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16. Stromal extract (260 ml, 1.3 g of protein) was fractionated by ammonium sulfate precipitation (20 to 40% saturation). Precipitated proteins were dissolved in 120 ml of buffer A [25 mM Hepes-KOH (pH 8), 50 mM KCl, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride] and applied to a 90-ml DEAE-Sepharose column. The column was washed with 200 ml of buffer A and eluted with a 50 to 350 mM KCl gradient (500 ml). CpSecA-containing fractions at ~170 mM KCl were mixed with an equal volume of buffer A and applied to a 45-ml hydroxylapatite column. After washing with 100 ml of 10 mM potassium phosphate (pH 7) and 100 ml of 1 M KCl (unbuffered), the column was eluted with a 10 to 300 mM potassium phosphate gradient (300 ml). CPSecA-containing fractions at ~150 mM potassium phosphate were pooled and concentrated to 1 ml on a Centriprep-10 (Amicon) and then applied to an 80-ml Sephacryl S-300 gel filtration column and eluted with 20 mM Hepes-KOH (pH 8), 65 mM KCl, 1 mM dithiothreitol, 1% ethylene glycol. Fractions with CPSecA were applied to a 1-ml Mono Q column. After washing with 10 ml of 20 mM Hepes-KOH (pH 8), 50 mM KCl, the column was eluted with 30 ml of a 50 to 300 mM KCl gradient. CPSecA-containing fractions (~225 mM KCl) were adjusted to 50 mM potassium phosphate (pH 7), 1.5 M ammonium sulfate and applied to a 7.85-ml polyethylene glycol Hydropore-HIC column (Rainin). After washing with 20 ml of the same buffer, the column was eluted with 50 ml of 1.5 to 0 M ammonium sulfate in a descending gradient. CPSecA eluted at ~0.6 M ammonium sulfate.
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25. Radiolabeled precursors were prepared by in vitro transcription and translation (*10*). Intact chloroplasts were isolated from 9- to 10-day-old pea (Laxton's Progress 9) seedlings (*24*). Chloroplast lysate, thylakoids, and SE were prepared as described (*13*). Assays for import into chloroplasts or for transport or integration into thylakoids were carried out for 10 min and 30 min, respectively, in white light and 4 mM MgATP as described (*10*, *13*). Import assays were terminated with $HgCl_2$ [J. E. Reed *et al.*, *Eur. J. Biochem.* **194**, 33 (1990)]. Sodium azide was added to chloroplasts and lysates from a stock of 0.6 M in import buffer, and the mixture was incubated for 10 min at 25°C in light (70 $\mu Em^{-2} s^{-1}$) before the addition of precursor. Samples were analyzed by SDS–polyacrylamide gel electrophoresis–fluorography and quantification carried out by extraction of radiolabeled proteins from gel slices and scintillation counting (*24*). Chlorophyll concentrations were determined according to D. I. Arnon [*Plant Physiol.* **24**, 1 (1949)].
26. A highly conserved region was identified by comparison of *secA* sequences from *P. lutherii*, *Antithamnion spp*, *E. coli*, and *B. subtilis*. This region of the *P. lutherii* chloroplast *secA* gene, from base pair 1043 to 1458, was amplified by polymerase chain reaction with primer 5′-GCTCCACCATATGAAAATCGCCGAGATGAAGACAGG-3′ containing an in-frame Nde I site and reverse primer 5′- GGAATGTTTCAAGCTTTCGGGAGATTATTAGTGG-3′ containing a Hind III site with pMAQ805 (*7*) as template and was cloned into an appropriately digested pET24b (Novagen) to allow in-frame fusion with the His-6 tag. The resulting clone was introduced into BL21(λDE3) and the protein expressed and purified from inclusion bodies as described (*10*). Antibody to the SDS-denatured peptide was prepared in rabbits by Cocalico Biologicals (Reamstown, PA). Protein assays were performed with bovine serum albumin as standard as described [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. Immunoblotting was conducted as described [L. Payan and K. Cline, *J. Cell Biol.* **112**, 603 (1991)].
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Experimental Evidence That Competition Promotes Divergence in Adaptive Radiation

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Interspecific competition driving divergence in adaptive radiation has not previously been tested experimentally. Natural selection on a morphologically variable species of stickleback fish was contrasted in the presence and absence of a close relative. Selection was nondirectional when the target species was alone, whereas addition of the second species favored individuals most different from it morphologically and ecologically. Disproportionately severe competition between similar phenotypes indicates frequency-dependent selection, verifying a crucial element of theory of competition and character divergence. The findings help resolve outstanding debates on the ecological causes of diversification and the evolutionary consequences of competitive interactions.

