17. In the gel electrophoresis–DNA binding assay a defined DNA fragment is incubated with the DNA binding protein and then the reaction mix is analyzed by electrophoresis through agarose as previously reported (*10*). DNA fragments bound to protein are identified by their reduced mobility relative to DNA fragments that have not been exposed to protein. Constant amounts of purified OTF-1 and an end-labeled, Ad Ori–containing DNA fragment were mixed with increasing amounts of various unlabeled test DNA fragments. The quantity of test DNA required in the reaction to decrease the amount of radioactive marker DNA bound to protein (shifted species) by a defined fraction (50%) is taken to be indicative of the relative affinity of OTF-1 for the test DNA.
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26. Unlabeled competitor DNA fragments were purified by gel electrophoresis. Various quantities of competitor DNA fragment were mixed with 5×10^{-15} mol of labeled marker fragment. The DNA was then mixed with 1.1 Ad Ori–specific DNA binding units of OTF-1, and incubated for 30 min. After electrophoresis of the reaction mix, the gel was dried and autoradiographed. The bands corresponding to bound and unbound DNA were excised from the gel and quantitated by liquid scintillation counting. The amount of radioactive DNA bound by OTF-1 in the absence of competitor DNA was determined, and the fraction of this DNA bound in the presence of competitor DNA was calculated.
27. The pUpm 46 contains the Ad Ori with an A to T transversion at base 46 in the NF-III binding site (C. R. Burrow and T. J. Kelly, unpublished construction). The poMA contains a double base substitution within the octamer consensus sequence, generating the novel sequence ATTCAACT (*15*).
28. The standard in vitro initiation reaction was performed as described (*10*). All reaction mixtures were adjusted to constant concentrations of NaCl (20 m*M*), glycerol (10%), Hepes (*p*H 7.5, 30 m*M*), and other buffer constituents. Reconstitution of the replication of DNA containing the cloned Ad Ori was performed by previously described techniques (*26*), with the following modifications. Each reaction contained 60 ng of plasmid DNA restricted with Eco RI and Ava II. All reactions were adjusted to constant concentrations of NaCl (20 m*M*), glycerol (8%), Hepes (*p*H 7.5, 40 m*M*), and other buffer constituents. The reactions were carried out at 37°C for 60 min. The largest restriction fragment (approximately 1450 bp) contains the adenovirus terminus positioned at one end.
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Mitogen-Induced Replication of Woodchuck Hepatitis Virus in Cultured Peripheral Blood Lymphocytes

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Peripheral blood lymphocytes (PBLs) isolated from woodchucks chronically infected with the woodchuck hepatitis virus (WHV) carry low levels of nonreplicating WHV DNA. When PBLs from chronic carrier woodchucks were activated in culture with the generalized mitogen lipopolysaccharide (LPS), WHV DNA replication was initiated in cells obtained from one of three animals examined. Intracellular WHV core particles, containing WHV DNA replication intermediates, RNA/DNA hybrid molecules, and an active endogenous DNA polymerase, appeared 3 days after the start of LPS stimulation. After 5 to 7 days of LPS stimulation, WHV DNA–containing particles, which displayed the properties of intact, mature virions, were released into the culture medium. These studies provide evidence for reactivation of a latent WHV infection of circulating lymphoid cells and indicate that the presence of nonreplicating hepadnaviral DNA in lymphoid cells represents a potentially active infection following cellular activation.

A NUMBER OF ACUTE AND CHRONIC viral infections involve a lymphatic phase with early replication at the site of entry and in regional lymph nodes followed by spread to nonlymphatic tissue, where the primary disease is manifested (*1*). In two well-studied chronic infections, measles virus in subacute sclerosing panencephalitis (SSPE) (*2*, *3*) and human immunodeficiency virus (HIV) in acquired immunodeficiency syndrome (AIDS) (*4*, *5*), recovery of virus in culture requires cellular activation. Thus, the lymphoid system may serve as a site of viral amplification and as a reservoir of latent virus.

Hepatitis B virus (HBV) causes acute and chronic liver disease and has been epidemiologically linked to primary hepatocellular carcinoma (HCC) (*6*). Recent studies on HBV and other members of Hepadnaviridae have demonstrated viral infection of lymhoid cells and other extrahepatic tissues, although the role of such infections in the pathogenesis of virus-induced disease is poorly understood (*7*, *8*). WHV and its natural host, the eastern woodchuck (*Marmota monax*), constitute the relevant animal model system for the study of HBV-induced disease, especially HCC and lymphatic infections (*7–10*).

