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A hepatitis B virus pre-S–retinoic acid receptor β chimera transforms erythrocytic progenitor cells *in vitro*

(nuclear receptor/hematopoiesis/tumorigenicity)

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ABSTRACT **In this report, we investigated the transforming properties of retinoic acid receptor β (RARβ). The v-erbA protein, which is the viral oncogenic homologue of the thyroid hormone receptor, was replaced by either the complete RARβ (βR) or a hepatitis B virus pre-S–RARβ (HβR) hybrid product in an avian erythroblastosis virus-based vector. In chicken hematopoietic cells, the HβR protein was able to transform erythroid progenitor cells, whereas no such transformation was observed with the wild-type βR protein. Moreover, the fully transformed phenotype was observed even in the absence of v-*erbB*, and HβR-transformed erythroid cells grew independently of growth factors and transforming growth factor α. The analysis of erythrocytic-specific proteins revealed that the transformed cells were blocked at the colony-forming unit–erythroid stage and that the expression of the carbonic anhydrase II gene, a gene normally regulated by thyroid hormones, was repressed by the HβR protein. Finally, hepatocarcinomas rapidly developed in some chickens infected *in ovo* with viruses encoding either the normal or the hybrid HβR, suggesting that an inappropriate expression of the RARβ gene may represent an important event in oncogenesis.**

Retinoic acid receptors (RARs) are members of a large family of ligand-regulated transcriptional factors that includes the thyroid hormone receptors (TRs) and steroid hormone receptors. This nuclear receptor family has a modular structure consisting essentially of a DNA-binding domain defined by two zinc finger motifs and a carboxyl-terminal hormone-binding domain. Upon binding of their respective ligands, these receptors interact with specific DNA sequences and regulate the transcription of adjacent genes (1). Three different RAR subtypes (α, β, and γ) have been isolated in humans and other animal species. They might exert their functions by regulating the transcription of various genes at different times of development and in specific cells (2). Interestingly, RARα and RARβ are more homologous to TRα and TRβ, respectively, than to any other members of the nuclear receptor family (3, 4). Moreover, their similarities are not only structural, but also functional: the facts that RARα binds to and activates transcription from thyroid responsive elements (5) and that TRα and TRβ can both form heterodimers with RARα (6) suggest that retinoic acid (RA) and thyroid hormones, acting through their respective receptors, could control overlapping gene networks involved in the regulation of vertebrate morphogenesis and homeostasis.

The v-erbA protein is the viral oncogenic homologue of c-erbA, the α form of the nuclear receptor for the thyroid hormone 3,3′,5-L-triiodothyronine (T_3). It contains an intact DNA-binding domain but has lost its ability to bind T_3 (7, 8). Recently, it was proposed that v-erbA can act as a constitutive repressor against both c-erbA and RAR (9–11). In this study, we have investigated the possibility that other nuclear receptors may be implicated in tumorigenesis. So far, two observations have raised the possibility that altered retinoid receptors may have oncogenic properties: (*i*) in one human hepatocellular carcinoma, the RARβ gene is rearranged as a result of a hepatitis B virus (HBV) integration (12); and (*ii*) in acute promyelocytic leukemias, the RARα subtype is truncated by the (15:17) translocation (13–15).

We have analyzed the transforming potential of RARβ by using avian erythroblastosis virus (AEV) as a model system. AEV induces erythroleukemias and fibrosarcomas in susceptible chickens and transforms erythroid progenitor cells and fibroblasts in culture (16). The transforming properties of AEV are determined by two host-derived oncogenes, v-*erbA* and v-*erbB* (17). The v-*erbB* product is a transmembrane glycoprotein showing extensive homology with the receptor of epidermal growth factor and transforming growth factor α (TGF-α) (18, 19). In this report, we constructed AEV-based retroviral vectors in which the v-*erbA* sequences were replaced by either the entire RARβ coding sequence or a chimeric HBV–RARβ form derived from a human hepatocellular carcinoma (12). We show that the HBV–RARβ form, but not its wild-type counterpart, was able to transform erythroid progenitor cells. The transforming potential of RARβ was further confirmed after *in vivo* injection, since some infected chickens developed hepatocarcinomas.

