tion column (Sephadex G-75) developed in 100 mM acetic acid (pH 3.0), in a discrete peak with an apparent molecular weight in the range of 25,000 to 40,000 daltons, well ahead of native bovine PTH-(1–84) (molecular weight, 9100).

Our studies indicate that cultured human keratinocytes produce a PTH-like factor that stimulates cyclic AMP formation in cultured rat osteosarcoma cells but differs from PTH both immunologically and with respect to apparent molecular weight. PTH receptor agonists derived from HHM-associated tumors share these characteristics (*4–6*), which suggests that such tumors may secrete a factor related to that produced by certain normal squamous epithelia, such as the skin. Further studies are necessary to determine whether the active component of KCM possesses bone-resorbing activity (the cardinal feature of any putative HHM factor) and to determine the precise structural relation between the keratinocyte- and tumor-derived adenylate cyclase–stimulating factors.

The determination of a possible physiologic role for this factor must also await further investigation. Although the dermis is not traditionally regarded as a PTH target organ, recent studies have demonstrated the presence of receptors that recognize both PTH and the putative HHM-factor on dermal fibroblasts (*12*). This suggests that the PTH-like substance produced by keratinocytes may play a role in normal skin physiology.

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Protein-Tyrosine Kinase Activity in *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* was examined for tyrosine kinase activity in vitro because this organism offers molecular and genetic approaches for analyzing the role of tyrosine phosphorylation in cellular growth control that are unavailable in higher eukaryotes. Yeast extracts phosphorylated a random copolymer (glutamic acid:tyrosine, 80:20) at tyrosine in a reaction that was linear with respect to time and protein concentration. In the absence of added copolymer, phosphotyrosine was 0.1 percent of the total phosphoamino acids labeled with [γ-^{32}P]adenosine triphosphate in endogenous yeast proteins. However, specific activities of these reactions were low (approximately 1 percent of those in extracts of chick embryo fibroblasts). Lack of significant incorporation of label from [α-^{32}P]adenosine triphosphate into the copolymer or endogenous yeast proteins demonstrated that nucleotide interconversion, adenylylation, and subsequent hydrolysis could not account for the generation of phosphotyrosine observed.**

PROTEIN PHOSPHORYLATION AT TYROSINE appears to be important in growth control and in malignant transformation of animal cells (*1*). A variety of cellular protein substrates for these enzymes have been identified (*2*), but the function of these target proteins in growth control or in oncogenesis remains unclear. Yeast is a eukaryote that provides several advantages for the study of growth regulation, including its short generation time, small genome size, the availability of many mutations that affect the cell division cycle (*3*), and facile application of recombinant DNA methods, such as gene replacement (*4*). Yeast has already proven valuable for investigating the function of the *ras* family of proto-oncogenes (*5, 6*). We describe here the use of a sensitive assay to determine if yeast extracts contain tyrosine kinase activity.

Braun *et al.* (*7*) first used a synthetic random copolymer of 80 percent glutamic acid and 20 percent tyrosine [poly(Glu^{80} Tyr^{20})] and [γ-^{32}P]adenosine triphosphate (ATP) for the assay of several purified tyrosine kinases. Our initial experiments with crude yeast extracts indicated that the presence of endogenous phosphoacceptor proteins made precipitation with acid (*7, 8*) an ineffective single method for measuring specific incorporation into the polymer. Hence we sought an alternative method for separating the phosphorylated polymer from the other reaction components. Although poly (Glu^{80}Tyr^{20}) is heterogeneous in size (molecular weight, 20K to 60K), all the species present have the same charge-to-mass ratio because of their constant composition. For this reason and because of their strongly negative net charge, we anticipated that the population of molecules should migrate rapidly as a relatively discrete band when subjected to electrophoresis in polyacrylamide gels in the absence of any ionic detergent, provided that the matrix imposes little sieving action. We were able to find conditions (Fig. 1) under which poly(Glu^{80}Tyr^{20}) migrated as a doublet just slightly slower than the dye front, as revealed by staining with Coomassie blue.

