Vertebrate glycoprotein hormone heterodimers are stabilized by a strand of their β-subunits known as the “seatbelt” that is wrapped around loop 2 of their α-subunits (α2). The cysteine that terminates the seatbelt is “latched” by a disulfide to a cysteine in the NH₂-terminal end of the β-subunit loop 2 (β1) of all vertebrate hormones except some teleost folliculotropins (teFSH), wherein it is latched to a cysteine in the β-subunit NH₂ terminus. As reported here, teFSH analogs of human chorionicadotropin (hCG) are assembled by a pathway in which the subunits dock before the seatbelt is latched; assembly is completed by wrapping the seatbelt around loop α2 and latching it to the NH₂ terminus. This differs from hCG assembly, which occurs by threading the glycosylated end of loop α2 beneath the latched seatbelt through a hole in the β-subunit. The seatbelt is the part of the β-subunit that has the greatest influence on biological function. Changes in its sequence during the divergence of lutropins, follitropins, and thyrotropins and the speciation of teleost fish may have impeded heterodimer assembly by a threading mechanism, as observed when the hCG seatbelt was replaced with its salmon FSH counterpart. Whereas wrapping is less efficient than threading, it may have facilitated natural experimentation with the composition of the seatbelt during the co-evolution of glycoprotein hormones and their receptors. Migration of the seatbelt latch site to the NH₂-terminal end of the β-subunit would have facilitated teFSH assembly by a wraparound mechanism and may have contributed also to its ability to distinguish lutropin and follitropin receptors.

The glycoprotein hormones have key roles in reproduction and thyroid function (1). These heterodimers have an unusual topology in which a strand of their β-subunits surrounds a loop of their α-subunits (2–4). Because the carboxyl-terminal end of this strand is “latched” by a disulfide to the β-subunit, it has been likened to a “seatbelt” (2). In addition to its role in stabilizing the heterodimer, the seatbelt is responsible for much of the influence of the β-subunit on human glycoprotein hormone activity (5–8).

Most glycoprotein hormone β-subunits appear to have evolved from a single ancestor (9) and their folding pattern is highly conserved in all vertebrates except for that in FSH of some teleost fish. Whereas in most species the seatbelt is latched to a cysteine in loop β1, in many teleosts it is latched to a cysteine in the NH₂-terminal end of the β-subunit corresponding to hCG residue βLeu8 (10). This reduces the size of the hole in the β-subunit through which loop α2 is straddled (Fig. 1). The smaller size of this space in teFSH would be expected to impede threading of the glycosylated end of loop α2 through the β-subunit, a phenomenon that explains the greater acid stability of the teFSH heterodimer (10, 11) relative to that of glycoprotein hormones such as hCG. The latter dissociate completely within 15–20 min at pH 2, 37 °C (12).

The human glycoprotein hormones hCG, hFSH, and hTSH are assembled in mammalian cells primarily by a process in which loop α2 and its attached oligosaccharide are threaded through a hole in the β-subunit. This hole is formed after the seatbelt is latched to loop β1 (19, 20). The smaller space available for passage of loop α2 beneath the teFSH seatbelt suggested that teFSH might be assembled by a mechanism in which the subunits dock before the seatbelt is latched. This would enable the seatbelt to be wrapped around loop α2 before it is latched to a cysteine in the β-subunit NH₂ terminus. Experiments described here were designed to learn how teFSH analogs of hCG are assembled in the endoplasmic reticulum and to identify factors that contribute to the use of the threading and wraparound pathways for glycoprotein hormone assembly. Because the topology of teFSH differs substantially from that of hCG, we anticipated these studies would also enable us to test our procedures for analyzing hormone assembly in living cells (19, 20). By studying the assembly of a series of salmon FSH-hCG chimeras that are readily monitored using antibodies to hCG, we found that hCG analogs having the teFSH fold cannot be assembled by a threading mechanism and, as a consequence, are formed by a wraparound mechanism. Forcing the seatbelt to be latched to a site in the NH₂ terminus enhanced the assembly of teFSH analogs, most likely by reducing the inherent tendency of the seatbelt to be latched before the subunits dock.

**EXPERIMENTAL PROCEDURES**

The α- and β-subunit analogs used in these studies are illustrated in Fig. 2. Chimeras of salmon FSH and hCG β-subunits are identified using the root term s/hCGβ. For example, s/hCGβ-NtSB,C26A is an hCG β-subunit analog that contains the salmon FSH NH₂-terminal and seatbelt and an alanine in place of βCy5. All other reagents and nadropin; hFSH, human follitropin; hTSH, human thyrotropin; e2, α-subunit loop 2; β1, β2, β3, β-subunit loops 1, 2, and 3, respectively; s/hCGβ chimera of chum salmon FSH and hCG β-subunits; NH₂ terminus, amino-terminal end of the chum salmon β-subunit; SB, chum salmon seatbelt; TL, chum salmon seatbelt tensor loop; St, chum salmon seatbelt strap; teFSH, teleost follitropin; LH, lutropin. The structures of the analogs used in this work can be determined by reference to Fig. 2.
Influence of Seatbelt on Assembly Pathway

Fig. 1. Comparison of the structures of hCG and salmon FSH showing the difference in the sizes of the holes in the β-subunits when the seatbelt is latched to β and the NH2 terminus. The Ca carbons of hCG (left panel) and a model of salmon FSH (right panel) are shown here. Atoms of β-subunit residues in the subunit core and seatbelt that surround α2 are highlighted. Note that the size of this hole appears to be considerably greater when the seatbelt is latched to a cysteine in β1 than when it is latched to a cysteine in the NH2 terminus. The smaller size of the β-subunit hole in salmon FSH would make it more difficult to thread loop α2 beneath the seatbelt, a phenomenon that would account for its greater acid stability than hCG (10, 11). Therefore, one might expect that the assembly of salmon FSH differs from that of hCG. Color code: white, α-subunit; dark gray, β-subunit; hatched patches, β-subunit hole straddled by loop α2.