MATERIALS AND METHODS

Viruses. A partially digested *Sau*3A–*Mae* I fragment, 1360 base pairs long and corresponding to the complete coding region of the human RARβ gene positions 318–1677 (3), was treated with Klenow polymerase and inserted in place of v-*erbA*, in phase with the *gag* sequences, in the genome of AEV (20). Thus, the resultant retroviral vector genome, βR-B, contained, in addition to the v-*erbB* gene, a fused *gag*–RARβ sequence, which is expressed from the viral long terminal repeat as a genomic RNA encoding a gag–RARβ fusion protein of approximately 82 kDa. The βR-N retroviral vector has the same structural organization as βR-B except that the v-*erbB* sequences have been replaced by the neomycin resistance gene (*neo*) from Tn*5* (20). The HβR-B and HβR-N retroviral vectors were constructed similarly to βR-B and βR-N, respectively, except that in the HβR-expressing viruses, the 74 N-terminal residues of RARβ were replaced by the first 30 amino acids of the pre-S_1 envelope protein of HBV as it occurred in the initial hepatocarcinoma (21). All

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Abbreviations: RA, retinoic acid; RAR, RA receptor; TR, thyroid hormone receptor; HBV, hepatitis B virus; AEV, avian erythroblastosis virus; TGF-α, transforming growth factor α; CFU(s)-E, colony-forming unit–erythroid; BMC, bone marrow cell; T_3, 3,3′,5-L-triiodothyronine.

viruses were rescued as Rous-associated virus 1 (RAV1) pseudotypes by transfection on chicken embryo fibroblasts (CEFs) as described (22).

The βR-BN and HβR-BN constructions are similar to the βR-B and HβR-B retroviruses, respectively, except that a *neo* gene was inserted downstream from the v-*erbB* sequences (23). The βR-BN and HβR-BN retroviruses were produced as helper-free retroviral vectors in an avian leukosis virus-based packaging cell line: the QT6 Isolde clone (24).

The viruses AEV(v-*erbA*, v-*erbB*), XJ12 (v-*erbA*, *neo*), and AEV-H (v-*erbB*) have been described (19, 22).

Protein Analysis. Cultured cells were lysed in 1% SDS lysis buffer, and protein extracts were analyzed by electrophoresis on SDS/12.5% polyacrylamide gels. After electrotransfer, the Western blots were incubated with specific antibodies, and detection of alkaline phosphatase was performed in the presence of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates (Promega).

Infection of Bone Marrow Cells (BMCs). BMCs from 1-day-old chickens were infected and grown in Methocel as described (25). After 7 days, colonies were picked and seeded in liquid culture containing α-MEM supplemented essentially with 20% fetal bovine serum and 5% chicken serum.

RESULTS

Genomic Structures of the Viruses. In one case of human hepatocellular carcinoma following HBV infection that we have studied, HBV integration generated a virus–host hybrid sequence in which the first codons of the viral pre-S_1 envelope gene were inserted in-frame, six codons upstream of the DNA-binding domain of the RARβ gene (12). The resulting chimeric protein, presumably expressed from the viral pre-S_1 promoter (26), was postulated to participate in the cellular transformation process, but so far there is no direct experimental data supporting this hypothesis. Fig. 1*A* outlines the junction site between HBV and RARβ protein in the original human hepatocellular carcinoma (patient D). The predicted hybrid retained most of the receptor part except for the first 74 amino acids, which had been substituted by the 30 amino-terminal residues of pre-S_1.

In this report, we investigated the transforming capacity of both the HBV–RARβ hybrid (HβR) and its wild-type RARβ counterpart (βR) and analyzed their possible cooperation with v-*erbB*. For that, we constructed avian retroviral vectors expressing one or the other protein based on the structural organization of the AEV genome. Fig. 1*B* shows the outline of the vectors used. βR-B carries both RARβ and v-*erbB* sequences, whereas βR-N contains only RARβ coding sequences associated with the *neo* gene. βR-BN was similar to βR-B except that an additional *neo* gene was inserted downstream from the v-*erbB* sequences. In these viruses, the RARβ sequences are translated from a genomic RNA as a gag–RARβ fusion protein. In the *gag*–RARβ gene, 765 nucleotides of the *gag* gene were fused in-frame with the intact RARβ coding sequence. The v-erbB and neo proteins encoded in βR-B and βR-N, respectively, are translated from subgenomic RNAs. The HβR constructions were essentially identical to the βR-containing proviruses except that in these cases the RARβ gene was fused to the HBV pre-S_1 sequence as previously mentioned. All these viruses were rescued on chicken embryo fibroblasts (CEFs) as RAV-1 pseudotypes or as helper-free retroviruses in Isolde cells (24).