To test the feasibility of this approach as an assay procedure for tyrosine kinase activity, extracts were prepared from uninfected

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chick embryo fibroblasts (CEF) and from CEF infected with Rous sarcoma virus (RSV), which contained the known tyrosine kinase $pp60^{v\text{-}src}$. In either case, when the reaction products were subjected to gel electrophoresis, poly($Glu^{80}Tyr^{20}$) was the most heavily labeled species and was well separated from all other labeled bands detected by autoradiography. In control reactions, where the polymer was added after the reaction was quenched, very little label ($\leq$0.0001 percent of the input radioactivity) was associated with the poly($Glu^{80}Tyr^{20}$) band. As expected, extracts from the RSV-infected cells had five to ten times more tyrosine kinase activity than that in extracts from uninfected CEF, as determined by excising the poly($Glu^{80}Tyr^{20}$) band from the gel and measuring the radioactivity present by liquid scintillation counting.

When extracts of *Saccharomyces cerevisiae* cells were assayed under the same conditions, apparent phosphorylation of poly ($Glu^{80}Tyr^{20}$) was detected (Fig. 1A). In controls in which the polymer was added after quenching of the reaction mixture, only a low level of radioactivity was associated with this region of the gel, presumably because of adventitious adsorption, or fortuitous comigration, of some other labeled material. To confirm that most of the label incorporated into poly($Glu^{80}Tyr^{20}$) was due to phosphorylation of tyrosine, the band was excised and the protein was eluted and subjected to phosphoamino acid analysis (Fig. 1B). Phosphotyrosine was the only phosphoamino acid detected.

When urea and the nonionic detergent NP-40 were used to reduce nonspecific adsorption to the polymer (Fig. 2), phosphorylation of poly($Glu^{80}Tyr^{20}$) was linear with respect to time (up to 12 minutes) and protein concentration (up to 300 μg/ml). Incorporation into the polymer represented about 1 percent of the total radioactivity incorporated into protein. The specific activity of yeast extracts was $\leq$1 percent that of extracts from uninfected CEF.

Because of the low level of incorporation, we were concerned that the apparent phosphorylation of poly($Glu^{80}Tyr^{20}$) in yeast extracts might be a product of some other reaction. At least one other covalent modification of proteins at tyrosine is known, adenylylation of glutamine synthetase in Gram-negative bacteria (*9*), and preliminary evidence suggests that adenylylation of yeast glutamine synthetase may occur in vivo (*10*). Furthermore, acid hydrolysis, like that utilized for phosphoamino acid analysis, is known to convert adenylyltyrosine to phosphotyrosine (*11, 12*). Because the label in the tyrosine kinase assay was provided in the form of [γ-^{32}P]ATP, however, a significant fraction of it would have to be converted to [α-^{32}P]ATP for adenylylation to be the source of the radioactivity incorporated into the polymer. To demonstrate that adenylylation was not responsible for the label incorporated into poly($Glu^{80}Tyr^{20}$) or for the generation of phosphotyrosine upon subsequent acid hydrolysis, yeast extracts were assayed with [α-^{32}P]ATP at the same concentration and at the same specific radioactivity as the [γ-^{32}P]ATP. No incorporation above background was detected under these assay conditions. Thus, labeling of the polymer is apparently due to authentic tyrosine phosphorylation.

To determine whether endogenous yeast proteins could serve as substrates for tyrosine phosphorylation in vitro, yeast extracts were incubated under the same conditions but in the absence of added polymer. Many yeast proteins were phosphorylated by [γ-^{32}P]ATP (lane 2 in Fig. 3A). In parallel reactions in which [α-^{32}P]ATP was substituted, relatively few yeast proteins were labeled, presumably by adenylylation (lane 1 in Fig. 3A). Phosphoamino acid analysis indicated that a significant fraction (about 0.1 percent) of the label incorporated into endogenous yeast proteins from [γ-^{32}P]ATP could be recovered as phosphotyrosine (Fig. 3C). No detectable phosphotyrosine was found in yeast proteins labeled with [α-^{32}P]ATP (Fig. 3B), again demonstrating that the level of adenylylation at tyrosine in vitro is too low to account for the amount of phosphotyrosine generated from reactions with [γ-^{32}P]ATP. Either nucleotide interconversion or adenylylation at serine, as has been observed for SV40 large T antigen (*13*), may account for the phosphoserine present.