RESULTS

Some Heterodimers Containing β-Subunits That Are Chimeras of Salmon FSH and hCG Are Assembled Differently from hCG—To study the assembly of heterodimers that have the telFsh folding pattern, we employed an hCG β-subunit analog that contained the salmon FSH β-subunit NH2 terminus and seatbelt. This β-subunit chimera, s/hCGβ-Nt,SB,C26A (Fig. 2), retained binding sites for most hCG monoclonal antibodies that recognize epitopes formed by the subunit core, which includes its cystine knot and loops β1, β2, and β3. Because this analog contains an alanine in place of Cys26, the normal hCG β-subunit seatbelt latch site, it was expected to latch its seatbelt to βCys5, a cysteine within its NH2-terminal salmon sequence. This β-subunit chimera was incorporated into heterodimers that contained the native human α-subunit, but much less efficiently than hCGβ (Table I, data rows 1 and 7). Unlike the hCG heterodimer, which dissociated completely at pH 2 within 30 min at 37 °C, only about 20% of the hCG/teFsh chimera heterodimer dissociated under these conditions. It was not as stable as heterodimers that contain an intersubunit disulfide, which do not dissociate under these conditions (13). These observations showed that we could distinguish heterodimers in which the seatbelt is latched to the α-subunit, heterodimers in which the seatbelt is latched to βCys5, and heterodimers in which the seatbelt is latched to βCys26 by measuring differences in their stabilities at pH 2, 37 °C. These differences become much more apparent following an overnight treatment at pH 2, 37 °C (Fig. 3).

The hCGβ and s/hCGβ-Nt,SB,C26A subunits differ in their NH2-terminal ends, their seatbelts, and in loop β1. Studies described next were designed to learn how each of these regions influence heterodimer assembly. Replacing hCG β-subunit residue βLeu5 with cysteine created hCGβ-L5C, an hCG analog that has a seatbelt latch site in an NH2-terminal location corresponding to that in salmon FSH. Heterodimers that contained the native human α-subunit and hCGβ-L5C dissociated rapidly at pH 2, 37 °C, and were recognized by B111 (Table I, row 2), indicating that their seatbelts were latched to βCys26 in loop β1, not to βCys5. Thus, introduction of a potential latch site into the hCGβ NH2 terminus was not sufficient to alter the manner in which the heterodimer is assembled. In contrast, elimination of the seatbelt latch site in loop β1 of hCGβ-L5C reduced its ability to form heterodimers with the native human α-subunit as can be seen by the lack of heterodimer formed when the α-subunit was co-expressed with hCGβ-L5C,C26A (Table I, row 3). This finding is consistent with the notion that the hCG seatbelt of hCGβ-L5C,C26A became latched to βCys5 prior to subunit docking and/or that the seatbelt did not become latched to βCys5 after the subunits had docked. The propensity of the hCG seatbelt to be latched prior to subunit docking (19) suggests that it may have become latched to this site prior to the start of assembly. This would indicate that it can interfere with threading when latched to a cysteine in the NH2 terminus.

To learn how the salmon FSHβ NH2 terminus affected assembly, we expressed s/hCGβ-Nt and s/hCGβ-Nt,C26A with the native human α-subunit (Table I, rows 4 and 5). The presence of the salmon FSHβ NH2 terminus appeared not to affect most heterodimer assembly. Consequently, 90% of the het-

FIG. 2. Sequences of the α- and β-subunit analogs used in these studies. Cysteines were substituted for residues in the α-subunit to create analogs whose names are indicated at the sites of the mutations. Changes to the hCG β-subunit are indicated above the hCG β-subunit sequence, which is boxed. The name of each mutation described in the text is indicated in parentheses following its sequence. For example, C26A indicates that cysteine residue 26 in loop β1 had converted to alanine; Nt indicates that the hCG β-subunit NH2-terminal sequence Lys-Glu-Pro-Leu-Arg-Pro-Arg had been replaced by the salmon NH2-terminal sequence Gly-Thr-Glu-Cys4-Arg-Tyr-Gly. Note that residues Cys26 and Cys5 are seatbelt latch sites in β1 and NH2 terminus, respectively. Some analogs are combinations of these mutations as suggested by their names. Thus, s/hCGβ-Nt,C26A,SB is a salmon FSH and hCG β-subunit chimera with the hCG β-subunit NH2-terminal and seatbelt sequences replaced with those from salmon FSH and the seatbelt latch site in loop β1 Cys26 replaced with alanine.
erodimers containing s/hCGβ-Nt dissociated within 30 min at pH 2, 37 °C, and were readily recognized by antibody B111 (Table I, row 4). The latter observation showed that their seatbelts were latched to βCys26 in loop β1. The remaining 10% survived pH 2 treatment, 37 °C for 30 min, however, indicating that their seatbelts were latched to βCys5 in the salmon FSHβ NH2 terminus. Preventing s/hCGβ-Nt from latching its seatbelt to loop β1 by converting βCys26 to alanine inhibited heterodimer assembly as seen by the lack of incorporation of s/hCGβ-Nt,C26A into heterodimers (Table I, row 5). Considered together, these observations suggested that the hCG seatbelt present in either s/hCGβ-Nt or s/hCGβ-Nt,C26A can become latched to βCys5 in the salmon FSH NH2 terminus before or after the subunits dock; latching it to βCys5 prior to subunit docking appeared to inhibit assembly.