Active replication of HBV, WHV, and duck hepatitis B virus (DHBV) DNA occurs in the spleen and in the liver (*8*, *11–13*). However, hepadnavirus genomes in PBLs are usually found in a nonreplicating state (*7*, *8*, *11*, *13*). Such observations could indicate that the viral genomes in these cells are no longer capable of sustaining a complete virus life cycle. Alternatively, these viral genomes could be replication competent, but the host cell environments may not be compatible with viral DNA replication.

To determine if the WHV genomes in PBLs were replication competent in an activated host cell environment, we stimulated PBLs from three chronically infected woodchucks, in culture, with LPS (Sigma), concanavalin A (Con A) (Difco), phytohemagglutinin (PHA) (Difco), or human recombinant interleukin-2 (IL-2) (Amgen). All four mitogens induced significant blast formation (as determined by microscopic examination) and variable levels of thymidine incorporation into cellular DNA (Table 1) in all the PBL preparations examined. The levels of incorporated exogenous thymidine were relatively low when compared to other cell systems (*14*), although these levels were significantly above the incorporation observed in unstimulated cells (Table 1). Maximal thymidine incorporation was observed during the first 24 hours of culture in the presence of LPS and at day 3 of culture for the other mitogens. Different PBL preparations varied considerably in their mitogen response; no consistent differences in the overall patterns of thymidine incorporation were observed between PBLs from an uninfected woodchuck (WC178) and PBLs from the chronically infected animals (WC139, WC192, WC195) (Table 1).

LPS maintained the viability of lymphoid cells in culture for several days (*15*). LPS treatment of PBLs from WC192 stimulated cell division, as evidenced by a twofold increase in total cell number over a 7-day period. Because the mitogenic activity of LPS is limited almost exclusively to B cells (*16*), a minor component of the total PBL population, the observed increase in total cell number is significant. The number of viable cells (as determined by the exclusion

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of trypan blue dye) was maintained at a high level (70 to 80%) for at least 7 days in the LPS-stimulated cultures. Viable cells comprised less than 30% of the unstimulated cell population after 5 days.

PBLs from each of three chronically infected woodchucks used in this study carried low levels of WHV DNA (0.1 to 0.5 genomic equivalent per cell) (*7*, *8*, *13*). Viral DNA in these PBLs was episomal, existing primarily as multimers of 7 to 12 kb in size (see Fig. 1a, lane US, day 0). Some monomeric episomal genomes (3.3 kb) were also occasionally observed. Three days after continuous stimulation of WC192 PBLs by LPS, electrophoretic DNA patterns representative of WHV DNA replication intermediates (*17 18*) were observed (Fig. 1a, LPS, day 3). The level of these intermediates remained the same until day 7 of culture and then disappeared during the next 4 days. This agrees with the loss of viable cells observed after 7 days in culture with LPS. No changes in the WHV DNA patterns were observed in unstimulated cells maintained in culture for up to 9 days. However, some low molecular weight DNA species, migrating as expected for single-stranded, minus-strand WHV DNA (*17*), were occasionally observed (Fig. 1). Stimulation of PBLs from WC192 with PHA, Con A, or IL-2 did not induce WHV DNA replication.

Stimulation by LPS, Con A, IL-2, or PHA of PBLs from WC139 or WC195 did not induce WHV DNA replication under comparable culture conditions. It is not known if the differential response of WHV in PBLs to LPS stimulation from these animals reflects a lessened response to LPS (Table 1), an inability of the WHV genomes to carry out a replication cycle, or some difference in the stage of the chronic infection in these two woodchucks. The failure of PHA, Con A, or IL-2 to induce WHV replication in WC192 PBLs may be related to an inability to induce critical host factors. Whether WHV replication occurs within the dividing lymphoid cells in direct response to LPS or takes place in primed or nondividing cells in response to soluble factors is unknown.