As additional controls for assessing the specificity and efficiency of the tyrosine phosphorylation observed in yeast extracts, the endogenous phosphoacceptor activities of extracts of *Escherichia coli* and uninfected CEF were also determined at equivalent protein concentrations. In agreement with

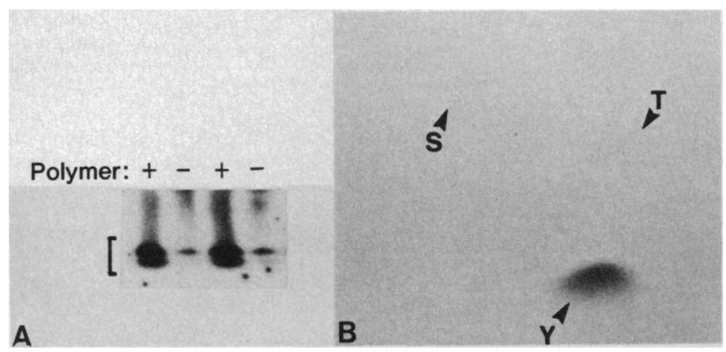

Fig. 1. Phosphorylation of synthetic poly($Glu^{80}Tyr^{20}$) at tyrosine by an activity in yeast extracts. Strain X2180-1A was grown at 30°C in YPD medium (*20*) to mid-exponential phase; collected by centrifugation; washed in 10 ml of 10 m*M* KCl, 1 m*M* EDTA, pepstatin (3 μg/ml), 1 m*M* phenylmethylsulfonyl fluoride, and 25 m*M* 2-(*N*-morpholino)-ethanesulfonic acid (MES), *p*H 6.5 (buffer A); resuspended in two volumes of ice-cold buffer A; and ruptured by breakage with glass beads (*21*). The lysate was made 1 percent in Triton X-100 and centrifuged for 10 minutes at 13,000*g*; the supernatant fraction was used immediately for assay. Protein concentrations were determined by the dye-binding method of Bradford (*22*). (A) Reaction mixtures (final volume, 30 μl) contained 10 m*M* $MnCl_2$, 10 percent glycerol, 1 percent Triton X-100, 10 m*M* 2-mercaptoethanol, 10 μ*M* Na_3VO_4 (*23*), 50 μg of poly($Glu^{80}Tyr^{20}$) (Sigma), 70 m*M* MES (*p*H 6.5), and crude extract. Reactions were initiated by addition of 270 μ*M* [γ-^{32}P]ATP (2.7×10^4 count/min per picomole) (*24*) and incubated at 30°C. Reactions were terminated by addition of excess unlabeled nucleotide (10 μl of 108 m*M* ATP), followed immediately by ice-cold 20 percent trichloroacetic acid (TCA) (0.5 ml). Five microliters of bovine serum albumin (10 mg/ml) and 5 μl of bovine γ-globulin (10 mg/ml) were added as carriers. For controls, to correct for nonspecific binding or coelectrophoresis of phosphorylated compounds with the product, reactions were conducted in the absence of poly($Glu^{80}Tyr^{20}$) and the polymer was added back after the reaction was stopped. After cooling on ice for 10 minutes, the precipitates were collected by centrifugation in a microcentrifuge, washed twice with 0.5 ml 20 percent TCA, and then twice with 0.5 ml of ethanol. Residual solvent was removed under vacuum and the precipitate was dissolved in electrophoresis sample buffer [2 percent Nonidet P-40, 9.4*M* urea, 62.5 m*M* tris-HCl (*p*H 6.8), and 0.002 percent bromphenol blue]. Gel electrophoresis (*25*) was performed in slabs of 5 percent polyacrylamide, omitting sodium dodecyl sulfate (SDS), but including 2 percent Nonidet P-40 and 9.4*M* urea. Gels were fixed (*26*), stained in 10 percent acetic acid with 0.002 percent Coomassie blue, and washed exhaustively in 10 percent acetic acid containing 50 m*M* sodium pyrophosphate. The region of the autoradiogram containing the copolymer is shown. Symbols: (+) duplicate reactions containing poly($Glu^{80}Tyr^{20}$) and yeast extract (1.67 mg/ml); (−) duplicate control reactions containing yeast extract, but with poly($Glu^{80}Tyr^{20}$) added back after quenching; and ([) migration position of polymer revealed by staining with Coomassie blue. (B) Labeled poly($Glu^{80}Tyr^{20}$) band was excised from the gel, rehydrated, and subjected to phosphoamino acid analysis by two-dimensional electrophoresis on cellulose thin-layer plates (*27, 28*). Abbreviations: S, phosphoserine; T, phosphothreonine; and Y, phosphotyrosine.