The tFSH seatbelt has a much greater influence than the NH2-terminal latch site on heterodimer assembly. Much less heterodimer was formed when the native human α-subunit was co-expressed with an analog of s/hCGβ-Nt in which the hCG seatbelt was replaced with the salmon seatbelt (i.e. s/hCGβ-Nt,SB, Table I, row 6). The small amount of s/hCGβ-Nt,SB that was assembled into heterodimers containing the native α-subunit was roughly as stable as that containing s/hCGβ-SB,C26A, suggesting that its seatbelt was latched to βCys5 in the NH2 terminus and not to βCys26 in loop β1. In fact, heterodimers containing s/hCGβ-Nt,SB,C26A, a β-subunit analog that has only a single seatbelt latch site, were produced more efficiently than those containing s/hCGβ-Nt,SB (Table I, data rows 6 and 7). This observation indicated that the salmon FSH seatbelt in s/hCGβ-Nt,SB became latched to each of these potential latch sites and that it may block threading when it is latched to βCys26. In subsequent studies, we converted βCys5 of s/hCGβ-Nt,SB to alanine, thereby creating s/hCGβ-Nt,SB,C5A, which lacks an NH2-terminal seatbelt latch site. This abolished heterodimer formation (not shown), confirming the notion that the seatbelt in heterodimers containing s/hCGβ-Nt,SB is latched to βCys5 and that latching the seatbelt to βCys26 in loop β1 suppressed assembly.

Assembly of Glycoprotein Hormones in Which the Seatbelt Is Latched to a Cysteine in the β-Subunit NH2 Terminus Occurs by a Wraparound Route—Heterodimers in which the seatbelt was latched to the β-subunit NH2 terminus dissociated at pH 2, 37 °C, albeit much slower than hCG (Fig. 3). This showed that during acid-induced dissociation of tFSH analogs the glycosylated end of loop α2 can pass through the space between the seatbelt and the β-subunit core. Theoretically, the reversal of this process would permit heterodimer assembly by a threading mechanism, albeit at a very slow rate. The small space between the seatbelt and the subunit core suggested that threading would be highly unlikely, even if the tensor disulfide were to be disrupted during threading as is the case during the assembly of hCG (20). The threading and wrapping pathways could be distinguished if we were able to determine whether the seatbelt had been latched before or after the subunits docked. As discussed next, this can be accomplished using disulfide cross-links to trap early assembly intermediates.

To trap early docking intermediates, we took advantage of the fact that the NH2-terminal ends of the subunits become stable at low pH. We expected that the seatbelt was latched to βCys5 in most of the free β-subunit but this could not be determined using existing antibodies due to the fact that B111 does not recognize the salmon seatbelt, even when it is latched to βCys26.

**Table I**

Relative influence of the β and N-terminal latch sites on heterodimer assembly

| Data row | Analog transfected                  | Total dimer (ng/50µl ± SEM) | Dimer detected by B111 | Acid-stable dimer | Probable seatbelt latch disulfide |
|----------|-----------------------------------|------------------------------|------------------------|-------------------|----------------------------------|
| 1        | Native hCGβ                       | 6.72 ± 0.36                 | 6.89 ± 0.26            | <0.1              | βCys110/βCys26                  |
| 2        | Native hCGβ-L5C                    | 7.32 ± 0.52                 | 4.81 ± 0.18            | <0.1              | βCys110/βCys26                  |
| 3        | Native hCGβ-L5C,C26A               | <0.1                        | ≥0.1                   | βCys110/βCys26    |
| 4        | Native s/hCGβ-Nt                   | 5.23 ± 0.20                 | 6.37 ± 0.13            | 0.42 ± 0.08       | βCys110/βCys26/βCys5            |
| 5        | Native s/hCGβ-Nt,C26A              | ≥0.1                        | 0.1                    | 0.12 ± 0.03       | βCys110/βCys5                  |
| 6        | Native s/hCGβ-Nt,SB                | 0.16 ± 0.01                 | ≥0.1                   | 0.74 ± 0.09       | βCys110/βCys5                  |
| 7        | Native s/hCGβ-Nt,SB,C26A           | 0.91 ± 0.08                 | ≥0.1                   | βCys110/βCys5     |

* a Heterodimer secreted into the culture medium was determined in A113/251-B110 sandwich assays.
  b Latching of the seatbelt to β1 was determined in A113/251-B111 sandwich assays. B111 does not recognize the salmon FSH seatbelt when it is latched to βCys26, however.
  c The acid stability of the heterodimer was determined in A113/251-B110 sandwich assays of media treated at pH 2 for 30 min at 37 °C.
  d Because we were unable to detect any heterodimer, we assumed the seatbelts of these analogs were either unlatched or, more likely, became latched to the cysteine in the N2-terminal end of the β-subunit prior to formation of the heterodimer.
  e The seatbelt was assumed to be latched to the NH2-terminal end of the β-subunit based on the finding that the small amounts of heterodimer formed are stable at low pH. We expected that the seatbelt was latched to βCys5 in most of the free β-subunit but this could not be determined using existing antibodies due to the fact that B111 does not recognize the salmon seatbelt, even when it is latched to βCys26.
Intersubunit disulfide cross-link that would prevent the heterodimer from dissociating upon treatment overnight at pH 2, 37 °C. In contrast, if assembly occurs by a threading mechanism instead of a wraparound mechanism, seatbelt residue βCys\(^{110}\) would have been cross-linked to βCys\(^{5}\) prior to docking. This would have prevented the formation of both the αβ-ββ and αβ-ββ110 disulfides and led to the formation of a heterodimer that dissociated with a half-life of 5–6 h during treatment at pH 2, 37 °C. Of course, it was also possible that during wrapping, seatbelt residue βCys\(^{110}\) would become latched to βCys\(^{5}\) to create a heterodimer that would also dissociate with a half-life of 5–6 h. Therefore, a finding that all the heterodimer dissociated with a half-life of 5–6 h would indicate but not prove that assembly occurs by a threading mechanism. However, the finding that a substantial fraction of the heterodimer was acid stable would show that most, if not all, heterodimer assembly had occurred by a wraparound mechanism.