The ecological causes of adaptive radiation are poorly understood. Especially contentious is the issue of whether rates and patterns of speciation and morphological divergence have been greatly affected by resource competition between species (*1*). This debate mirrors a long-standing issue in ecological research: whether differences between coexisting species are commonly the outcome of ecological character displacement (evolutionary change resulting from interspecific competition) (*2*–*4*). Conflicting views have been difficult to resolve because evidence is scarce and entirely correlative. I addressed the problem experimentally by measuring the strength of divergent natural selection between closely related, morphologically similar species.

Threespine sticklebacks (*Gasterosteus aculeatus* complex) inhabiting small lakes of coastal British Columbia, Canada, were used for the study. The collection of species diversified very recently, mainly at the end of the Pleistocene (≤13,000 years ago) (*4*, *5*). Earlier work suggested that coexisting pairs of species were character-displaced (*4*): One species (the "benthic") feeds on benthic invertebrates in the littoral zone and is large and deep-bodied with few, short gill rakers and a wide gape; the other species (the "limnetic") feeds on plankton, is small and slender, and has long, numerous gill rakers and a narrow gape. Species occurring alone in lakes are intermediate in body form and exploit both benthic and plankton habitats. These morphological differences are strongly associated with feeding efficiency and growth rate in the two habitats (*6*, *7*). The pattern is replicated over several watersheds and is a general characteristic of radiations of fish taxa that inhabit low-diversity post-Pleistocene lakes (*8*).

The experiment was carried out in summer 1993 in two divided 23 m by 23 m ponds on the University of British Columbia campus (*9*). The solitary species from Cranby Lake, Texada Island, was the target of the experiment; it is morphologically intermediate between benthic and limnetic species (*4*). The limnetic species from nearby Paxton Lake was the potential competitor. This species is morphologically and ecologically most similar to one extreme of the range of phenotypes in the Cranby species (*4*) (Table 1). The goal of the experiment was to test the prediction from theory that individuals at this extreme should suffer disproportionately when the limnetic species is added, generating natural selec-

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tion in favor of phenotypes at the opposite extreme (*3*, *10*). The combination of morphological forms used in this experiment (that is, limnetic plus intermediate) recreates those thought to have been initially present 10,000 to 13,000 years ago in lakes colonized twice by ancestral forms (*4*, *5*).

The study took place within a single generation. Selection was assessed by comparing growth and survival of different Cranby phenotypes in the presence and absence of the limnetic species. Growth rate in fish is highly correlated with food intake (*7*) and is closely linked to fitness through its effects on overwinter size and survival (*11*), time of breeding (*12*), and fecundity (*7*, *13*). The most critical comparison was that between extreme phenotypes of the Cranby population; yet individuals at these extremes are inevitably rare in nature. I used interspecific hybridization to increase the frequency of these individuals and, therefore, the sensitivity of the test. Hybridization is a valid manipulation because all previous crosses between closely related freshwater sticklebacks have not revealed any intrinsic reduction in offspring viability (*14*). F_1 hybrids were raised from artificial crosses between the Cranby species and the Paxton benthic species (C × B), and between the Cranby and the Paxton limnetic species (C × L). The target experimental population was a mixture of equal numbers of C × B, C × L, and offspring from crosses between Cranby individuals (C × C).

A potential problem when comparing fitness of phenotypes between treatments was that growth and development of several trophic traits are affected by diet (*15*). For this reason I instead used two hard parts of the anatomy, gill raker number and body armor, as markers for trophic phenotype. Growth of these traits is unaffected by diet (*15*). Each Cranby cross is distinct from the other two in these markers, and a combination of markers yields an index that substitutes for underlying differences in trophic morphology (Table 1). All analyses were carried out on this index. This step is conservative, because the patterns reported were stronger when plastic traits such as gape width were used directly.

A paired design was used in which 1800 individuals of the Cranby experimental population were introduced to both sides of each pond. Then 1200 limnetic individuals were added to one randomly chosen side of each pond (*16*). Addition of limnetic fish to one side caused the treatments to differ in the total density of fish and in the combined frequencies of different phenotypes present. However, theory is explicit about which phenotypes in the target species should be most affected by the addition. If the prediction is upheld, then it may be concluded that selection was frequency-dependent as required by theory (*3*, *10*).