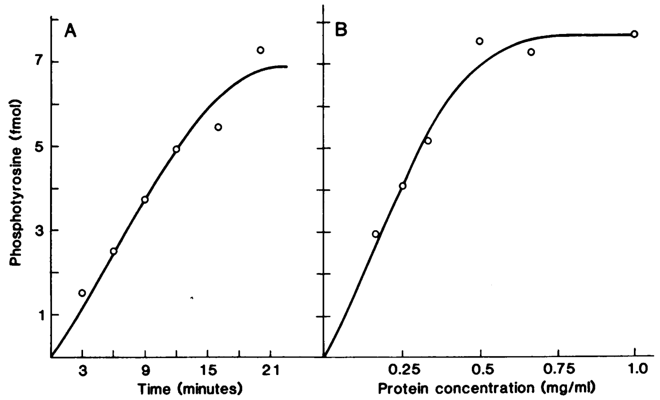

Fig. 2. Kinetics and dependence on protein concentration of the phosphorylation of poly($Glu^{80}Tyr^{20}$) by yeast extracts. Incubations were conducted as described in the legend to Fig. 1, except that reactions were terminated by adding 80 μl of 166 m*M* ATP, 9.4*M* urea, 3.2 percent Nonidet P-40, 0.1*M* tris-HCl (*p*H 6.8), and 0.002 percent bromphenol blue. In controls, in which polymer was omitted during reaction, the quench also contained poly($Glu^{80}Tyr^{20}$) (625 μg/ml). After electrophoresis and staining of the gel, a wide horizontal strip including the poly($Glu^{80}Tyr^{20}$) bands was cut out, washed overnight, and then washed five more times as described in the legend to Fig. 1. After autoradiography the individual polymer bands were excised and suspended in NCS solubilizer (New England Nuclear), and radioactivity was measured by scintillation counting. Background incorporation at each point, obtained from the control reactions in which polymer was omitted until the reaction was quenched, was subtracted from all the samples. Values reported represent the average of duplicate determinations, which did not vary by more than 6 percent. (A) Time course of the phosphorylation of poly($Glu^{80}Tyr^{20}$) by yeast extract (0.3 mg/ml). (B) Relationship between reaction velocity and concentration of yeast extract added in a 10-minute incubation.