There was one other caveat to this approach. Co-expression of α-Q5C and hCG-β-R8C, analogs in which cysteines are substituted for hCG residues αGln\(^{5}\) and βArg\(^{6}\) has been shown to form a disulfide cross-linked heterodimer (14). Formation of this disulfide is explained by the distances between the Ca and Cβ atoms of these residues (i.e. roughly 5.2 and 3.9 Å), which are similar to those in typical glycoprotein hormone disulfides (i.e. roughly 5.6–5.8 Å, respectively). The location of the salmon FSH β-subunit latch site corresponds to hCG β-subunit residues βLeu\(^{5}\), not βArg\(^{6}\), however. The distances between the Ca and Cβ atoms of αGln\(^{5}\) and βLeu\(^{6}\) are ~9.1 and 11.1 Å, suggesting that when these residues are replaced with cysteines, their positions in the heterodimer might prevent them from forming a disulfide.

To learn if an αβ-ββ disulfide bridge can form during heterodimer assembly, we co-expressed hCGβ-L5C with the native α-subunit and with α-Q5C. Heterodimers formed with the native α-subunit were unstable at acid pH and dissociated within 30 min (Table I, data row 2); those formed when hCGβ-L5C was co-expressed with α-Q5C remained intact following an overnight incubation at pH 2, 37 °C (Table II, data row 1). Both heterodimers were recognized by antibody B111 (not shown), demonstrating that their seatbelts were latched to residue βCys\(^{5}\). This showed that heterodimers containing α-Q5C and hCGβ-L5C are stabilized by an αβ-ββ disulfide bridge.

We reasoned that if seatbelt residue βCys\(^{110}\) is latched to βCys\(^{5}\) before the teFSH β-subunit docks with α-Q5C, neither of these β-subunit cysteines can be cross-linked to αCys\(^{5}\). We tested this possibility by co-expressing α-Q5C with hCGβ-L5C,C26A, an analog that has a teFSH folding pattern and in which the seatbelt appears to be latched efficiently to βCys\(^{5}\) before the subunits dock. This analog did not form detectable amounts of heterodimer with the native α-subunit (Table I, data row 3) and only small amounts were incorporated into heterodimers containing α-Q5C (Table II, row 2). Apparently, the seatbelt of hCGβ-L5C,C26A became latched to βCys\(^{5}\) before the subunits dock, which precluded the formation of an αCys\(^{5}\)-βCys\(^{5}\) disulfide bridge. The finding that small amounts of hCGβ-L5C,C26A were incorporated into heterodimers containing α-Q5C (Table II, row 2) showed that α-Q5C can be used to trap β-subunits that contain unlatched seatbelts, even when these represent a small fraction of the total β-subunit population. Based on the finding that less than 10% of these heterodimers dissociated after an overnight incubation at pH 2, 37 °C (Table II, data row 2), we anticipate that 9 of 10 heterodimer molecules appeared to contain an intersubunit disulfide bridge. This showed that heterodimers containing α-Q5C and hCGβ-L5C,C26A were stabilized by an αβ-ββ disulfide bridge.

These findings showed that the pathway used to assemble heterodimers containing teFSH analogs can be deduced from
COS-7 cells were transfected with \( \alpha \)-Q5C and hCG\( \beta \) analogs with their NH\(_2\)-terminal and/or seatbelt sequences replaced by the counterparts of their salmon FSH counterparts as indicated. The loop \( \beta \) seatbelt latch site was also removed in some analogs by replacing \( \beta \)Cys\(^{5\text{th}} \) with alanine. Heterodimer secreted into the culture medium was quantified in A113\(^{\alpha=9} \)-B110 sandwich assays. The stabilities of the heterodimers were determined in A113\(^{\alpha=9} \)-I-B110 sandwich assays of media treated at pH 2 for 30 min or overnight at 37 °C. All values are mean ± S.E. of triplicate transfections.

| Data row | Analog | Total dimer ng/50\mu l ± S.E. | pH 2, 0.5 hours % total ± S.E. | pH 2, 16 hours % total ± S.E. |
|----------|--------|-------------------------------|-------------------------------|-------------------------------|
| 1        | \( \alpha \)-Q5C + hCG\( \beta \)-L5C | 20.02 ± 1.94                  | 102.1 ± 6.1                   | 100.3 ± 3.6                   |
| 2        | \( \alpha \)-Q5C + hCG\( \beta \)-L5C,C26A | 1.12 ± 0.97                   | 92.7 ± 0.7                    | 91.9 ± 1.8                    |
| 3        | \( \alpha \)-Q5C + s/hCG\( \beta \)-Nt | 7.26 ± 0.09                   | 97.1 ± 1.5                    | 102.9 ± 2.5                   |
| 4        | \( \alpha \)-Q5C + s/hCG\( \beta \)-Nt,C26A | 0.73 ± 0.10                   | 91.9 ± 3.7                    | 86.0 ± 3.5                    |
| 5        | \( \alpha \)-Q5C + s/hCG\( \beta \)-Nt,SE,C26A | 1.98 ± 0.05                   | 89.9 ± 1.0                    | 89.0 ± 0.9                    |
| 6        | \( \alpha \)-Q5C + s/hCG\( \beta \)-L5C,SE,C26A | 8.22 ± 0.12                   | 104.1 ± 2.8                   | 102.7 ± 0.9                   |

The ability of \( \alpha \)Cys\(^{5\text{th}} \) to compete for the seatbelt of the \( \beta \)-subunit N-terminal latch site

\[ \text{Influence of Seatbelt on Assembly Pathway} \]