WHV DNA levels in LPS-stimulated PBLs from WC192 increased (relative to the levels of co-electrophoresed WHV DNA standards and the amount of cellular DNA loaded in each lane) approximately 10- to 100-fold, to about ten copies per cell. It is likely that the replicating WHV DNA in cultured WC192 PBLs was concentrated in only a few cells as has been demonstrated for WHV DNA in the total PBL population in chronically infected woodchucks (*13*). WHV-specific RNA was also found in LPS-stimulated WC192 PBLs (Fig. 1b). The primary transcripts expected for WHV [approximately 2.3 and 3.6 kb (*8*, *19*)] were present, as were other, uncharacterized, WHV-specific RNA species. Overall, the appearance and relative levels of the WHV-specific RNA transcripts were correlated with the changes in WHV DNA. As observed for the WHV DNA replication intermediates, the WHV-specific RNA transcripts disappeared rapidly as the number of viable cells declined in prolonged culture (Fig. 1).

WHV DNA replication proceeds by reverse transcription of a 3.6-kb RNA pregenome within immature, intracellular core particles (*17*, *18*). WC192 PBLs stimulated with LPS in culture for 3 to 7 days were disrupted in the presence of 0.2% NP-40, and the lysates were analyzed by density gradient centrifugation in CsCl (*8 17*, *20*) (Fig. 2). WHV DNA replication intermediates were present in particles with a buoyant density of 1.30 to 1.34 g/cm^3 (Fig. 2c), the density expected for intracellular core particles (*21*). When these particles were utilized in an endogenous DNA polymerase reaction (*8*, *17*, *20*), Cs_2SO_4 density gradient analysis demonstrated the presence of RNA/DNA hybrid molecules (*15*). These results are consistent with the production of hepadnavirus DNA from an RNA template within core particles in lymphoid cells.

To look for WHV DNA–containing particles, we pooled culture media from two separate PBL cultures and subjected them to centrifugation over a 43% sucrose cushion (*8*, *17*, *20*). Nucleic acids were extracted from the pelleted material and examined by DNA hybridization analysis (Fig. 1c). Media pooled from LPS-stimulated WC192 PBLs (5 to 9 days of culture) contained approximately 1×10^4 WHV DNA–containing particles per milliliter (lane C). There was not enough virus for antigen analysis or electron microscopy. No WHV DNA–containing particles were detected in culture media obtained from unstimulated WC192 PBLs cultured for 5 to 9 days or from LPS-stimulated WC192 PBLs cultured for 1 to 3 days (lanes A and B). These results are consistent with those observed for in vitro DHBV infection of primary duck hepatocytes, in which release of infectious DHBV particles occurs several days after the appearance of DHBV DNA replication intermediates (*22*, *23*).