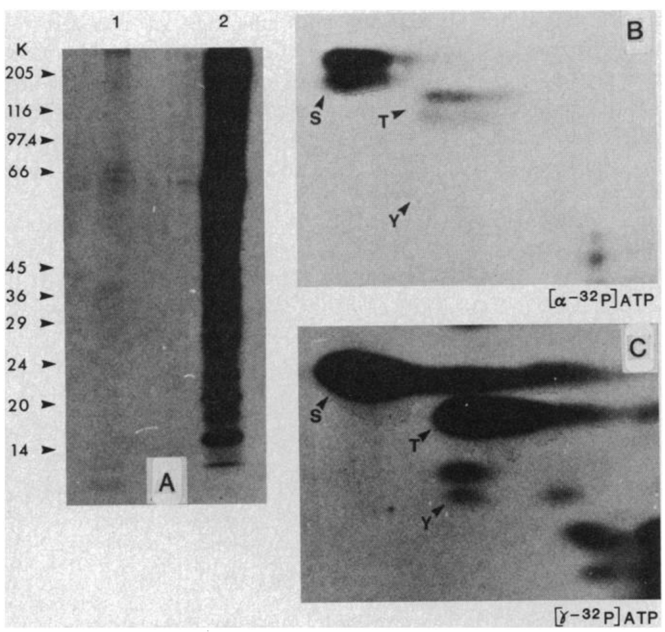

Fig. 3. Phosphorylation at tyrosine of endogenous yeast proteins in vitro. (A) Reactions contained 50 μg of yeast protein and were conducted as described in the legend to Fig. 1, except that polymer was omitted. One incubation contained [α-^{32}P]ATP (Amersham) (lane 1) and another [γ-^{32}P]ATP (lane 2) at the identical concentration and specific radioactivity. Reactions were terminated by adding 200 μl of 2 percent SDS followed by boiling for 5 minutes. Phosphoproteins were extracted with phenol, precipitated with TCA, and washed (*28, 29*). Samples were analyzed by electrophoresis in slabs of 7.5 percent polyacrylamide containing SDS. Numbers at left mark the migration positions of protein standards. (B) Phosphoproteins recovered from the reaction with [α-^{32}P]ATP were subjected to partial acid hydrolysis and two-dimensional thin-layer electrophoresis. (C) Phosphoproteins recovered from the reaction with [γ-^{32}P]ATP were subjected to partial acid hydrolysis and two-dimensional thin-layer electrophoresis as in Fig. 1. Abbreviations: S, phosphoserine; T, phosphothreonine; and Y, phosphotyrosine.

previous results (*14*), no phosphotyrosine was detectable in the *E. coli* proteins labeled in vitro with [γ-^{32}P]ATP. When [α-^{32}P] ATP was used, phosphotyrosine was present in the products, presumably because of the adenylylation at tyrosine known to occur in bacteria (*9, 11*). Phosphotyrosine constituted 5 percent of the total phosphoamino acids in the proteins labeled in extracts from CEF, in agreement with the amount of tyrosine phosphorylation in vitro observed by other investigators (*15*). This level is nearly 100 times greater than that observed in vivo (*16*) and may be due to the increased accessibility of potential substrates, or more favorable reaction conditions, for the kinases in lysates. By comparison, therefore, phosphorylation at tyrosine (measured in counts per minute per microgram of protein) in yeast extracts was inefficient (approximately 0.5 percent of the level in CEF extracts).

There are several possible reasons for the low apparent specific activity of yeast extracts. Although the reaction conditions (Fig. 1) were chosen to favor tyrosine phosphorylation on the basis of the properties of protein-tyrosine kinases from animal cells (*1*), these conditions may be far from optimal for the yeast activity. Alternatively, yeast may contain lower levels, or fewer species, of such enzymes. Finally, the tyrosine phosphorylation observed may reflect a secondary activity of an enzyme with another function. The clearest way to resolve these issues will be to purify the tyrosine phosphorylating activity from yeast. If the yeast activity is analogous to the protein-tyrosine kinases that are the products of certain cellular proto-oncogenes in animal cells (*17*), the finding of such an enzyme (or enzymes) in a single-celled eukaryote would push the origin of tyrosine kinases much farther back in evolutionary time and would suggest that this class of enzymes has a critical role in metabolic regulation or in control of the cell division cycle.

Note added in proof: While this report was in preparation, it was reported (*18*) that hydrolysates of yeast proteins labeled in vivo with [^{32}P]PO_4^{3-} contained a low level of phosphotyrosine and that crude plasma membranes from yeast were able to phosphorylate endogenous proteins and exogenously added casein at tyrosine (*19*). Our results support the conclusion that yeast cells have authentic protein-tyrosine kinase activity.