**Table II**

The observation that \( \alpha \)Cys\(^{5\text{th}} \) became cross-linked to the \( \beta \)-subunit during the assembly of heterodimers containing \( \alpha \)-Q5C and \( \beta \)-subunits having the teFSH\( \beta \) folding pattern strongly supports the notion that these teFSH analogs are assembled by a wraparound mechanism. To test this result in an alternative fashion, we repeated these studies using \( \alpha \)-subunit analogs having unpaired cysteines located at other sites (Table III). We have found that hCG seatbelt residue \( \beta \)Cys\(^{110\text{th}} \) can be latched to cysteines added to the \( \alpha \)-subunit when it is prevented from being latched to \( \beta \)Cys\(^{5\text{th}} \) in loop \( \beta \)1 (13). To learn if the salmon FSH seatbelt behaved similarly to the hCG seatbelt in either regard, we compared the stabilities of heterodimers containing \( \alpha \)-L41C or \( \alpha \)-S43C and either s/hCG\( \beta \)-Nt,SE,C26A or s/hCG\( \beta \)-L5C,SE,C26A (Table III). Heterodimers containing the native \( \alpha \)-subunit (Table III, data rows 3 and 6) were more stable than hCG (Table III, data row 1), but not nearly as stable as the cross-linked heterodimers that contained \( \alpha \)-L41C and hCG\( \beta \)-C26A in which the seatbelt was latched to \( \alpha \)Cys\(^{111\text{th}} \) (Table III, row 2) or heterodimers that contained cysteines added to parts of loop \( \alpha \)2 (Table III, rows 4, 5, 7, and 8). This observation shows that cysteines added to the \( \alpha \)-subunit compete with \( \beta \)Cys\(^{5\text{th}} \) as a seatbelt latch site, confirming the notion that hCG analogs in which the seatbelt is latched to a cysteine in the NH\(_2\)-terminal end of the \( \beta \)-subunit are formed by a wraparound pathway. This suggests that all glycoprotein hormone analogs having the teFSH architecture are likely to be formed by a wraparound mechanism, not by threading.

These studies revealed that teFSH\( \beta \) analogs containing the salmon FSH seatbelt and an hCG\( \beta \) NH\(_2\)-terminal latch site were incorporated into heterodimers better than those in which both regions were derived from salmon FSH\( \beta \) (Table III, data rows 3–5 and 6–8). This observation contradicted our expectations that the salmon NH\(_2\)-terminal would serve as the more efficient latch site. One explanation for this observation is that the salmon FSH seatbelt has a greater tendency to be latched to the salmon FSH\( \beta \) NH\(_2\)-terminal before the subunits dock, a phenomenon that would reduce assembly. Another explanation is that residues at the NH\(_2\)-terminal end of the \( \beta \)-subunit have a role in subunit docking (15, 16). Those in hCG\( \beta \) may be more effective in promoting subunit docking than those in salmon FSH\( \beta \).

**The Composition of the Seatbelt Can Influence Threading**—Differences in the assembly of heterodimers containing s/hCG\( \beta \)-Nt and s/hCG\( \beta \)-Nt,SE indicated that the composition of the seatbelt might have a substantial influence on the mechanism of heterodimer assembly. Although each of these \( \beta \)-subunits has two potential seatbelt latch sites, the hCG seatbelt of s/hCG\( \beta \)-Nt became latched to \( \beta \)Cys\(^{5\text{th}} \) and permitted assembly by a threading mechanism. As had been seen earlier (Table I, data row 6), assembly of heterodimers containing the native

**Observation That Confirms Assembly Occurs by a Wraparound Mechanism**—The observation that \( \alpha \)Cys\(^{5\text{th}} \) became cross-linked to the \( \beta \)-subunit during the assembly of heterodimers containing \( \alpha \)-Q5C and \( \beta \)-subunits having the teFSH\( \beta \) folding pattern strongly supports the notion that these teFSH analogs are assembled by a wraparound mechanism. To test this result in an alternative fashion, we repeated these studies using \( \alpha \)-subunit analogs having unpaired cysteines located at other sites (Table III). We have found that hCG seatbelt residue \( \beta \)Cys\(^{110\text{th}} \) can be latched to cysteines added to the \( \alpha \)-subunit when it is prevented from being latched to \( \beta \)Cys\(^{5\text{th}} \) in loop \( \beta \)1 (13). To learn if the salmon FSH seatbelt behaved similarly to the hCG seatbelt in either regard, we compared the stabilities of heterodimers containing \( \alpha \)-L41C or \( \alpha \)-S43C and either s/hCG\( \beta \)-Nt,SE,C26A or s/hCG\( \beta \)-L5C,SE,C26A (Table III). Heterodimers containing the native \( \alpha \)-subunit (Table III, data rows 3 and 6) were more stable than hCG (Table III, data row 1), but not nearly as stable as the cross-linked heterodimers that contained \( \alpha \)-L41C and hCG\( \beta \)-C26A in which the seatbelt is latched to \( \alpha \)Cys\(^{111\text{th}} \) (Table III, row 2) or heterodimers that contained cysteines added to parts of loop \( \alpha \)2 (Table III, rows 4, 5, 7, and 8). This observation shows that cysteines added to the \( \alpha \)-subunit compete with \( \beta \)Cys\(^{5\text{th}} \) as a seatbelt latch site, confirming the notion that hCG analogs in which the seatbelt is latched to a cysteine in the NH\(_2\)-terminal end of the \( \beta \)-subunit are formed by a wraparound pathway. This suggests that all glycoprotein hormone analogs having the teFSH architecture are likely to be formed by a wraparound mechanism, not by threading.
Heterodimers secreted into the culture medium by COS-7 cells co-transfected in triplicate with the indicated α- and β-subunits were concentrated 10-fold and quantified in A13/S17/B110 assays. The samples were then treated at pH 2, at 37 °C for 30 min, 120 min, and overnight before being assayed a second time. Values shown (mean ± S.E. for triplicates) represent the amount of material in the concentrated medium and the percentage that remained as heterodimer following acid treatment. The least acid-stable heterodimer is hCG, in which the seatbelt is latched to βCys26 in loop 1. The stability of α-L41C/hCG-C26A is typical of heterodimers in which the seatbelt is latched to a cysteine in the α-subunit. The stabilities of heterodimers having the seatbelt latched to βCys26 (i.e. those containing the native α-subunit and s/hCGβ-Nt,SB,C26A, s/hCGβ-L5C,SB,C26A, s/hCGβ-Nt,SB, or hCGβ-L5C,C26A-KDEL) is between that of hCG and α-L41C/hCG-C26A. The increased stability of chimeras containing α-subunit analogs having an additional cysteine is due to the presence of an intersubunit disulfide. Formation of this cross-link can occur only if the seatbelt is latched by a wraparound mechanism. (Note that in two other experiments, we observed that 15.2 and 19.2% of the s/hCG α-subunit and s/hCGβ-Nt,SB,C26A containing heterodimer was stable after overnight incubation, values that indicate that the heterodimers containing the native human α-subunit and s/hCGβ-L5C,SB,C26A or s/hCGβ-Nt,SB,C26A have approximately the same stability.)