The Chimeric HβR Protein Can Transform Erythroid Cells. The v-*erbA* oncogene is responsible for blocking the differentiation program of erythroid progenitor cells at the colony-forming unit–erythroid (CFU-E) stage (25). Therefore, we investigated whether RARβ was able to interfere similarly with any differentiation process in hematopoietic cells.

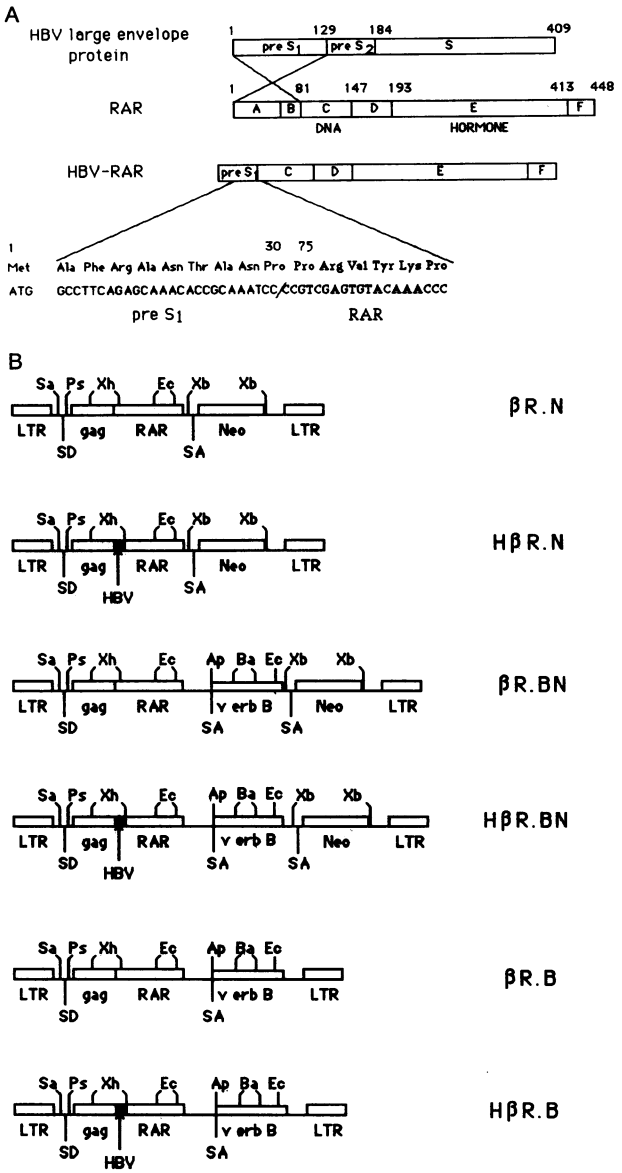

FIG. 1. (*A*) Schematic structure of the HBV large envelope protein, RARβ, and the predicted HBV–RARβ gene products. The large envelope protein of HBV is divided into pre-S_1, pre-S_2, and S regions. Amino acid positions are indicated (26). The RARβ product is divided into six regions, A–F; the C and E regions correspond, respectively, to the DNA- and hormone-binding domains. Numbers denote amino acid positions (12). The chimeric protein, presumably produced in the original liver tumor of patient D, is represented (12). The DNA sequence of the pre-S_1–RARβ junction is shown. (*B*) Genomic structures of the different viruses. In all constructions, the gag–RAR or gag–HBV–RAR fusion proteins are translated from genomic RNA. The v-*erbB* and the *neo* genes are transcribed from the viral long terminal repeat (LTR) as subgenomic RNAs. Ap, *Apa* I; Ba, *Bam*HI; Ec, *Eco*RI; Ps, *Pst* I; Sa, *Sac* I; Xb, *Xba* I; Xh, *Xho* I; SD, splice donor; SA, splice acceptor.

