Cooperation of *v-jun* and *v-erbB* Oncogenes in Embryo Fibroblast Transformation In Vitro and In Vivo

MADELEINE GARCIA* AND JACQUES SAMARUT

Laboratoire de Biologie Moléculaire et Cellulaire, UMR 13 Centre National de la Recherche Scientifique, Ecole Normale Supérieure de Lyon, 46, Allée d'Italie, 69364 Lyon Cédex 07, France

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Retroviral vectors carrying either the *v-jun* and *v-erbB* sequences or the *v-jun* gene linked to the neomycin resistance gene were constructed on the basis of the structural genome organization of avian erythroblastosis virus (AEV). These viruses, called JB and JN, respectively, were rescued as Rous-associated virus-1 pseudotypes, and they were shown to successfully transform chicken embryo fibroblasts in vitro. However, in agar, colonies developed from JB-infected fibroblasts were three to five times larger than those obtained after infection with JN or with AEV Pst124 carrying only a functional *v-erbB* gene. In vivo, on chorioallantoic membrane (CAM) assays, JB produced fibrosarcomas that were more rapidly growing and much larger than those induced by JN or AEV Pst124. Moreover, in chickens infected in ovo with JB, multiple fibrosarcomas arose in different organs a few days after birth, whereas no tumor could be detected in parallel experiments in either JN- or AEV Pst124-infected animals. These results demonstrate that in embryo fibroblast cells, *v-jun* and *v-erbB* can act synergistically to enhance the transformation potential of either oncogene alone both in vitro and in vivo.

Tumorigenesis is generally believed to be a multistep process involving independent events (reviewed in reference 6). Hence, the study of neoplastic transformation of primary fibroblasts in culture has shown that a fully malignant phenotype can be induced by the cooperative action of two or more transforming genes (22, 29, 31). Avian erythroblastosis virus (AEV) provides a model system for studying oncogene cooperativity. AEV induces erythroleukemias and sarcomas in chickens and transforms erythroid progenitor cells and fibroblasts in vitro (16). The genome of AEV carries two oncogenes, *v-erbA* and *v-erbB* (35). The *v-erbA* gene has been shown to encode the nuclear receptor for the thyroid hormone triiodothyronine (32, 38). The *v-erbB* gene product is a transmembrane glycoprotein showing extensive homology with the receptor for epidermal growth factor (EGF) (10, 34, 39). This oncoprotein displays a tyrosine-specific protein kinase activity (15). The cooperation of *v-erbB* with *v-erbA* is necessary to fully transform fibroblasts or erythroblastic cells either in vivo or in vitro (11-13). To further investigate oncogene cooperation in neoplastic transformation, we have examined the ability of the *v-erbB* oncogene product to cooperate with v-Jun, another nuclear oncoprotein. The *v-jun* oncogene carried by avian sarcoma virus 17 (ASV17) is derived from the proto-oncogene *c-jun*, which encodes the transcription activator protein AP1 (2, 5, 24, 37).

The mechanism by which the majority of nuclear oncogenes act is still a matter of speculation. Some of them appear to encode proteins that interact directly with transcriptional *cis*-regulating elements; others encode proteins that deregulate gene expression without direct binding to DNA, modifying the normal behavior of the cell by interaction with normal transcription factors. In the case of Jun and ErbA, transcriptional activity results from the direct interaction of these proteins with precise nucleotide sequences. In addition, both proteins are able to form homodimers

(Jun-Jun, ErbA-ErbA) or heterodimers (Fos-Jun). These interactions then lead to conformational modifications of the proteins, resulting in a modulation of their transcriptional activity. However, several differences exist between these proteins: Jun contains an intrinsic transactivating activity and a helix-turn-helix structure responsible for DNA recognition, whereas ErbA possesses a hormone-dependent transactivating activity and has a zinc finger motif responsible for its interaction with the target DNA sequence. Transcription factors can thus be separated into several classes on the basis of their mode of interaction with specific *cis*-acting promoter elements.