The experiment was run for 3 months (*17*). All remaining fish were removed, stained, and preserved. A random sample of 60 to 70 Cranby individuals was taken from each half pond for measurement. Because fish were introduced at a very small size, growth rate (millimeters per 90 days) was calculated as 90 × (final body length)/(duration of experiment).

Mean growth rate in the target population inversely matched total fish density within and between ponds (Table 2), indicating that competition was density-dependent [one-tailed F test on independent contrasts (*18*), $r = 0.95$, $F(1,2) = 17.5$, $P = 0.026$]. Different Cranby phenotypes used different pond resources: Individuals most like the limnetic species in morphology were also most similar to it ecologically. This pattern further justifies the prediction that phenotypes most like the limnetic species should be most greatly affected by its addition.

As predicted, the presence of the limnetic species altered natural selection in the target species (Table 3 and Fig. 1). Different

Table 1. Mean attributes of populations and species. Measurements are means from 120 laboratory-raised fish (±SE). Offspring from the three crosses C × B, C × C, and C × L make up the experimental Cranby population. Trophic morphology is a composite shape variable (first principal component) based on size-adjusted ln-transformed measurements of body length, body depth, gape width, and number and length of gill rakers, calculated as described in (*4*). Fish having a negative value are relatively deep-bodied with a wide gape and few, short rakers; fish having a positive value are more slender and narrow-gaped with long, numerous rakers. The gill raker number is counted on the long arm of the first gill arch. The armor is a composite variable (first principal component calculated from the correlation matrix) of the number of plates and the size-adjusted ln-transformed lengths of the pectoral spines and pelvic girdle. Traits were size-adjusted with residuals of regressions of each trait on standard length. All the armor traits contributed positively and approximately equally to the combined variable. The morphological index is a linear combination (first principal component) of armor and gill raker number. Negative values indicate fish with low armor and few gill rakers; positive values indicate high armor and numerous gill rakers.

Trait	Cranby			Paxton limnetic
	C × B	C × C	C × L	
Trophic morphology	−1.02 (0.50)	0.00 (0.52)	0.53 (0.46)	1.04 (0.40)
Gill raker number	13.8 (0.8)	13.8 (0.6)	15.3 (0.9)	17.0 (0.9)
Armor	−1.66 (0.75)	0.27 (0.50)	0.27 (0.49)	0.49 (0.49)
Morphological index	−0.97 (0.75)	0.00 (0.37)	0.75 (0.73)	1.79 (0.48)

Table 2. Pond conditions at the end of the experiment. The pond was treated as a fixed effect in the statistical tests, and degrees of freedom reflect the number of fish sampled rather than the number of ponds. This was done to describe conditions in the individual ponds, not to test experimental effects (which is carried out in the text). The number of fish is the total number surviving to the end of the experiment. Initial numbers of fish were 3000 on the experimental side, of which 40% were limnetics (1200) and 1800 were on the control side (all Cranby). The mean growth rate (±SE) is based on 60 to 70 randomly sampled Cranby individuals per half pond. Two-way analysis of variance (ANOVA) revealed that overall mean growth rate (ln-transformed) differed between treatments [$F(1,260) = 8.37$, $P = 0.004$] but not between ponds [$F(1,260) = 0.28$, $P = 0.602$]. The magnitude of the difference between treatment sides also varied between ponds [interaction $F(1,260) = 13.92$, $P < 0.001$]. Correcting for three simultaneous tests with the sequential Bonferroni method (*21*) did not alter these conclusions. The diet fraction is the number of cladocerans and copepods in the stomach as a proportion of all prey items (remaining items were predominantly benthic ostracods and amphipods). This fraction was measured on 10 to 15 individuals sampled from the lower third of the frequency distribution of phenotypes, and on 10 to 15 individuals from the upper third (presumably mainly C × B and C × L crosses, respectively). Means are averages of individual fractions. The diet fraction in limnetic fish was 0.99 in both ponds ($n = 30$). Three-way ANOVA showed that diet fraction differed between the two morphological extremes of the Cranby population [$F(1,101) = 23.8$, $P < 0.001$], between treatments [$F(1,101) = 3.06$, $P = 0.041$], and between ponds [$F(1,101) = 24.0$, $P < 0.001$]. The data suggest that a diet shift between treatment and control sides was limited to pond 2, but the interaction between pond and treatment was not significant, after correcting for four simultaneous tests (*21*).