Since the v-*erbB* oncogene cooperates with v-*erbA* by inducing a mitogenic stimulation and releasing the cells from their dependence on specific growth factors (27), we first tested the RARβ-containing viruses carrying the v-*erbB* oncogene. BMCs from 1-day-old chickens were infected *in vitro* with the βR-B and HβR-B retroviruses and their respective helper-free retroviral forms βR-BN and HβR-BN. The cells were then seeded in semisolid methylcellulose cultures containing essentially 20% fetal bovine serum and 5% normal chicken serum and G418 in the case of βR-BN and HβR-BN infections. This medium supports the growth of several dif-

ferent lineages including erythroid, myeloid, and megakaryocytic lineages. Although no transformed colony was observed with the βR-B or βR-BN viruses, colonies of transformed cells rapidly developed in BMCs infected with HβR-B and HβR-BN viruses. These colonies were compact, were made of round cells, and were very similar to the erythroid colonies developed from BMCs infected with AEV (28). Cells isolated from these colonies and grown in liquid culture exhibited the same cytological features as AEV-transformed erythroid cells (data not shown). The transformation was directly mediated by the RAR-containing retrovector since the helper-free virus could also transform these cells.

The same transformation assays were then performed using the retroviral constructions expressing the HBV–RARβ hybrid product in the absence of the v-*erbB* oncogene. For that, the growth conditions applied were the same as those described for HβR-B and HβR-BN infections. Again, identical erythrocytic-transformed cells developed in semisolid methylcellulose using the HβR-N retrovirus, and these cells could be grown in liquid culture in the presence of G418 (data not shown; see Fig. 4). Moreover, the transforming efficiency of HβR-N viruses, as assessed by the number of transformed colonies, was 7 times higher than that of the XJ12 retrovirus expressing only the v-*erbA* oncogene (data not shown).

To check that retroviruses expressing the HBV–RARβ protein have not undergone rearrangements in transformed BMCs, the synthesis of the appropriate HβR protein was checked in HβR-B-transformed hematopoietic cells. By immunoblot analysis, these cells expressed an 82-kDa protein specifically recognized by a monoclonal antibody directed against an HBV pre-S_1 epitope (Fig. 2*A* and ref. 29).

Expression of Proteins Characteristic of the Erythrocytic Lineage. HβR-N- and HβR-B-transformed BMCs were further characterized by analyzing the expression of proteins specific to the erythrocytic lineage. Expression of histone H5 is restricted to the avian erythroid lineage. It is normally expressed in v-*erbA*- and AEV-transformed CFU-E. Immunoblot analysis of protein extracts from HβR-N- and HβR-B-transformed BMCs revealed that these cells expressed high levels of H5 (Fig. 2*B*). The amount of H5 was similar to that observed in AEV-transformed BMCs and in 6C2 cells, an AEV-transformed erythroid cell line. As expected, H5 was not found in HβR-B-transformed chicken embryo fibroblasts.

The erythrocytic nature of the transformed BMCs was further confirmed by the presence of the Im antigen on the cellular membrane, suggesting that HβR-transformed BMCs represented cells blocked at the CFU-E stage (data not shown; ref. 28).

Repression of the Carbonic Anhydrase II Gene by the HBV–RARβ Hybrid Protein in Erythrocytic Cells. The v-*erbA* oncogene blocks erythrocytic differentiation at the CFU-E stage (25), and it has been shown that the v-*erbA* product specifically represses the gene encoding the carbonic anhydrase II in these cells (30, 31). To test whether regulation of the carbonic anhydrase II gene is also impaired by the mutant RA receptors, immunoblot analyses were performed on HβR-transformed erythroid cells. Like 6C2, HβR-N-transformed CFU-E showed a barely detectable level of carbonic anhydrase II (Fig. 3). In this case, erythroid cells were grown for only a few generations before protein extraction, and very little spontaneous erythroid differentiation occurred. In contrast, when primary erythroid cells transformed by either HβR-N or AEV were grown longer, the presence of the carbonic anhydrase II product in these cells could be detected resulting from some spontaneous differentiation during their expansion.