To analyze the possible cooperation between *v-jun* and *v-erbB* and gain more insight into the various mechanisms leading to cell growth, we constructed an AEV-based retroviral vector in which the *v-erbA* sequences were replaced by the *v-jun* oncogene. This vector was able to transfer and express both *v-jun* and *v-erbB* sequences in chicken embryo fibroblasts (CEFs). Our results indicate that *v-jun* and *v-erbB* can act synergistically to transform embryo fibroblasts both in vitro and in vivo.

MATERIALS AND METHODS

Viruses. ASV17 (clone 241) and plasmids pG551 and pJun were kindly provided by P. Vogt (University of Southern California, Los Angeles). These plasmids, carrying the *v-jun* oncogene, have been described previously (24). pG551 was used to isolate *v-jun* sequences after digestion with *Xho*I and *Eco*RI. The 1.7-kilobase (kb) fragment recovered by electroelution represents part of *gag* sequences (3' end of p19 and deleted p10) fused to the complete *v-jun* gene. This fragment was inserted into the pCBXN vector between *Xho*I and *Xba*I sites after filling noncohesive ends with DNA polymerase (Klenow fragment). The pCBXN vector is a pAEV-based vector in which *v-erbA* sequences have been deleted. After ligation with *v-jun*, the resultant retroviral vector genome, JB, contained, in addition to the *v-erbB* gene, all of the *v-jun* oncogene, which is expressed from the viral long terminal

* Corresponding author.

repeat as a genomic RNA encoding a Gag-Jun fusion protein. The JN retroviral vector has the same structural organization as JB except that the *v-erbB* sequences have been replaced by the neomycin resistance (*neo*) gene from Tn5 (4).

AEV Pst124 is a mutant of AEV ES4; it produces an altered Gag-ErbA protein lacking *v-erbA* activity. It was derived from plasmid pAEV11 by deleting the 487 base-pair *PstI-PstI* fragment within the *v-erbA* sequences (12).

All viruses were rescued as Rous-associated virus-1 (RAV-1) pseudotypes by transfection on CEFs as previously described (12).

Cell culture. Primary CEFs were prepared as described previously (17) from 10-day-old C/E SPAFAS chicken embryos. The cells were grown in Ham F10 medium (GIBCO Laboratories) supplemented with 10% tryptose phosphate broth (GIBCO), 2.5% fetal bovine serum, 2.5% calf serum, 1% normal chicken serum, 0.2% NaHCO_3 , and 1% antibiotics. Transforming particles were recovered 12 to 15 days after transfection and titrated on CEFs by the transforming focus assay (36).

Protein labeling and immunoprecipitation. Cultured cells were labeled for 3 h in 2 ml of methionine-free alpha minimal essential medium containing 50 μCi of [^{35}S]methionine (>1.2 mCi/mmol). The cells were then washed with Tris-glucose and lysed in 1 ml of buffer containing 0.15 M NaCl, 0.05 M Tris hydrochloride, 1 mM EDTA, 1% Triton, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% phenylmethylsulfonyl fluoride. The protein extract was clarified for 1 h at 13,000 rpm, and the supernatant was immunoprecipitated for 4 h with anti-Jun antibodies (Oncogene Science). Extract (2×10^6 cpm) was then incubated with 50 μl of a 50% suspension of Sepharose-bound protein A. The precipitate was extensively washed and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel, followed by autoradiography.

RNA analysis. Cellular RNA was purified from cell cultures or frozen tumors essentially as previously described (14). Polyadenylated RNA was isolated by chromatography on an oligo(dT)-cellulose column. After denaturation at 50°C with glyoxal (25), the samples were fractionated by electrophoresis on a 1.5% agarose gel, transferred to a nylon filter, and hybridized to a random-primed labeled probe. Filters were finally washed under highly stringent conditions and exposed to Kodak X-OMAT films.

Colony formation in agar. The colony-forming assay was performed as described by Moscovici et al. (26). Briefly, infected cells (5×10^4) were seeded in soft agar in 35-mm dishes. After 5 days, an additional soft agar overlay was added, and colonies were scored 5 days later.