Treatment	Number of fish	Percent limnetics	Cranby mean growth rate (mm/90 days)	Diet fraction (C × L, C × B)
		Pond 1		
Experiment	1058	33	48.0 (0.7)	0.89, 0.68
Control	751		48.4 (0.7)	0.95, 0.63
		Pond 2		
Experiment	1136	40	46.2 (0.8)	0.55, 0.24
Control	403		50.7 (0.8)	0.71, 0.58

Cranby phenotypes grew at similar rates on the control sides of the ponds. This is in contrast to the treatment sides, where Cranby individuals closest to the limnetic species in morphology suffered a substantial growth depression (one-tailed paired t test on regression slopes; $df = 1$, $P = 0.016$). This effect on the Cranby species diminished gradually (rather than suddenly) with increased morphological distance away from the limnetic competitor. Intensity of directional selection away from the limnetic species was correlated with final limnetic density [one-tailed test of independent contrasts, $r = 0.96$, $F(1,2) = 25.6$, $P = 0.018$]. Individuals morphologically closest to the limnetic species also tended to have reduced survival in one of two ponds (Table 3), but no overall experimental effect was identified (one-tailed paired t test on survival differentials; $df = 1$, $P = 0.31$).

Table 3. Experimental results. The growth differential is calculated from the slopes of linear regressions of ln[growth rate] on the morphological index. Significance levels refer to tests of the null hypothesis that the slope is zero. No significant curvilinearity was detected with quadratic regression. Estimates were similar when robust methods less sensitive to outliers were used instead (*22*). Significance levels were confirmed with the unsmoothed bootstrap method recommended in (*23*). Only the growth differential in pond 2, experimental side, is significant after correcting for four tests of slope with the sequential Bonferroni method (*21*). The survival differential is the difference between treatments (experiment minus control) in mean value of the morphological index at the end of the experiment. Significance levels are from two-sample t tests between pond sides, with individual fish as replicates (see Table 2 for justification).

Pond	Growth differential		Survival differential
	Experiment	Control	
1	−0.025**	−0.004	0.03
2	−0.050***	−0.006	−0.18*

*$P \leq 0.10$. **$P \leq 0.05$. ***$P \leq 0.01$.

These findings constitute experimental evidence that resource competition promotes morphological diversification in a radiating lineage. They support the view developed from ecological, genetic, and biogeographic data (that is, indirect evidence) that competition played a large role in the rapid diversification of sticklebacks (*4*–*8*). The current morphological differences between sympatric species are consistent with the selection intensities recorded herein and with the presumed duration of sympatry: If total fitness is proportional to growth rate, then a persistent ln[growth] differential of −0.025 (Table 3) is sufficiently strong to produce the observed difference between limnetic and benthic species in about 500 years (or generations) (*19*). Detection of frequency-dependent selection on trophic traits also confirms a crucial element of mathematical theory of competition and character divergence (*3*, *10*). Such selection also provides a simple mechanism for adaptive peak shifts and has been implicated in the process of speciation itself (*20*). Finally, the results emphasize the experimental advantages of studying adaptive radiations in their early stages, when species diversities are low and intermediate stages can be re-created.