α-subunit and s/hCGβ-Nt,SB was very inefficient (Table IV, row 3). Both s/hCGβ-Nt and s/hCGβ-Nt,SB were incorporated efficiently into heterodimers that contain α-Q5C (Table IV, row 1 and 2), showing that their seatbelts were latched primarily to loop β1 residue βCys26 prior to subunit docking. The difference in the amount of heterodimer formed when s/hCGβ-Nt,SB was expressed with the native α-subunit and α-Q5C suggested that the salmon FSH seatbelt inhibited threading when it was latched to βCys26. Studies described next were initiated to test this notion and to identify the portion of the seatbelt that was most likely to be responsible for its ability to interfere with threading.

We monitored the formation of heterodimers containing the native α-subunit and s/hCGβ-SB or s/hCGβ-SB,L92K; the former seatbelt has two C-HC residues at its NH2 terminus, namely βAla51 and βLeu92. In the chum salmon FSH seatbelt, these residues are isoleucine and lysine and, to learn how a positively charged residue adjacent to the tensor loop would affect assembly, we replaced βLeu92 with lysine. The amount of heterodimer made when either of these β-subunit chimeras was co-expressed with the native α-subunit was much lower than that of hCG (Table V, data rows 1–3). This showed that the presence of the salmon FSH seatbelt reduced assembly, most likely by impeding threading.

The NH2- and COOH-terminal halves of the hCG and salmon FSH seatbelts differ significantly and we studied each of these regions separately. The NH2-terminal half of the seatbelt contains a small loop that is stabilized by a disulfide that we term the “tensor” because of its ability to change the length of the seatbelt during heterodimer assembly. The tensor disulfide is disrupted during threading, which elongates the seatbelt and facilitates passage of the glycosylated end of loop α2 through the β-subunit (20). Reformation of the tensor disulfide following threading stabilizes the heterodimer. To test the notion that differences in the stabilities of the hCG and salmon FSH tensor loops were responsible for the reduced ability of the salmon FSH seatbelt to permit threading, we replaced the hCG tensor loop with its salmon FSH counterpart and monitored heterodimer assembly. The salmon FSH tensor loop had little or no inhibitory influence on heterodimer assembly. Consequently, s/hCGβ-TL was incorporated into heterodimers as well as or better than hCGβ and much better than s/hCGβ-SB or s/hCGβ-SB,L92K, analogs that have nearly the entire salmon FSH seatbelt (Table V, data rows 1–4).
Influence of Seatbelt on Assembly Pathway

### Table IV

| Data row | Analog                  | Total heterodimer | pH 2 stable, 30 min | pH 2 stable, 16 h |
|----------|-------------------------|-------------------|---------------------|------------------|
|          | ng/50 µl ± S.E.         | % total ± S.E.    |                     |                  |
| 1        | α-Q5C + s/hCGβ-Nt       | 5.48 ± 0.44       | 99.4 ± 3.7          | 103.7 ± 4.4      |
| 2        | s/hCGβ-NT                | 4.73 ± 0.09       | 112.9 ± 8.3         | 99.2 ± 4.6       |
| 3        | α + s/hCGβ-Nt            | 0.12 ± 0.01       | 67.5 ± 3.1          | 34.9 ± 3.7       |

### Table V

| Influence of the salmon seatbelt tensor loop and strand on heterodimer assembly |
|---------------------------------|-----------------|-----------------|-----------------|
|                                  | Analog          | Total % Stable  | ng/50 µl ± S.E. | % total ± S.E. |
| 1                                | hCGβ            | 2.34 ± 0.28     | 0.4 ± 0.3       |
| 2                                | s/hCGβ-SB       | 0.39 ± 0.12     | 0.2 ± 0.1       |
| 3                                | s/hCGβ-SB,L92K  | 0.67 ± 0.12     | 0.7 ± 0.3       |
| 4                                | s/hCGβ-TL       | 2.27 ± 0.26     | 0.3 ± 0.2       |
| 5                                | s/hCGβ-St       | 0.28 ± 0.07     | 5.2 ± 0.5       |

### Table VI

| Stability of heterodimers containing the salmon seatbelt strap region following co-expression with α-S43C and α-S92C |
|---------------------------------------------------------------|-----------------|-----------------|-----------------|
|                                                                 | Analog          | Total % Stable  | ng/50 µl ± S.E. | % stable ± S.E. |
| 1                                                                 | hCGβ            | 7.27 ± 0.49     | 103 ± 0.6       |
| 2                                                                 | s/hCGβ-St       | 1.58 ± 0.04     | 44.4 ± 1.4      |
| 3                                                                 | s/hCGβ-St,C93A  | 0.44 ± 0.03     | 102.9 ± 1.2     |
| 4                                                                 | s/hCGβ-St,C93A,C100A | Not detected | Not done       |

### Table VII

| Species                    | Stability of heterodimers containing the salmon seatbelt strap region following co-expression with α-S43C and α-S92C |
|---------------------------|----------------------------------------------------------------------------------------------------------------|
| COS-7 cells               | Formation of the α5-β strand cross-link rescued the formation of heterodimers containing s/hCGβ-Nt,SB |

The indicated β-subunit analogs were co-transfected into COS-7 cells with the native α-subunit. Production of heterodimer was monitored in the medium three days later. The stability of the heterodimer was measured following an incubation at pH 2, 37 °C for 30 min. Values shown are mean ± S.E. for triplicate transfections.