In vivo tumorigenicity assay. (i) **CAM assay.** Eggs were prepared as described by Poste and Flood (27). After the eggs were incubated for 10 days, the blunt end was pierced and a small window was opened in the shell overlaying the chorioallantoic membrane (CAM). Infected or uninfected cells (2×10^6) were layered onto the surface of the CAM. The window was then sealed, and the eggs were incubated horizontally. After 7 days, the shell was opened, and the CAM was removed, fixed, and processed for histological examinations.

(ii) **In ovo inoculation.** Viral suspension (100 to 200 μl) was inoculated into the chorioallantoic vein of 10-day-old embryos. After birth, chickens were checked periodically for signs of disease. One to five weeks later, the animals were sacrificed and the tumors were fixed for histological examination or frozen at -80°C for nucleic acid analysis.

FIG. 1. Genome structures of JN and JB viruses. In both viruses, the Gag-Jun fusion protein is translated from genomic RNA. The *v-erbB* and the neomycin resistance (*Neo*) genes are transcribed from the viral long terminal repeat as subgenomic RNA in JB and JN proviruses, respectively. Restriction endonuclease cleavage sites: Ap, *ApaI*; Ba, *BamHI*; Eco, *EcoRI*; Sa, *SacI*; Xb, *XbaI*; Xh, *XhoI*; SD, Splice donor; SA, splice acceptor.

RESULTS

Genomic structures of the viruses. To investigate the possible cooperation between *v-jun* and *v-erbB*, we constructed avian retroviral vectors based on the structural organization of the AEV genome. As controls, we used viral constructions expressing either the *v-jun* or the *v-erbB* oncogene alone. The genomic configurations and the restriction maps of the JB and JN viruses are shown in Fig. 1. JB carries both *v-jun* and *v-erbB* sequences, whereas JN contains only the *v-jun* oncogene. In JN, the *v-erbB* gene of JB has been replaced by the *Neo* gene isolated from previously constructed retrovirus vectors (4). In both viruses, the *v-jun* sequences are translated from the genomic RNA as a Gag-Jun fusion protein. This protein is identical to the Gag-Jun protein synthesized in ASV17, the natural viral vector of the *v-jun* oncogene. *v-ErbB* and *Neo* proteins in the JB and JN proviruses, respectively, are translated from a 3.4-kb subgenomic RNA. In addition to Δgag and Δenv sequences, these viruses contain all of the transcriptional regulation signals from AEV. Three other viruses were also used as controls: AEV ES4, carrying the normal *v-erbA* and *v-erbB* oncogenes, and AEV Pst124 and AEV H, synthesizing only the *v-ErbB* oncoprotein. AEV Pst124 is a deletion mutant of AEV ES4 (12), and AEV H is a natural virus carrying only the *v-erbB* oncogene (39). Both AEV Pst124 and AEV H have reduced transforming efficiency on fibroblasts and erythroid cells (11, 12, 39). All of the viruses were rescued on CEFs by cotransfection of the plasmids containing the respective proviruses with a plasmid containing the RAV-1 helper provirus.

Viral expression in cells infected in vitro. JN and JB cloned DNAs were cotransfected independently with RAV-1 DNA into secondary CEFs. Transformed foci started to appear 7 to 10 days later. The cells in these foci showed the same morphological features as ASV17-infected CEFs: they appeared fusiform and refractile and grew in parallel orientation and multiple layers (data not shown; 24). The transformed JN and JB cells were subcultivated for more than 30 generations without any decrease in their growth potential. Moreover, their growth properties were very similar, and they were both able to multiply in low-serum conditions (data not shown).