Growth Characteristics of HβR-N-Transformed BMCs. v-*erbA*-transformed CFU-E express functional TGF-α receptors and require TGF-α to grow in culture (27). We thus further checked whether TGF-α might control the growth of HβR-transformed erythroid cells. Growth curves analyses were performed in the absence or presence of recombinant human TGF-α at 1 ng/ml. As shown in Fig. 4, HβR-N-transformed erythroid cells grew independently of TGF-α with a doubling time of ≈21 hr. In contrast, XJ12-transformed erythroid cells were strongly dependent on TGF-α for their growth (27). As expected, AEV erythroleukemia cells did not require TGF-α for growth.

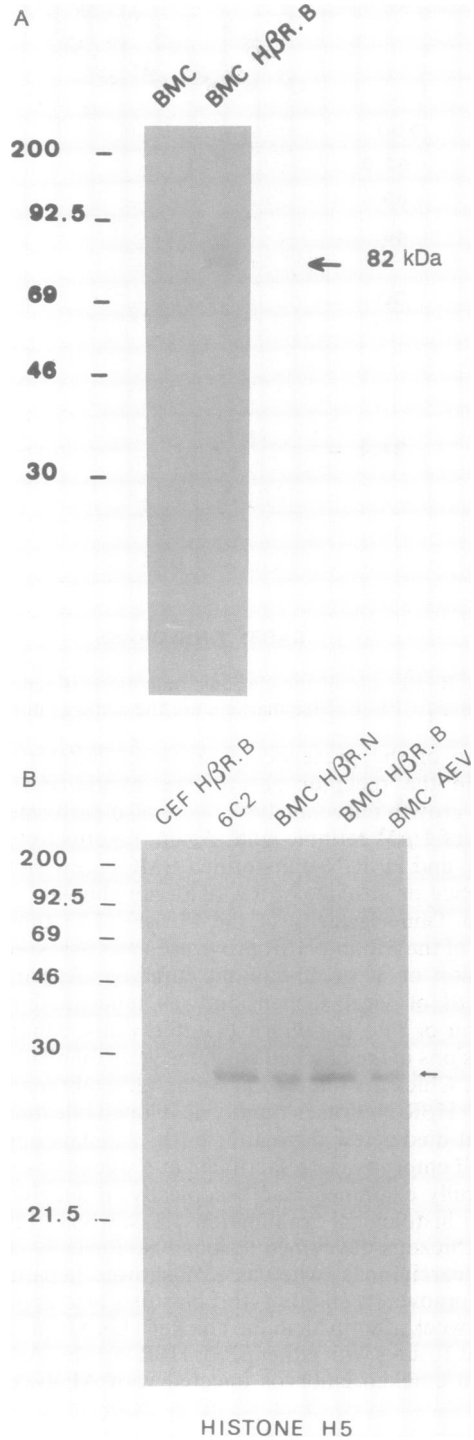

FIG. 2. (*A*) Detection of the gag–HBV–RARβ fusion protein in BMCs transformed by HβR-B virus. (*B*) Detection of histone H5 in transformed BMCs. The size markers indicated on the left are myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21.5 kDa).

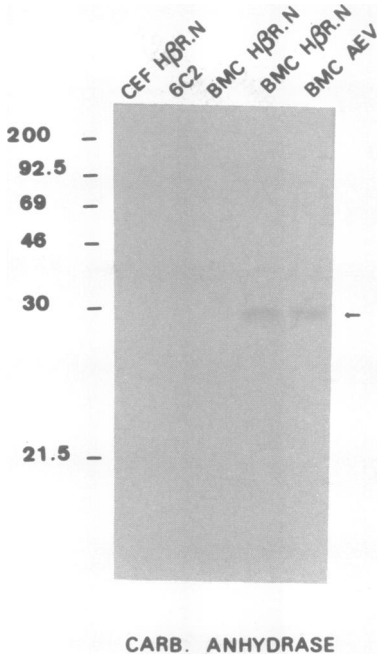

FIG. 3. Repression of the carbonic anhydrase II gene in HβR-N-transformed CFU-E. Size markers are the same as in Fig. 2.