### Discussion

Mechanisms of Glycoprotein Hormone Assembly in Vertebrates—Because of the tendency of the seatbelt to be latched to a cysteine in loop β1 before the subunits dock, we anticipate that most vertebrate glycoprotein hormones are assembled by a threading mechanism. Indeed, as shown here, even the salmon FSH seatbelt tends to be latched to a cysteine in loop β1 when it has a choice of seatbelt latch sites. When the seatbelt can only be latched to an NH2-terminal β-subunit site, however, as it is in the case for salmon FSH and other related teleost species (Table VII), heterodimer assembly can occur by a wraparound mechanism, but not by threading. This appears due to the combination of the low ability of the salmon seatbelt to be latched to the NH2 terminus and the difficulty of threading loop Ω2 beneath these seatbelts once they are latched. The finding that the salmon seatbelt can impede threading when it is latched to loop β1 suggests that some glycoprotein hormones may be assembled by a wraparound pathway even when their seatbelts are latched to loop β1. We would expect this process to be inefficient, however.

Implications of these Observations for the Evolution of tFSH and the Interaction of tFSH with Piscine FSH Receptors—The finding that hCG analogs which have the tFSH folding pattern are assembled by a wraparound mechanism suggest that hormones such as salmon FSH are also assembled in this fashion. Why would the wraparound pathway, an assembly mechanism that appears to be relatively inefficient (19, 20), be used to produce hormones that are usually thought to be critical for the reproduction of vertebrates? The wraparound pathway permits formation of heterodimers that cannot be assembled by threading. As such, it would have facilitated natural experimentation with the seatbelt during co-evolution of these heterodimeric ligands and their receptors. The seatbelt is responsible for much of the influence of the hormone-specific β-subunit on biological activity (5–8) and, as shown here, the efficiency of threading. When changes in the β-subunit create seatbelts that block threading, the use of the wraparound mechanism offers the organism a mechanism of producing het-
We anticipate that all glycoprotein hormones originated from a precursor in which the seatbelt was latched to a cysteine in β1. This would permit assembly by either a threading or wraparound mechanism. Selection pressure to optimize reproduction and thyroid function led to the duplication and reduplication of the β-subunit to create β-subunits found in lutropins, follitropins, and thyrotropins. Subsequent divergence of the resulting β-subunits occurred in response to selection pressures on reproduction and development. The seatbelt is the portion of the β-subunit that has the greatest influence on hormone activity (5–8), making it more sensitive to selection pressure than other parts of the protein. Its roles in heterodimer assembly and stability would also have influenced β-subunit evolution. Mutations of the seatbelt that prevented assembly would have been lost regardless of their abilities to regulate receptor function. The existence of the wraparound assembly mechanism would have permitted mutations to the seatbelt that prevented threading, even though wrapping appears to be much less efficient than threading. In part, this is due to the tendency of the seatbelt to become latched to the β-subunit before it docks with the α-subunit. Factors that drove the evolution of teFSH remain unknown.

In teleosts, these are likely to have involved seatbelt mutations that affected the interaction of teleost FSH with FSH, LH, and TSH receptors. Relocation of the seatbelt to the NH₂ terminus of the β-subunit would be expected to disrupt high affinity interactions between the COOH terminal end of the seatbelt and any of these receptors. Based on the studies of Yan et al. (18), which show that salmon FSH does not bind the salmon LH receptor, we propose that the evolution of the teFSH structure was driven by the need to reduce cross-reactivity with the LH receptor and/or to increase the stability of the heterodimer. Shown here (single letter code) are parts of the ligands that created the interaction of teFSH with FSH, LH, and TSH receptors.

| Species and β-subunit | Acquisition number | Assembly pathway | Amino terminus | Residue in β1 | Seatbelt region |
|-----------------------|-------------------|-----------------|---------------|---------------|----------------|
| Human hCGβ            | NP_000728         | Threading       | SKEPLPRFC     | C             | AL CRRTSTDC    |
| Human FSHβ            | 1P7/8             | Threading       | NSC           | C             | GK CSDSTDC     |
| Human TSHβ            | AA830828          | Threading       | F C           | C             | GK CNDYSDC     |
| Shark FSHβ            | CAC43253          | Threading       | NRC           | C             | GM CNETTDC     |
| Japanese eel          | Q9YKGK3           | Threading       | RASTSC        | C             | SK CNSTDC      |
| Conger eel            | CAB93518          | Wrapping        | RACSSC        | W             | SR CNSTDC      |
| Black carp FSHβ       | AAK7415           | Threading       | GSECRSSC      | C             | SK CNSIDAC     |
| Common carp FSHβ      | O13095            | Unknown-T       | GSECRSSC      | C             | SK CNSIDTC     |
| Goldfish FSHβ         | Q98848            | Unknown-T       | GTEECRGSC     | S             | IK CKDTNDTC    |
| Chum salmon           | P10257            | Wrapping        | GTEECRGSC     | S             | IK CKDTNDTC    |

The evolutionary pressures that drove the changes in glycoprotein hormone topology that created teFSH are enigmatic, particularly because hormones having this structure appear to be more difficult to assemble. One factor that may have contributed to the development of teFSH is its increased stability, which may offset the additional difficulty of assembling the heterodimer. More likely, the changes in the teFSH β-subunit occurred during the continual co-evolution of the gonadotropins and their receptors (6). Both salmon LH and salmon FSH interact with the salmon FSH receptor (18), indicating that some of the actions of salmon FSH can be replaced by salmon LH. In contrast, salmon FSH does not interact with the salmon LH receptor, a property that may reflect its altered seatbelt latch site. The observation that the FSH strap region of the seatbelt occupies two very different positions in the Japanese eel and the conger eel (Table VII) suggests that the strap may not participate in high affinity contacts with the FSH receptor in either of these related species. If the strap region contributed to the ability of FSH to bind to LH receptors when it was latched to loop β1, changing its position to the NH₂ terminus would reduce binding to the LH receptor without affecting binding to the FSH receptor.

