fig. 3). PCR amplification of these

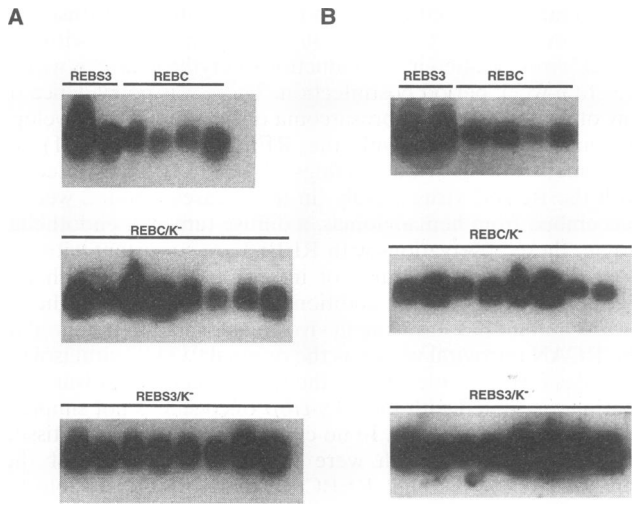

FIG. 3. PCR amplification of integrated proviral sequences from genomic DNA of infected birds. To confirm that birds which did not develop tumors were infected with virus, unaffected tissues were collected from virally infected birds and genomic DNA was extracted for analysis of specific integrated proviral sequences by PCR amplification. By using the oligonucleotide primers and DNA hybridization probes depicted in Fig. 1, two specific sequences were amplified. One sequence encompassed the catalytic kinase domain which hybridizes specifically with probe A, and the second sequence encompasses the carboxyl-terminal portion of c-*erbB1* through specific retroviral sequences of the 3′ LTR of the retroviral vector RCAN which hybridizes specifically with probe B. Panel A represents genomic DNA from infected chickens amplified with primers 1 and 2 and subsequent Southern hybridization of amplified DNA with hybridization probe A. Panel B represents the same genomic DNA from infected chicks amplified with primers 3 and 4 and subsequent hybridization of amplified DNA with hybridization probe B. Results demonstrate the presence of amplified sequences in birds infected with all four of the retroviral constructs REBS3, REBC, REBC/K$^-$, and REBS3/K$^-$. Lanes contain genomic DNA from individual chicks infected with the recombinant retrovirus indicated.

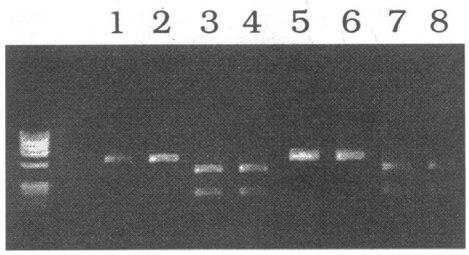

FIG. 4. *Nru*I restriction enzyme digestion of PCR-amplified integrated provirus from genomic DNA. PCR products of amplified integrated proviral sequences which encompass the kinase domain of each mutant retrovirus (619 bp) were digested with the restriction endonuclease *Nru*I, and digestion products were resolved on a 1.0% agarose gel. Each lane represents digested PCR products of amplified genomic DNA from an individual chick infected with the following retroviruses: REBC (lanes 1 and 2), REBC/K$^-$ (lanes 3 and 4), REBS3 (lanes 5 and 6), and REBS3/K$^-$ (lanes 7 and 8). Sequences amplified from birds infected with either REBC or REBS3 contain no *Nru*I sites and yield a single band of 619 bp, while sequences amplified from birds infected with either REBC/K$^-$ or REBS3/K$^-$ contain a single *Nru*I restriction site resulting in the generation of two products of 443 and 176 bp upon digestion.

two distinct segments enabled us to distinguish between amplification of integrated recombinant proviral DNA and potential amplification of endogenous c-*erbB1* sequences. In addition, the 619-bp PCR products which encompass the kinase domain were subsequently gel purified and digested with the restriction endonuclease *Nru*I to confirm the presence of the substitution mutation in birds infected with the kinase-defective recombinant retroviral constructs (Fig. 4). Digestion of amplified PCR products from birds infected with the kinase-defective recombinant viral constructs REBC/K$^-$ and REB S3/K$^-$ resulted in two bands of 443 and 176 bp (Fig. 4). As expected, PCR products from birds infected with the kinase-intact retroviral constructs REBC and REBS3 were not digested by *Nru*I (Fig. 4).

Several distinct structural alterations in the avian c-*erbB1* gene have been shown to lead to the expression of mutant receptors which cause tissue-specific transformation leading to the induction of at least three distinct tumors in chickens (23). Interestingly, to date, transformation in all three avian tissues appears to be mediated by a ligand-independent mechanism (23). While the products of the human and avian c-*erbB1* genes share significant structural homology, identification of structural alterations which may activate the tumorigenic potential of the human receptor has been elusive. There is, however, evidence of structural rearrangement of the human c-*erbB1* gene in glioblastomas (12, 25, 46, 48). Extensive analysis of malignant glioblastomas has revealed that the human c-*erbB1* gene is amplified in 40 to 50% of all tumors examined, and that structural alterations of the c-*erbB1* gene are present in as many as 60 to 70% of these amplified genes (12, 25, 46, 48). The most frequently identified structural alteration of the human c-*erbB1* gene in these tumors is deletion of the extracellular ligand-binding domain (12, 25, 46, 48). Analogous c-*erbB1* deletions in glioblastomas have been identified by several independent laboratories (12, 25, 46, 48), and these deletions share remarkable similarity to the ligand-binding domain mutations identified in AEV- and ALV-transformed cells. Interestingly, a second type of structural alteration of the human c-*erbB1* gene has recently been identified in glioblastomas by Ekstrand et al. (12) and involves an internal in-frame deletion of sequences within the carboxyl-terminal regulatory domain of the receptor. This structural alteration of the human c-*erbB1* gene is also strikingly similar to the in-frame carboxyl-terminal deletion that occurs in the S3 v-*erbB* mutant that we have characterized in previous studies (35, 36). These studies of the hEGFR demonstrate that there are direct structural parallels between known activating mutations of the avian c-*erbB1* gene and structural rearrangements of the human c-*erbB1* gene in malignant glioblastomas and suggest that the activating mutations defined for the avian system may ultimately provide insights (both structural and functional) into the mechanism of oncogenic transformation mediated by the hEGFR. Moreover, these studies also suggest that the c-*erbB1*-encoded receptor may be involved in tumorigenic pathways via ligand-independent activation in human malignancies, and it is, therefore, imperative that the properties of ligand-independent transforming mechanisms mediated by the avian v-*erbB* products be systematically defined.