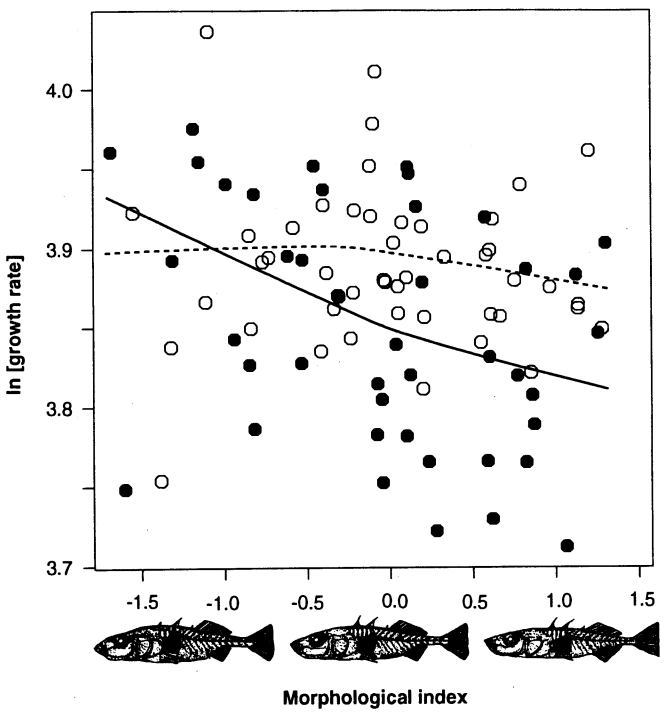

Fig. 1. Growth rate of Cranby phenotypes in the presence (filled symbols, solid line) and absence (open symbols, dotted line) of the limnetic species. The morphological index reflects the gradation of forms within the experimental population, from more benthic on the left (deep body, wide gape, and short gill rakers) to more limnetic on the right (slender body, narrow gape, and long gill rakers). Drawings slightly exaggerate differences in profile among the three cross types (from left to right: C × B, C × C, and C × L). Original growth measurements are millimeters per 90 days. Data from both ponds are combined; growth rates within each treatment were pooled to the same mean. Each curve is a cubic spline (*24*), a nonparametric regression function that may assume any shape the data warrant. For clarity, each symbol is an average of three adjacent points.

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9. Ponds had a sandy bottom sloping gradually (3:1) to a maximum depth of 3 m. They were constructed in 1991, seeded with plants and invertebrates from Paxton Lake on Texada Island, and left standing for 2 years. Sampling indicated that benthic invertebrates and plankton were abundant before fish introduction. Most natural predators were also present (insects and piscivorous birds, but not predatory fish). Each pond was divided into two equal sections with a plastic sheet immediately before the experiment. A 2 m by 2 m mesh window (100 μm) allowed exchange of water but not prey between sides.
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16. All fish were raised from eggs in the laboratory and introduced into ponds at 2 to 3 weeks of age. Densities were set such that growth rate of pond fish would equal that in the wild, as judged from an earlier pilot experiment, and allowing for mortality of 50 to 70% of individuals soon after introduction (D. Schluter, unpublished observations).
17. The experiment was run from the beginning of June to September (pond 1) and the beginning of August to October (pond 2). Laboratory constraints prevented me from simultaneously raising large numbers of fish for two replicates. Fish were collected at the end of the experiment with rotenone.
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Hydroxyurea as an Inhibitor of Human Immunodeficiency Virus–Type 1 Replication

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Hydroxyurea, a drug widely used in therapy of several human diseases, inhibits deoxynucleotide synthesis—and, consequently, DNA synthesis—by blocking the cellular enzyme ribonucleotide reductase. Hydroxyurea inhibits human immunodeficiency virus–type 1 (HIV-1) DNA synthesis in activated peripheral blood lymphocytes by decreasing the amount of intracellular deoxynucleotides, thus suggesting that this drug has an antiviral effect. Hydroxyurea has now been shown to block HIV-1 replication in acutely infected primary human lymphocytes (quiescent and activated) and macrophages, as well as in blood cells infected in vivo obtained from individuals with acquired immunodeficiency syndrome (AIDS). The antiviral effect was achieved at nontoxic doses of hydroxyurea, lower than those currently used in human therapy. Combination of hydroxyurea with the nucleoside analog didanosine (2′,3′-dideoxyinosine, or ddI) generated a synergistic inhibitory effect without increasing toxicity. In some instances, inhibition of HIV-1 by hydroxyurea was irreversible, even several weeks after suspension of drug treatment. The indirect inhibition of HIV-1 by hydroxyurea is not expected to generate high rates of escape mutants. Hydroxyurea therefore appears to be a possible candidate for AIDS therapy.

Further attempts to design drugs for therapy of AIDS are necessary (*1*). Despite their differences in structure, antiviral activity, and pharmacokinetic properties, ddI, zidovudine (azidothymidine, or AZT), noncompetitive HIV-1 reverse transcriptase inhibitors, and HIV-1 protease inhibitors (*2*) share a common feature: They directly target viral proteins. As an alternative approach, we have suggested targeting one or more cellular components (*3*). The rationale for this strategy is to avoid triggering the onset of viral escape mutants as a result of direct selective pressure against viral proteins. Another rationale is to achieve specific antiviral effects of the drug with low or no toxic effects on the cell.