The most ancient species for which a sequence of the FSH β-subunit is known is the shark. The seatbelt of this follitropin appears to be latched to a site in loop β1. We have found that hCG-shark FSH chimeras containing the shark FSH seatbelt are assembled into heterodimers readily and that this is not affected by adding the salmon NH₂-terminal seatbelt latch site.³ The finding that the shark FSH seatbelt is readily latched to loop β1 and that it does not inhibit threading suggests strongly that shark FSH is assembled by a threading mechanism similar to β-subunit analog s/hCG-β-Nt (Table II, row 3). The FSH β-subunit in goldfish and the common carp appears to have two seatbelt latch sites, one in the NH₂ terminus and one in loop β1, but it is not known which of these latch sites are used (Table VII). Indeed, whereas it is conceivable that these fish produce two forms of FSH, we expect that similar to s/hCG-β-Nt most of their FSH will be assembled by a threading mechanism and its seatbelt will be latched to loop β1. This is supported by the finding that black carp FSH, which has a seatbelt that is similar to that of the common carp and goldfish, appears to have only the seatbelt latch site in loop β1 (Table VII). Thus, it would be expected that this heterodimer forms by threading. The presence of two potential latch sites within the goldfish and common carp β-subunits suggests that the precursor of teFSHβ may have also had two potential latch sites. Based on our finding that s/hCGβ-β-Nt,SB is assembled into heterodimers poorly, we expect that the site in loop β1 was eliminated to enhance heterodimer assembly. Loss of the loop β1 latch site would have reduced premature latching of the seatbelt, thereby facilitating assembly by the wraparound pathway.

The Strategies Devised to Study the Assembly of hCG and Analogs Having the Folding Pattern of teFSH Can Be Used to Study the Assembly of Other Vertebrate Glycoprotein Hormones—The amino acid sequences of several vertebrate hormones have been reported, but it remains to be determined how

---

² Fish gonadotropins often differ from their mammalian counterpart. In many cases, the piscine lutropin, which is also known as GTH-II is capable of interacting with LH and FSH receptors. This may have also had a role during the evolution of teFSH.

³ M. P. Bernard, R. V. Myers, D. Cao, and W. R. Moyle, unpublished data.
these are assembled. Considerable variations occur in the sizes of the α- and β-subunit cores and the seatbelt regions of many glycoprotein hormones, particularly in fish. These have the potential to provide new insights into the mechanisms of protein folding within the endoplasmic reticulum. We anticipate that the approaches described here for studying the assembly of tFSH will be applicable to studies of the assembly of glycoprotein hormones from most vertebrates including fish. The most important aspect of our approach is the use of α- and β-subunit analogs that contain unpaired cysteines. Although we took advantage of a well characterized panel of monoclonal antibodies to hCG, we anticipate that any procedure capable of measuring the amounts of heterodimers produced following transfection of cells with subunit analogs containing appropriate unpaired cysteines would suffice. This includes the use of epitope tagged α- and β-subunit analogs. Many of the studies described in this and in the accompanying articles (19, 20) were performed to check the internal consistency of our findings. We anticipate that only a few of the analogs that we produced and characterized would be required to distinguish most assembly pathways. These would include analogs corresponding to α-Q5C, α-S43C, α-S92C, and hCGβ-C26A.

Acknowledgments—We thank Dr. William Munroe (Hybritech, Incorporated, San Diego, CA, a subsidiary of Beckman Coulter, Inc.) for antibodies A113 and B111, and Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA) for purified recombinant hCG. We thank Drs. Robert Campbell and Penny Swanson (Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, Seattle, WA) for valuable suggestions.

REFERENCES
1. Pierce, J. G., and Parsons, T. F. (1981) Annu. Rev. Biochem. 50, 465–495
2. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, P. J., and Isaac, N. W. (1984) Nature 309, 455–461
3. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) Structure 2, 545–559
4. Fox, K. M., Dias, J. A., and Van Roey, P. (2001) Mol. Endocrinol. 15, 378–388
5. Campbell, R. K., Dean Emig, D. M., and Moyle, W. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 760–764
6. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Hsu, Y., and Wang, X. (1994) Nature 368, 251–255
7. Dias, J. A., Zhang, Y., and Liu, X. (1994) J. Biol. Chem. 269, 25289–25294
8. Grossmann, M., Szekdinski, M. W., Wong, R., Dias, J. A., Ji, T. H., and Weintraub, B. D. (1997) J. Biol. Chem. 272, 15532–15540
9. Li, M. D., and Ford, J. J. (1998) J. Endocrinol. 156, 529–542
10. Garcia-Hernandez, M. F., Roche, Y., Diaz, M. V., and Kawauchi, H. (1997) Gen. Comp. Endocrinol. 106, 389–399
11. Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991) Biol. Reprod. 44, 29–38
12. Abuj, S. M., Edelhock, H., Ingham, K. C., Morgan, F. J., Canfield, R. E., and Ross, G. T. (1973) Arch. Biochem. Biophys. 159, 497–504
13. Xing, Y., Lin, W., Jiang, M., Myers, R. V., Cao, D., Bernard, M. P., and Moyle, W. R. (2001) J. Biol. Chem. 276, 46953–46960
14. Heikoop, J. C., van den Boogaart, P., Mulders, J. W. M., and Grootenhuis, P. D. J. (1997) Nat. Biotech. 15, 658–662
15. Chen, F., and Puett, D. (1991) J. Biol. Chem. 266, 6904–6908
16. Slautter, S., Wang, Y. H., Myers, R. V., and Moyle, W. R. (1995) Mol. Cell. Endocrinol. 112, 21–25
17. Campbell, R. K., Bergert, E. R., Wang, Y., Morris, J. C., and Moyle, W. R. (1997) Nature Biotech. 15, 439–443
18. Yan, L., Swanson, P., and Dickhoff, W. W. (1992) Biol. Reprod. 47, 418–427
19. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) J. Biol. Chem. 279, 35426–35436
20. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) J. Biol. Chem. 279, 35437–35448