The WHV-containing particles in the

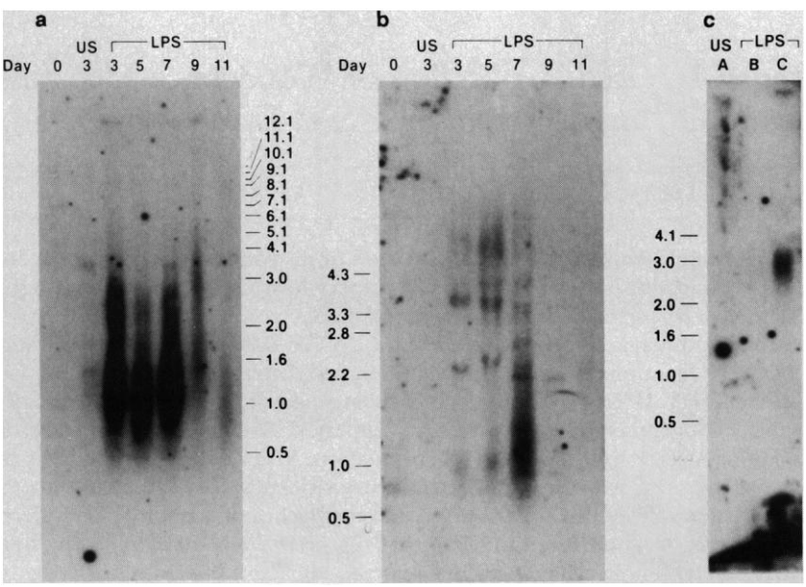

Fig. 1. Examples of (**a**) DNA and (**b**) RNA blot hybridization analysis of WHV nucleic acids in cultured WC192 PBLs. Nucleic acids were extracted from cells and pelleted media particles (**c**) and analyzed by DNA blot hybridization as described (*7*, *8*, *13*). Sizes (kilobases) in (a) and (c) are based on co-electrophoresis of 1-kb ladder DNA standards (BRL, Gaithersburg, Maryland) and in (b) on co-electrophoresis of denatured, WHV DNA standards (*7*). Each lane of (a) and (b) corresponds to the same nucleic acid preparation from a single population of cells. The ^{32}P-labeled hybridization probe used was a 3.3-kb Bam HI WHV DNA fragment purified from a representative DNA clone (WHV *7*) of a standard virus infection pool, which was subsequently sequenced (*27*). Day 0, WC192 PBLs before culture; days in culture in the absence (US) or presence (LPS) of LPS (10 μg/ml) are shown. Amounts of total cell nucleic acids used in each lane of (a) and (b) were: day 0 and day 3 (US), 10 μg of DNA, 3 μg of RNA; all LPS lanes, 30 μg of DNA, 10 μg of RNA. In (c), nucleic acids extracted from material pelleted from pooled culture media were analyzed by DNA blot hybridization: lane A, 9 ml of pelleted culture media from WC192 PBLs maintained for 3 to 7 days without LPS; lanes B and C, 9 ml of pelleted culture media from WC192 PBLs maintained in culture with LPS (10 μg/ml) for 3 days (lane B) or 5 to 9 days (lane C). All panels represent 7-day film exposures.

Table 1. Mitogen-induced incorporation of exogenous thymidine into PBL DNA in culture. Woodchuck PBLs were isolated by centrifugation over Ficoll as described in (*7, 8*). PBLs were cultured in 96-well, U-bottomed plates at 2×10^5 to 4×10^5 cells per well (1×10^6 to 2×10^6 cells per milliliter). Culture medium was changed at days 3, 5, 7, and 9 of culture. Growth medium used was Dulbecco's minimum essential medium containing 20% heat-inactivated fetal bovine serum (Biofluids, Rockville, Maryland), 5×10^{-5} *M* 2-mercaptoethanol, 2 m*M* glutamine, and gentamycin sulfate (50 μg/ml). Medium was filtered through a 0.22 μ*M* Nalgene cellulose acetate membrane before use. Mitogens were added at the initiation of the cell cultures and maintained throughout the experiments. Incorporation of [^{3}H]thymidine (1 μCi/ml; specific activity, 6.7 Ci/mmol, New England Nuclear) was performed as described in (*14*). Exogenous [^{3}H]thymidine was added 24 hours before harvesting on day 3 after the initiation of the cultures. For mitogen-treated cells, data represent $\bar{x} \pm$ SEM from five separate wells and unstimulated control values represent $\bar{x} \pm$ SEM from ten separate wells in each experiment. Thymidine incorporation levels for Con A were only determined for WC178 PBLs and were 1322 ± 205 cpm on day 2 of culture (unstimulated cell values were 207 ± 50 cpm). The mitogen concentrations used were determined to be optimal (by thymidine incorporation levels) on the basis of empirical testing of several mitogen concentrations. Mitogen concentrations used were: LPS (10 μg/ml), PHA (10 μg/ml), IL-2 (250 IU/ml), and Con A (20 μg/ml). Woodchucks used in these experiments were caught in the wild (Cocaleco, Reamstown, Pennsylvania) and had preexisting, chronic WHV infections (WC192, WC195), had a chronic infection experimentally induced (WC139), or were uninfected (WC178) (Cocaleco). Chronic WHV infections were determined by the persistence (for at least 2 years) of specific viral serologic markers (WHV surface antigen, antibody to WHV core antigen, WHV DNA) (*7, 25, 26*).