Because of the presence of an intact hormone-binding domain in the receptor part of the hybrid HBV–RARβ proteins, growth curve analyses were also performed in the presence of 1 μM retinoic acid. Again, no effect of RA was observed, and HβR-N-transformed BMCs grew similarly in the presence or absence of RA (data not shown).

***In Vivo* Tumorigenicity of the Viruses.** The tumorigenic potential of the βR and HβR proviruses was assessed *in vivo* by infection of 3- or 10-day-old embryos. Approximately 100–200 μl of viral suspension was inoculated near the blastoderm or into the chorioallantoic veins. Major pathological lesions in the hatched chickens were found in the liver (Table 1). Chickens infected as 3-day-old embryos with the βR-N and HβR-N viruses rapidly developed bile duct hyperplasia and died a few days after birth. Animals infected as 10-day-old embryos were sacrificed at 4 weeks posthatching and carefully examined macroscopically; tissues were processed for histological examinations. As a whole, 12% of the injected chickens developed lesions histologically classified as hepatocarcinomas, whereas 13% showed hepatic hyperplasia. Moreover, a slight erythroblastosis also appeared as soon as 1 week after birth in the chickens born from embryos infected at 10 days with either βR or HβR viruses. As control, 10-day-old chicken embryos injected with AEV-Pst 124, a virus carrying the v-*erbB* oncogene as the sole oncogene (22), developed erythroblastosis between 10 and 17 days of age, with no other type of detectable tumors.

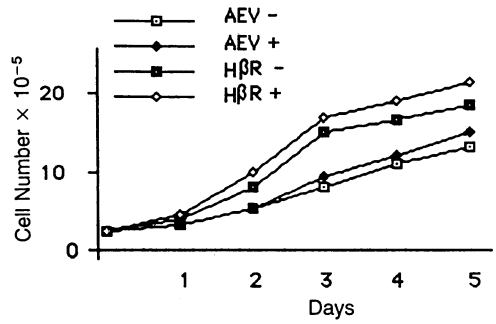

FIG. 4. Growth characteristics of HβR-N-transformed BMCs. The cells were seeded at 2 × 10^5 cells per dish in the presence (+) or absence (−) of TGF-α (1 ng/ml), and each day the cells were counted. Each point represents the mean of duplicate cultures.

Table 1. Tumorigenicity assay after *in ovo* inoculation

Virus	Number with pathological lesions/total* Day 3	Day 10	Age at autopsy,† days	Apparent tumors or affections
βR-N	1/1		3	Hepatitis
		3/3	3	Hepatic steatosis and bile duct hyperplasia
			27	Hepatocarcinoma
			28	Cholangiosarcoma
HβR-N	3/3		3	Hepatic steatosis
			3	Hepatic steatosis and bile duct hyperplasia
			6	Hepatic steatosis
		3/6	28	Hepatocarcinoma
			31	Hepatosarcoma
			39	Bile duct hyperplasia
βR-B		3/11	29	Hepatic steatosis and bile duct hyperplasia
			29	Hepatic steatosis
			30	Hepatocarcinoma
HβR-B		2/15	29	Hepatocarcinoma
			30	Ellipsoid hyperplasia

*Viral suspensions were injected either in the chorioallantoic veins of 10-day-old chicken embryos or near the blastoderm of 3-day-old chicken embryos.

†After birth the animals were checked periodically for signs of disease and sacrificed for histological examinations. Apparently healthy animals were autopsied after 4 weeks.