The expression of JN and JB proviral genomes was analyzed in transformed CEFs. Cells were labeled with [^{35}S]methionine for 3 h, and protein extracts were immunoprecipitated with a rabbit antiserum directed against the DNA-binding domain of the c-Jun-AP1 protein. After analysis by electrophoresis on an SDS-polyacrylamide gel, the 65-kilodalton (kDa) protein was readily detected in CEFs

FIG. 2. The v-Jun oncoprotein in transformed CEFs. Cells grown for 3 h in [35 S]methionine were immunoprecipitated with a Jun antiserum. Immunoprecipitates were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel and subsequent autoradiography. The size markers indicated on the left are as follows: myosin, 200 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa. The position of the 65-kDa protein is shown on the right.

infected with JN and JB (Fig. 2, lanes 3 and 4). The same 65-kDa protein corresponding to the v-Jun oncoprotein was observed in CEFs infected with ASV17 (lane 1), whereas no such protein could be immunoprecipitated in uninfected

CEF (lane 2). Thus, the v-Jun oncoprotein is correctly synthesized in CEFs transformed with JN and JB retroviruses.

As a next step toward characterization of the expression of these viruses, the supernatants of infected CEFs were tested for the presence of transforming viral particles in a focus assay. The JN- and JB-transformed cells released infectious particles at approximately the same titer: 1.5×10^5 focus-forming units per ml.

In summary, these results demonstrate that JN and JB viruses can successfully be expressed in chicken fibroblasts and that they both can efficiently transform these cells.

Growth properties in soft agar of CEFs transformed in vitro. One of the properties of transformed cells is the formation of colonies in soft-agar suspensions. We thus asked whether CEFs infected with either JN or JB would present different growth characteristics in soft agar. CEFs infected with the JN virus developed anchorage-independent transformed colonies (Fig. 3C), whereas uninfected CEFs did not grow in soft agar (Fig. 3A). The JN-transformed colonies exhibited the same morphological characteristics as those formed by CEFs infected with ASV17 (data not shown). As expected, CEFs infected with JB, containing both *v-jun* and *v-erbB* oncogenes, also developed colonies in soft agar (Fig. 3D). However, these colonies were approximately five times larger than those obtained after infection with JN. These JB-transformed colonies displayed a widely diffused structure. In contrast, the colonies that developed from CEFs transformed by AEV Pst124, carrying only a functional *v-erbB* gene, were usually three times smaller than those originating from JN-infected CEFs (Fig. 3B). Moreover, the frequencies of colony-forming cells were much higher in cultures infected with JB (0.6%) than in

FIG. 3. Colony formation in agar. Colonies observed with uninfected CEFs (A) and with CEFs infected with AEV Pst124 (B) were compared with colonies obtained with JN-infected (C) and JB-infected (D) CEFs.

TABLE 1. Tumorigenicity assay of infected CEFs on CAM^a

CEFs infected with:	Incidence of tumors (%)	Diam of tumors (mm)
JN	2/8 (25)	1
JB	17/18 (94)	6–12
ASV17	4/12 (33)	2
AEV	24/42 (57)	3–4
No virus	0/16 (0)	

^a CEFs were infected with the indicated viruses, and 2×10^6 infected cells were then implanted into the CAM of 10-day-old embryos. Tumor formation was checked 9 days later.

cultures infected with either JN (0.07 to 0.13%) or AEV Pst124 (0.26%). Hence, these results show that v-jun can act synergistically with v-erbB to induce a more potent transformation of the fibroblastic cells.

In vivo tumorigenicity of the viruses. (i) CAM assay. The ability of the CEFs infected with these viruses to develop tumors was subsequently analyzed on CAM. Cells infected with either JN or JB were implanted into CAM of 10-day-old embryos. As controls, uninfected CEFs and CEFs infected with ASV17 or AEV were tested in parallel. All infected cells developed tumors on CAM (Table 1). However, depending on the virus, different efficiencies and tumor sizes were observed. Although JN- and ASV17-infected CEFs had approximately the same incidence of tumors (25 and 33%, respectively), JB-infected cells showed a much higher incidence (94%). Moreover, tumors induced by JB-infected CEFs were three to six times larger in diameter than tumors developed from either JN- or ASV17-infected cells.