Hydroxyurea has been widely used over the last 30 years for the treatment of human malignancies, especially chronic myelogenous leukemia and other myeloproliferative syndromes (*4*). More recently, hydroxyurea has been proposed for the treatment of sickle cell anemia (*5*). High doses of hydroxyurea are commonly used in leukemia treatment (*4*). Oral administration of the drug at a dose of 500 mg/m^2 every 4 hours generated plasma peak concentrations ranging from 0.5 to 2.5 mM and trough concentrations of 0.2 to 0.5 mM (*6*). Hydroxyurea is a free radical quencher and inhibits the cellular enzyme ribonucleotide reductase [a rate-limiting enzyme in the synthesis of deoxynucleoside triphosphates (dNTPs)]. We have shown that, by decreasing the intracellular pool of dNTPs, hydroxyurea inhibits HIV-1 DNA synthesis, resulting in the generation not only of decreased amounts of viral DNA, but also mainly incomplete chains (*3*). Goulaouic *et al.* (*7*), with Moloney murine leukemia virus, confirmed that inhibition of reverse transcription by hydroxyurea depends on the intracellular nucleotide pool (rather than on the precise arrest of the host cell cycle). High single doses of hydroxyurea delay HIV-1 spread in vitro (*8*). Furthermore, by decreasing the amount of cellular dNTPs, hydroxyurea was expected to increase the uptake and metabolism of nucleoside analogs, such as ddI or AZT, and consequently to enhance the effect of these compounds, hopefully in a synergistic manner. We now demonstrate that low, subtoxic doses of hydroxyurea, alone or in combination with AZT or ddI, block HIV-1 replication.

Because nonstimulated lymphocytes are not productively infected by HIV-1, but only allow viral entry and reverse transcription (*3*, *9*), we assessed HIV-1 infection in peripheral blood mononuclear cells (PBMCs) by monitoring HIV-1 DNA synthesis (*3*, *10*). Compared to untreated cells, HIV-1 DNA synthesis was slower and less efficient, and the final DNA was mostly incomplete, in hydroxyurea-treated quiescent lymphocytes (Fig. 1). A similar phenomenon has been described for activated lymphocytes (*3*). The effect of hydroxyurea was dose-dependent, especially for the synthesis of the full-length minus strand DNA (R-*gag*, the longer DNA synthesis product analyzed in our experiments). The block of DNA synthesis was almost complete at 1 mM. The DNA shown in Fig. 1 mainly represents DNA carried by the incoming virions (*3*, *10*); the amount of DNA did not vary during the time course and remained mostly incomplete. More elongation was observed at 0.1 mM, although at much lower levels compared to the untreated control. No cytotoxic effects were observed at the drug concentrations used in these experiments (*11*) because quiescent cells do not undergo genomic DNA synthesis.

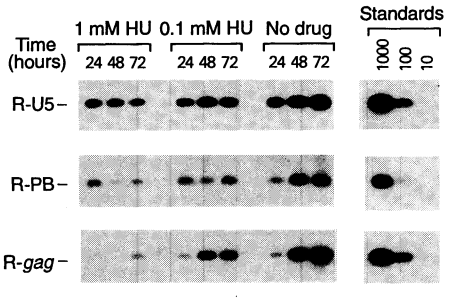

Fig. 1. Time course of inhibition of HIV-1 DNA synthesis by hydroxyurea in quiescent PBMCs. PBMCs were isolated from healthy donors and infected after 2 days with the HIV-1 strain HTLV-III$_B$ (*18*) at a multiplicity of infection of 1 in the absence of cell stimulation. After 2 hours at 37°C, the cells were washed, and fresh medium containing hydroxyurea (HU) at the indicated concentrations was added. Cells were harvested after 24, 48, and 72 hours and analyzed by quantitative polymerase chain reaction (PCR). Primers were used as described (*3*) to amplify different regions of the HIV-1 genome: R-U5 [between the R and U5 regions of the long terminal repeat (LTR)], R-PB (between the R region of the LTR and the primer binding site), and R-*gag* (between the R region of the LTR and the *gag* gene). After 30 cycles of PCR amplification and subsequent electrophoresis on 2% agarose, the samples were blotted on a nylon membrane and hybridized with a ^{32}P-labeled oligonucleotide as described (*3*). Quantitation of HIV-1 DNA during PCR amplification was achieved by comparison with a standard curve of serial dilutions of pHXB2(Rip7) plasmid DNA (*19*). The numbers above the lanes labeled "Standards" indicate the number of plasmid copies.

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