We have, therefore, initiated a series of experiments to determine what properties of receptor biochemistry and function are shared by ligand-activated and ligand-independent c-*erbB1* products. Chen et al. (5) and Honegger et al. (17) have shown previously that the intrinsic tyrosine kinase activity of the hEGFR is required for ligand-stimulated autophosphorylation, phosphorylation of cellular substrates, influx of intracellular calcium, DNA synthesis, and mitogenesis. In our

studies, we have analyzed the tumorigenic potential of kinase-defective v-*erbB* mutants known to induce tumors in a ligand-independent manner in three distinct tissues. These studies clearly demonstrate that intrinsic tyrosine kinase activity is required for ligand-independent v-*erbB*-mediated stimulation of anchorage-independent growth in primary cultures of CEF. Furthermore, intrinsic tyrosine kinase activity is also absolutely essential for the v-*erbB*-mediated transformation of all three potential avian target tissues as assayed by tumor formation in vivo. Since we and others (this study and reference 29) have demonstrated that all c-*erbB1* tissue-specific mutants exhibit tyrosine kinase activity in vitro yet do not transform all tissues in which they are expressed, we conclude that tyrosine kinase activity may be necessary but is not sufficient for c-*erbB1*-mediated tumorigenicity.

While the results reported here were not unexpected, they were also not predictable. A significant number of studies using point mutants of the ATP-binding sites of certain receptor tyrosine kinases, including the hEGFR (5, 17), neu (c-*erbB2*) (8, 44), insulin receptor (IR) (6, 11, 26), insulin-like growth factor receptor (IGF1-R) (19), and colony-stimulating factor receptor (CSF-1R) (9), have demonstrated that intrinsic protein tyrosine kinase activity is required for many downstream signaling events. However, several more recent studies have demonstrated that at least certain downstream signaling events can occur through apparently kinase-independent mechanisms. For example, studies by Campos-Gonzalez and Glenney (4) and by Selva et al. (37) have both recently demonstrated that ligand stimulation of cells expressing kinase-inactive mutants of the hEGFR results in phosphorylation and activation of mitogen-activated protein (MAP) kinase. Recent studies in our laboratory further demonstrate that the kinase-defective hEGFR is capable of signaling transcriptional activation mediated by the c-*fos* serum response element (SRE) in a ligand-dependent manner (13). Further, ligand-dependent kinase-independent signaling of DNA synthesis has also recently been reported by Coker et al. (7).

Although the mechanism(s) of kinase-independent signal transduction by these proteins has not yet been determined, potential mechanisms have been postulated for the EGFR and IR families (4, 7, 13, 27, 37). In both cases, there is evidence to suggest that the conformation of these receptor proteins may be important for their ability to signal via kinase-independent mechanisms. For example, a recent study by Sung et al. (40) showed that S6 kinase can be activated by monoclonal antibodies directed against the ligand-binding domain of the IR. Interestingly, however, these monoclonal antibodies do not activate the tyrosine kinase activity of the receptor in vitro or in vivo. These investigators have speculated that the interaction of these antibodies with the IR stimulates a conformational change in the receptor and that this conformational change is sufficient to "activate" the receptor, resulting in stimulation of S6 kinase phosphorylation.

These results become particularly important in the context of a receptor that can apparently be activated to an oncogenic state via ligand-independent mechanisms. In fact, before the present study, it remained formally possible that overexpression of v-*erbB*, even in the absence of catalytic activity, could transform cells via heterodimeric activation of endogenous c-*erbB*-related products. Our results clearly demonstrate that kinase-defective v-*erbB* products cannot transform cells through a simple heterodimerization mechanism. It remains possible, however, that the formation of heterodimers between v-*erbB*-encoded products and wild-type c-*erbB*-encoded receptors contributes to oncogenic transformation mediated by the kinase-intact v-*erbB* products, and this hypothesis is experimentally testable.

In summary, mutant forms of the avian c-*erbB1* gene can induce tumor formation in three distinct tissues. In all three tissues, transformation occurs via a ligand-independent mechanism. Numerous examples of ligand-dependent cellular responses that do not require intrinsic tyrosine kinase activity, including ligand-stimulated DNA synthesis, have recently been reported. We, therefore, examined the requirement for the intrinsic tyrosine kinase activity of v-*erbB* in ligand-independent transformation. The results presented in this study demonstrate that the intrinsic tyrosine kinase activity of these v-*erbB* mutants is absolutely essential for their transforming properties in all three tissues and further suggest that these kinase-defective v-*erbB* products cannot activate the transforming potential of native endogenous receptors via heterodimerization.

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