As a comparison, AEV- and AEV Pst124-infected cells induced about half as many tumors as did JB-infected cells (57 and 56%, respectively; Table 1; 12). Tumor sizes were also significantly smaller: a two- to fourfold size amplification was noted in JB-infected cells as opposed to AEV-infected CEFs. As shown previously, tumors developed from AEV-transformed CEFs are larger than those originating from AEV Pst124-transformed cells (12). Histological analyses of tumors developed from JN- and JB-transformed CEFs are shown in Fig. 4. A dense fibrosarcoma invading the mesodermal layer of the CAM was characteristic of tumors arising from JB-transformed CEFs (Fig. 4C). Many cells undergoing mitosis were also visible among the tumor cells. In contrast, no cellular growth was detectable in CAM grafted with uninfected CEFs (Fig. 4A). The tumors that developed in the mesodermal layer from the JN-infected CEFs (Fig. 4B) were soft fibrosarcomas with poor cell density, and mitotic cells were rare. The tumor (arrowheads in Fig. 4B) was surrounded by a wide collagenous surface. A similar histological picture was detected in CAMs implanted with AEV Pst124-infected cells (12).

(ii) In ovo inoculation. As a last step in the characterization of the in vivo tumorigenic potential of JN and JB viruses, infection of 10-day-old embryos was achieved by inoculation of 100 to 200 μ l of viral suspensions into a chorioallantoic vein. After birth, all infected animals were periodically tested for viremia by analysis of blood smears stained with Wright-Giemsa (Table 2). No apparent sign of disease was detected in the JN-infected chickens, although virus titration allowed us to check that chickens were successfully infected with JN. The animals were sacrificed at 5 weeks posthatching, and careful macroscopic examination of all organs showed a small subcutaneous outgrowth in only one animal. This subcutaneous nodule was histologically classified as a soft fibrosarcoma showing irregular bundles of fusiform cells

FIG. 4. Histological analysis of tumors developed on CAM. CAM were inoculated with control CEFs (A) or with CEFs infected with JN (B) or JB (C). Arrowheads in panel B mark the tumor. The slides were stained with hematoxylin. Magnification, $\times 100$.

(data not shown). A similar situation was obtained in ASV17-infected animals, in which a soft fibrosarcoma developed in the liver of a 28-day-old animal (Table 2).

A completely different picture appeared in JB-infected chickens. A few days after birth, infected animals started to die, and autopsies revealed tumors in several organs, predominantly liver but also spleen, kidneys, skeletal muscles, gizzard, and ovaries. Explants of tumors from these chickens were assessed for their ability to grow in culture. These cells exhibited the same morphology and growth pattern as the CEFs infected in vitro with JB. These observations were in agreement with the histological examinations showing a well-differentiated fibrosarcoma in all of the infected organs (data not shown and Table 2). The presence of viral particles was also checked in blood collected from JB-infected chickens. In all animals tested, high titers ($>5 \times 10^5$ focus-forming units per ml) of viral particles could be demonstrated by using the focus-forming assay. These results thus indicated that the defective viruses spread in the infected chickens.

TABLE 2. Tumorigenicity assay after in ovo inoculation^a

Virus	Age of chicken (days) at autopsy	Apparent tumors	Diam of tumors (mm)
JN	1	No	
	16	No	
	21	No	
	30	No	
	30	Subcutaneous	<2
ASV17	7	No	
	7	No	
	15	No	
	20	No	
	28	No	
	28	Liver	<2
	28	Liver	5–10 mm
JB	6	No	
	8	No	
	13	Liver	>15
	13	Liver, kidneys, ovaries, muscle	>15
	14	Liver, spleen, gizzard, muscle	>15
	15	Liver	>15
	15	Spleen	>15
	17	Liver, spleen, gizzard	>15
	17	Liver	>15

^a Viral suspensions were inoculated into the chorioallantoic veins of 10-day-old embryos. After birth, the animals were checked periodically for signs of disease and sacrificed for histological examinations. Apparently healthy animals were autopsied after 28 days.