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The Context Effect Does Not Require a Fourth Base Pair

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The translational activity of a transfer RNA at a codon varies at different message sites, although the codon does not vary. The source of this effect, which may help to determine the level of gene expression, is generally agreed to be in nearby message sequences. By making every possible nucleotide combination between position 33 of the transfer RNA and the major context nucleotide of the message, it was shown that base-pairing between the two nucleotides is not the source of this context effect on translation in vivo.

The efficiency of translation of polypeptide chain termination codons (UAA, called ochre; UAG, amber; and UGA, opal) by their transfer RNA (tRNA) suppressors depends on other message nucleotides near the nonsense codon (*1*). This is termed the context effect, and nucleotides 3′ to the codon are most strongly implicated. By selecting for increased activity of a weakened amber suppressor, Bossi and Roth (*2*) showed that *supE* in *Salmonella typhimurium* suppressed a particular UAG in *hisD* tenfold more efficiently when UAGC was mutated to UAGA. More recently, this pattern has been observed in several amber mutations (*1, 3*). In general, amber codons followed by pyrimidines are less efficiently translated than are amber codons followed by purines. Bossi and Roth (*2*) have adapted a hypothesis originally formed by Taniguchi and Weissman (*4*), which explains these observations by suggesting that a fourth base pair occurs between the tRNA's anticodon loop and the message.

All elongator tRNA's have a U at position 33 (just 5′ to the anticodon), which could base-pair with the nucleotide on the 3′ side of the codon, forming a U-A or U-G pair (Fig. 1). Such a fourth base pair would favor elongation of the nascent polypeptide chain, rather than termination at UAG purine.

In order to test this possibility, we used Su7 (the amber-suppressing variant of $tRNA^{Trp}$) and three amber-suppressing, nucleotide-33 derivatives of this tRNA to suppress amber codons in four contexts within *lacI-Z* fusion genes. The suppressor mutants used in the study are identical to Su7 except that U33 in Su7 has been replaced by C, G, or A (Fig. 1). These tRNA's will be called Su7 U33, Su7 C33, Su7 G33, and Su7 A33, respectively. The *lacI-Z* message sequences were chosen to complement nucleotide 33 of this set of tRNA's and to keep the remainder of the context of the UAG codon consistent. All four nucleotides occur 3′ to the UAG in this set of contexts (Table 1). Therefore, we made strains containing the *lacI-Z* fusions and the suppressors in all combinations. When all suppressor activities on all messages are determined (by measuring β-galactosidase activity), every possible combination of nucleotides at the position of the hypothetical fourth base pair can be compared.

The *lacI-Z* genes we used also conserve the nucleotide 5′ to the amber codon and the nucleotide that is the next-nearest 3′ neighbor of the codon (Table 1). This normalizes the effects of other nucleotides that might have a role in the context effect. Miller and Albertini (*3*) have shown that the next-nearest 3′ neighbor has an influence on the translation of amber codons. The 5′ neighbors of sense codons are correlated with the sequence of the codon, and therefore might have functional effects (*5*). In all measurements in Table 1, both potential "context" positions are always C.

The *lacI-Z* fusions were created by a deletion which fused the NH_2 terminus of *lacI*, where the amber resides, to the COOH-terminus of *lacZ* (*6*). There is no substantial level of reinitiation of translation after termination at the amber codon. Translation of the amber codon in *lacI* is required for active *lacI-Z* product (β-galactosidase), and enzyme levels should be proportional to the fraction of message transits in which the codon is translated by the suppressor tRNA. This fusion system for the measurement of suppressor efficiency (the fraction of transits in which the tRNA acts) has been extensively characterized by Miller and Albertini (*3*). The β-galactosidase was measured as described by Miller (*7*), with the modifications of Raftery *et al.* (*8*).

Enzyme levels for the 16 strains in this experiment are shown in Table 1. Pyrimidines at tRNA position 33 resulted in more efficient suppressors, and U33, which is always found in natural sequences, is the most efficient pyrimidine (*9, 10*). This pyrimidine preference was clear and similar in all contexts. In comparisons of different contexts, we observed that the sequence GAC UAG ACA contains the most efficiently translated amber codon for all tRNA's, in agreement with the expected

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