Animal number	Thymidine incorporation (cpm)			
	LPS	PHA	IL-2	Unstimulated
WC178	496 ± 76	530 ± 91	1144 ± 130	223 ± 56
WC192	1203 ±195	551 ± 89	1144 ± 130	208 ± 57
WC195	538 ± 70	364 ± 65	417 ± 58	161 ± 26
WC139	655 ±129	462 ± 82	377 ± 49	185 ± 49

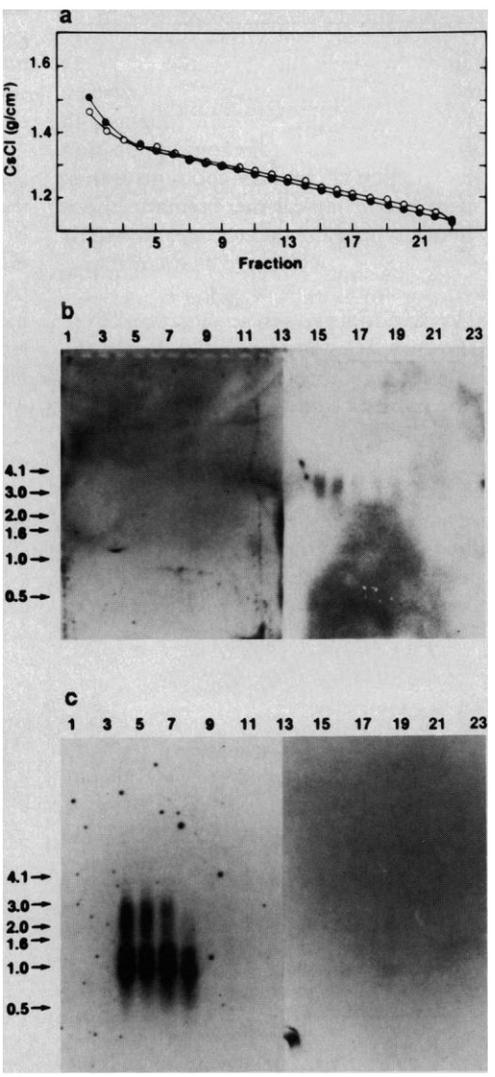

Fig. 2. Density-gradient analysis of intracellular and extracellular WHV particles in CsCl. WC192 PBLs were maintained in culture with LPS as described in the legend to Table 1. Cells were lysed, viral particles were concentrated by centrifugation over a 43% sucrose cushion, and particles were subjected to density-gradient centrifugation in CsCl as described (*8, 17, 20*). (**a**) The CsCl density profiles of the intracelllular (solid line) and extracellular (dashed line) particle gradients. Extracellular particles (**b**) were isolated from 11 ml of pooled culture media from WC192 PBLs maintained in culture with LPS for 5 to 9 days (see legend to Fig. 1). Intracellular particles (**c**) were isolated from 5×10^7 WC192 PBLs maintained in culture with LPS for 80 hours.

PBL culture media were most likely intact, mature virions. The partially double- and single-stranded, circular DNA molecules are common to all hepadnaviruses (*19*); a completely double-stranded WHV DNA genome would migrate as a discrete, sharp band (*13*). The WHV DNA–containing particles in the culture media had a buoyant density of 1.21 to 1.26 g/cm^3 in CsCl (Fig. 2b), the buoyant density expected for complete WHV virions (*21*). In addition, no WHV DNA replication intermediates were observed in the culture media (Fig. 1c), indicating little or no release of immature WHV core particles due to cell export or lysis [these particles would also pellet under the centrifugation conditions used (*8, 17, 20*)]. LPS induction of WHV replication does not appear to be a phenomenon unique to WC192 PBLs. Preliminary experiments have indicated that LPS was capable of inducing WHV replication in cultured PBLs obtained from two of six additional chronic carrier woodchucks.

This study provides direct evidence for reactivation of a latent WHV infection of circulating lymphoid cells and indicates that the presence of hepadnaviral DNA in lymphoid cells represents a potentially active infection and alternative source of infectious virus other than the liver. WHV has been shown to be a model system for many aspects of HBV infection of humans (*7–10*). Although the presence of HBV DNA in lymphoid cells has been observed, mitogenic activation of HBV replication in circulating lymphocytes has not yet been reported. Lymphatic sources of virus may be involved in viral transmission or in the reactivation of viral infection and exacerbation of disease in long-term asymptomatic HBV carriers (*24*). Because the WHV genomes in PBLs are initially in a quiescent state, this system, like the in vitro DHBV infection of duck hepatocytes (*22, 23*), should be useful in the analysis of the early events in the initiation of hepadnaviral DNA replication.