As a comparison, 10-day-old chicken embryos injected with AEV Pst124 only developed erythroblastosis between 10 and 17 days of age, with no other type of tumors being detected (P. Jurdic, personal communication).

To ensure that the tumors in JB-infected chickens grew from cells transformed by the virus, liver tumors were analyzed by Northern (RNA) blot. Polyadenylated RNA was purified from the livers of two chickens that had been infected as embryos. Hybridization of the filter with a *v-jun* probe revealed a strong 5.6-kb band in the liver of one infected bird (Fig. 5A, lane 3). A similar band was observed in another infected liver (lane 2) after longer exposure of the blot (data not shown). As a control, normal liver from 10-day-old chickens did not show any hybridization (lane 1).

The expression of RNAs containing *v-erbB* sequences was also checked after extensive washing of the previous filter and rehybridization with a *v-erbB* probe (Fig. 5B). Again, *v-erbB* transcripts were not detected in normal liver (Fig. 5B, lane 1). However, two bands of 5.6 and 3.4 kb, respectively, were seen in the liver of one JB-infected chicken (lane 3). The 5.6-kb transcript was the expected genomic mRNA, whereas the 3.4-kb transcript corresponded to the subgenomic RNA encoding the v-ErbB oncoprotein.

Endogenous *c-jun* and *c-erbB* mRNAs were not detected in Fig. 5, which shows a filter exposed for only 2 to 3 h. However, longer exposure of these blots clearly showed these messages (data not shown).

Overall, we have shown that multiple tumors arose in JB-infected chickens. The tumor cells contained JB proviruses integrated in their genomes (data not shown), and they expressed high levels of viral transcripts and viral particles. These results suggested that JB virus was involved in the formation of fibrosarcomas in chickens infected in ovo. In contrast, tumors rarely develop at the mean time in the JN-infected animals, supporting the view that the *v-jun*

FIG. 5. Viral transcription in liver tumors of JB-infected chickens. Polyadenylated RNA (1 to 2 μ g) was fractionated on a 1.5% agarose gel, transferred to a nylon filter, and hybridized to a *v-jun* probe (A) or to a *v-erbB* probe (B). Lanes: 1, mRNA from normal liver; 2 and 3, mRNA from two different liver tumors.

oncogene alone was probably not sufficient to induce tumors in chickens infected as embryos.

DISCUSSION

We have constructed a retroviral vector called JB from the AEV ES4 genome by replacing the *v-erbA* coding sequence by the *v-jun* oncogene, a different type of nuclear oncogene. As a control, we also constructed the JN virus, in which the *v-erbB* gene of the JB virus was replaced by the *neo* gene, a dominant selectable marker. We show here that these two vectors are efficient in transforming CEFs in vitro. These transformed cells synthesize the expected 65-kDa v-Jun protein, and high titers of infectious particles are recovered in the supernatant of both JN- and JB-infected cells.

After infection with JN, CEFs showed a transformed phenotype identical to that of cells infected with ASV17, the natural host of the *v-jun* oncogene. However, the vector carrying both the *v-jun* and *v-erbB* oncogenes displayed a higher transformation capacity than did those containing the *v-jun* gene alone (such as JN or ASV17) or those expressing *v-erbB* alone as functional oncogene (such as AEV Pst124). Our results clearly show that in embryo fibroblasts in vitro, expression of both *v-jun* and *v-erbB* strongly enhances the efficiency of colony formation and the size of the transformed colonies in soft agar.

These results were subsequently confirmed by in vivo assays. Hence, whereas the retroviral vector carrying the *v-jun* gene linked to the *neo* gene had the same behavior as ASV17, the association of the *v-jun* oncogene with *v-erbB* allowed the JB virus to acquire a much higher transformation efficiency in vivo. More dramatic results were obtained after in ovo inoculation of 10-day-old chicken embryos. Indeed, JB-infected animals developed multiple large and dense fibrosarcomas in several organs, and the animals died soon after birth. In contrast, in JN (or ASV17)-infected chickens, only rare small and soft fibrosarcomas appeared, and they did so late in life with nonlethal effects. In addition, AEV Pst124-infected chickens never developed fibrosarcomas after in ovo inoculation, although transient erythroblastosis appeared 2 to 3 weeks after birth.