DISCUSSION

Both RA and T_3 are important modulators of development and morphogenesis (32, 33). Direct evidence for the oncogenic potential of nuclear hormone receptors comes from analysis of the properties of the v-*erbA* oncogene, a natural retroviral oncogene encoding an altered version of the chicken TR (7, 8). v-erbA contributes to cellular transformation by inhibiting the function of its normal T_3 and RA receptors (9, 11). The finding that in one human hepatocellular carcinoma the HBV genome was inserted in the RARβ gene first raised the possibility that altered retinoid receptors might exhibit oncogenic properties (12). Moreover, it has been reported recently that the translocation breakpoint in human acute promyelocytic leukemia fuses the RARα gene to a novel locus, named PML (13, 15, 34), and that the resulting PML–RARα hybrid exhibits altered transactivating properties (35, 36). Hence, the possibility that RAR genes could be involved in oncogenesis would not be surprising in view of these observations and due to the fundamental role played by vitamin A derivatives in differentiation and cell proliferation (33). We have investigated the oncogenic potentialities of the RARβ gene by transferring RARβ coding sequences derived either from the normal human cDNA (βR) or from the rearranged form (HβR) found in the original human hepatocellular carcinoma (12) into avian cells by retrovirus vectors. In HβR, the amino terminus of RARβ was replaced by a coding sequence from the pre-S_1 large envelope protein of HBV. To mimic the structure of the v-*erbA* oncogene, these two RARβ-derived sequences were fused in-frame with *gag* retroviral sequences.

In chicken erythrocytic progenitor cells, the v-*erbA* oncogene blocks the differentiation program at the CFU-E stage

(25) and inhibits the transcription of erythrocyte-specific genes (30, 31). In these cells, the v-*erbA* oncogene cooperates with the v-*erbB* oncogene to induce full leukemogenic transformation *in vitro* and *in vivo* (37). In this report, we show that, like v-*erbA*, the HβR form was able to block the differentiation of chicken erythrocytic cells at the CFU-E stage, whereas the wild-type RARβ was not. The HβR-expressing virus induced the growth of the blocked CFU-E in the absence of TGF-α, a growth factor of chicken erythrocytic progenitor cells normally required for the growth of v-*erbA*-transformed CFU-E (27). When tested *in vivo*, both the intact and the HBV–RARβ induced hepatocellular hyperplasias and carcinomas in chickens. Such tumors never arise in AEV-infected chickens. The slight erythroblastosis observed in RARβ-injected animals was never as dramatic as the severe erythroblastosis usually induced by AEV. This finding together with the low frequency of appearance of liver tumors in βR- and HβR-infected chickens may reflect the requirement of a second genetic event involving the activation of one or more additional oncogenes. Our results allow us to establish a direct link between an inappropriate expression of the RARβ gene and the development of liver carcinoma. Altogether, our data demonstrate that the RARβ and HBV–RARβ proteins are both tumorigenic by themselves and that the mechanisms of cellular transformation by RARβ may be different from that of v-*erbA*. The difference between the normal and the mutant forms of RARβ used here relies on the presence of a peptide sequence derived from the HBV large envelope protein fused at the amino terminus of RARβ. It is then likely that this viral sequence strongly enhances the oncogenic potential of the rearranged RAR in erythroid cells. What are the molecular mechanisms governing the oncogenic effect of HBV–RARβ? It is surprising that this form is oncogenic even in the presence of RA, although it contains a normal carboxyl-terminal hormone-binding domain. v-erbA oncoproteins in which the normal hormone-binding domain is restored display their oncogenic effect only in the absence of thyroid hormone T_3. In the presence of T_3, the chimeric oncoprotein binds the hormone and is no longer oncogenic (30, 31). We may imagine that the HBV pre-S_1 sequence in the chimeric receptor induces strong modifications in the structure and function of the receptor, which remains oncogenic even in the presence of RA.

The role of retinoids in avian erythropoiesis has not been established. However, recent results suggest that v-*erbA* may transform erythrocytic cells by interfering with a retinoid-mediated differentiation process (11). At least two explanations could be put forward to explain erythroid transformation by HBV–RARβ. First, one may imagine that the mutant RARβ binds competitively with normal RARβ to the specific response element, thereby arresting transcription of genes normally required for erythrocytic differentiation. The second possibility is the formation of inactive heterodimers between c-erbA and HBV–RARβ that could not activate the genes required for the differentiation of CFU-E. In conclusion, this work demonstrates that a RA receptor can be activated into an oncogene and suggests that rearrangements of RARβ might contribute to the development of cancers.

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