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Gibberellins: A Phytohormonal Basis for Heterosis in Maize

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Four commercially important maize parental inbreds and their 12 F_1 hybrids were studied to investigate the role of the phytohormone gibberellin (GA) in the regulation of heterosis (hybrid vigor). All hybrids grew faster than any inbred. In contrast, all inbreds showed a greater promotion of shoot growth after the exogenous application of GA_3. Concentrations of endogenous GA_1, the biological effector for shoot growth in maize, and GA_{19}, a precursor of GA_1, were measured in apical meristematic shoot cylinders for three of the inbreds and their hybrids by gas chromatography–mass spectrometry with selected ion monitoring; deuterated GAs were used as quantitative internal standards. In 34 of 36 comparisons, hybrids contained higher concentrations of endogenous GAs than their parental inbreds. Preferential growth acceleration of the inbreds by exogenous GA_3 indicates that a deficiency of endogenous GA limits the growth of the inbreds and is thus a cause of inbreeding depression. Conversely, the increased endogenous concentration of GA in the hybrids could provide a phytohormonal basis for heterosis for shoot growth.

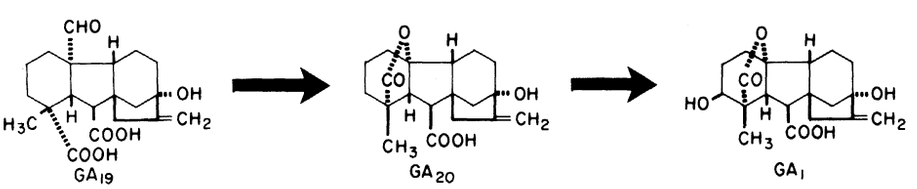
IN ANIMALS AND PARTICULARLY IN plants, it is commonly observed that hybrids—that is, genotypes resulting from the crossing of dissimilar parents—outperform either of their parental genotypes. This phenomenon is referred to as heterosis, or hybrid vigor, and underlies much of the improvement in crop yields achieved in the 20th century. Although heterosis is economically important for agriculture, horticulture, and silviculture, its physiological basis is unclear.

At least three lines of evidence support a role for endogenous gibberellins (GAs) in the regulation of heterosis in maize: (i) Inbreds, homozygous lines resulting from repeated self-pollinations, are particularly responsive to the exogenous application of GA_3 (*1, 2*). GA_3 can accelerate shoot growth in many maize genotypes, but inbreds are most responsive. The correlation between inbreeding and the responsiveness to exogenous GA_3 suggests that the growth of inbreds is limited by a deficiency of endogenous GAs. (ii) The endogenous GA-like substances have been quantified by bioassay from two inbreds and their heterotic hybrid (*3*), and significantly higher concentrations of GA-like activity were observed in the hybrid over a sequence of harvests (*3*). (iii) Heterosis for early seedling growth of maize is frequently observed (*4, 5*), and the rapid production of hydrolytic enzymes such as α-amylase and protease is essential to provide respiratory substrates for this heterosis of early seedling growth (*4, 6*). GAs participate in regulating the levels of hydrolytic enzymes, in germinating cereal seeds and young seedlings (*7*), and this argument for the relation between GAs, hydrolytic enzymes, and heterosis for seedling growth was independently proposed by Paleg (*8*) and by Sarkissian *et al.* (*4*).

A definitive experiment to evaluate the role of GAs in regulating heterosis in the growth of the maize shoot must include more than a single hybrid and its parents, and the assessment of endogenous GA concentration must be unequivocal.

Although maize shoots contain at least eight endogenous GAs (*9, 10*), only GA_1 is the biologically active "effector" in the regulation of maize shoot elongation (*10*). Other GAs serve as precursors to GA_1 (for example, $GA_{19} \rightarrow GA_{20} \rightarrow GA_1$) (Fig. 1), are metabolites of GA_1, or are the results of branch points in the GA metabolic pathway (*10*). Thus, the analysis of GA_1 is particularly relevant.

In the present study, we crossed four maize inbreds in diallel combinations to obtain a matrix of all possible single-cross hybrids and their inbred parents (Table 1). The selected inbreds represent some of the economically most important maize inbreds available; the inbreds, A632, B73, and



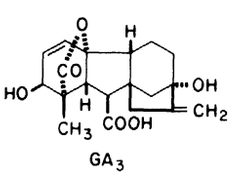


Fig. 1. Chemical structures and metabolic relationships of the GAs included in the present study. GA_3 is a commercially available analog of GA_1, which is the biological effector for shoot growth in maize (*10*).

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