Taken together, these data show that CEFs expressing both *v-jun* and *v-erbB* oncogenes display a higher transforming potential than do cells expressing either *v-jun* or *v-erbB* alone. This situation was somewhat homologous to that observed in AEV-transformed cells, in which the *v-erbA* nuclear oncogene can potentiate the transformation capacity of *v-erbB* (16). More precisely, *v-erbA* was shown to activate fibroblast proliferation in vitro and to cooperate with *v-erbB* to enhance tumor growth in vivo (12). Although the *v-jun* oncogene appears as a more potent transforming gene than *v-erbA*, they are both transcription factors. Thus, these observations show that the association of a nuclear transcription factor with a tyrosine kinase receptor oncogene leads to a fully transformed cell phenotype. What feature might these oncogenes present to enable them to work cooperatively?

The v-ErbB protein corresponds to a truncated form of the EGF receptor. It is deleted in the extracellular ligand-binding domain, but it retains the transmembrane and the cytoplasmic tyrosine kinase domain of the EGF receptor. Hence, v-ErbB could represent an unregulated receptor in a constitutively active state. Receptor tyrosine kinases are key components of the biological control network that govern cellular growth and differentiation. It has been shown that the interaction of EGF stimulates the tyrosine kinase activity of its receptor and secondarily the phosphorylation of specific proteins in the vicinity of the cell membrane through pathways involving protein kinase C (reviewed in reference 40). The *v-erbB* gene might modulate the activity of multiple *trans*-acting factors, thus promoting long-term proliferation of the infected cells. Indeed, several oncogenes have been shown to participate in signal transduction; in several cases, mitogenic signals are transmitted by cytoplasmic proto-oncogenes, and some nuclear proto-oncogene products, such as c-Fos and c-Myc, are potential targets of protein kinase C (reviewed in reference 30). Although we have not addressed the roles of *c-fos*, *c-myc*, or *c-jun* in embryo fibroblasts infected by *v-erbB*, these proto-oncogenes may all play some part in the mechanism of oncogenesis by *v-erbB*.

One of the properties shared by some nuclear oncoproteins is the ability to bind to DNA in vitro. Recent evidence indicates that the v-ErbA protein is a competitive repressor. It inhibits the activation of thyroid hormone response elements by competing with its normal cellular counterpart, c-ErbA (9, 33). The mechanisms by which v-Jun act are not known. However, it appears that the c-Fos oncoprotein can form a complex in vitro with the products of *c-jun* and *v-jun* (8). These heterodimers then can bind to Jun-AP1 sites with high affinity and regulate the transcription of various genes containing this DNA element in their promoter regions (3, 18, 23). Although the oncogenically relevant genes that are differentially affected by v-Jun are not yet known, v-Jun could represent a constitutively active transcriptional factor modulating the expression of specific genes involved in the program of cell proliferation. Epidermal growth factor has been shown to cause transient induction of *jun* mRNA in fibroblasts (28). The *c-jun* product then stimulates its own transcription through an AP1-binding site present in its own promoter (2); in this manner, it could possibly convert the primary transient signal into long-term effects on gene expression (30).

The protein kinase C pathway may be one of several pathways leading to growth stimulation of cells. Our results, showing in vivo and in vitro the cooperative action of *v-jun* and *v-erbB*, suggest that the *jun* oncogene might modulate

gene expression through a distinct signal pathway. The fact that *v-jun* and *v-erbB* have synergistic effects in CEFs suggests that they affect parallel signal pathways whose functions are probably complementary in normal cells. Thus, the control of normal cell proliferation involves stimulation of cellular receptors and activation (or suppression) of gene transcription activities. Deregulation of any of these processes simultaneously with a constitutively active receptor (such as v-ErbB) and transcription factor (such as v-Jun) would result in uncontrolled cell growth through multiple nonconvergent pathways.

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